1999

Characterization of the Microsomal Mixed-Function Oxidase System of Several Species of Sea Anemones (Phylum: Cnidaria).

Linda Marie Heffernan
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/7046

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

UMI
800-521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
CHARACTERIZATION OF THE MICROSOMAL MIXED-FUNCTION OXIDASE SYSTEM OF SEVERAL SPECIES OF SEA ANEMONES (PHYLUM: CNIDARIA)

A Dissertation

Submitted to Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biochemistry

by

Linda Marie Heffernan
A.S., Russell Sage, 1985
B.S., University of Maine, 1987
December 1999
Dedication

I dedicate this dissertation to my parents, William J. Heffernan and Dorothy M. Heffernan, who have always treated me with love and respect. They also instilled in me a love for the outdoors, enthusiasm for science, and a sense of the importance of education.
Acknowledgments

Each of my committee members were helpful towards the completion of my research and the requirements for the degree. In particular, my advisor, Dr. Gary W. Winston, was encouraging and gave me wide discretion in the focus of my research. He also gave me opportunities to present my research at several professional meetings, both national and international. In addition to Dr. Gary Winston, Dr. Grover Waldrop, Dr. Barbara Shane, and Dr. Patrick DiMario provided me with constructive criticism, challenged me in our meetings, and always encouraged me to take the extra steps in my research. As the dean’s representative, Dr. Daniel Burba fulfilled his responsibilities commendably. Approximately two years prior to the completion of this degree, Gary Winston took a position in the Department of Toxicology at North Carolina State University. During this time, Grover Waldrop allowed me access to his equipment to complete my research. Finally, a few days prior to my defense, Grover Waldrop was called out of town and Dr. William Stickle took his place on short notice. Throughout the research, William Stickle also provided a site to house my sea anemones and gave me his insights on maintaining healthy animals.

There were also many people beyond my committee members who assisted in my research. Dr. Hershel F. Morris, Jr., Director of the Agricultural Chemistry Center, allowed me to use the Residue Analysis Facility to perform the aldrin epoxidation studies. Several members of this facility, especially Amy B. Hernandez and Nina M. Huffstetler, were very helpful in optimizing the conditions of the Gas Chromatographer to improve the sensitivity of the assay and in teaching me the program’s parameters for peak integration.

Dr. Wayne R. StochaJ (Hoefer-Pharmacia) ran the 2-dimensional gels for the partial purification of the 40 KDa protein. He also provided consultation on maintenance of healthy sea anemones and collected sea anemones from California. My husband, Scott W. Herke, assisted in the production of more microsomes than he will ever care to remember and in the aldrin studies.
Scott was also very supportive throughout this process and read multiple versions of this dissertation. Kristina Daberry (student worker) performed protein assays and assisted in extraction of dieldrin. Jeff Tamplin collected the Alaskan *A. elegantissima*. I also appreciate the help, encouragement, and friendship of the people the Biochemistry Department and in our laboratory, especially Billy F. Dudley, Caroline A. Metosh-Dickey, and Robin P. Ertl.

Cindy Henk examined the TEM images of the microsomal and cytosolic fractions. Ron Bouchard assisted in production of the photographs for the dissertation and the various presentations throughout my stay at Louisiana State University. Dr. Harold Silverman (Chairman of Biological Sciences Department) and Dr. John Fleeger (Graduate Student Advisor) were both helpful in providing support after my advisor moved to North Carolina.

The various cytochrome P450 antibodies were provided by: Dr. Donald R. Buhler (anti-CYP2K1 and CYP3A1), Oregon State University, Corvallis, OR; Dr. John J. Stegeman (anti-CYP1A1 and anti-CYP2B), Woods Hole Oceanographic Institution, Woods Hole, MA; Dr. Stelvio Bandiera (anti-CYP2C11*), University of British Columbia, Vancouver, BC; Dr. Arthur Cedarbaum (anti-CYP2E1), Mount Sinai School Medicine, NY; and Dr. G. Gordon Gibson (anti-CYP4A), University of Surrey, Guildford, UK. This research was supported in part by a grant 14-35-0001-30660/#19916 from the U.S.A. Department of Interior, Mineral Management Survey Unit and the Department of Biological Sciences and Department of Agricultural Chemistry.
# Table of Contents

Dedication ............................................................................................................................... ii  

Acknowledgments ................................................................................................................... iii  

Abstract .................................................................................................................................. vi  

Chapter 1: Introduction ........................................................................................................ 1  

Chapter 2: Spectral Analysis and Catalytic Activities of the Mixed-Function Oxidase System in Several Species of Sea Anemones .............................................................. 11  

Chapter 3: Distribution of Microsomal CO-binding Chromophores and EROD Activity in Sea Anemone Tissues from *Anthopleura xanthogrammica* ....................................................... 51  

Chapter 4: Analysis and Partial Purification of Cytochrome P450 Immunoreactive Proteins in Sea Anemone Microsomes ..................................................................................................... 66  

Chapter 5: Summary ............................................................................................................. 113  

References Cited ................................................................................................................... 119  

Appendix A: Aldrin Epoxidation .......................................................................................... 131  

Appendix B: Letter of Permission ......................................................................................... 132  

Vita ...................................................................................................................................... 133
Abstract

Several studies have demonstrated detrimental effects of anthropogenic compounds on coral reef communities. These compounds are known to be bioaccumulated rapidly, but eliminated slowly by anthozoans (i.e., corals and sea anemones). The cytochrome P450-dependent mixed function oxidase (MFO) system is the primary pathway for initial oxidation of many hydrophobic exogenous compounds; however, its presence had not been well documented in anthozoans. The studies reported herein show that a functional cytochrome P450-dependent MFO is present in the microsomal fraction of the sea anemones *Anthopleura xanthogrammica*, *A. elegantissima*, and *Bunodosoma cavernata*. The evidence is based on the presence of classical microsomal MFO components (e.g., cytochrome P450 and flavin-containing reductases) and their ability to catalyze P450-dependent monooxygenase reactions (e.g., aldrin epoxidation and ethoxyresorufin O-dealkylation (EROD)) in the presence of NAD(P)H. The P450 specific contents and MFO activities are similar to values found in many other marine invertebrates. NADPH- and NADH-dependent EROD activity was consistently observed in *A. elegantissima* and *A. xanthogrammica*; however, it was below the detection limit of the assay for *B. cavernata*. In contrast, NADPH- and NADH-dependent aldrin epoxidation activity was consistently observed in *B. cavernata* and *A. elegantissima*, but it was less consistently observed in *A. xanthogrammica*. Despite the much higher NADH-cytochrome c and ferricyanide reductase activities, sea anemone monooxygenase reactions consistently preferred NADPH as a cofactor for EROD. The aldrin epoxidation activity tended to be slightly higher with NADPH versus NADH; however, there were a few preparations that strongly preferred either NADH or NADPH. The difference in preference of electron donors between microsomal preparations may result from differential expression of P450 isoforms capable of metabolizing aldrin. Further, the presence of multiple microsomal P450 isoforms in these sea anemones was indicated by immunodetection. Several antibodies raised against rat or fish P450 isoforms of the CYP 1, 2,
and 3 families recognized protein(s) between 50-60 KDa in the microsomal fraction of each species studied that were characteristic of P450. Anti-CYP2K also recognized a 40 KDa protein that was not characteristic of P450.
Chapter 1: Introduction

The effect of anthropogenic compounds, such as organochloride pesticides and polyaromatic hydrocarbons, on the fragile coral reef communities is a subject of growing concern. Sea anemones and corals (Phylum Cnidaria; Class Anthozoa) are both important members of this marine community. Field studies on hard corals have demonstrated that acute exposure to pollutants, such as oil, often results in massive mortality. Exposure to sublethal levels results in a reduction in reproductive capacity, changes in lipid metabolism, lower growth rates, reduction or elimination of recolonization, and extensive mucus production. The extensive mucus production enhances bacterial production on the corals, which often leads to death (Burns & Knap, 1989; Loya & Rinkevich, 1980; Rinkevich & Loya, 1979).

Several studies have demonstrated rapid uptake and slow elimination rates of xenobiotics in anthozoans as compared to other marine invertebrates (Knap et al., 1982; Solbakken et al., 1982; 1983; 1984; 1985). However, none of these studies examined the ability of the anthozoans to metabolize these compounds to more hydrophilic substances that can be eliminated. The long half-life of many xenobiotic compounds including polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and chlorinated hydrocarbon insecticides suggests that detoxification or elimination may be especially slow in cnidarians. For instance, Solbakken et al. (1984) demonstrated that the radiolabeled 2,4,5,2',4',5'-hexachlorobiphenyl (PCB) was still present one year after exposure. The proportions of remaining radioactivity as parent PCB or PCB metabolites were not determined.

The initial oxidation of many hydrophobic exogenous compounds is performed by the cytochrome P450-dependent mixed-function oxidase (MFO) system. These reactions are often the first step in increasing the hydrophilicity of lipophilic compounds, allowing their excretion from the organism. Thus, this pathway was originally thought of as a detoxification pathway; however, it is currently known that this pathway may actually lead to more toxic intermediates.
through formation of reactive electrophiles or free radicals, which readily attack nucleophilic centers of tissue macromolecules (e.g., Okey, 1990). Despite the production of potentially harmful intermediates, this pathway is often the first step necessary for excretion of xenobiotic compounds through primarily oxidation reactions. The addition of a reactive functional group (e.g., -OH, -NH₂, -SH, -COOH) by P450 is often a prerequisite for phase II conjugation reactions, which render lipophilic molecules more hydrophilic by addition of highly polar groups (e.g., glutathione, sulfate, glucuronate) at the oxidized sites (e.g., Livingstone, 1991; Stegeman & Hahn, 1994).

The presence of a cytochrome P450-dependent MFO system has been demonstrated in most phyla, including several marine invertebrate phyla (e.g., reviews by den Besten, 1998; James & Boyle, 1998; Livingstone, 1991; Lee, 1998); however, many of the more basic questions still need to be addressed, such as the inducibility of P450 and identification of regulatory pathways, both activation and deactivation. Invertebrate studies are complicated by the presence of low P450 contents, endogenous inhibitors to P450, and low reductase activities (e.g., Bend et al., 1981; James, 1989; James et al., 1979; Lindstrom-Seppa et al., 1982; 1983; Livingstone, 1991; Payne, 1977). As a result, early molluscan studies failed to discover an MFO system (Lee, 1972, Payne, 1977, Vandermeuleun et al., 1978), yet molluscs are now accepted as containing cytochrome P450 (e.g., Livingstone, 1991). Similarly, most early cnidarian studies reported an absence of cytochrome P450 monooxygenase activity or its characteristic spectral properties (Lee, 1975; 1981; Payne, 1977). However, several recent studies have indicated the presence of a functional cytochrome P450-dependent MFO system in two cnidarian classes—hydrozoa and anthozoa (Gassman & Kennedy, 1992; Heffernan et al., 1996; Heffernan & Winston, 1998; Khan et al., 1972b; Winston et al., 1998).

The vast majority of the P450 literature has focused on mammalian systems (particularly the rat), although the catalytic cycle has been examined primarily with the only two
cytosolic cytochromes P450 (i.e., in bacteria) known to exist. Due to their direct impact on humans, insects have also been heavily studied. Only recently has significant progress been made on elucidating P450 mechanisms in the invertebrate (non-insect) world. Further, the road to discovery has been difficult because analytical procedures for studying P450 have been optimized for mammalian systems, which are very different from invertebrate systems. In many cases, research has suggested that invertebrates have low levels of P450 and/or P450 activity. It remains an open question as to whether the P450 content and MFO activities are as low as some of the literature has suggested, or if the low levels are an artifact of mammalian-based analytical procedures. Examples have already demonstrated that additional heme-proteins, which interfere with quantitation the P450 content, resulted in detection of a lower P450 content (Berghout et al., 1991, Livingstone et al., 1989; Nelson et al., 1976; Singer et al., 1980). Further, endogenous inhibitors found in several species of crustacean have interfered with MFO activities (Bend et al., 1981; James et al., 1979; Lindstrom-Seppa et al., 1982; 1983; Payne, 1977).

The focus of this dissertation research was to characterize the microsomal MFO system in the cnidarian (i.e., sea anemone) more rigorously through: (1) identification of the presence of the components necessary for a functional MFO system; (2) characterization of the spectral properties of cytochrome 450 and other potentially active heme-proteins of sea anemone microsomes; and, (3) assessment of the ability of the MFO system to metabolize benchmark substrates for cytochrome P450. The rest of this introductory chapter will focus on general background information on P450 and the cnidarian, particularly the sea anemone.

**CYTOCHROME P450**

Cytochromes P450 are a family of heme-centered enzymes capable of performing a diverse number of predominantly oxidation reactions on numerous substrates. These reactions include oxidation, peroxidation, hydroxylation, dealkylation, desulfuration, and dehalogenation. Generalized examples of these reactions are shown in Table 1-1. P450 isoforms are also capable
Table 1-1. Classes of reactions catalyzed by P450.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliphatic Hydroxylation</td>
<td>( R-\text{CH}_2-\text{CH}_2-\text{CH}_3 \rightarrow R-\text{CH}_2-\text{CHOH}-\text{CH}_3 )</td>
</tr>
<tr>
<td>Aromatic Hydroxylation</td>
<td><img src="image" alt="Aromatic Hydroxylation" /></td>
</tr>
<tr>
<td>Epoxidation</td>
<td>( R-\text{CH}==\text{CH}-R' \rightarrow R-\text{CH}==\text{CH}-R' )</td>
</tr>
<tr>
<td>N-, S-, or O- Dealkylation</td>
<td>( R-(\text{NH}, \text{O}, \text{S})-\text{CH}_3 \rightarrow R-(\text{NH}_2, \text{OH}, \text{SH})-\text{CH}_3 + \text{CH}_2\text{O} )</td>
</tr>
<tr>
<td>Deamination</td>
<td>( R-\text{CH}_2-\text{NH}_2 \rightarrow R-\text{C}==\text{H} + \text{NH}_3 )</td>
</tr>
<tr>
<td>N-hydroxylation</td>
<td>( R-\text{NH}==\text{C}==\text{CH}_3 \rightarrow R-\text{NOH}==\text{C}==\text{CH}_3 )</td>
</tr>
<tr>
<td>Sulfoxidation</td>
<td>( R-\text{S}==\text{R}' \rightarrow R-\text{S}==\text{R}' )</td>
</tr>
<tr>
<td>Desulfuration</td>
<td>( R_1R_2\text{P}==\text{X} \rightarrow R_1R_2\text{P}==\text{X} + \text{S} )</td>
</tr>
<tr>
<td>Oxidative Dehalogenation</td>
<td>( R-\text{C}==\text{H} \rightarrow R-\text{C}==\text{OH} \rightarrow R-\text{C}==\text{H} + \text{HX} )</td>
</tr>
</tbody>
</table>
of performing reduction reactions on some substrates under certain conditions; however, these reactions are less common. They can metabolize both endogenous substrates, such as steroids, fatty acids, bile acids, prostaglandins, and leukotrienes, and exogenous compounds (also referred to as xenobiotic compounds), such as phytoalexins, polyaromatic hydrocarbons, and organochlorine pesticides. The general reaction catalyzed by P450 can be written as:

$$\text{NAD(P)H} + \text{H}^+ + \text{O}_2 + \text{SH} \rightarrow \text{NAD(P)}^+ + \text{H}_2\text{O} + \text{S-OH}$$

where SH represents the substrate and S-OH represents the product. All of these oxidation reactions involve the insertion of one oxygen atom derived from molecular oxygen into the substrate; thus, P450 has also been referred to as a monooxygenase. The insertion of the oxygen may be followed by rearrangement of the compound to yield a final product that does not incorporate the oxygen atom (e.g., O, N, and S dealkylation reactions). Some isoforms of P450 are specific for a particular substrate and the kind of reaction catalyzed, whereas many other isoforms have a broad specificity and catalyze a myriad of oxidative processes. A characteristic feature of various P450 isoforms is their ability to be induced by the compounds that they are capable of metabolizing. These various substrates can promote induction of cytochromes P450 at the transcriptional, post-transcriptional and translational levels (e.g., Okey, 1990).

Based on the most recent published list of P450 isoforms, there are 481 genes and 22 pseudogenes representing 74 families (Nelson et al., 1996). There are estimated to be 60 to 200 isoforms existing in a single mammalian species (Nebert & Nelson, 1991). To simplify the identity of these isoforms, cytochromes P450 have been designated as CYP # letter #, where the first number refers to its family, the letter refers to its subfamily, and the last number refers to the specific isoform. For instance, one commonly studied P450 is CYP1A1. The various isoforms have been designated to the particular family and subfamily based on sequence
homology. In mammals, a family typically has greater than 40% sequence homology, while a subfamily has greater than 55% homology. The full name, CYP1A1, is reserved for isoforms and species where sequence information confirms its identity. Without such sequencing information, the isoform is referred to as CYP1A.

Cytochromes P450 contain a single heme prosthetic group (iron protoporphyrin IX) with the central iron atom liganded to a cysteiny1 residue, which can exist in the thiol (-SH) or thiolate (-S') state. P450 is a b-type cytochrome; however, the ligation of the fifth ligand proximal to this cysteiny1 residue provides P450 with spectral properties that are atypical of other cytochrome b-type proteins. Under reduced conditions in the presence of carbon monoxide (CO), native cytochrome P450 has a characteristic 450 nm peak (Fig. 1-1); hence, it was named P450. Based on the extinction coefficient determined from rat samples, cytochrome P450 can be quantitated from the difference between the absorbance at 450 nm and 490 nm, which is the isosbestic point between the CO-liganded, sodium dithionite (DTN)-reduced form of cytochrome P450 and the unliganded, DTN-reduced form of cytochrome P450 (Omura & Sato, 1964b).

Denaturation of mammalian P450 results in the loss of the characteristic 450 nm peak and the production of a 420 nm peak; hence, denatured P450 has been referred to as P420. In contrast to vertebrates, a peak with a relatively large amplitude near 418 nm has been detected in many of the aquatic invertebrates, particularly the echinoderm, mollusc and cnidarian (e.g., den Besten, 1998; Heffernan & Winston, 1998; Livingstone & Farrar, 1984). Based on the mammalian literature, the identity of this 420 nm peak is classically considered to be denatured P450 (e.g., Schenkman & Kupfer, 1982; Omura & Sato, 1964b); however, its actual identity in invertebrates is unknown. Several studies have suggested that the invertebrate 418 nm chromophore may actually result from the presence of additional heme-centered protein(s).

Cytochromes P450 are the terminal oxidase in the MFO system. They are typically membrane-bound proteins, which are most abundant in the endoplasmic reticulum. There are
also several membrane-bound mitochondrial isoforms and two bacterial cytosolic isoforms. In vertebrates, cytochrome P450 has been found in most tissues; however, it tends to be more concentrated in the liver (e.g., Schenkman & Kupfer, 1982; Lewis, 1996; Okey, 1990). In invertebrates, P450 tends to be principally concentrated in tissues associated with processing of food and pollutants (e.g., Jewell & Winston, 1989; Livingstone & Farrar, 1984; McElroy, 1990). The other components of the MFO system that are critical or ancillary for a functional P450-dependent MFO system in vivo are the NADPH-cytochrome P450 reductase, cytochrome b₅, and NADH-cytochrome b₅ reductase. The membrane composition is also an important property of functional MFO systems. Preparation of the P450 isoforms from the endoplasmic

Fig. 1-1. CO-difference spectra of DTN-reduced rat liver microsomes. Microsomes were DTN-reduced and background corrected prior to addition of CO.
reticulum for analysis involves homogenization of the tissue, which breaks the membrane and allows it to reform as vesicles (referred to as microsomes), and differential centrifugation, which separates the microsomal fraction from other cellular components.

The early steps involved in the mechanisms of P450 catalysis, including substrate binding, heme reduction and oxygen binding are relatively well understood; however, much of the latter steps, especially the O – O bond cleavage in molecular oxygen and oxygen insertion steps, are still not well understood. These steps have been studied predominantly with the cytosolic bacterial camphor-metabolizing P450cam. However, due to the highly conserved nature of the functional regions of this enzyme, this pathway appears to be a reasonable description of all cytochromes P450 (Ortiz de Montellano, 1995; Lewis, 1996). In the presence of no substrate, CYP is predominantly present in the low spin oxidized (Fe^{3+}) form (Fig. 1-2; Step 1). The binding of the substrate to P450 usually causes a transition to the high spin form and a concurrent shift in the reduction potential that favors reduction of the iron (2). The iron is reduced by transferring an electron from the NADPH-cytochrome P450 reductase (3). The reduction of the iron (Fe^{2+}) favors binding of oxygen (4). After molecular oxygen is bound, the second electron is transferred to P450 sometimes through cytochrome b_{5} (5). The exact steps beyond the addition of the second electron to the hydroxylation of the substrate are still unknown. The addition of the second electron is thought to result in the formation of a peroxo-P450 complex, which ultimately leads to the cleavage of the O – O bond of molecular oxygen through heterolytic cleavage. Controversy remains as to whether cleavage is always heterolytic. The final steps in the reaction involve formation of an activated electrophilic substrate intermediate (e.g., a substrate radical, hydroxylation of that radical) and release of the product (1).

In vertebrates, the first electron is transferred from NADPH-cytochrome P450 reductase directly to P450. The second electron can be transferred directly or indirectly through cytochrome b_{5} to P450. Cytochrome b_{5} can also be reduced by NADH-cytochrome b_{5}
Fig. 1-2. Cytochrome P450 Catalytic Cycle

reductase. In contrast to vertebrates, there are many instances where invertebrates will more readily utilize NADH in lieu of NADPH as an electron donor. The preference for these cofactors appears to vary between substrates and species. Invertebrates contain both NADPH-cytochrome P450 reductase activity and NAD(P)H-cytochrome b5 activities. These reductase activities are very low as compared to vertebrate values. Currently, relatively little is known about the invertebrate reductase(s) involved in this cycle.

CNIDARIANS

Cnidarians are primarily marine organisms that live in a wide diversity of habitats from tropical to arctic regions and from intertidal to pelagic zones. The phylum contains over 10,000 living species and is composed of four classes: Hydrozoo (e.g., hydra); Scyphozoo (e.g., jellyfish that lack a true velum); Cubozoo (e.g., jellyfish); and, Anthozoo (e.g., sea anemone, sea fan, coral, sea pansy). These organisms play important roles in marine environments, particularly in
the coral reef communities. Cnidarians are among the most primitive eumetazoa and lack discrete organs, having evolved only to the tissue level of development. Further, they exhibit radial symmetry, a simple nerve network, and a thin body wall that surrounds a sac-like digestive cavity. The medusa (free-swimming) and the polyp (sedentary) are the two basic body designs of cnidarians. Finally, cnidarians are unique in containing within their tentacles stinging cells called nematocysts, which are used to immobilize prey (Barnes, 1980; Shick, 1991).

This dissertation research has focused on the sea anemone as opposed to coral to take advantage of several beneficial aspects of that organism. The sea anemone contains a greater tissue mass per number of individuals, so fewer sea anemones are required for research. Unlike coral, removal of several sea anemones will not damage or stress the whole colony. In addition, it is easier to maintain stress-free and healthy sea anemones in the laboratory. Finally, in U.S. waters, there are more federal restrictions on the collection of coral as compared to those for the sea anemone. Finally, due to the biological similarities of these organisms, results found in the sea anemone should be relatively similar to those in coral. For instance, both groups typically contain an algal or diatom symbiont beneficial to the anthozoan host; further, both hosts tend to expel the symbionts (i.e. bleaching) upon prolonged stress. Bleaching of coral reef communities is currently a chronic problem, which results in the death of corals.

Loss of coral reefs due to overproduction of mucus or to bleaching is devastating to these ecosystems. Some of the causative factors might be better addressed by understanding how these anthozoans deal with stress resulting from environmental pollutants. Thus, there is a need to examine biochemical mechanisms such as the cytochrome P450 dependent-MFO system. Some anthozoans reproduce clonally, so studies on those species could eliminate a major experimental variable by allowing a researcher to expose groups of genetically identical animals to various pollutants. However, before such studies can proceed, it is necessary to first identify the presence of the MFO system and characterize it in these organisms.
Chapter 2: Spectral Analysis and Catalytic Activities of the Mixed-function Oxidase System in Several Species of Sea Anemones

INTRODUCTION

The presence of a functional cytochrome P450-dependent mixed function oxidase (MFO) system has been clearly demonstrated in several marine invertebrate phyla (e.g., Livingstone, 1991); however, its presence has been far less rigorously documented in cnidarians (Gassman & Kennedy, 1992; Heffernan et al., 1996; Khan et al., 1972b; Winston et al., 1998). In fact, most early cnidarian studies reported an absence of cytochrome P450 monooxygenase activity (Lee, 1975; 1981; Payne, 1977). For instance, Lee (1975) did not detect in vivo metabolism of naphthalene, benzo[a]pyrene (B[a]P), or 3-methylcholanthrene (3MC) in either the jellyfish (genus unknown) or in the ctenophore, Pleurobrachia pileus. In the sea anemone, Metridium sp., Payne (1977) reported an absence of in vitro metabolism of B[a]P (fluorometric assay), and Lee (1981) found no in vivo metabolism of unidentified polyaromatic hydrocarbons (PAH). More recently, Firman (1995) failed to detect the presence of cytochrome P450 in the reef-building coral, Montastraea faveolata, by either the carbon monoxide (CO)-binding spectra or by metabolism of P450 substrates (i.e., chlordane [in vivo] and ethoxyresorufin [in vitro]).

However, even though molluscs are now accepted as containing cytochrome P450 (e.g., Livingstone, 1991), early molluscan studies also failed to identify an MFO system (Lee et al., 1972; Payne, 1977; Vandermeuleum & Penrose, 1978). A similar reversal has occurred for cnidarians, with several recent studies indicating the presence of a functional cytochrome P450-dependent MFO system. For instance, the freshwater hydrozoan, Hydra littoralis, can catalyze in vivo epoxidation of aldrin, presumably by MFO activity (Khan et al., 1972b). Also, in anthozoa, in vitro metabolism of B[a]P was detected in the sea anemone, Bunodosoma

**The spectral, reductase, and EROD analysis sections have been reprinted with permission from Comparative Biochemistry and Physiology Part C 121 (1998) 371-383. Copyright 1998 by Elsevier Science Inc. This publication does not include the aldrin epoxidation study.**
cavernata (Winston et al., 1998), and in the scleractinian coral, Favia fragum (Gassman & Kennedy, 1992). Based on the CO-liganded, sodium dithionite (DTN)-reduced microsomes, F. fragum, contained a cytochrome P-450 content between 60 and 350 pmol per mg microsomal protein (Gassman & Kennedy, 1992—P450 units in their figures are correct, but are misprinted in their text [N. Gassman, personal communication]). Finally, although western blot analyses suggested that several isoforms of P450 exist in the sea anemone (Heffernan et al., 1996), the evidence for the presence of a functional cytochrome P450 system in cnidarians was still equivocal. Thus, the purpose of this study was to further investigate the presence of P450 in the sea anemone with respect to three features: (1) spectral characteristics of the CO-liganded, DTN-reduced microsomes; (2) presence of flavin-containing reductases (i.e., additional MFO components) in microsomes of several species of sea anemone; and (3) ability of these sea anemone microsomes to metabolize two classical P450 reactions, ethoxyresorufin O-dealkylation (EROD) and aldrin epoxidation.

The specific metabolic reactions were chosen because several vertebrate studies had shown that ethoxyresorufin and aldrin are metabolized through the P450-dependent MFO system (Burke et al., 1985; 1994; Wolff et al., 1979; 1980) and both EROD and aldrin epoxidation activity has been demonstrated in other marine invertebrates (e.g., Livingstone 1991; Khan et al., 1972a; 1972b; Porte & Escartin, 1998). Further, both assays can detect low activity levels, particularly with regard to the epoxidation of aldrin to dieldrin. Ethoxyresorufin is metabolized to resorufin through an O-dealkylation reaction (Burke et al., 1985; 1994), and the production of resorufin can be monitored by a fluorescent assay. While EROD activity was not detected in the coral, Favia fragum (Firman, 1995), it has been observed in molluscs, crustaceans, and annelids (references in Table 2-4). Aldrin, a chlorinated cyclodiene insecticide, is converted to dieldrin through an epoxidation reaction (Wolff et al., 1980) and the production of dieldrin can be measured through gas chromatography using a Ni$^{49}$ electron capture detector (ECD). In vivo
aldrin epoxidation has been detected in the freshwater cnidarian, *Hydra littoralis* (Khan et al., 1972b), and NADPH-dependent aldrin epoxidation activity has been observed in the microsomal fraction of several other invertebrates (Burns, 1976; Carlson et al., 1974; Khan et al., 1972a, 1972b; Kreiger et al., 1979; Nelson et al., 1976). Although dieldrin has been shown to be further metabolized in the rat at a very slow rate (Wolff et al., 1979), dieldrin was the only metabolite detected in the earthworm, *Lumbricus terrestris* L. (Nelson et al., 1976).

**METHODS**

**Animals**

*Bunodosoma cavernata* were collected from the Gulf of Mexico at Fourchon, Louisiana. *B. cavernata* is sometimes confused with the morphologically-similar species, *Bunodactis texensis*, but the blue stripe and reddish coloration (as opposed to gray streaks) on the tentacles of the organisms collected for this study is consistent with *B. cavernata* (Fotheringham & Brunenmeister, 1975). *Anthopleura elegantissima* and *A. xanthogrammica* were obtained from Pacific Bio-Marine Laboratories and North Coast Invertebrate Collectors in California. All sea anemones were maintained in a recirculating system with Instant Ocean™ sea water. *A. elegantissima* and *A. xanthogrammica* were kept at 34% salinity and 12 °C, while *B. cavernata* were kept at 25% salinity and 23 °C.

**Microsomal Preparation**

Each sea anemone microsomal preparation consisted of approximately 50 animals for the smaller *A. elegantissima*, 30 animals for *B. cavernata*, and only 1 – 2 animals for the larger *A. xanthogrammica*. The tentacles were discarded, and diced sections of the columnar region of the sea anemone were immediately submerged in homogenization buffer. Microsomes were prepared by homogenizing the tissue in four volumes homogenization buffer with a hand-cranked plastic meat grinder, a Tekmar Tissumizer™ (3 – 4 passes), and then a Potter-Elvehjem tissue homogenizer (3 – 4 passes). The hand-cranked meat grinder was not used to prepare the *A.
*elegantissima* microsomes. The homogenization buffer was 100 mM potassium phosphate pH 7.6, containing 125 mM sucrose, 1 µg/mL aprotinin, 1 mM ethylene diaminetetraacetic acid (EDTA), 1 µg/mL leupeptin, 1 µg/mL pepstatin, 0.1 mM ortho-phenanthroline, 1 mM para-methylsulfonylfluoride (PMSF), 1 mg/mL soybean trypsin inhibitor, and 1 mM dithiothreitol (DTT). Homogenates were serially centrifuged for 20 min at 8,500 x g and 15 min at 14,000 x g. The resulting pellets were discarded. The 14,000 x g supernatant was then centrifuged for 1 hour at 105,000 x g to pellet the microsomal fraction. The microsomal pellet was washed once in homogenization buffer and centrifuged again at 105,000 x g for 1 hour. Microsomes were resuspended and stored at -80 °C in 100 mM potassium phosphate pH 7.6, containing 250 mM sucrose at a protein concentration of approximately 10 mg/mL. Protein concentrations were determined by the 96-well microplate reader fluorescamine assay as described by Lorenzen and Kennedy (1993), with two exceptions: the fluorescamine was prepared in HPLC-grade dioxane; and, the buffer used for the assay was 250 mM sodium phosphate, pH 8.5.

**Spectral Properties**

A Perkin Elmer lambda 5 dual beam spectrophotometer was used to examine the spectra of the sea anemone microsomal cytochrome P450 in terms of both the carbon monoxide (CO)-difference spectrum of sodium dithionite (DTN)-reduced samples and the DTN-difference spectrum of CO-liganded samples (Omura & Sato, 1964a). These spectra can be performed either by (1) adding the DTN and background-correcting prior to the addition of the CO, or (2) adding the CO and background-correcting prior to the addition of the DTN. The CO-difference and DTN-difference spectra were examined both ways. Prior to the addition of either DTN or CO, microsomal samples (200 µL) were solubilized with 100 µL of 10 mM potassium phosphate, pH 7.4 (containing 1 mM EDTA, 1 mM DTT, 150 mM NaCl, and 1.5% Triton N-101) and diluted immediately with 900 µL of 100 mM potassium phosphate (pH 7.4) to a final protein concentration of approximately 2 mg/mL. This sample was split between the reference
and test cuvettes for analysis. After the addition of the CO and DTN, the solubilized microsomes were scanned immediately and then at 5 min intervals while maintaining the sample at room temperature. The extinction coefficient used to determine the cytochrome P450 content (450-490 nm) was 91 cm⁻¹mM⁻¹. This value was determined from a mammal and is typically used to quantitate P450 content; however, the actual extinction coefficient for the sea anemone may be different.

**Reductase Activities**

The NAD(P)H cytochrome c (P450) reductase activities were measured as an increase in cytochrome c absorbance over time at 550 nm (Lake, 1987). The NAD(P)H-cytochrome c reductase reactions contained a final concentration of 0.048 mM cytochrome c, 1 mM potassium cyanide, approximately 200 µg microsomal protein, and either 0.1 mM NADH or 1 mM NADPH in 100 mM potassium phosphate, pH 7.7. The NADPH-dependent reactions were performed in tandem cuvettes to correct for the rate of non-enzymatic activity and noise contribution from the microsomes. The NADH-dependent reactions had a non-enzymatic component. Thus, NADH reactions were performed in standard cuvettes with a correction for the background noise reading of the microsomes. Activities were calculated based on an extinction coefficient of reduced cytochrome c, 21.0 cm⁻¹mM⁻¹.

The activity of the NADH potassium ferricyanide (b₃) reductase was measured as a decrease in ferricyanide absorbance over time at 420 nm (Ichikawa et al., 1969). The NADH-ferricyanide activity reactions contained a final concentration of 0.97 mM potassium ferricyanide, 1 mM potassium cyanide, approximately 200 µg microsomal protein, and 0.4 mM NADH in 1 mL of 100 mM potassium phosphate, pH 7.7. The NADH-dependent reactions were performed in standard cuvettes with a correction for non-enzymatic activity. Any noise contribution from the microsomes was negligible; thus, the microsomal contribution was not
corrected. Activities were calculated based on an extinction coefficient of reduced ferricyanide, 1.02 cm$^{-1}$mM$^{-1}$. All reductase activities were initiated by the addition of the cofactor.

**Ethoxyresorufin O-Dealkylation (EROD) Analysis**

Ethoxyresorufin O-dealkylation activity was measured as a continuous fluorimetric assay performed with a 96-well microplate reader (Eggens & Galgani, 1992). The optimized reaction and standards contained 50 mM Tris-HCl pH 7.2, 4 mM NAD(P)H, and 4.5 μM ethoxyresorufin in a final volume of 110 μL. The microsomal protein, 150 to 300 μg per well, was included in the reactions, but not in the standards. Enzymatic reactions were initiated by the addition of the ethoxyresorufin, and plates were incubated at 37 °C in an orbital shaker set at 220 rpm. As discussed below in more detail, EROD activities were not always linear over the entire incubation time. Therefore, plates were read every 15 min between 0 to 90 min with the Cytofluor 2300 system at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The ethoxyresorufin and resorufin were freshly diluted in 50 mM Tris-HCl, pH 7.2 from stocks (w/v DMSO; each reaction contained less than 2% DMSO), based on an extinction coefficient for ethoxyresorufin of 22.83 cm$^{-1}$mM$^{-1}$ at 464.0 nm and for resorufin of 40.0 cm$^{-1}$mM$^{-1}$ at 571.3 nm. When microsomes were incubated with ethoxyresorufin in the absence of cofactor, there was a linear loss in fluorescence over time. Therefore, to calculate the EROD activity, the fluorescence from microsomes incubated without cofactor was subtracted from the fluorescence produced when microsomes were incubated with cofactor. EROD activity was quantified based on the resorufin extinction coefficient mentioned above. The conditions of the EROD assay were optimized with *A. xanthogrammica* microsomes.

**Aldrin Epoxidation Analysis**

Aldrin epoxidation activity was determined based on the production of dieldrin, which was measured by gas chromatography (Khan & Terriere, 1968). In a final volume of 2 mL, the optimized reaction contained 65 mM potassium phosphate pH 7.5, 50 μM aldrin (w/v hexane;
final concentration hexane per reaction was 5%), 4 – 9 mg microsomal protein (300 μL/mL reaction), and an NAD(P)H-regenerating system (final concentrations in reaction: 3 mM NAD(P)⁺, 15 mM glucose-6-phosphate, 1.25 units/mL glucose-6-phosphate dehydrogenase).

The glucose-6-phosphate dehydrogenase in the NADPH-regenerating system was prepared from torula yeast (Sigma G-8878), while the dehydrogenase in the NADH-regenerating system was prepared from *Leuconostoc mesenteroides* (Sigma G-5760). The NAD(P)H-regenerating systems were prepared approximately 15 min prior to its addition to the reaction. The reaction was initiated by the addition of the cofactor, and incubated for 60 – 70 min at 30 °C in an incubator shaking at 100 rpm. The reaction was stopped by the addition of 4 mL pesticide-grade hexane (vortexed 45 seconds). The hexane layer was filtered through sodium sulfate in a small glass funnel plugged with glass wool. Samples were extracted two more times with 4 mL hexane. Due to the low dieldrin levels produced, any variations in extraction conditions (e.g., vortexing time or length of time in hexane) resulted in higher variation among replicates; thus, all samples were extracted at the same time, vortexed 45 seconds for each of the three extractions, and extractions were completed within an hour after incubation. Samples were dried under nitrogen and redissolved in 1 mL with pesticide grade hexane. Sodium sulfate, glass wool, and glassware for reactions and extractions were rinsed well with either hexane or petroleum ether.

The dieldrin was quantitated on a Hewlett Packard 6890 gas chromatograph equipped with an HP-608 special analysis column (30 m x 0.53 mm x 0.50 μm) and an HP-50 50% phenyl methyl siloxane column (30 m x 0.53 mm x 1.0 μm). Each column was coupled to its own Ni⁶⁵ electron capture detector (ECD). There was a single inlet port with a temperature of 270°C. For each sample, the oven temperature of the column was held at 80°C for 1 min, ramped 30 °C min⁻¹ to 190 °C, then ramped 3.6 °C min⁻¹ to 260 °C, and finally held at 260 °C for 15 min. Both ECD detectors were set at 350 °C. The carrier gas, helium, had a total flow rate of 11.0 mL per min, which was split between the two columns.
The identity of the dieldrin peak was verified and quantitated on both columns based on a known dieldrin standard (Fig 2-1). Both columns demonstrated an increase in NAD(P)H-dependent activity with respect to an increase in protein concentration per reaction; however, for *B. cavernata*, the increase was linear over 0 – 10 mg protein for the HP-50+ column versus 0 – 4 mg protein for the HP-608 column (see results). On the HP-608 column, aldrin epoxidation gradually decreased at protein concentrations exceeding 4 mg per reaction and significantly decreased in the presence of 10 mg protein per reaction. The lack of a linear increase in activity at these higher protein concentrations appeared to be an artifact of the *B. cavernata* microsomes, for which an additional compound eluted from the HP-608 column as a shoulder off the dieldrin peak (Fig. 2-2). The size of the shoulder increased linearly as the microsomal protein concentration was increased from 0 to 10 mg, but it was unaltered over the incubation time; thus, this unknown compound was already present in *B. cavernata* microsomes (i.e., it was not a metabolite of the reaction). The shoulder was not observed in *A. elegantissima* and *A. xanthogrammica*; however, the assay was optimized using *B. cavernata* due to limited quantities of *A. elegantissima* and to the particularly low activity in *A. xanthogrammica*.

The amount of dieldrin detected on the HP-608 and HP-50+ columns was similar as long as the protein concentration was not above 7 mg protein per reaction. Above this value, it was difficult to identify the appropriate integration parameters between the dieldrin peak and the shoulder on the HP-608 column. The HP-50+ column did not contain a shoulder off the dieldrin peak; however, it was a less sensitive column. The lower sensitivity raised the detection threshold of the assay and hence reduced the ability to detect the low rates of activity in some of the *A. xanthogrammica* microsomal preparations. To eliminate any interference observed at higher protein concentrations and yet ensure enough enzyme was added to remain above the detection limit of the assay, all samples were analyzed with 4 – 6 mg microsomal protein per reaction. Further, all samples within a run were analyzed with the same integration parameters.
Fig. 2-1. Identification of dieldrin peak in NADPH-aldrin epoxidation reaction (with B. cavernata) based on retention time of a known dieldrin standard as observed by the ECD detector of both columns, HP-608 and HP-50+. A similar chromatograph was observed for the NADH-reaction. Details of column run are given in the Methods.
Fig. 2-2. Comparison of dieldrin peak of NADPH aldrin epoxidation reaction (with 0.8 mL *B. cavernata* microsomes) as observed by the ECD detector of both columns. A similar peak was observed on each column with the NADH-dependent reaction.
The aldrin substrate contained low amounts of contaminating dieldrin and the microsomal protein concentration altered the extraction efficiency of the assay. Thus, all calculations for the rate of activity were determined as the difference between the NAD(P)H-dependent reaction and the reaction without any cofactor (after incubating for the same length of time and with the same microsomal protein concentration). These features were particularly important to consider in *A. xanthogrammica* because the activity was close to the detection limit of the assay. In fact, some of the *A. xanthogrammica* replicates had artifactualy negative values. Replicates with negative values were not considered in the calculations of the activity; the final activities without these data points were reproducible between different batches of microsomal preparations.

**RESULTS**

**Spectral Properties**

The spectral properties of the CO-liganded, DTN-reduced microsomes indicated the presence of cytochrome P450 in the sea anemone (Fig. 2-3; Table 2-1). A small 450 nm peak was consistently observed in the CO-difference spectra in three species of sea anemones: *A. elegantissima*, *A. xanthogrammica*, and, *B. cavernata*. Quantification of P450 requires that the spectrum be free of interference at 490 nm, the isosbestic wavelength between CO-liganded and unliganded reduced microsomes. A positive absorbance around 490 nm was often observed in the sea anemone microsomes; thus, the cytochrome P450 content in the majority of the microsomal preparations could not be accurately quantified by this method. Based on the microsomal preparations that did not contain a positive absorbance at 490 nm, the columnar region of the sea anemone, *B. cavernata*, had a microsomal cytochrome P450 content of about 52 pmol per mg protein.

In addition to the 450 nm peak, a predominant peak with a wavelength maximum between 417 to 420 nm was consistently observed in the sea anemone spectra in the
Fig. 2-3. CO-difference spectra of DTN-reduced *A. xanthogrammica* columnar solubilized microsomes. Solubilized microsomes [100 mM potassium phosphate, 0.15% Triton N101, 15 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol] were DTN-reduced and background corrected prior to addition of CO. Spectra were recorded at time intervals over a 40 minute period. (A) Spectrum recorded immediately after addition of CO. (B) The spectrum was recorded at 0, 5, 10, 20, and 40 minutes after addition of CO (418' amplitude increased with time).
Table 2-1. Microsomal mixed-function oxidase components of sea anemone columnar tissue.

<table>
<thead>
<tr>
<th>Species</th>
<th>NADPH(^b) cyto c R</th>
<th>NADH(^b) cyto c R</th>
<th>NADH(^b) ferri R</th>
<th>418(^c)</th>
<th>P450(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. elegantissima</td>
<td>3.4 ± 0.5</td>
<td>12 - 17</td>
<td>175 - 240</td>
<td>45.1</td>
<td>---</td>
</tr>
<tr>
<td>A. xanthogrammica</td>
<td>2.0 ± 0.2</td>
<td>15 - 22</td>
<td>73 - 232</td>
<td>30.9</td>
<td>---</td>
</tr>
<tr>
<td>B. cavernata</td>
<td>2.1 ± 0.3</td>
<td>9 - 25</td>
<td>114 - 165</td>
<td>114.0</td>
<td>52</td>
</tr>
</tbody>
</table>

Mean ± standard deviation.

\(^a\) nmol/min/mg protein; cyto c R = cytochrome c reductase; ferri R = ferricyanide reductase; KCN was included in each reductase assay.

\(^b\) NADPH cytochrome c reductase assays were performed in tandem cuvettes to eliminate non-enzymatic activity.

\(^c\) (490-418 nm) x 1000 x mg\(^{-1}\).

\(^d\) pmol/mg microsomal protein.

DTN-reduced CO-liganded microsomes. In contrast to the expectations for a denatured P450 (i.e., P420), the presence of the 418 nm chromophore was not altered by the inclusion of antioxidants (DTT), chelators (EDTA, ortho-phenanthroline), and proteolytic inhibitors (aprotinin, leupeptin, pepstatin, PMSF, soybean trypsin inhibitor) in the sucrose (or glycerol) homogenization medium (data not shown). A large 418 nm chromophore has been observed in many aquatic invertebrates; however, currently its identity is unknown.

The sea anemone microsomal cytochrome P450 spectra were examined by both CO-difference and DTN-difference spectra (Fig. 2-4). The large 418 nm chromophore was detected by both types of spectra regardless of the order of addition of the DTN and CO; its amplitude was not altered by the method. In contrast to the 418 nm chromophore, the detection of the 450 nm peak was altered by the method. In the DTN-difference spectra, the 450 nm chromophore was less consistently detected and when it was detected, it was not well resolved from the 418 nm peak. The ability to detect the 450 nm peak was particularly difficult in the DTN-difference spectra when CO was added prior to DTN.
Fig. 2-4. (A) CO-difference and DTN-difference spectra of *A. xanthogrammica* columnar solubilized microsomes: (1) CO-difference spectra (CO added prior to DTN); (2) CO-difference spectra (DTN added prior to CO); (3) DTN-difference (DTN added prior to CO); and, (4) DTN-difference spectra (CO added prior to DTN). (B) Experimental protocols for determination of cytochrome P450-binding spectra. Background was corrected immediately after the first addition of either DTN or CO; equal quantities of solubilized microsomes were added to test and reference cuvettes. Samples were read immediately after the last addition of DTN or CO.
In the CO-difference spectra, the 418 and 450 nm peaks were well resolved regardless of the order of addition of DTN and CO. However, a larger 450 nm peak was more consistently observed when DTN was added prior to CO. Regardless of the CO-difference method used, the maximum 450 nm peak for the sea anemone was attained within 5 min and it typically was stable for the entire 90 min assay (Fig. 2-3B). Further, the 418 nm peak slowly increased over 10 min (CO added prior to DTN) or 20 min (DTN added prior to CO), and then began to decrease about 40 – 60 min later. There was no corresponding increase in the 450 nm peak as the 418 nm peak decreased.

**Reductase Activities**

The sea anemones, *A. elegantissima*, *A. xanthogrammica*, and *B. cavernata*, each contained NAD(P)H-dependent cytochrome c reductase activity and NADH-dependent ferricyanide (b₅) reductase activity (Table 2-1). Each of these reactions was linear with respect to protein concentration and time. At 1 mM NADPH, the NADPH-dependent cytochrome c (P450) reductase activity was 1.8 – 3.9 nmol/min/mg protein for each species of sea anemone. In *B. cavernata*, the activity increased significantly as the concentration of NADPH was increased. The reaction initially plateaued between 10 – 15 mM NADPH, and then continued to increase (maximum concentration examined was 25 mM NADPH). Thus, the reductase activity reported here is not the maximum level. Further, these data indicated the presence of both a low and high Kᵣ NADPH-dependent cytochrome c reductase activity. In contrast, the NADH-dependent reductase reactions contained excess cofactor. For each species of sea anemone, the NADH-ferricyanide reductase activity ranged from 73 to 232 nmol/min/mg protein, and the NADH-cytochrome c reductase activity was 9 to 25 nmol/min/mg protein.

**Ethoxyresorufin O-Deethylation**

Both *A. elegantissima* and *A. xanthogrammica* contained a low, but reproducible level of microsomal NAD(P)H-dependent EROD activity (Table 2-2). The microsomal EROD activity
Table 2-2. Microsomal NAD(P)H-dependent EROD activities in the sea anemone columnar tissue.

<table>
<thead>
<tr>
<th>Species</th>
<th>NADPH ( ^b )</th>
<th>NADH ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. elegantissima</em></td>
<td>2.28 ± 0.23 (4)</td>
<td>1.24 ± 0.30 (3)</td>
</tr>
<tr>
<td><em>A. xanthogrammica</em></td>
<td>0.80 ± 0.17 (7)</td>
<td>0.37 ± 0.04 (7)</td>
</tr>
<tr>
<td><em>B. cavernata</em></td>
<td>ND (6)</td>
<td>ND (6)</td>
</tr>
</tbody>
</table>

Mean ± std. dev. (number of microsomal preparations examined in triplicate).  
* pmol/min/mg protein.  
ND = not detected.

required the presence of either NADH or NADPH as a cofactor; thus, there was no cofactor-independent activity similar to that observed in the mussel (e.g., Livingstone et al., 1989) (Fig. 2-5). Despite the higher NADH-dependent cytochrome c and ferricyanide reductase activities observed in the sea anemone, the EROD activities were consistently higher with NADPH in each species. In both *A. elegantissima* and *A. xanthogrammica* microsomal reactions, the NAD(P)H-EROD activity was linear from approximately 30 to 90 min and often decreased between 90 to 120 min (Fig. 2-5). Curiously, during the initial 15 or 30 min, a decrease in fluorescence was often observed. This "lag period" was primarily observed in reactions with lower EROD activities, while more active samples tended to be linear over the entire reaction time. Consistent with this finding, the length of the "lag period" decreased as the protein concentration in the reaction increased.

All calculations for the rate of activity were determined from only the linear portion of the time course. If measured over the entire assay (versus only the linear region), NAD(P)H-EROD activity was often not detectable in the sea anemone. This feature could explain why Firman (1995) reported the absence of EROD activity in the reef-building coral, *M. faveolata*. Finally, the NAD(P)H-EROD activity increased linearly with respect to protein concentration (Fig. 2-5; NADPH, 150 to 300 µg protein per well; NADH, 150 to 400 µg protein per well). Below the
Fig. 2-5. Effect of cofactor concentration, protein concentration, and time on NADH- (---) and NADPH-dependent (—) ethoxyresorufin O-dealkylase activity in A. xanthogrammica columnar microsomes. Bars represent the mean ± 1 standard deviation of triplicate determinations with one representative microsomal preparation. The same trends were observed in several other microsomal preparations.
minimum protein ranges, EROD activity could be detected; however, the values were very close to the detection limit of the assay.

In both *A. elegantissima* (~50 animals/sample) and *A. xanthogrammica* (1 – 2 animals/sample), NAD(P)H-dependent EROD activity was detected in most of the microsomal preparations (Table 2-2). In *A. elegantissima*, the NAD(P)H-dependent activities were about three times higher than the typical values for *A. xanthogrammica*. In *A. xanthogrammica*, the typical EROD activity was 0.80 ± 0.17 pmol/min/mg protein with NADPH and 0.37 ± 0.04 pmol/min/mg protein with NADH; however, there was also one microsomal batch that did not contain EROD activity and one preparation that contained activity comparable to that observed in *A. elegantissima*. Compared to NADPH, the NADH-dependent EROD activity levels were about two times lower for both species. For *B. cavernata*, any increase in fluorescence observed in the presence of NADPH was so gradual that it could not be discerned as activity. Microsomes were examined between 0 – 450 µg protein per well over 0 – 120 min, and regardless of the reaction conditions, the EROD values were close to the detection limit of the assay. In the presence of NADH, there was definitely no increase in fluorescence.

**Aldrin Epoxidation**

Microsomal preparations from both *A. elegantissima* and *B. cavernata* were consistently capable of metabolizing aldrin to dieldrin through an epoxidation reaction at 30 °C; aldrin epoxidation was also observed in most *A. xanthogrammica* microsomal preparations (Table 2-3). While similar activities were detected on both columns, the values reported in Table 2-3 were obtained from the HP-608 column. The production of dieldrin was relatively linear with respect to time between 0 – 75 min and protein concentration (see methods; Figs. 2-6 and 2-7); dieldrin was the only metabolite detected for this reaction. In each species, epoxidation of aldrin required the presence of either NADH or NADPH (Figs. 2-6 and 2-7). In most of the microsomal preparations examined (~ 65%), the NADPH-dependent activity tended to be slightly higher than
Table 2-3. Microsomal NAD(P)H-dependent aldrin epoxidation activities in the sea anemone columnar tissue. Values are given as the range of activity detected between different microsomal preparations (Mean activity). *a*

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>pmol/hr/mg protein</th>
<th>pmol/hr/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NADH</td>
<td>NADPH</td>
</tr>
<tr>
<td>A. elegantissima</td>
<td>4</td>
<td>0.5 - 2.7 (1.4)</td>
<td>0.7 - 4.2 (2.0)</td>
</tr>
<tr>
<td>B. cavernata</td>
<td>5</td>
<td>1.1 - 2.3 (1.6)</td>
<td>0.4 - 2.7 (1.5)</td>
</tr>
<tr>
<td>A. xanthogrammica d</td>
<td>8</td>
<td>0.2 - 1.3 (0.5)</td>
<td>0.5 - 1.5 (0.9)</td>
</tr>
</tbody>
</table>

*a* Aldrin activity (mean ± 1 standard error) for each microsomal preparation are given in Appendix A (Table A-1).

n = number of microsomal preparations examined in triplicate for activity expressed per mg protein.

n = number of microsomal preparations examined in triplicate for activity expressed per g tissue.

Due to *A. xanthogrammica* being at the detection limit of the assay, any variation in extraction efficiency strongly affected the amount of dieldrin detected in that sample. Thus, samples that contained negative values or were much different than others after subtracting the control (reaction without cofactor) were removed from calculation of mean. Table A-1 contains the exact number of values removed to calculate the *A. xanthogrammica* activity. No values were removed determination of the epoxidation activity in either *A. elegantissima* or *B. cavernata* microsomes.

8 of 24 values were not used to calculate the mean; 1 microsomal preparation had no NADH-dependent activity.

5 of 24 values were not used to calculate the mean.

6 of 18 values were not used to calculate the mean.

4 of 18 values were not used to calculate the mean.
Fig. 2-6. HP-608 column: Effect of cofactor concentration, protein concentration, and time on NADH- (-----) and NADPH-dependent (—) aldrin epoxidation activity in *B. cavernata* columnar microsomes. Error bars represent the mean ± 1 standard error of triplicate determinations with one representative microsomal preparation. The same trends were observed in several other microsomal preparations.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Fig. 2-7. HP-50\textsuperscript{+} column: Effect of cofactor concentration, protein concentration, and time on NADH- (-----) and NADPH-dependent (\textarrow) aldrin epoxidation activity in B.\textit{cavernata} columnar microsomes. Error bars represent the mean ± 1 standard error of triplicate determinations with one representative microsomal preparation. The same trends were observed in several other microsomal preparations.
the NADH-dependent activity. In contrast, there were a few microsomal preparations from *A. elegantissima* and *A. xanthogrammica* that strongly preferred NADPH as an electron donor over NADH (~30%) and only one preparation from *B. cavernata* that significantly preferred NADH over NADPH (~5%).

*A. elegantissima* and *B. cavernata* both contained a similar rate of NAD(P)H-dependent aldrin epoxidation activity (Table 2-3). While the *A. xanthogrammica* activity was not significantly lower than either *A. elegantissima* or *B. cavernata*, many of the microsomal preparations had low enough activity that any alteration in extraction efficiency of a particular sample would result in a replicate that appeared to be inactive (see methods). If these individual replicates were ignored, the remaining samples typically had similar activities even between replicates from different microsomal preparations. Thus, when calculating the mean activities for *A. xanthogrammica* in Table 2-3, any value that had no activity or had either extremely high or low activity as compared to the other two replicates was removed (as noted in the tables) from the calculations for mean activity. This procedure was only done for *A. xanthogrammica* because the activities for *A. elegantissima* and *B. cavernata* were substantially greater than the detection limit of the assay.

In *B. cavernata* and *A. xanthogrammica*, most of the microsomal preparations had activities similar to the mean for that particular species (Table 2-3). The few samples that deviated from the mean accounted for the wide range of activity observed. Variation in activity between microsomal preparations was higher in *A. elegantissima* than either *A. xanthogrammica* or *B. cavernata*. Thus, the differences among species in variability are probably not the result of variation between individual specimens as *A. elegantissima* had the greatest number of individuals per microsomal preparation (~50 individuals).
DISCUSSION

These studies demonstrate that the sea anemone columnar microsomes contain the major components of a cytochrome P450-dependent MFO system. These properties include the presence of a characteristic CO-binding spectra similar to that observed in other marine invertebrates as well as reductase activities associated with NAD(P)H-dependent cytochrome c and NADH-dependent ferricyanide. Sea anemone microsomes also exhibited NAD(P)H-dependent metabolism of classical P450-substrates ethoxyresorufin O-dealkylation (EROD) and aldrin epoxidation, although the three species differed in their ability to metabolize these substrates. With regard to other aquatic invertebrates, the EROD activities (i.e., for A. elegantissima and B. cavernata) and P450 contents were comparable; however, the aldrin epoxidation activities were significantly lower than expected.

Spectral Properties

According to the CO-difference spectra of DTN-reduced microsomes, each species of sea anemone contained a 450 nm peak, characteristic of cytochrome P450. In addition to the 450 nm peak, there was a predominant 418 nm chromophore. A chromophore near 418 nm has also been observed in four other groups of marine invertebrates: mollusc (Cheah et al., 1995; Kirchin et al., 1992; Koivusaari et al., 1980; Livingstone & Farrar, 1984; Livingstone et al. 1985; Livingstone et al., 1989; Sole et al., 1994; 1995; Stegeman, 1985; Weinstein, 1995), echinoderm (den Besten et al., 1990), crustacean (James, 1990; Koivusaari et al., 1980; Lindstrom-Seppa et al., 1983; Quattrochi & Lee, 1984a; 1984b; Singer et al., 1980), and annelid (Achazi et al., 1998; Berghout et al., 1991; Nelson et al., 1976). The presence of the 418 nm chromophore is also consistent with results seen in the coral, F. fragum (Gassman & Kennedy, 1992); however, the amplitude of the 418 nm peak in the coral appears to be smaller than that typically seen in the sea anemone.
The presence of a large 418 nm peak has been shown to affect the ability to detect the P450 peak. In several invertebrates, the amount of interference by this 418 nm peak can be altered by the use of DTN-difference spectra as compared to CO-difference spectra as well as by the order of addition of the CO versus DTN. For instance, the order of addition of the DTN and CO was important in the mussel (*Mytilus edulis*) digestive gland microsomes with a large 418 nm peak. In the CO-difference spectra (DTN was added prior to CO), particularly high 418 nm peak concentrations interfered with the development of the 450 nm peak over time and resulted in more inconsistent estimates of P450 concentrations. In contrast, when the mussel 418 nm peak was small, the maximum size for the P450 peak was independent of the order in which the CO and DTN were added to the CO-difference spectra (Livingstone & Farrar, 1984; Livingstone et al., 1989).

Despite the presence of a large 418 nm peak in the sea anemone microsomes, the above issues were not relevant because the 418 and 450 nm peaks were well resolved in the CO-difference spectra, regardless of the order of addition of DTN and CO (Fig. 2-4). In the sea anemone, a maximum 450 nm peak was obtained by a CO-difference spectra as compared to a DTN-difference spectra. The larger 450 nm peak was more consistently observed in the CO-difference spectra when DTN was added prior to CO. Similarly, in the digestive gland microsomes of the pond snail, *Lymnaea stagnalis*, the CO-difference spectrum resulted in a larger and better resolved 450 nm peak than did the DTN-difference spectrum (Wilbrink et al., 1991). In contrast, James and colleagues (1979) demonstrated that the DTN-difference spectra was necessary to obtain the maximum cytochrome P450 content in hepatopancreas microsomes of the spiny lobster, *Palinurus argus*.

According to the CO-difference spectra of DTN-reduced microsomes, the columnar region of the sea anemone, *B. cavernata*, had a cytochrome P450 content of about 52 pmol per mg protein (Fig. 2-3; Table 2-1). This is about a third of the P450 content typically observed in
the scleractinian coral, *F. fragum* (Gassman & Kennedy, 1992), although during the month (October) in which the species was tested the coral values fluctuated randomly between 60 and 350 pmol P450 per mg microsomal protein. The authors suggested that any pattern of cytochrome P450 content over the reproductive cycle (which coincides with the lunar cycle) was masked by the small sample size, high individual variation, or effects of pollutants. In contrast to *B. cavernata* and *F. fragum*, no cytochrome P450 was detected in the reef-building coral, *M. faveolata* (Firman, 1995).

The cytochrome P450 contents observed in the above representatives of anthozoans are consistent with results found in annelids, echinoderms, molluscs, and in some crustaceans (Achazi et al., 1998; Berghout et al., 1991; den Besten et al., 1990; Fries & Lee, 1984; James, 1989; Jewell & Winston, 1989; Livingstone, 1991; Livingstone et al., 1989). For instance, most aquatic invertebrates have cytochrome P450 levels between 20—140 pmol P450 per mg microsomal protein. However, the anthozoan levels were lower than those found in the hepatopancreas (310 pmol P450 per mg microsomal protein) of the freshwater crayfish, *Astacus astacus* L. (Lindstrom-Seppa et al., 1983). Further, microsomal P450 contents comparable to that of the rat liver were found in the hepatopancreas of the red swamp crayfish, *Procambarus clarkii* [577 pmol/mg protein (Escartin & Porte, 1996); 720 pmol/mg protein, (Jewell & Winston, 1989)].

The P450 content for the other two species of sea anemone (*A. elegantissima* and *A. xanthogrammica*) could not be determined by the standard method because of interference by the absorbance at 490 nm. The 490 nm wavelength is the isosbestic point between the CO-liganded, DTN-reduced form of cytochrome P450 and the unliganded, DTN-reduced form of cytochrome P450. At the isosbestic point, the two spectra have the same absorbance value. As a result, the background-corrected DTN-reduced spectra can be autozeroed on 490 nm and the addition of CO will not alter that zero absorbance value; hence, the addition of CO will not affect the
absorbance value and the difference between the 450 and 490 nm absorbance values will reflect the P450 content (450 minus 490 nm absorbance). Thus, anything that alters the absorbance at 490 nm will interfere with the ability to quantitate the P450 content.

Interference at 490 nm has also been observed in the octopus, Octopus pallidus (Cheah et al., 1995), and in the mussel, Anodonta cygnea (Koivusaari et al., 1980), although it was not reported for the CO-binding spectra of coral, F. fragum (Gassman & Kennedy, 1992). Interference at 490 nm could be the result of the order of addition of CO and DTN in the spectral analysis or of an additional compound in the microsomes that absorbs around 490 nm. In several echinoderms (i.e., sea star, sea cucumber, and sea urchin), the CO-difference spectra where CO was added prior to DTN resulted in better spectra because background correcting after the addition of CO (prior to DTN) eliminated any baseline drift (den Besten, 1998). Drift could cause the overall spectra to be tilted (i.e., slope); hence, artifactually increasing the absorbance at 490 nm. While baseline drift could account for the interference observed in several of the sea anemone spectra (particularly in A. elegantissima and A. xanthogrammica), the sea anemone 490 nm region often resembled a broad peak. The presence of a peak suggests that this 490 nm absorbance was the result a compound present in the microsomes, rather than simply baseline drift.

Some investigators have suggested the invertebrate 418 nm peak is denatured cytochrome P450 (e.g. Gilewicz et al., 1984; Stegeman, 1985; Wilbrink et al., 1991). In contrast, other investigators have suggested the 418 nm chromophore is another heme-centered protein that interferes with the detection of the 450 nm peak (Berghout et al., 1991; Livingstone et al., 1989; Nelson et al., 1976; Singer et al., 1980). Unless the invertebrate P420 has a different extinction coefficient than does the vertebrate P420 model (the chromophore classically ascribed to denatured P450 in various vertebrate studies), it is unlikely that the 418 nm peak is entirely denatured P450. If it were, the cytochromes P450 specific content in the sea anemone
would be higher than that of many marine invertebrates, given that the sea anemone has a particularly high 418 nm peak. Further, the sea anemone P450 content would be roughly equivalent to that of mammalian microsomes. It is more likely that the 418 nm chromophore is another heme protein [e.g., a cytochrome or a peroxidase (Applebly, 1969, Lindenmeyer & Smith, 1964)] that may interfere with the observation of the 450 nm peak. This hypothesis is further supported by the presence of the high 418 nm peak even when antioxidants, proteolytic inhibitors, and glycerol were included in the homogenization media (e.g., Koivusaari et al., 1980).

In the mammal, the conversion of P450 to denatured P450 can be observed as a coinciding decrease in the 450 nm peak and increase and in the 420 nm peak. Similar to the sea anemone, Livingstone and Farrar (1984) observed that the peaks obtained in the mussel microsomal CO-difference spectra (CO added before DTN) attained their maximum amplitude by 5–10 min after the addition of DTN (Fig. 2-3B). In contrast, the mussel CO-difference spectra (DTN added before CO) required more than 20 min to fully develop; during that time, the 418 nm peak slowly decreased as the 450 nm peak developed. This coinciding increase in the 450 nm peak and decrease in the 418 nm peak was also reported in the pond snail, L. stagnalis, and in additional studies in the mussel, M. edulis (Stegeman, 1985; Wilbrink et al., 1991), but reported as not present in the octopus, O. pallidus (Cheah et al., 1995), sea star, A. rubens and M. glacialis, or sea cucumber, H. forskali (den Besten, 1998). Stegeman (1985) suggested that the coinciding increase in the 450 nm peak and decrease in the 418 nm peak is indicative of a conversion process. Such a conversion process does not appear to occur in the sea anemone. In the earthworm, Lumbricus terrestris, Berghout et al. (1991) detected a significant increase in the 450 nm peak with time, especially in their semi-purified P450 fractions. However, these authors did not mention a coinciding change in the 418 nm peak even though it was a major chromophore in their crude microsomal preparations. They suggested that the increase in the 450 nm peak over time was a result of the invertebrate cytochromes P450 being reduced at a very slow rate.
Regardless of the identity of the 418 nm chromophore and the interference at 490 nm, each species of sea anemone contained a 450 nm peak indicative of cytochrome P450. However, the estimated P450 values may not reflect the true values because several studies have demonstrated that additional heme-centered proteins in the microsomes can interfere with the detection of the P450 content (e.g., Achazi et al., 1998; Ade et al., 1982; Berghout et al., 1991; Liimatainen & Hanninen, 1982; Nelson et al., 1976; Schenkman & Kupfer, 1982). Thus, it may be necessary to remove this compound(s) to accurately estimate the P450 content in these sea anemones. The presence of the 418 nm chromophore and the interference of the 490 nm absorbance may be the result of the same compound.

Reductase Activity

The presence of the microsomal MFO components in the sea anemones provide further evidence for the existence of cytochrome P450 in the cnidaria. *A. elegantissima*, *A. xanthogrammica*, and *B. cavernata* each contained reductase activity associated with NAD(P)H-dependent cytochrome c and NADH-dependent ferricyanide (b$_5$) in the microsomal fraction (Table 2-1). There are no other reports of these MFO components in cnidarians.

The NADPH-dependent cytochrome c (P450) reductase activity was 1.8 – 3.9 nmol/min/mg protein for each species of sea anemone at 1 mM NADPH. The range of NADPH-dependent cytochrome c reductase activity in most invertebrates is approximately 2 – 12 nmol/min/mg protein; thus, the reductase activity in the sea anemones is at the lower end of the range observed in most invertebrates studied (Escartin & Porte, 1996; Kirchin et al., 1992; Livingstone, 1991; Sole et al., 1994; Yawetz et al., 1992). Reductase activities much higher than this range have been reported in the earthworm (*L. terrestris*), the barnacle (*Balanus eburneus*), and several molluscs (Berghout et al., 1991; Michel et al., 1993; Sole et al., 1994; Stegeman & Kaplan, 1981; Vrolijk & Targett, 1992).
For each species of sea anemone, the NADH-ferricyanide reductase activity ranged from 73 to 232 nmol/min/mg protein, while the NADH-cytochrome c reductase activity was 9 to 25 nmol/min/mg protein. These sea anemone reductase activities were similar to activities observed in the sea urchin, *Echinus esculentus*, but significantly lower than those observed in other invertebrates (den Besten et al., 1990). The NADH-ferricyanide reductase activities in molluscs and other echinoderms were between 360 – 2300 nmol/min/mg protein, while the NADH-cytochrome c reductase activities in the molluscs, crustaceans, and other echinoderms were between 32 – 400 pmol/min/mg protein. Although most invertebrates fell in the lower end of these ranges, their activities were still significantly higher than the activity observed in the sea anemone (den Besten et al., 1990; Jewell & Winston, 1989; Kirchin et al., 1992; Lindstrom-Seppa et al., 1982; 1983; Livingstone, 1985; Livingstone & Farrar, 1984; Livingstone et al., 1985; Stegeman, 1985; Stegeman & Kaplan, 1981).

In vertebrates, the critical reductase in P450 activity is the NADPH-cytochrome P450 reductase (activity measured as NADPH-cytochrome c reductase), while the NADH-reductases are ancillary reductases (e.g., Lewis). In invertebrates, the MFO reductases have not been characterized; however, in contrast to vertebrates, invertebrates can often readily utilize either NADH or NADPH as an electron donor for MFO activities. In sea anemones, the NADPH-cytochrome c reductase activities were comparable to the other marine invertebrate values, whereas the NADH-cytochrome c and ferricyanide reductase activities were significantly lower than other marine invertebrates. Despite these low NADH-reductase activities, these sea anemone species were capable of utilizing both NADPH and NADH as an electron donor for metabolism of both ethoxyresorufin and aldrin. Relatively low reductase activities in invertebrates as compared to vertebrates are thought to be part of the cause for relatively low P450-dependent activities in the invertebrates. In support of this idea, refortification of
crustacean microsomes with purified mammalian cytochrome P450 reductase significantly increased the rate of EROD activity (James, 1984; 1989; 1990).

**Ethoxyresorufin O-deethylation**

The sea anemone, *A. elegantissima* and *A. xanthogrammica*, contained microsomal NADPH-dependent EROD activities relatively similar to rates observed in most aquatic invertebrates (e.g., annelids, crustaceans, various molluscs) (Tables 2-2 and 2-4). The activities for these invertebrates ranged between 0 – 6 pmol/min/mg protein. In contrast, sea anemone EROD activities were 10 – 20 times lower than rates reported in gastropods, *Patella caerulea* and *Avicularia gibbosula*, bivalves, *Brachidontes variabilis* and *Donax trunculus* (Yawetz et al., 1992), and the sandworm, *Nereis virens* (Reily et al., 1992). In the molluscs, the activity varied with the collection site from 0 to 60 pmol/min/mg protein; thus, the particularly high EROD activities may have been due to exposure to xenobiotics (Yawetz et al., 1992). A similar effect of exposure could also account for the higher activity in the sandworm, *N. virens*. Comparisons with the NADH-dependent EROD activity in *A. elegantissima* and *A. xanthogrammica* cannot be made because it has not been examined in other marine invertebrates.

Endogenous inhibitors have been cited as the reason for low EROD activity in crustacean hepatopancreas (Bend et al., 1981; James et al., 1979; Lindstrom-Seppa et al., 1982; 1983; Payne, 1977). When spiny lobster hepatopancreas microsomes were incubated with sheepshead liver microsomes, the fish liver microsomes showed a decrease in the NADPH-dependent EROD and B[a]P hydroxylase activities (James et al., 1979). In contrast, when sea anemone microsomes were incubated with rat liver microsomes, the NADPH-dependent EROD activity was actually greater than the expected value based on an additive effect (data not shown). Thus, unlike in the spiny lobster, the presence of endogenous inhibitors in the sea anemone was not apparent.
<table>
<thead>
<tr>
<th>Species</th>
<th>pmol/min/mg protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cnidarian</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coral (Montastraea faveolata)</td>
<td>below detection</td>
<td>Firman, 1995</td>
</tr>
<tr>
<td><strong>Mollusc</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octopus (Octopus pallidus)</td>
<td>1.4 - 4.82</td>
<td>Cheah et al., 1995</td>
</tr>
<tr>
<td>Octopus (O. pallidus)</td>
<td>0.18 - 0.32</td>
<td>Butty &amp; Holdway, 1997</td>
</tr>
<tr>
<td>Mussel (M. edulis)</td>
<td>5</td>
<td>Stegeman, 1985</td>
</tr>
<tr>
<td>Calico clam (M. maculata)</td>
<td>below detection</td>
<td>Stegeman, 1985</td>
</tr>
<tr>
<td>Bermuda mussel (A. zebra)</td>
<td>below detection</td>
<td>Stegeman, 1985</td>
</tr>
<tr>
<td>Pond snail (L. stagnalis)</td>
<td>below detection</td>
<td>Wilbrink et al., 1991</td>
</tr>
<tr>
<td>Gastropod (C. gibbosum)</td>
<td>below detection</td>
<td>Vrolijk &amp; Targett, 1992</td>
</tr>
<tr>
<td>Chiton (Cryptochiton stelleri)</td>
<td>below detection</td>
<td>Schlenk &amp; Buhler, 1989</td>
</tr>
<tr>
<td>Gastropod (L. stagnalis)</td>
<td>95 ± 33</td>
<td>Meimberg et al., 1997</td>
</tr>
<tr>
<td>Gastropod (P. caerulea)</td>
<td>53 ± 25</td>
<td>Yawetz et al., 1992</td>
</tr>
<tr>
<td>Gastropod (A. gibbosula)</td>
<td>35 ± 39</td>
<td>Yawetz et al., 1992</td>
</tr>
<tr>
<td>Bivalve (B. variabilis)</td>
<td>48 ± 22</td>
<td>Yawetz et al., 1992</td>
</tr>
<tr>
<td>Bivalve (D. trunculus)</td>
<td>24 ± 7</td>
<td>Yawetz et al., 1992</td>
</tr>
<tr>
<td><strong>Crustacean</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crayfish (P. clarkii)</td>
<td>0.7 ± 0.6</td>
<td>Escartin &amp; Porte, 1996</td>
</tr>
<tr>
<td>Crayfish (P. clarkii)</td>
<td>0.51 ± 0.24</td>
<td>Porte &amp; Escartin, 1998</td>
</tr>
<tr>
<td>Crayfish (A. astacus)</td>
<td>below detection</td>
<td>Lindstrom-Seppa et al., 1983</td>
</tr>
<tr>
<td>Crab (Carcinus aestuarii)</td>
<td>0-50 (exposed)</td>
<td>Fossi et al., 1998</td>
</tr>
<tr>
<td>Blue crab (C. sapidus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stomach</td>
<td>2.6</td>
<td>Singer et al., 1980</td>
</tr>
<tr>
<td>hepatopancreas</td>
<td>below detection</td>
<td>Singer et al., 1980</td>
</tr>
<tr>
<td>Spiny lobster (P. argus)</td>
<td>28 - 98</td>
<td>James, 1984; 1989; 1990; 1994;</td>
</tr>
<tr>
<td>Annelid</td>
<td></td>
<td>James &amp; Little, 1984</td>
</tr>
<tr>
<td>Earthworm (L. terrestris)</td>
<td>below detection</td>
<td>Berghout et al., 1991;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liimatainen &amp; Hanninen, 1982</td>
</tr>
<tr>
<td>Sandworm (N. virens)</td>
<td>30</td>
<td>Reily et al., 1992 (abstract)</td>
</tr>
<tr>
<td>Tiger worm (Eisenia f. fetida)</td>
<td>traces levels</td>
<td>Achazi et al., 1998</td>
</tr>
<tr>
<td>Pot worm (Enchytraeus crypticus)</td>
<td>3.98 ± 0.55</td>
<td>Achazi et al., 1998</td>
</tr>
<tr>
<td>Terrestrial annelid</td>
<td>15.41 ± 9.48</td>
<td>Hellwig*</td>
</tr>
</tbody>
</table>

*a* Microsomes were prepared from mollusc digestive gland, crustacean hepatopancreas (except blue crab), earthworm and sandworm midgut, tiger worm and pot worm whole body.

*b* Microsomes were sodium cholate-solubilized, then fortified with mammalian reductase.

*c* Data given as personal communication in Achazi et al., 1998.
Large variation between individuals in MFO activity is common in studies of marine invertebrates (e.g., Kreiger et al., 1979; Michel et al., 1991), and may have played a role in the NAD(P)H-dependent EROD activities in this study. For instance, values were consistent between different microsomal batches of *A. elegantissima*; in contrast, while the typical activity of *A. xanthogrammica* was about one-third that of *A. elegantissima*, one sample had almost no activity and another had activity comparable to the level observed in *A. elegantissima* (Table 2-2). These differences could have resulted from the *A. xanthogrammica* microsomes being prepared from one or two individuals instead of approximately 50 individuals, as occurred with the smaller species, *A. elegantissima*.

In the vertebrates, there is an extremely strong preference for NADPH as the electron donor in the MFO system, but several aquatic invertebrates can use NADH instead (e.g., den Besten et al., 1994; Jewell & Winston, 1989; Lindstrom-Seppa et al., 1983; Livingstone, 1991; Wilbrink et al., 1991). In fact, NADH was shown to be the preferred cofactor for B[a]P hydroxylase activity in the crayfish, *P. clarkii*, and in the sea star, *Asterias rubens* (den Besten et al., 1994; Jewell & Winston, 1989). Nevertheless, the preference for NADH in aquatic invertebrates appears to vary between substrates, species, and tissues. For instance, the NADPH-dependent EROD activity was about two times higher than the NADH-dependent EROD activity in *A. elegantissima* and *A. xanthogrammica* (Table 2-2). The preference for NADPH in these species is consistent with the NAD(P)H-dependent hydroxylation of B[a]P activity in sea anemone microsomes (Winston et al., 1998).

Microsomal NADPH-dependent EROD activity appeared to be lacking in a third species, *B. cavernata*, suggesting that the ability to detect P450 activity in sea anemones can vary depending on the species examined. While lacking EROD activity, *B. cavernata* is capable of catalyzing B[a]P hydroxylation (Winston et al., 1998) and its microsomes contain a protein recognized by the monoclonal mouse anti-Scup CYP1A (Heffernan et al., 1996). In vertebrates,
ethoxyresorufin and B[a]P are both specifically metabolized by the CYP1A isoforms (Burke et al., 1994; Lewis, 1996); thus, given the presence of CYP1A in *B. cavernata*, it is surprising that this species does not appear to have EROD metabolism.

Currently, it is unclear whether the marine invertebrate cytochromes P450 are inducible because induction studies in those organisms have yielded conflicting results (e.g., Livingstone, 1991). However, based on western blots probed with a fish CYP1A antibody, the putative 1A isoform is not induced in the sea anemone, *B. cavernata*, after exposure for three days to 3-methylcholanthrene (20 mg/kg) (Heffernan et al., 1996). In contrast, B[a]P hydroxylation activity reported for coral, *F. fragum*, collected in the Florida Key Largo Marine Sanctuary from the polluted Kemphouse Reef was over three times greater than for coral collected from the relatively pristine South Caryfort Reef. The more inshore site, Kemphouse Reef, contained 3–15 times more polyaromatic hydrocarbons and pesticides than did the South Caryfort Reef; thus, these authors suggested that the increase in activity was associated with an induction in MFO activity in the coral. However, there was no coinciding increase in P450 content with the increase in B[a]P activity (Gassman & Kennedy, 1992). In any event, the *B. cavernata* were collected from a site heavily contaminated with petroleum products, whereas both *A. elegantissima* and *A. xanthogrammica* were collected from relatively pristine sites. Thus, if induction were a factor in the EROD results for these species, the results were contrary to expectations.

**Aldrin Epoxidation**

In addition to metabolizing ethoxyresorufin through an O-dealkylation reaction, the sea anemone can convert aldrin to dieldrin through an epoxidation reaction (Table 2-3; Figs. 2-6 and 2-7). Dieldrin was the only metabolite produced, which is consistent with results found in the earthworm, *Lumbricus terrestris* L. (Nelson et al., 1976). The presence or absence of additional metabolites was not mentioned in other aquatic invertebrate aldrin epoxidation studies (Burns,
1976; Carlson et al., 1974; Khan et al., 1972a, 1972b; Kreiger et al., 1979). Dieldrin has been shown to be further metabolized in the rat, but at such a slow rate that the concentration of additional metabolites was negligible during the incubation times of an *in vitro* study (Wolff et al., 1979).

Aldrin epoxidation activity was detected in each microsomal preparation from *A. elegantissima* and *B. cavernata* and most microsomal preparations from *A. xanthogrammica* (Table 2-3). In each species, the epoxidation of aldrin to dieldrin required either NADH or NADPH (Table 2-3; Figs. 2-6 and 2-7). While most of the microsomal preparations did not have a strong preference for either NADPH or NADH, there were some microsomal preparations that either strongly preferred NADPH or NADH. The difference in preference for a particular cofactor as an electron donor may be a result of aldrin being metabolized by multiple P450 isoforms. It is impossible to compare these aldrin results to other marine invertebrates because other studies only examined NADPH as a potential cofactor for aldrin epoxidation (Burns, 1976; Carlson et al., 1974; Khan et al., 1972a; 1972b; Kreiger et al., 1979; Nelson et al., 1976). Those studies were done prior to the recognition that invertebrates are capable of effectively using NADH as an electron donor in the metabolism of P450-substrates (e.g., den Besten et al., 1994; Jewell & Winston, 1989; Lindstrom-Seppa et al., 1983; Livingstone, 1991; Wilbrink et al., 1991; Winston et al., 1998).

In addition to a difference in preference for a particular cofactor between different microsomal preparations, there were several preparations that varied more from the mean activity for that species. This variability does not seem to be the result of individual variation as the highest degree of variability was observed in *A. elegantissima*, which also contained the greatest number of individuals within a microsomal preparation (~ 50 individuals). In addition to individual variation, there are many factors that could contribute to variation in the MFO properties. For instance, the P450 contents and activities have been shown to vary with season,
exposure to pollutants, developmental status, and availability of food (e.g., Kirchin et al., 1992; Sole et al., 1995; Stegeman & Hahn, 1994; Weinstein, 1995). The variability within some preparations along with the difference in preference for a particular cofactor could result from the sea anemone microsomes containing multiple differentially-expressed P450 isoforms that are capable of metabolizing aldrin.

There was some variation between samples due to the extraction differences; however, most of these differences were eliminated by taking extreme care in extracting the samples under identical conditions. The literature is of limited value in resolving the cause of the wide variation in activities. In addition to only examining the effect of NADPH on aldrin epoxidation, most such studies on marine invertebrates also reported only an average value of activity (Khan et al., 1972a; 1972b; Kreiger et al., 1979; Nelson et al., 1976). Low variation was seen in the fiddler crab (*Uca pugnax*), but given that high variation was reported in the lobster (*H. americanus*) the sea anemone results are probably not artifactual (Burns, 1976; Carlson et al., 1974).

The presence of aldrin epoxidation activity in the sea anemone (i.e., *A. elegantissima*, *A. xanthogrammica*, and *B. cavernata*) is consistent with an *in vivo* study that demonstrated the freshwater hydra, *H. littoralis*, can convert aldrin to dieldrin, presumably by MFO activity (Khan et al., 1972b). *In vivo* exposure of the hydra to 0.1 ppm aldrin for 2 hours resulted in uptake of 46.2 ng aldrin per animal and 2.6% of the aldrin was metabolized to dieldrin. This rate of aldrin epoxidation in the hydra was comparable to that observed in planaria, annelida, and crustacea (Isopoda), but approximately 2 to 3 times less than for the other classes of crustacea and about 6 to 8 times lower than in the freshwater mussel (*Anodonta* sp.) and snail (*Lymnaea* sp.) (Khan et al., 1972b). In contrast to this *in vivo* study, the rate of *in vitro* aldrin metabolism by the microsomal fraction of each of these sea anemone species was significantly lower than values reported in other marine invertebrates (Burns, 1976; Carlson et al., 1974; Khan et al., 1972a; 1972b; Kreiger et al., 1979; Nelson et al., 1976).
There are many potential explanations for the lower activity in the sea anemone in addition to the possibility that the sea anemone is not particularly efficient at this reaction. For instance, the sea anemone microsomes may contain an endogenous inhibitor to the P450 isoform(s) involved in aldrin epoxidation. Endogenous inhibitors have been found in several species of crustacean (Bend et al., 1981; James et al., 1979; Lindstrom-Seppa et al., 1982; 1983; Payne, 1977). Although endogenous inhibitors were not found in the sea anemone for isoforms that metabolize ethoxyresorufin, their presence was not examined with the aldrin reaction. P450 inhibitors are known to have different degrees of specificity for a particular P450 isoform (Ortiz de Montellano, 1995); thus, an endogenous inhibitor may inhibit aldrin epoxidation and not EROD activity. Further, in other organisms, the ability to obtain high, reproducible activities required specific assay conditions. For instance, the epoxidation of aldrin in a crayfish, *Cambarus*, a mussel, *Anodonta*, and the sandworm, *N. virens* has been shown to have a very narrow ideal pH range (Khan et al., 1972b; Nelson et al. 1972); the activity was much lower outside of this pH range. While the pH range used in this study was identical to the pH from these studies, it is possible that the optimum pH for the sea anemone is different. Further analysis would need to be performed prior to eliminating either of these possible explanations.

Of course, the simplest explanation is that the sea anemone does not metabolize aldrin as well as most marine invertebrates. The particularly low rate of metabolism in the sea anemone would be consistent with several field studies on coral. These field studies reported that coral rapidly bioaccumulate polynuclear aromatic hydrocarbons and pesticides, but slowly eliminate these compounds (Knap et al., 1982; Solbakken et al., 1982; 1983; 1984; 1985). Given the physiological similarities between sea anemones and corals, field studies would probably demonstrate similar results in sea anemone. Even so the sea anemone is capable of enzymatically converting aldrin to dieldrin. There is no disputing that the activity was linear.
with respect to time and protein concentration, required either NADH or NADPH, and was consistently observed in both *A. elegantissima* and *B. cavernata*.

Conclusions

These results demonstrate that the sea anemone columnar microsomes contain the major components characteristic of a cytochrome P450-dependent MFO system. These properties include the presence of a characteristic CO-binding spectra similar to that observed in other marine invertebrates and reductase activities associated with NAD(P)H-dependent cytochrome c and NADH-dependent ferricyanide. The ability of sea anemone microsomes to metabolize two classical P450-dependent MFO substrates, ethoxyresorufin and aldrin, clearly demonstrates that the sea anemone microsomes contain a functional P450. In each of these species, there was no NAD(P)H-independent activity similar to that observed in mollusc (e.g., Livingstone et al., 1989). There was a strong preference for NADPH as compared to NADH as a cofactor for the EROD activity. In contrast, only minor differences in NADH- versus NADPH-dependent aldrin activity were detected in most microsomal preparations.

The differences in metabolism of ethoxyresorufin and aldrin could be explained by the presence of multiple P450 isoforms in the sea anemone. For instance, they could account for the marked difference in cofactor preference between the EROD and epoxidation reactions. Further, the ability of several of these isoforms to metabolize aldrin would explain the variability in rate of activity as well as the cofactor preference between the different microsomal preparations within a species. Finally, there were clearly differences in the ability of these three species to metabolize these substrates, suggesting that the isoforms present in each of these species may be different or at least expressed to different degrees.

The estimates of P450 content and activity in the anthozoans may be considerably lower than the true values. In the anthozoan, the cytochrome P450 content was determined from microsomes prepared from either the entire animal (corals) or the columnar region of the
organism (sea anemones), while microsomes of other organisms were prepared from specific organs where the cytochromes P450 tend to concentrate. The cytochromes P450 may be concentrated in a particular tissue; if so, they would be diluted in the sea anemone microsomal preparations. However, due to the lack of organs in the cnidarian and to their amorphic and non-rigid structure, it is difficult to isolate a specific tissue for microsomal preparation. Microsomes from the anthozoan and other invertebrates also suffer from P450 being more closely associated with the digestive system. Therefore, the P450 is more susceptible to degradation by endogenous proteolytic enzymes.

The CO-binding spectral properties, reductase activities, and NAD(P)H-dependent metabolism of classical cytochrome P450 substrates (i.e., aldrin epoxidation, B[a]P hydroxylation, ethoxyresorufin O-dealkylation) of earlier sea anemone microsomal studies (Gassman & Kennedy, 1992; Heffeman et al., 1996; Khan et al., 1972b; Winston et al., 1998) and this study support the presence of cytochrome P450 in the cnidarians. The failure of some studies to detect the presence of an MFO system in the cnidarian may have been due to problems with the conditions of the assays rather than to a true lack of activity. A similar situation occurred when Payne (1977) did not detect B[a]P activity in the mussel (M. edulis), while several more recent studies with this mussel have reported B[a]P activity (e.g. Lemaire et al., 1991; Livingstone et al., 1991; Livingstone et al., 1989). Prior studies reporting an absence of P450 in the cnidarian, did so based a single species with a single substrate. This study indicates that a broad conclusion, such as the absence of P450 in a phylum, cannot be drawn from the lack of metabolism of one substrate in one species.

Several potential explanations exist for why early studies did not detect the MFO system in the cnidarian. First, in most metazoans, cytochrome P450 tends to concentrate in particular organs. As previously discussed, it is difficult to isolate P450 from discrete regions of the cnidarians. Second, in experiments that tested many organisms from several different phyla,
enzymatic assays and tissue preparation were typically optimized for one organism and all other animals were analyzed under the same conditions. Particularly in organisms with low MFO activity, establishing optimal conditions can be critical. For instance, this research indicates that the detection of MFO activity in the cnidarians requires longer incubation times, during which the MFO activity remained linear. Third, because the invertebrate cytochromes P450 trend to concentrate in the digestive and reproductive system, the lack of optimal buffer conditions (i.e., protease inhibitors and reducing agents) could prevent isolation of a functional protein (e.g., Livingstone, 1991). This problem is further compounded in the cnidarian because it is essentially a large digestive sac. Finally, Lee (1981) suggested that sea anemones may contain endogenous inhibitors of cytochrome P450 that are released upon homogenization. The presence of endogenous inhibitors of the MFO system has been reported in the hepatopancreas of several crustaceans (Bend et al., 1981; James et al., 1979; Lindstrom-Seppa et al., 1982; 1983; Pohl et al., 1974). While the EROD data indicated that the sea anemone columnar region microsomes do not contain any endogenous to P450 isoforms involved in ethoxyresorufin metabolism, their presence was not examined with the aldrin epoxidation reaction. Endogenous inhibitors to P450 isoforms involved in aldrin epoxidation could account for the low epoxidation activity.

In conclusion, despite the lack of MFO activity reported in anthozoans and scyphozoans by several cnidarian studies (Firman, 1995; Lee, 1975; Lee, 1981; Payne, 1977), other studies indicate that anthozoans and hydrozoans do contain a functional cytochrome P450-dependent MFO system (Gassman & Kennedy, 1992; Heffernan et al., 1996; Khan et al., 1972b; Winston et al., 1998; present study). The evidence is based on the presence of proteins that cross react with cytochrome P450 antibodies, a characteristic CO-difference spectra in DTN-reduced coral and sea anemone microsomes, active compliments of MFO components (i.e., P450 and b5 reductases), and metabolism of classical cytochrome P450-catalyzed reactions (i.e., ethoxyresorufin O-dealkylation, B[a]P hydroxylation, and aldrin epoxidation). Although the
aldrin epoxidation activity was lower than values obtained from other marine invertebrates, it is interesting that the P450 content and EROD activity does not differ substantially from most other invertebrates, despite the fact that cnidarian microsomes are composed of either the whole animal (i.e., hydra, coral) or the entire columnar region (i.e., sea anemone).
Chapter 3: Distribution of Microsomal CO-binding Chromophores and EROD Activity in Sea Anemone Tissues from *Anthopleura xanthogrammica*

**INTRODUCTION**

Cytochromes P450 have a wide tissue distribution in aquatic invertebrates; however, they tend to be most concentrated in tissues associated with processing food or pollutants. This has been demonstrated in several species of molluscs (Livingstone & Farrar, 1984; Schlenk & Buhler, 1989; Stegeman, 1985), crustaceans (Burns, 1976; James, 1989; Jewell & Winston, 1989; Khan et al., 1972a; 1972b; Lindstrom-Seppa et al., 1983; Stegeman & Kaplan, 1981), and annelids (Fries & Lee, 1984; McElroy, 1990; Nelson et al., 1976). Based on these studies, the P450 content and mixed-function oxidase (MFO) activities are often 2 to 10 times lower or even not detectable in tissues that do not process food or pollutants. In contrast, two aquatic invertebrate studies have reported that the P450 content and P450-dependent activities are not concentrated in any particular organ (Schlenk & Buhler, 1989; den Besten et al., 1990).

Recent studies have demonstrated the presence of a functional cytochrome P450-dependent MFO system in at least five species of sea anemone and in a scleractinian coral (Phylum Cnidaria; Class Anthozoa). The evidence is based on the presence of the P450-dependent MFO system components (i.e., cytochrome P450 and flavin-containing reductase activities) in the microsomal fraction of these organisms, and on their ability to catalyze three reactions (ethoxyresorufin O-dealkylation, benzo[a]pyrene hydroxylation, and aldrin epoxidation) that are specific for cytochrome P450 (Gassman & Kennedy, 1992; Heffernan & Winston, 1998; Heffernan et al., 1996; Winston et al., 1998; previous chapter). However, those cnidarian P450 studies were performed on microsomal preparations from either the whole animal (coral) or the entire columnar region (sea anemone) rather than on microsomes from specific tissues that might concentrate P450.
The cnidarians have evolved only to the tissue level of development; thus, they lack discrete organs. Sea anemones are morphologically a large digestive sac surrounded by a nerve network, a gonadal region, a thick muscular region, and a tough outer wall (Barnes, 1980; Shick, 1991). Based on its physiology, the sea anemone could be expected to concentrate P450 in either the mesentery filaments (important for internal and external digestion) or the imperfect and perfect mesentery (important for absorption of nutrients, further processing of food, and lipid storage). The particularly high concentration of lipids in the perfect mesentery would tend to concentrate pollutants; thus, this tissue could play a role in metabolism of these pollutants.

The purpose of this study was to provide a preliminary analysis of the relative distribution of P450 in four general tissue regions. *A. xanthogrammica* was analyzed because its large size made it amenable to dissection. This species has been shown to contain spectral properties characteristic of P450 and to perform a classical P450 metabolic reaction, ethoxyresorufin O-dealkylation (EROD).

**METHODS**

*Anthopleura xanthogrammica* were obtained from North Coast Invertebrate Collectors (Bodega Bay, CA) and maintained in a recirculating system with Instant Ocean™ sea water at approximately 34% salinity and 12 °C. The amorphic and non-rigid structure of these organisms makes it difficult to dissect out distinct tissues; thus, the four regions studied herein do not consist of one tissue. Instead, they consist of four mixed tissue regions that are referred to as the tentacle, soft, inner, or outer region. To isolate these regions, the animal was cut vertically through the center of the column (Fig 3-1). The tentacles, which include the nematocysts and most individuals of the symbiont (algae or diatom), were removed as the first region. The second tissue region consisted of the softest tissues, which had absolutely no structure (i.e., digestive sac, gonads, mesentery filaments, and acontia with their associated nematocysts). After
Fig. 3-1. Cross-section of sea anemone showing intertwining of connective tissue with other tissues. The algae or diatom symbiont is predominantly in the tentacles with some also concentrated in the oral disc (modified from Shick, 1991).
removing as much of the soft tissues as possible, the fibrous inner region (i.e., perfect and
imperfect mesentery and retractor muscle) was separated from the tough outer layer (i.e., tough
outer muscle and skin, oral disk, and basal disk). As described in Chapter 2, the various tissue
regions were immediately immersed in homogenization buffer. Microsomal fractions were
prepared from each of the four tissue regions of two individuals, except that the tentacular region
was not saved from the second animal. These microsomal fractions were analyzed for their
protein concentration, EROD activity, and CO-difference, DTN-reduced spectral properties
(DTN added prior to CO) between 390-700 nm.

RESULTS

The CO-difference spectra of DTN-reduced sea anemone microsomes contained a 450 nm
chromophore indicative of P450 in each of the tissue regions (Fig. 3-2). Further, the 450 nm
peak always reached its maximum amplitude immediately and did not change within 40 min.
Quantification of the P450 content by classical methods requires an absence of interference with
the absorbance at 490 nm (see Discussion). The 490 nm absorbance was altered by two
features: the presence of a broad 490 nm peak in some of the tissue regions, similar to that
observed in the sea anemone microsomes prepared from the entire columnar region; and, a
tendency for the baseline of the spectra to slope. Therefore, the P450 content was not based on
the absorbance difference between 450 and 490 nm, but on the change in the absorbance
maximum at 450 nm and a baseline drawn tangent to that of the spectrum (Fig. 3-2). Based on
this method, the P450 content in each of these tissue regions was estimated to be between
16 – 31 pmol/mg microsomal protein (Table 3-1). There was no significant tendency for P450
to concentrate in any particular tissue region, although the P450 content was a little higher in
both the soft and inner regions.

There was a predominant chromophore at 418 nm associated with each tissue region (Fig.
3-2; Table 3-1). In contrast to the 450 nm chromophore, the 418 nm chromophore was
Fig. 3-2. CO-difference spectra of DTN-reduced solubilized microsomes (*A. xanthogrammica*) prepared from four tissue regions: soft, S; inner, I; outer, O; and tentacle, T. Tangent lines used for quantifying P450 content are shown. (A) Comparison of Soret region (380-500 nm) for all four tissue regions. (B) Enlargement of spectra obtained from the soft, inner, and tentacle regions to improve measurements. Microsomes were solubilized in 100 mM potassium phosphate, 0.15% Triton N101, 15 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol.
Table 3-1. Quantitation of microsomal cytochrome P450, 418 nm chromophore, and NAD(P)H-EROD activities in *A. xanthogrammica* tissue regions.

<table>
<thead>
<tr>
<th>Tissue Regions</th>
<th>P450&lt;sup&gt;a&lt;/sup&gt;</th>
<th>418&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NADPH&lt;sup&gt;c&lt;/sup&gt; EROD</th>
<th>NADH&lt;sup&gt;c&lt;/sup&gt; EROD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; individual</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>outer muscle</td>
<td>22</td>
<td>64</td>
<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td>inner region</td>
<td>29</td>
<td>4</td>
<td>0.66</td>
<td>0.27</td>
</tr>
<tr>
<td>soft region</td>
<td>22</td>
<td>3</td>
<td>1.09</td>
<td>0.26</td>
</tr>
<tr>
<td>tentacles</td>
<td>16</td>
<td>4</td>
<td>2.61</td>
<td>0.36</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; individual&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>outer muscle</td>
<td>16</td>
<td>89</td>
<td>1.69</td>
<td>0.27</td>
</tr>
<tr>
<td>inner region</td>
<td>31</td>
<td>9</td>
<td>1.48</td>
<td>0.47</td>
</tr>
<tr>
<td>soft region</td>
<td>31</td>
<td>15</td>
<td>1.46</td>
<td>0.48</td>
</tr>
</tbody>
</table>

<sup>a</sup> pmol/mg protein; calculation from baseline, instead of 490 nm absorbance.
<sup>b</sup> (490-418 nm) x 1000 x mg<sup>-1</sup>.
<sup>c</sup> pmol/min/mg protein; performed in triplicate.
<sup>d</sup> Tentacle region removed, but microsomes were not prepared.

significantly higher in amplitude in the tough outer region of the sea anemone than in any of the other regions. Further, the amplitude of the 418 nm peak slowly increased over 20 to 30 min after the addition of CO to the sample to a final amplitude that was almost two times larger than the initial peak. The 450 nm peak reached its maximum amplitude immediately after addition of CO and remained stable for the entire assay (data not shown).

Cytochromes contain characteristic chromophores in the 500-700 nm region of the spectrum as well as between 380-500 nm (soret region). The inner, outer, and soft tissue regions contained chromophores in the 500-700 nm range under CO-difference, DTN-reduced conditions; however, the spectra detected were quite different for each tissue region (Fig. 3-3).

The outer region contained two distinct peaks at 535 and 569 nm with a trough at 555 nm. The soft region only contained one well-defined peak with a wavelength maximum (λ<sub>max</sub>) at 548 nm, at least two overlapping peaks between 570-640 nm (λ<sub>max</sub> 576 and 599 nm), and a peak at about 660 nm (no clear λ<sub>max</sub>). Finally, the inner region contained one distinct peak at 615 nm and some
Fig. 3-3. CO-difference spectra (500-700 nm) of DTN-reduced of solubilized microsomes representing three of the four tissue regions of *A. xanthogrammica*: outer, O; inner, I; and, soft, Microsomes were solubilized in 100 mM potassium phosphate, 0.15% Triton N101, 15 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
positively absorbing chromophores above 660 nm. The tentacle region was not analyzed. The presence of different spectral properties within this spectral region is indicative of the presence of additional heme-centered proteins in the sea anemone microsomes.

NAD(P)H-dependent EROD activity was detected in each tissue region, with NADPH being the preferred cofactor (Table 3-1). There was no cofactor-independent activity. Unlike the columnar microsomes, there was no initial decrease in fluorescence between 0 – 15 min prior to detecting a linear increase in fluorescence between 20 – 60 min. Instead, the EROD activity was linear for the entire reaction (0 – 60 min) for each tissue. Within each animal, results for the inner and soft regions were comparable, although values were somewhat higher for animal #2. The outer region had very low NAD(P)H-EROD activity in animal #1, but high activity in animal #2. The tentacle region had the highest NADPH-EROD activity and high NADH-EROD activity.

Each of the tissue regions was capable of metabolizing ferulic acid, presumably by peroxidase(s); however, the differences in the rates of metabolism were minor. In contrast, while a low rate of caffeic acid metabolism was detected in sea anemone microsomes of the entire columnar region, the caffeic acid activity in these tissue regions was below the detection limit of the assay (data not shown; peroxidase assay described in Chapter 4).

DISCUSSION

The CO-difference spectra of DTN-reduced sea anemone microsomes indicated that P450 is present in each tissue region (Fig. 3-2; Table 3-1). Quantification of P450 by standard methods used for vertebrate analysis of P450 ($\epsilon_{450-490} = 91 \text{ cm}^{-1} \text{ mM}^{-1}$) requires that the spectrum be free of interference at 490 nm. This wavelength is the isosbestic point between CO-liganded reduced microsomes and unliganded reduced microsomes, and it is classically used to quantify the change in absorbance of the 450 nm chromophore. At the isosbestic point, the two spectra have the same absorbance value, so the background-corrected DTN-reduced spectra can be
autozeroed on 490 nm and the addition of CO will not alter that zero absorbance value. As a result, the difference between the 450 and 490 nm absorbance values will reflect the P450 content (450 minus 490 nm absorbance), not any effect from the addition of CO.

Since the 490 nm absorbance is the point used to quantitate P450, anything that alters the absorbance at 490 nm will interfere with quantitation of the P450 content. Thus, the tendency of the overall baseline of the spectrum to slope and the presence of a broad 490 nm chromophore prevented the quantification of P450 by the classical method. Similar complications have also been observed in other marine invertebrates (e.g., Cheah et al., 1995; Heffernan & Winston, 1998; Koivusaari et al., 1980; Livingstone & Farrar, 1984). In octopus microsomes (*Octopus pallidus*), P450 was quantified from the absorbance difference between the 450 nm chromophore and a baseline drawn tangent to the slope of the spectrum (Cheah et al., 1995). This procedure was used to quantify P450 content in the sea anemone microsomes reported herein.

Livingstone (1991) noted that the concentrations of most invertebrate enzymes are low relative to vertebrates, which possibly reflects the lower metabolic rates of these animals. Similarly, the sea anemone P450 content was between 16 – 31 pmol/mg microsomal protein (Table 3-1), which is within the range of values detected in most other aquatic invertebrates (20 – 140 pmol/mg microsomal protein), but substantially lower than that of mammals and non-mammalian vertebrates.

In most aquatic invertebrate studies, P450 concentrates in tissues associated with processing of food and pollutants (Burns, 1976; Fries & Lee, 1984; Jewell & Winston, 1989; James, 1989; Khan et al., 1972a; 1972b; Lindstrom-Seppa et al., 1983; Livingstone & Farrar, 1984; McElroy, 1990; Nelson et al., 1976 Schlenk & Buhler, 1989; Stegeman, 1985; Stegeman & Kaplan, 1981). Thus, the highest P450 content would be expected to be in either the soft region, which contains the mesentery filaments, or the inner region, which contains the imperfect...
and perfect mesenteries (high lipid content). The P450 content was slightly higher in the soft and inner region, but the differences between all of the tissue regions were small.

A predominant 418 nm chromophore was associated with each of the sea anemone tissue regions. The amplitude of the 418 nm peak was approximately 10-fold greater in the outer region of the sea anemone than in any of the other tissue regions, whether the spectrum was read immediately or after the 418 nm peak reached its maximum amplitude. In mammals, the 420 nm peak detected in the difference spectra of CO-liganded, DTN-reduced microsomes is the result of denatured P450 (i.e., P420). This is evident from the coinciding increase in the 420 nm peak and decrease in the 450 nm peak as mammalian P450 is converted to P420. However, the mammalian results do not seem relevant to the 418 nm peak in most invertebrates. For instance, in each of these sea anemone tissue regions, as the 418 nm chromophore increased over time, there was no corresponding decrease in the 450 nm chromophore (data not shown). The pattern seen in the sea anemone has also been observed in several other invertebrates (Cheah et al, 1995; den Besten, 1998; Heffernan & Winston, 1998), although a coinciding shift in the 420 and 450 nm peaks has been observed in the bivalves M. edulis and L. stagnalis (Livingstone & Farrar, 1984; Stegeman, 1985; Wilbrink et al., 1991).

There are several additional characteristics of the invertebrate 418 nm chromophore that are atypical of a vertebrate denatured P450. For instance, while P420 content may be present in some vertebrate tissues (e.g., lung), it is typically negligible in most microsomal preparations. In contrast, the 418 nm chromophore is consistently observed in molluscs, cnidarians, and echinoderms, despite the addition of protein protectants (i.e., protease inhibitors, antioxidants, and glycerol) in the microsomal preparation and prevention of over-homogenization of the tissue (e.g., den Besten, 1990; Heffernan & Winston, 1998; Livingstone & Farrar, 1984; Koivusaari et al., 1980). Further, unlike the mammalian microsomal spectra (Figs. 1-1), the invertebrate spectra (Fig. 3-2) contain a distinct trough between the 420 and 450 nm peaks and have a very
broad peak with a wavelength maximum that varies from 415 to 428 nm between different organisms.

Based on other studies, P450 contents are typically much lower in the invertebrates as compared to vertebrates. But if the P420 were due to denatured P450, many of the lower invertebrates would have P450 contents equivalent to those found in mammalian microsomes. The extinction coefficients of P420 and P450 have only been determined in a mammal (e.g., Omura & Sato, 1964; Schenkman & Kupfer, 1982); thus, it is possible that the P420 extinction coefficient in these invertebrates is significantly different from mammals. If so, that would explain the general discrepancy noted above. However, the outer region of the sea anemone would still contain significantly more P450 than each of the other regions. According to other invertebrate studies, the outer region (i.e., gill, muscle, mantle, or integument) would be expected to have the lowest P450 content (Khan et al., 1972b; Livingstone & Farrar, 1984; Nelson et al., 1976). Given all of the above characteristics, the invertebrate 418 nm chromophore might primarily result from another heme-centered protein(s) rather than solely from denatured P450.

In the sea anemone, the 500-700 nm spectral properties of the DTN-reduced, CO-difference spectra were not characteristic of native or denatured P450. Under these spectral conditions, purified cytochromes P450 from fish (mullet, Liza saliens) displayed at most one peak and its presence was ambiguous (Sen & Arinc, 1998). Further, the presence of any peaks in this 500-700 nm region were not specified for either native or denatured purified mammalian cytochrome P450 (Schenkman & Kupfer, 1982; Omura & Sato, 1964b). In contrast, the outer and soft regions of the sea anemone both displayed two peaks between 500-700 nm in the DTN-reduced, CO-difference spectra. The presence of two peaks that do not appear to be the result of either P450 or P420 indicates that there are additional heme-centered proteins in the sea anemone microsomes. Along these lines, these peaks were similar to chromophores found in a peroxidase isolated from Halobacterium sp. (Fukumori et al., 1985). The presence of a peroxidase in the
microsomes could account for the large 418 nm chromophore in the sea anemone. Finally, additional heme protein(s) may also be present in other marine invertebrates. In the DTN-reduced, CO-difference spectra, two chromophores were also observed in the mollusc digestive gland microsomes ($\lambda_{\text{max}}$: 535, 569 nm; Livingstone & Farrar, 1984) and crayfish, *P. clarkii*, hepatopancreas microsomes ($\lambda_{\text{max}}$: 548, 576 nm; Jewell & Winston, 1989).

Beyond the spectral properties noted above, several catalytic studies also indicated the 418 nm chromophore may be associated with a peroxidase. For instance, sea anemone microsomes (from the whole columnar region) catalyzed the oxidation of several classical peroxidase substrates (i.e., ferulic acid, caffeic acid, vanillin). Also, Nelson and colleagues (1976) detected peroxidase activity in earthworm (*Lumbricus terrestris*) microsomes, which contained a 418 nm chromophore in the CO-difference, DTN-reduced spectrum; however, in rat liver microsomes, they did not detect a 418-420 nm peak nor peroxidase activity. Further, the bean 420 nm chromophore has been isolated and identified as containing two peroxidases (Rogers et al., 1993). These authors mention that substantial unpublished data indicates one of the peroxidases is actually a contaminant from the cell wall. The largest 418 nm peak seen in the sea anemone was from the tough outer region. Thus, if the 418 is the result of a peroxidase, that region ought to have the highest peroxidase activity. While microsomal fractions of each tissue region isolated from the sea anemone were capable of oxidizing ferulic acid, the differences in the rate of oxidation were minor. Nevertheless, these results do not necessarily argue against the 418 being a peroxidase. There are many peroxidase substrates and ferulic acid may be an inappropriate substrate for the putative peroxidases in these tissues.

The spectral analysis (500-700 nm) indicated that the inner, outer, and soft tissue regions contained distinctive additional heme-proteins (Fig. 3-3). The presence of additional heme-proteins, including P420, has been shown to interfere with the detection of the P450 content (e.g., Achazi et al., 1998; Ade et al., 1982; Berghout et al., 1991; Liimatainen & Hanninen, 1982;
Nelson et al., 1976; Schenkman & Kupfer, 1982). Thus, regardless of the exact identity of the 418 nm chromophores, its removal might alter the sea anemone spectral properties of the cytochrome P450. If so, it is possible that higher P450 contents would be apparent in the sea anemone. Finally, this interference also could prevent detection of small differences in P450 content between tissue regions, and it might account for some of the difficulty in obtaining spectral characteristics in the sea anemone microsomes.

NAD(P)H-dependent EROD activity was detected in each tissue region, with NADPH being the preferred cofactor (Table 3-1). Based on the EROD activity, none of the regions clearly concentrated P450. The tentacle region had the highest NADPH-EROD activity, suggesting that at least some P450 isoforms concentrate in the tentacles. However, this activity might result from the algal/diatom symbiont, which is most concentrated in the tentacles. The presence of a functional P450 has been demonstrated in unicellular algae, *Euglena gracilis*, and in several marine macroalgae (Briand et al., 1993; Pflugmacher & Sandermann, 1998; Thies et al., 1996). The rest of the symbiont is present in the oral disk (i.e., outer region), and differing amounts of the symbiont could account for the large difference in EROD activity seen in the outer region between the two individuals. Although there was no corresponding difference in the P450 content between the two animals, the algal symbiont might contribute a more active NADPH-dependent P450 isoform for EROD metabolism.

Currently, there are no methods for differentiating the P450 contributions made by the host as compared to those of the symbiont; however, it is possible to experimentally separate the symbiont from the host by exposing them to stressful conditions, such as prolonged starvation, continuous darkness, extremely bright light, high temperature, or abruptly altered salinity (Shick, 1991). A draw-back of experimentally inducing mass expulsion of the symbiont is that it does not remove any proteins present in the sea anemone that result from the symbiotic relationship. Further, the symbiont is a natural part of the sea anemone; thus, its contribution to metabolism...
of xenobiotics absorbed by the sea anemone could be important. However, in regards to analyzing the distribution of P450 in the sea anemone, it may be useful to analyze either a symbiont-free species or naturally-occurring aposymbiotic individuals (i.e., individuals that lost the symbiont, possibly as a result of living under a rock or in a cave).

The lack of any tissue-specific concentration of P450 in the sea anemone, *A. xanthogrammica*, is in agreement with findings in the sea star (den Besten, 1990). In the sea star (*A. rubens*), the pyloric caeca microsomes contained more P450 and MFO activity than the stomach or gonads; however, the difference was relatively small as compared to most other marine invertebrates (e.g., mollusc). More importantly, each of the other sea star tissues contained P450 and MFO activity, whereas in *M. edulis*, P450 was only found in the digestive gland and MFO-associated activities were relatively low in other tissues (Livingstone & Farrar, 1984). den Besten (1990) suggested that the presence of a circulatory system may play a role in the distribution of P450 in invertebrates. Organisms without a circulatory system may not concentrate P450 because no particular region plays a more dominant role in processing of food or pollutants, whereas organisms with a circulatory system would have one organ with a more dominant role in these processes.

The study reported here is the first to analyze the localization of cytochromes P450 in a cnidarian. If marked differences in P450 content existed between different tissues of the sea anemone, they should have been detectable in the experimental design used here. However, subtle differences in P450 content may have been missed if the dissection procedures did not completely separate tissues of different P450 contents. In addition, subtle differences may have been missed due to the effect of any contribution of the algal symbiont or interference of other heme-proteins in the detection of the P450.

In most marine invertebrates, large differences in MFO properties (content and activities) were observed between different organs, while in a few organisms the differences were very
small. For instance, Stegeman (1985) found the specific content of P450 to be about 400% higher in digestive gland than gill in *M. edulis*, whereas there was less than a 16% difference in P450 content of the digestive gland and gill in *Arca zebra*. The same trend in these bivalves was observed for the NADPH-B[a]P hydroxylation and NAD(P)H-cytochrome *c* reductase activities. These results suggest that some species may not have the same distribution of P450 between different tissues as do other species. Thus, another species of sea anemone may display larger differences in the MFO properties between the different tissue regions. Future studies should focus on additional individuals, symbiont-free sea anemones, and the examination of additional MFO properties, as well as other members of the phylum. Cytochemistry may prove to be useful for further elucidation of the distribution of P450 in these organisms. However, prior to such studies, the specificity of P450 probes and the elimination of non-specific binding would need to be addressed in the sea anemone.
Chapter 4: Analysis and Partial Purification of Cytochrome P450 Immunoreactive Proteins in Sea Anemone Microsomes

INTRODUCTION

Several studies have demonstrated the presence of a cytochrome P450-dependent mixed-function oxidase (MFO) system in two of the four cnidarian classes, anthozoa and hydrozoa (Gassman & Kennedy, 1992; Heffernan et al., 1996; Heffernan & Winston, 1998; Khan et al., 1972b; Winston et al., 1998). The evidence is based on the presence of proteins that immunoreact with P450 antibodies, a characteristic CO-difference spectra of DTN-reduced coral and sea anemone microsomes, active MFO components (i.e., flavin-containing reductases), and metabolism of classical cytochrome P450-catalyzed reactions (i.e., ethoxyresorufin O-dealkylation (EROD), benzo[a]pyrene hydroxylation, and aldrin epoxidation).

An interesting feature of the MFO system of marine invertebrates is its ability to use both NADPH and NADH as electron donors (e.g., den Besten et al., 1994; Heffernan and Winston, 1998; Jewell & Winston, 1989; Lindstrom-Seppa et al., 1983; Livingstone, 1991; Wilbrink et al., 1991), whereas mammalian MFO is NADPH-dependent (e.g., Lewis, 1995). In our cnidarian studies (i.e., sea anemone), NADPH-EROD activity was consistently higher than the NADH-EROD activity. However, there was only a slight preference for NADPH over NADH in ~ 65% of the microsomal preparations examined. The rest of the microsomal preparations had a strong preference for either NADPH (~ 30%) or NADH (~ 5%). This varying electron-donor preference for aldrin metabolism in the sea anemone could result from differential expression (in batches of microsomes) of multiple isoforms that are able to catalyze aldrin epoxidation. The presence of multiple isoforms in the sea anemone has also been suggested by the ability of antibodies prepared from different P450 isoforms to cross-react with sea anemone microsomal proteins (Heffernan et al., 1996).
Cytochrome P450 is a multi-gene family (Nelson et al., 1996; Stegeman & Hahn, 1994). Nelson et al. (1996) listed 481 genes and 22 pseudogenes that represented 74 families of P450, although more isoforms and families have been identified since that publication. Several P450 genes have been sequenced from aquatic invertebrates (non-insect): e.g., CYP2L1 and CYP2L2 in spiny lobster, *Panulirus argus* (James et al., 1996; Boyle et al., 1998a); CYP30 in clam, *Mercenaria mercenaria* (Brown et al., 1998); CYP10 in pond snail, *Lymnaea stagnalis* (Teunissen et al., 1992); and, CYP45 in lobster, *Homarus americanus* (Snyder, 1998a). In addition, several partial sequences of the CYP4 family were obtained from mussel (*Mytilus galloprovincialis*), abalone (*Haliotis rufescens*), shrimp (*Penaeus setiferus*), lobster (*Homarus americanus*), and sea urchin (*Lytechinus anamesis*) (Snyder, 1998b). These sequences demonstrate that multiple isoforms from several P450 families exist within the aquatic invertebrates. The presence of P450 isoforms in marine invertebrates has also been demonstrated by Western, Southern, and/or Northern blots in annelids (Achazi et al., 1998; Lee, 1998), cnidarians (Heffernan et al., 1996), crustaceans (Boyle & James, 1996; Boyle et al., 1998b; James & Boyle, 1998), echinoderms (den Besten, 1998), and molluscs (Livingstone et al., 1989; 1997; Matsumota et al., 1997; Obser dorster et al., 1998; Peters et al., 1998a; 1998b; Porte et al., 1995; Schlenk & Buhler, 1989; Sole et al., 1996; Wootton et al., 1995).

Western blot studies have indicated that aquatic invertebrates contain similar amino acid sequences (i.e., epitopes) to P450 isoforms of several species of fish (CYP1A, 2B, 2K, and 3A), the rat (CYP2B, 2C, 2E, and 4A), and the spiny lobster (CYP2L). Most of the proteins detected were between the 46-60 KDa molecular weight (e.g., den Besten, 1998; Peters et al., 1998a; 1998b; James & Boyle, 1998; Lee, 1998). Recent papers have also reported the presence of lower molecular proteins that are strongly recognized by several P450 antibodies (Boyle & James, 1996; James & Boyle, 1998; Peters et al., 1998a; 1998b). For instance, in the mussel (*Mytilus edulis*), a 42 KDa protein was recognized by polyclonal anti-perch CYP1A and a 44
KDa protein was recognized by polyclonal anti-trout CYP3A, polyclonal anti-rat CYP2B, and polyclonal anti-rat CYP4A (Peters et al., 1998a). The 44 KDa protein was also detected in *M. galloprovincialis* by the polyclonal anti-rat CYP4A (Peters et al., 1998b). Further, the polyclonal anti-spiny lobster CYP2L cross-reacted with a 30 KDa protein in the Florida spiny lobster, *P. argus*, and a 40 KDa protein in clear nose skate, *Raja eglanteria* (Boyle and James, 1996). These authors suggested that the lobster 30 KDa protein might be a translated product from a spliced (truncated) variant of CYP2L or a breakdown product of P450.

Purification procedures have been used to further identify and characterize the P450 isoforms present in the marine invertebrates (e.g., Achazi et al., 1998; Batel et al., 1986; Berghout et al., 1991; Conner & Singer, 1981; James, 1989; 1990; Kirchin, 1988; Lee, 1986; Livingstone et al., 1989; 1997; Quattrochi & Lee, 1984a; 1984b; Peters et al., 1998a; Porte et al, 1995). These studies have focused on identifying the presence of P450 isoforms and elucidating their role in metabolizing both endogenous and exogenous compounds. However, purification of P450 isoforms has typically been complicated by the presence of low P450 contents, high concentrations of degradative enzymes, and endogenous P450 inhibitors.

The above studies demonstrated the presence of multiple P450 isoforms in several marine invertebrates. The purpose of this study was to examine the microsomal fraction of several species of sea anemone for the presence of different P450 isoforms. This paper includes some of the antibodies examined previously (Heffeman et al., 1996) and analyses of additional P450 antibodies. In addition to the recognition of 50-60 KDa proteins, the polyclonal anti-trout CYP2K antibody strongly recognized a 40 KDa protein in the sea anemone microsomes. Partially purified samples from *A. xanthogrammica* were analyzed to assist in further identification of these immunoreactive proteins. Due to the particularly strong recognition of the 40 KDa protein by the CYP2K antibody and its uniqueness to the marine invertebrates, particular emphasis was focused on purifying and identifying this 40 KDa protein.
METHODS

Sample Preparation

_Bunodosoma cavernata_ were collected from the Gulf of Mexico at Pass Fourchon, Louisiana. _B. cavernata_ is sometimes confused with the morphologically-similar species, _Bunodactis texaensis_, but the blue stripe and reddish coloration as opposed to gray streaks on the tentacles of the organisms collected for this study is consistent with _B. cavernata_ (Fotheringham & Brunenmeister, 1975). _Anthopleura elegantissima_ and _A. xanthogrammica_ were obtained from Pacific Bio-Marine Laboratories and North Coast Invertebrate Collectors in California. Alaskan _A. elegantissima_ were collected near Little Port Walter, Baranoff Island. _Condylactis gigantea_ were obtained from Gulf Specimen Marine Laboratories in Florida (collected from reefs in the Florida Keys). All sea anemones were maintained in a recirculating system with Instant Ocean™ sea water. _A. elegantissima_ and _A. xanthogrammica_ were kept at 34‰ salinity and 12 °C, while _B. cavernata_ and _C. gigantea_ were kept at 25‰ salinity and 23 °C. Each sea anemone microsomal preparation consisted of ~50 animals for _A. elegantissima_, 30 animals for _B. cavernata_, 1–2 animals for _A. xanthogrammica_, and only 1 animal for _C. gigantea_. Tentacles were discarded and the columnar region of the sea anemone was immediately submerged in homogenization buffer. Procedures described in Chapter 2 and 3 were followed for microsome preparation and determination of protein concentration.

Western Blots

Proteins were separated on 10% SDS-polyacrylamide gels (Laemmli, 1970) and transblotted for 50 min onto nitrocellulose with 10 mM Tris-HCl, 100 mM Glycine, and 10% methanol transfer buffer (Towbin, 1979). The blot was blocked overnight with 5% Carnation powdered milk in 50 mM Tris-HCl with 20 mM sodium chloride (TBS), incubated with the appropriate antibody for 1 hour, the appropriate secondary antibody containing a biotinylated conjugate for 1–2 hour, and Sigma ExtrAvidin™ for 30 min. Between each of these incubation
steps, the blot was washed with TBS four times for 5 min intervals. Prior to developing the color of the bands, the blot was rinsed well with distilled water. The color was developed with 100 mM sodium bicarbonate (pH 9.8) containing 1 mM magnesium chloride plus 5-bromo 6-chloro 3-indolyl phosphate (15 mg BCIP per 500uL DMF) and nitroblue terazolium (30 mg NBT per 500 uL 70% DMF). The developer was prepared just prior to staining. The color development was stopped by rinsing and then soaking the blot in distilled water for 10 min.

**Purification Procedures**

The primary focus of the purification efforts for this study was to isolate the 40 KDa protein. Thus, the purity of the 50-60 KDa proteins was monitored only during the design stages of the purification procedure while the 40 KDa protein was monitored throughout the entire process. All purification steps were performed at 4 °C. Protein concentrations were determined by a 96-well microplate reader fluorescamine assay (see Chapter 2). Since a flat baseline could not be maintained from a UV monitor, the absorbance was read for each fraction at 280, 295, and 417 nm on a Hitachi dual beam U-3110 spectrophotometer. One-dimensional (1D) gels and Western blots were used to monitor the purification steps. Gels were coomassie blue stained (0.1% coomassie, 40% methanol, 10% acetic acid) for 1 hour and then destained (25% methanol, 10% acetic acid) for several hours.

**Partial Purification of 40 KDa Protein:** The distribution of the 40 KDa immunoreactive protein was examined in each of the *A. xanthogrammica* tissue regions (described in Chapter 3) through Western blots probed with the CYP2K antibody (Fig 4-1). The antibody strongly recognized a 40 KDa band in each tissue region. Any differences between the intensity of recognition by the 2K antibody in the different tissue regions appeared to be only minor. Thus, the microsomes used for the 40 KDa purification were prepared from the entire columnar region of *A. xanthogrammica.*
Fig. 4-1. Western blots probed with CYP2K1 antibody (polyclonal rabbit anti-trout) showing recognition of a 40 KDa protein in *A. xanthogrammica* tissue regions: O, outer; I, inner; S, soft; and T, tentacular. All lanes contain 30 µg protein.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
A. xanthogrammica microsomes (12-14 mg protein /mL) were initially solubilized with 1.5% sodium cholate. These microsomes were slowly inverted about five times until the solution was clear, and then immediately diluted with buffer for a final concentration of 100 mM potassium phosphate (KPO₄; dibasic brought to appropriate pH with monobasic) pH 7.4 containing 0.75% sodium cholate, 20% glycerol, 1 mM EDTA, 1 mM CaCl₂, and 1 mM NaCl. This solution was mixed slowly with a stir plate for 40 min and then centrifuged for 65 min at 100,000 x g. The supernatant was removed immediately.

After solubilization of the microsomes, a buffered solution of 2% E911 was slowly added to the supernatant for a final concentration of 0.2% E911. The solubilized microsomes (~ 30 mL; 8 mg protein/mL) were mixed with 60 mL DEAE (diethylaminoethyl) sephacel material. The DEAE sephacel (60 mL) had been prerinsed with 120 mL of Buffer A (20 mM KPO₄ pH 7.8 w/ 10% glycerol, 0.2% E911). Each solution added to the DEAE was mixed slowly by hand for 20 min prior to filtering it through a buchner funnel under low water pressure. The DEAE was washed by mixing it with 30 mL Buffer A. This wash was combined with the flow through from the column (DEAE wash). Due to rinsing of the column material, the DEAE wash had a final volume of approximately 80 mL. The remaining protein was eluted from the DEAE by mixing it with 30 mL Buffer A containing 0.4 M KCl (DEAE elution).

The CM (carboxymethyl) sepharose column (1.5 cm x 12.5 cm) was preequilibrated with two column volumes of Buffer B (20 mM KPO₄ pH 6.4 w/ 10% glycerol, 0.2% E911). The pH of the DEAE wash (~ 80 mL; 0.5 mg protein/mL) was adjusted to pH 6.4 and loaded on the CM sepharose column at a flow rate of 0.5 mL/min. The fraction size collected was 1 mL. The column was washed with two column volumes of Buffer B (CM wash), then eluted with two column volumes of Buffer B containing 0.1 M KCl (CM 0.1 M elution), and eluted again with two column volumes Buffer B containing 0.4 M KCl (CM 0.4 M elution). This procedure resulted in the partial purification of the 40 KDa protein.
An additional step examined for 40 KDa purification scheme, but not used in the final protocol, was the ability of the chloramphenicol column material to retain the 40 KDa protein. This was performed by gently mixing either 100, 200, or 300 uL solubilized microsomes with 1.5 mL chloramphenicol column material for 5 min after preequilibrating the resin with Buffer C (100 mM KPO₄ (pH 7.4) containing 20% glycerol and 0.3% sodium cholate). The solubilized microsomes were removed after allowing the resin to settle; then the resin was rinsed with Buffer C, and finally mixed with 300 uL Buffer C containing 0.4 M KCl. Each of these samples was analyzed through Western blots probed with the polyclonal CYP2K antibody.

Steps Towards Purification of 50-60 KDa Proteins: The A. xanthogrammica microsomes (12-14 mg protein/mL) were initially solubilized as described above. These solubilized microsomes were either polyethylene glycol 6000 (PEG) precipitated or run on an n-octylamine column. For the PEG precipitation, the solubilized microsomes (~ 9 mg protein/mL) were mixed with a known percentage of PEG for 20 min and then centrifuged at 100,000 x g for 40 min. The precipitate was saved and the supernatant mixed again with a higher concentration of PEG. This procedure was repeated until all of the PEG precipitated samples were prepared. An example of ranges of PEG precipitates collected from a batch of solubilized microsomes is: (1) 0-6%, 6-10%, 10-14%, and 14-18% or (2) 0-8%, 8-12%, 12-16%, and 16-20%. PEG precipitation is described in detail by Ingham (1990).

In regards to the n-octylamine column, the column was preequilibrated with Buffer D (10 mM KPO₄ pH 7.4 containing 20% glycerol, 0.1% sodium cholate). The solubilized microsomes were diluted with distilled water prior to loading on the column, such that the buffer of the solubilized microsomes was the same concentration as the equilibration buffer. These microsomes (~ 15 mg protein at ~ 2 mg protein/mL) were loaded on the n-octylamine column (1.5 cm x 11 cm) at 0.4 mL/min. The fraction size collected was 1 mL. The column was washed with two column volumes of the Buffer D, eluted with two column volumes of Buffer D
containing 0.1% E911, eluted again with Buffer D containing 0.2% E911, and finally eluted with 100 mM KPO₄ pH 7.4 containing 20% glycerol, 0.2% sodium cholate, 0.2% E911. The fractions containing a positive 295 nm absorbance were combined. The wash and elutions were each mixed with 0.2 g Amberlite XAD-2 beads/mL sample for 1 hour (to remove detergent), filtered through nylon, and finally concentrated about 10-fold with Amicon Centriprep 30 as recommended by the manufacturer.

2-Dimensional (2D) Gel Electrophoresis

TCA/Acetone Precipitation: The samples were precipitated in 90% TCA and 10% acetone overnight at -20 °C. After precipitation, the samples were centrifuged at 42,000 x g on a Beckman Ultracentrifuge. The pellets were washed twice with 80% ice cold acetone/water and then centrifuged again. Pellets were air dried for 10 min, and then solubilized in 120 – 350 µL (depending on the size of the pellet) of 8M urea and 4% CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate). The Pierce micro BCA protein assay was used to determine protein concentration, using BSA as the standard.

2D Gels (pH 3-10): Isoelectric focusing was carried out using 3-10 Immobline DryStrips on an IPGphor isoelectric focusing instrument (Amerham Pharmacia Biotech, Uppsala Sweden). An appropriate volume of extract was mixed with rehydration buffer (6M Urea, 2% CHAPS, 20 mM DTT, 0.5% V/V 3-10 IPG buffer, and trace of bromophenol blue) to a final volume of 125 µL and the sample rehydration was loaded onto the IPG strip for a period of 10 hours as instructed by the manufacturer (loaded 35 µg protein). The sample was focused using the following run conditions: 500 V for 250 Vhr, 1000V for 500 Vhr and 8000V for 8000 Vhr. Samples were then frozen at -40 °C until the second dimension was run.

Strips were prepared for second dimension separation by shaking for 15 min in reducing SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6M urea, 30% V/V glycerol, 2% SDS,
trace bromophenol blue, 20 mM DTT) and 15 min in alkylation SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6M urea, 30% V/V glycerol, 2% SDS, trace bromophenol blue, 135 mM iodoacetamide). The second dimension was run on a Mini VE (Amersham Pharmacia Biotech) electrophoresis system using 12.5% PAGE gels (1 mm thick). Gels were run at 35 mA for a period of 1.5 hours and subsequently stained using the Plus One Protein Silver staining kit (Amersham Pharmacia Biotech) in a Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech). Gels for blotting were transferred to either Hybond C plus nitrocellulose membrane or Hybond P PVDF membranes (Amersham Pharmacia Biotech) using the Mini VE blotting Unit (Amersham Pharmacia Biotech). Transfers were at 35 V for 1 hr in 20 mM Tris-HCl, 200 mM Glycine, and 20% methanol (Towbin et al., 1979).

2D Gels (pH 6-11): Isoelectric focusing of basic proteins was carried out using 7 cm 6-11 Immobiline Dry Strips on a MultiPhor isoelectric focusing unit (Amersham Pharmacia Biotech). Strips were rehydrated in rehydration buffer (6M Urea, 2% CHAPS, 20 mM DTT, 0.5% V/V 6-11 IPG buffer, and trace bromophenol blue) per instruction of the manufacturer, using Immobiline DryStrip reswelling tray and 100 µL of sample cup loaded onto the strips (loaded 35-40 µg protein). Strips were focused using the protocol recommended by the manufacturer.

Strips were prepared for second dimension separation by shaking for 15 min in reducing SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6M urea, 30% V/V Glycerol, 2% SDS, trace bromophenol blue, 20 mM DTT) and 15 min in alkylation SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6M urea, 30% V/V glycerol, 2% SDS, trace bromophenol blue, 135 mM iodoacetamide). Second dimension for the 6-11 strips were electrophoresed as stated above. The Strips were either coomassie blue stained or transferred as stated above. These studies indicate that a 1 hour transfer time at 1A was more effective that a transfer time of 2 hours.
N-terminal Sequence Analysis

Blots used for N-terminal sequence analysis were transferred to PVDF Protein Sequencing Membrane (BioRad) as described above, except 0.1 mM thioglycolate was included in the upper running buffer for the 1D gel and the running and transfer buffers were kept cool for both 1D and 2D gels. These membranes were coomassie stained (0.025% coomassie, 40% methanol, 6 drops HCl/100 mL stain) for 1 – 2 min and then destained with 50% methanol. The band or spot was cut from the sequencing membrane and sent for N-terminal sequence analysis to Dr. Richard Cook at Baylor College of Medicine in Houston, Texas.

Spectral Properties

An Hitachi dual beam U-3110 spectrophotometer was used to examine the sea anemone microsomal cytochrome P450 spectra by the carbon monoxide (CO)-difference spectrum of sodium dithionite (DTN)-reduced samples (Omura & Sato, 1964a). The spectra were performed by adding the DTN and background-correcting prior to the addition of the CO. The microsomal samples (600 μL) were diluted with 600 μL 100 mM potassium phosphate (pH 7.4) containing 10% glycerol; final protein concentrations were highly variable between different samples. This sample was split between the reference and test cuvettes for analysis. After the addition of the CO and DTN, the solubilized microsomes were scanned immediately.

Peroxidase Activity

The rate of peroxidation of caffeic and ferulic acid was examined as an increase in absorbance over 5 min (Bernal et al., 1995) on a Hitachi dual beam U-3110 spectrophotometer (caffeic acid, 312 nm; ferulic acid, 310 nm). The caffeic acid peroxidation reactions contained a final concentration of 0.15 mM caffeic acid, 160 – 260 μg microsomal protein, and 4.0 mM H₂O₂ in 1.1 mL 100 mM Tris-acetate, pH 5.0, whereas the ferulic acid peroxidation reactions contained a final concentration of 0.2 mM ferulic acid, 160 – 260 μg microsomal protein, and 1.0 mM H₂O₂ in 1.1 mL 100 mM Tris-acetate, pH 5.0. A 10 mM stock solution of either caffeic
or ferulic acid was prepared fresh in HPLC-grade methanol. To eliminate background noise, the Tris-acetate buffer was degassed prior to diluting the substrate with this buffer and both reactions were performed in tandem cuvettes. Activities were calculated based on an extinction coefficient for caffeic acid of 11.2 cm$^{-1}$mM$^{-1}$ at 312 nm and ferulic acid of 11.0 cm$^{-1}$mM$^{-1}$ at 310 nm. Reactions were initiated with the addition of microsomes.

RESULTS

Immunodetection with Cytochrome P450 Antibodies

Several cytochrome P450 antibodies from mammalian and fish sources recognized proteins in the sea anemones Anthopleura elegantissima, Anthopleura xanthogrammica, and Bunodosoma cavernata. A microsomal protein between the typical 50-60 KDa molecular weight was weakly recognized between 30-40 µg protein in Western blots (Figs. 4-2 to 4-7) probed with monoclonal mouse anti-scup CYP1A1, polyclonal rabbit anti-rat CYP2E1, and polyclonal rabbit anti-trout CYP3A1. Some of these species were further examined with several additional P450 antibodies: polyclonal rabbit anti-scup CYP2B1; polyspecific rabbit anti-rat CYP2C11$^+$; and, polyclonal rabbit anti-trout CYP2K1. The molecular weights of these 50-60 KDa immunoreactive proteins were estimated based on biotinylated standards (Table 4-1). In contrast to the other P450 antibodies, a 50-60 KDa protein was not detected by the polyclonal sheep anti-rat CYP4A at 30 µg protein in A. elegantissima (data not shown).

Recognition of the 50-60 KDa protein was weak, but relatively consistent in A. elegantissima and A. xanthogrammica between 30-40 µg protein. In contrast, it was not as consistent in B. cavernata and was often not detected in Condylostomis gigantea between 30-40 µg protein. Longer staining with the NBT/BCIP resulted in a darker band within the 50-60 KDa region; however, there was also a coinciding increase in non-specific recognition of protein bands (>65 KDa). Detection of the 50-60 KDa proteins was not noticeably enhanced by lengthening the incubation time with the primary antibody to over 1 hour. While the use of detergents may

77

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Fig. 4-2. Western blots probed with CYP1A antibody (monoclonal mouse anti-scup) showing recognition of a 50-60 KDa protein in several species of sea anemone microsomes: BC, B. cavernata; AE\textsuperscript{CA}, A. elegantissima from California; AE\textsuperscript{AK}, A. elegantissima from Alaska; AX, A. xanthogrammica; and, CG, Condylactis gigantea. All lanes contain 30 μg protein.
Fig. 4-3. Western blot probed with CYP2E1 antibody (polyclonal rabbit anti-rat) showing recognition of a 50-60 KDa protein in several species of sea anemone microsomes as compared to rat liver microsomes: BC, *B. cavernata*; AE<sup>AK</sup>, *A. elegantissima* from Alaska; AX, *A. xanthogrammica*; CG, *Condylactis gigantea*; and, R, rat. All of the sea anemone lanes contain 30 µg protein and the rat lane contains 2 µg protein. The lower molecular weight protein detected in the rat microsomes is most likely a result of degradation.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Fig. 4-4. Western blot probed with CYP3A antibody (polyclonal rabbit anti-trout) showing recognition of a 50-60 KDa protein and a lower molecular weight protein (~28 KDa) in several species of sea anemone microsomes: BC, *B. cavernata*; AE<sup>CA</sup>, *A. elegantissima* from California; AE<sup>AK</sup>, *A. elegantissima* from Alaska; AX, *A. xanthogrammica*; and, CG, *Condylactis gigantea*. All lanes contain 30 µg protein.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Fig. 4-5. Western blot probed with CYP2B1 antibody (polyclonal rabbit anti-scup) showing recognition of a 50-60 KDa protein in sea anemone microsomes as compared to trout liver microsomes: AX, *A. xanthogrammica* and T, rainbow trout. The *A. xanthogrammica* lane contains 40 μg protein and the trout lane contains 5 μg protein.
Fig. 4-6. Western blot probed with CYP2C11 \textsuperscript{+} antibody (polyspecific rabbit anti-rat) showing recognition of a 50-60 KDa protein in several species of sea anemone microsomes as compared to rat liver microsomes: AE\textsubscript{CA}, *A. elegantissima* from California; AE\textsubscript{AK}, *A. elegantissima* from Alaska; and, R, rat. All of the sea anemone lanes contain 30 \(\mu\)g protein and the rat lane contains 2 \(\mu\)g protein.
Fig. 4-7. Western blots probed with CYP2K1 (polyclonal rabbit anti-trout) showing recognition of a 50-60 KDa protein and a 40 KDa protein in sea anemone as compared to: (A) Liver microsomes of several vertebrates: AE, _A. elegantissima_; R, rat; T, rainbow trout; G, alligator; and, D, muscovy duck. The rat and trout lanes contain 1 µg protein and the alligator, duck, and sea anemone lanes contain 5 µg protein. (B) Microsomes from several marine invertebrates and trout liver: AX, _A. xanthogrammica_; m, mussel; cb, crab; cw, crawfish; ss, sea star; and, T, trout. All invertebrate lanes contain 40 µg protein and the trout lane contains 5 µg protein.
Table 4-1. Estimated molecular weights of immunoreactive bands of several sea anemone species detected by western blots with several vertebrate P450 antibodies.

<table>
<thead>
<tr>
<th></th>
<th>AE&lt;sup&gt;AK&lt;/sup&gt;</th>
<th>AE&lt;sup&gt;CA&lt;/sup&gt;</th>
<th>AX</th>
<th>BC</th>
<th>CG</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-scup CYP1A</td>
<td>52</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>52</td>
</tr>
<tr>
<td>anti-trout CYP3A</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>anti-rat CYP2E</td>
<td>52</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>n.d.</td>
</tr>
<tr>
<td>anti-scup CYP2B</td>
<td>n.d.</td>
<td>59</td>
<td>59</td>
<td>59</td>
<td>n.d.</td>
</tr>
<tr>
<td>anti-trout CYP2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

AE, <i>A. elegantissima</i>; AX, <i>A. xanthogrammica</i>; BC, <i>B. cavernata</i>; CG, <i>C. gigantea</i>

* molecular weights were estimated based on biotinylated standards from one gel.

<sup>b</sup> BC and CG were run at 10 µg protein (too dilute to detect the 50-60 KDa protein). CYP2C11<sup>+</sup> blots have only been examined with prestained standards; thus, the molecular weights are not included in these values.

prove to be useful in eliminating the non-specific binding, the addition of 0.1% Triton X-100 in the wash steps resulted in the loss of all recognition by anti-CYP2C11<sup>+</sup>.

For all samples analyzed, the recognition pattern in each species was identical for the CYP1A, 2E, and 3A antibodies (Figs. 4-2 to 4-4). These antibodies recognized one protein between 50-60 KDa with the same molecular weight in <i>A. elegantissima</i> (from California), <i>A. xanthogrammica</i>, and <i>B. cavernata</i>. In contrast, there were two more strongly recognized proteins of a slightly higher molecular weight detected by these antibodies in the <i>A. elegantissima</i> collected from Alaska. The Alaskan <i>A. elegantissima</i> may have also contained the slightly lower molecular weight protein, but it was a very faint band. Most striking was that the doublet was not observed in <i>A. elegantissima</i> from California. Although the blot probed with anti-CYP2E only contained the higher band of the doublet of the Alaskan <i>A. elegantissima</i> (Fig. 4-3), the doublet was present at higher protein concentrations (data not shown).
The recognition pattern for CYP2C11* was similar (Fig. 4-6), but not identical to the CYP1A, 2E, and 3A antibodies. Unlike these antibodies that detected a doublet only in the Alaskan *A. elegantissima*, the CYP2C11* antibody detected a doublet in *A. elegantissima* from both California and Alaska. Curiously, the doublet detected in the Alaskan *A. elegantissima* had a higher molecular weight than did the doublet in the Californian *A. elegantissima*. The recognition of multiple bands between 50-60 KDa in the sea anemone is consistent with the polyclonal CYP2C11* antibody being a polyspecific antibody in the rat (Bandiera et al., 1995).

The CYP2B and CYP2K antibodies’ cross-reactivity has only been shown at 40 µg protein with *A. xanthogrammica*; however, these antibodies have been examined in two other species (Table 4-1). The recognition pattern of anti-CYP2B was clearly quite different from each of the other antibodies (Fig. 4-5). There was only one 50-60 KDa protein detected by anti-CYP2B, and it was of a much higher molecular weight (~ 59 KDa) than the 50-60 KDa bands recognized by the other P450 antibodies. Finally, the CYP2K also only detected one protein between 50-60 KDa at approximately 55 KDa. While additional species were tested with the CYP2K antibody to examine its recognition of a 40 KDa protein (discussed below), the protein concentration was typically too low to detect the 50-60 KDa protein in those blots.

Several higher molecular weight proteins (>65 KDa) were also regularly detected in the sea anemone microsomes. Such proteins have also been reported in the spiny lobster, *P. argus* (Boyle & James, 1996) and worm, *E. fetida* (Achazi et al., 1998). In the case of the sea anemone, these proteins were detected even when the blot was not incubated with the primary antibody – indicating that they were the result of non-specific binding. Similarly, substantial low molecular (< 28 KDa) weight, non-specific binding was detected in digestive gland microsomes from the mussel, *M. edulis* (Peters et al., 1998a). Decreasing the incubation times with the secondary antibody and the ExtrAvidin™ minimized non-specific binding in the sea anemone. In contrast to the high molecular weight proteins, the 50-60 KDa sea anemone proteins did not

---

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
result from non-specific binding because they required staining with the primary antibody prior to the secondary staining system. In addition, the intensity of the 50-60 KDa protein recognized by each of the antibodies increased as the microsomal protein loaded on the gel was increased.

Relatively unique molecular weight microsomal proteins were strongly recognized in the sea anemone by both anti-CYP3A1 (Fig. 4-4) and anti-CYP2K1 (Fig. 4-7). Anti-CYP3A1 strongly recognized a protein of ~28 KDa at 30 µg protein and anti-CYP2K1 strongly cross-reacted with a protein of ~40 KDa at only 10 µg protein. Interestingly, the 40 KDa protein is also recognized by the CYP2K1 antibody in several other marine invertebrates, but it was not even weakly detected in the rainbow trout (Oncorhynchus mykiss), muscovy duck (Cairina sp.), alligator (Alligator mississippiensis), or rat (Rattus rattus) (Fig. 4-7A). The 40 KDa protein was recognized at equal intensity in microsomes of the mussel digestive gland, M. edulis, and crayfish green gland, Procambarus clarkii, while it was only weakly detected in the crab hepatopancreas, Callinectes sapidus, and not detected in the sea star pyloric caeca, Asterias rubens (Fig. 4-7B). In contrast to the 40 KDa protein, the 28 KDa protein detected in the sea anemone by anti-CYP3A was not detected in the microsomal fraction of these invertebrates (i.e., crab, sea star, mussel, and crayfish) or the rainbow trout. However, the CYP3A antibody detected a slightly lower molecular weight protein in the crab and a slightly higher molecular weight protein in the sea star (data not shown). It is possible that either the 28 or 40 KDa proteins detected in these sea anemones is a degraded P450 protein; however, the size or quantity of the protein detected was not altered by the use of various protease inhibitors, reducing agents, and chelators in the microsomal preparations.

Steps Examined Towards Purification of 50-60 KDa Protein

Based on the CO-difference (DTN-reduced) spectra, the microsomes solubilized equally well over a wide range of mg protein:mg sodium cholate. The 450 nm peak was consistently observed at a 1:1 ratio of protein to detergent concentration. However, slightly more P450 was
observed in microsomes solubilized at a 1.0:1.5 concentration of detergent (8 mg protein: 12 mg sodium cholate per mL) for ~30 s and then further solubilized for 40 min after diluting 1:2 with 100 mM potassium phosphate buffer (containing 20% glycerol). The P450 did not appear to be too sensitive to high detergent concentrations; there was only slightly less P450 observed after solubilizing for 40 min in a 1:3 ratio of detergent (8 mg protein: 24 mg sodium cholate per mL).

Polyethylene glycol 6000 (PEG) precipitation resulted in the precipitation of 50-60 KDa proteins in the 14-20% PEG pellet that cross-reacted with the polyclonal goat anti-rabbit CYP2B1/2 (Oxford Biomedical), polyclonal rabbit anti-rat CYP2E, and polyclonal rabbit anti-trout CYP3A antibodies (Fig. 4-8). The DTN-reduced, CO-difference spectra also indicated that P450 was present in this 14-20% PEG pellet, which contained these 50-60 KDa immunoreactive bands, and in the 8-12% PEG pellet. For the 0-8% pellet, there was too much noise in the spectra to assess if P450 was present. While P450 was present in the 8-12% PEG pellet, the coomassie blue gel indicated that this pellet contained a lot more contaminating proteins than the 12-18% PEG pellet. Thus, based on a compromise between purity and yield, the optimal precipitation range for the sea anemone P450 in general appeared to be 12-18% PEG 6000. This does not preclude the possibility that a particular P450 isoform would precipitate more readily at a slightly different percentage range. Western blots would need to be performed on each of the PEG pellets to optimize for precipitation of particular isoforms.

DEAE and n-Octylamine chromatography are commonly used in P450 purification protocols because of their ability to bind numerous P450 isoforms. The n-octylamine column retained a protein between 50-60 KDa that was weakly recognized by the polyclonal goat anti-rabbit CYP2B1/2 (Oxford Biomedical) and polyclonal rabbit anti-rat CYP2E (data not shown). However, there was no indication of a 417 nm absorbance in any of the fractions; thus, the CO-difference binding spectra were not examined. As for DEAE chromatography, the coomassie blue stained gel indicated that several 50-60 KDa proteins were retained by the DEAE column.
Fig. 4-8. Western blots of PEG 6000 precipitated sodium cholate solubilized sea anemone, *A. xanthogrammica*, microsomes probed with the following antibodies: CYP1A (monoclonal mouse anti-scup), CYP2B1/2 (polyclonal goat anti-rabbit – Oxford Biomedical), CYP2E1 (polyclonal rabbit anti-rat), or CYP3A (polyclonal rabbit anti-trout). Lanes: 1, *A. xanthogrammica* microsomes; 2, solubilized *A. xanthogrammica* microsomes; 3, 0-14% PEG pellet; and, 4, 14-20% PEG pellet.
material used for the purification of the 40 KDa protein (Fig. 4-9A). These 50-60 KDa proteins may prove to be P450. Currently, Western blots have not been performed with the various P450 antibodies beyond anti-CYP2K (Fig. 4-9B). Even the CYP2K blots were developed to provide recognition of the 40 KDa protein, not the 50-60 KDa protein. Finally, DTN-reduced, CO-difference spectra demonstrated that the DEAE eluate contained a 418 nm chromophore, but not a 450 nm chromophore.

**Partial Purification of CYP2K Immunoreactive 40 KDa Protein**

The first step in the purification of the 40 KDa protein involved solubilization of the *A. xanthogrammica* microsomes with sodium cholate. These solubilized microsomes were then mixed with the DEAE sephacel column material (at pH 7.8). Based on Western blots probed with the CYP2K antibody, the 40 KDa protein was not retained by the DEAE sephacel; instead, it was found in the wash (Fig. 4-9B). However, due to the volume of the DEAE wash, most proteins (including the 40 KDa protein) were too dilute to observe on the coomassie blue stained gels (Fig. 4-9A). Most of the proteins in the DEAE elution were also faint, but there was a clear 50-60 KDa band. Further purification was achieved by loading the DEAE wash onto a CM sepharose column. Initial tests of the CM sepharose column had shown that a pH of 6.4 was required for retention of the 40 KDa protein; therefore, prior to loading the wash, its pH was adjusted appropriately. Based on the 417 nm absorbance, there were heme-centered proteins present in the wash, the 0.1 M KCl eluate, and the 0.4 M KCl eluate (Fig. 4-10). The Western blot and coomassie blue stained gels indicated that the 40 KDa protein was present in the 0.1 M KCl eluate. According to the coomassie blue gels (Fig. 4-9A), there were additional proteins in the vicinity of the 40 KDa protein, a few lower molecular weight proteins (< 20 KDa), and several higher molecular weight proteins (> 65 KDa). The Western blots probed with anti-CYP2K indicated that the 40 KDa protein was completely eluted at 0.1 M KCl (Fig. 4-9A). There was no indication of cross reactivity in either the CM wash or the 0.4 M KCl eluate.
Fig. 4-9. Partial purification of the 40 KDa protein from the sea anemone, *A. xanthogrammica*. (A) Coomassie blue stained gel and (B) Western blot probed with CYP2K1 antibody (polyclonal rabbit anti-trout). Lanes: 1, solubilized microsomes (diluted 1:10); 2, DEAE wash; 3, DEAE elution; 4, CM wash fraction 89; 5–9, CM 0.1 M elution fractions 32, 34; 36; 38; and, 40.
Fig. 4-10. CM Chromatograph of 280, 295, and 417 nm absorbing material in fractions following application of solubilized sea anemone, *A. xanthogrammica*, microsomes to DEAE-sephacel. The 40 KDa protein is eluted with 0.1 M KCl.
Based on the DTN-reduced, CO-difference spectra, the only step in the 40 KDa purification scheme that contained a 450 nm peak was the solubilized microsomes (data not shown). The 418 nm chromophore was detected in the solubilized microsomes and DEAE eluate, but not in the DEAE wash. The CM wash contained a very small, broad 410-460 nm peak. While the characteristic P450 spectral properties were not detected through the purification procedure, the presence of the 40 KDa protein could be monitored by a 417 nm absorbance.

In the process of designing a purification procedure, several additional procedures were examined that have been more typically used to purify P450 isoforms. These procedures included PEG 6000 precipitation, n-octylamine chromatography, and chloramphenicol chromatography. Based on Western blot analysis with the CYP2K antibody, most of the 40 KDa protein precipitated between 12-18% PEG (data not shown). Coomassie blue stained gels indicated that there was a reduction in the concentration of many proteins (including the 40 KDa protein), but that there was not a substantial enough decrease in the overall number of proteins present to justify the amount of 40 KDa protein lost. These results were not altered by narrowing the percentage range of PEG used to precipitate the protein to 12-16%, 12-14%, 14-18%, or 15-18% PEG. The 40 KDa protein was not retained by the n-octylamine column, which is a general hydrophobic column that binds numerous P450 enzymes, or by the chloramphenicol column material, which is a commonly-used affinity column that binds P450 isoforms from the CYP1, 2, and 3 families of vertebrates. In regards to n-octylamine column, there was also no indication of a 417 nm absorbance in any of the fractions.

Analysis of the Partially Purified 40 KDa Protein

The purification scheme described above resulted in a partial purification of the 40 KDa protein, which was further characterized by N-terminal sequence analysis. The first 15 amino
acids obtained from the N-terminal region of the partially purified 40 KDa protein were: T I K F G I N G F G R I G R L. This N-terminal sequence most closely matched glyceraldehyde 3-phosphate dehydrogenase (GAPD), but also had sequence identity with thymidine phosphorylase, ubiquinol cytochrome c reductase, 20 α-hydroxysteroid dehydrogenase, and lactate dehydrogenase (Table 4-2). In contrast, there is no sequence similarity with the N-terminal region of trout CYP2K, lobster CYP2L, or clam CYP30 (Brown et al., 1998; Buhler et al., 1994; James et al., 1996) or the internal region of the trout CYP2K. After this finding, the polyclonal trout CYP2K antibody and a monoclonal mouse anti-rabbit GAPD antibody (Biogenesis, NH; # 4699-9555) were analyzed for their ability to cross-react with the cytosolic and microsomal fractions of sea anemone and trout.

The polyclonal trout CYP2K antibody did not recognize a purified rabbit muscle GAPD (Sigma G-5262) nor a 40 KDa protein in microsomal or cytosolic fraction of the trout (Fig. 4-11A). The expected 54 KDa protein was the only protein detected in trout microsomes by the CYP2K antibody. In contrast, the trout CYP2K antibody recognized a 40 KDa protein in the sea anemone microsomes and cytosol. The recognition was at least 5-fold stronger in the cytosol than in the microsomes. In regards to the GAPD antibody, this antibody recognized a purified rabbit muscle GAPD; however, it did not detect a 40 KDa protein in either the microsomal or cytosolic fraction of trout or sea anemone (Fig. 4-11B). It also did not recognize the partially purified 40 KDa protein from sea anemone microsomes. Finally, there were a few high molecular weight bands (> 65 KDa) faintly recognized by both of these antibodies in some of the samples; however, as mentioned above, these bands appear to be due to non-specific binding.

Comigration of a GAPD with the CYP2K-immunoreactive protein on 1D gels could account for the immunoreactivity of the band and the N-terminal sequence obtained; thus, 2-dimensional gel electrophoresis was performed (Fig 4-12 to 4-14). Because it is unlikely that two proteins would have the same molecular weight and isoelectric focusing points, they should
Table 4-2. N-terminal sequence comparison of sea anemone 40 KDa protein with several P450 and GAPD proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Residue Number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 KDa protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1D protein</td>
<td>T I K F G I N G F G R I G R L</td>
<td></td>
</tr>
<tr>
<td>2D (pI ~ 8.7)</td>
<td>T I K F G I N G F G R I G R L</td>
<td></td>
</tr>
<tr>
<td>2D (pI ~ 8.5)</td>
<td>T I K F G I N G F G - - - - -</td>
<td></td>
</tr>
<tr>
<td>GAPD*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lobster</td>
<td>- - K F G I N G F G R I G R L</td>
<td>P00357&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. elegans</td>
<td>- - - - G I N G F G R I G R L</td>
<td>P17330&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>yeast</td>
<td>V R V A I D G F G R I G R L</td>
<td>Buehner et al., 1974</td>
</tr>
<tr>
<td>pig</td>
<td>V K V G V D G F G R I G R L</td>
<td>Buehner et al., 1974</td>
</tr>
<tr>
<td>lobster</td>
<td>S K I G I D G F G R I G R L</td>
<td>Buehner et al., 1974</td>
</tr>
<tr>
<td>additional proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>thymidine phosphorylase</td>
<td>- - V K G I N E F G R I G R L</td>
<td>P19663&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ubiquinol cytochrome c reductase</td>
<td>I R I G F D G F G R I N R</td>
<td>S20014&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 α-hydroxysteroid dehydrogenase</td>
<td>V K V A I N G F G R</td>
<td>A44755&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>lactate dehydrogenase</td>
<td>M K I G I V G L G R V G</td>
<td>P16115&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P450 isoforms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trout CYP2K1</td>
<td>M S L I E D I L Q T S S S T V T</td>
<td>Buhler et al., 1994</td>
</tr>
<tr>
<td>lobster CYP2L</td>
<td>M L T G L L L L L L - V V I V Y</td>
<td>James et al., 1996</td>
</tr>
<tr>
<td>clam CYP30</td>
<td>M E I L G M V N L P T W L V C</td>
<td>Brown et al., 1998</td>
</tr>
</tbody>
</table>

* GAPD, glyceraldehyde 3-phosphate dehydrogenase.
<sup>b</sup> Ascension number from GenBank.
Fig. 4-11. Western blot probed with (A) CYP2K1 antibody (polyclonal rabbit anti-trout) and (B) glyceraldehyde 3-phosphate dehydrogenase (GAPD) antibody (monoclonal mouse anti-rabbit). The samples probed were: AE, *A. elegantissima* cytosol; AE, *A. elegantissima* microsomes; T, trout liver cytosol; T, trout liver microsomes; and, GAPD. Blot B contained the partially purified *A. xanthogrammica* 40 KDa protein designated as 0.1 M. The protein concentration loaded per lane for *A. elegantissima* cytosol and microsomes was 20 μg, for trout cytosol or microsomes lanes was 2 μg, and for GAPD was 10 μg.
Fig. 4-12. Silver stained 2D gel (pH 3-10). The gel contains 35-40 μg of the partially purified 40 KDa A. xanthogrammica protein (CM 0.1 M eluate). The 3 and 10 above the gel mark the edge of range for the isoelectric focusing dimension; outside of these pH values the gel runs as a 1-dimensional gel.
Fig. 4-13. Western blot 2D gel (pH 3-10) probed with CYP2K1 antibody (polyclonal rabbit anti-trout). The gel contains 35-40 μg of the partially purified 40 KDa *A. xanthogrammica* protein (CM 0.1 M eluate).
Fig. 4-14. Coomassie stained 2D gel (pH 6-11). The gel contains 35-40 μg of the partially purified 40 KDa _A. xanthogrammica_ protein (CM 0.1 M eluate).
not comigrate on 2D gels. Each of the 2D gels were performed under denaturing conditions.

The silver-stained 2D gel (pH 3-10; 35-40 µg protein) demonstrated that there were multiple proteins at ~ 40 KDa (Fig. 4-12). The CYP2K antibody cross-reacted with about four 40 KDa spots between pI 8.5 and 9.5 and with one spot at about pI 8.0 (Fig. 4-13). The two center spots of the four were very strongly recognized, while the other spots were more weakly detected. There were several additional 40 KDa spots detected by the silver stain that are not recognized by the CYP2K antibody. Finally, three additional spots were detected by the CYP2K antibody and on the silver stained gel at a slightly lower molecular weight between pI 7 and 8. Although these additional spots look of equal intensity, they did not appear in the staining until well after the four 40 KDa proteins were recognized. When reading these gels, it is important to note that the pH values 3 and 10 above the gel mark the edges of the focusing region for the second dimension. Any protein on the IPG Strip outside this region runs like a 1D gel, not like a 2D gel.

The 2D coomassie blue stained gel (pH 6-11; 35-40 µg protein) indicated that three of the four spots (recognized by the CYP2K antibody) between pI 8.5 and 9.5 contained enough protein for N-terminal sequence analysis (Fig. 4-14). The estimated pIs of these denatured 40 KDa proteins are ~ 8.5, ~ 8.7, and ~ 8.9. Unlike 2D gels prepared with tube gels, the IPG strips are consistently linear over the entire pH range examined (Berkelman & Stenstedt, 1998). The N-terminal sequence was determined for two of the three spots. The first 15 amino acid residues for the center spot (pI ~ 8.7) were: T I K F G I N G F G R I G R L. These residues were identical to the first 10 residues of the 2D-gel protein at a pI of ~ 8.5 and the 15 residues obtained for the 40 KDa protein from the 1D-gel (Table 4-2).

**DISCUSSION**

This study indicates that multiple isoforms of P450 exist within the sea anemone. Several lines of evidence support this assertion. First, Western blots probed with several P450 antibodies demonstrated that sea anemone microsomes contained proteins between 50-60 KDa.
with epitope regions (i.e., similar amino acid sequences) common to multiple vertebrate P450 isoforms (i.e., CYP1A, 2B, 2C11*, 2E, 2K, and 3A). Second, these proteins are within the expected molecular weight range (i.e., 50-60 KDa) for P450. Third, the procedures examined towards purifying the 50-60 KDa proteins revealed proteins with additional physical characteristics similar to known P450 isoforms; for instance, they are precipitated by PEG and they bind to an n-octylamine column. Finally, the P450 binding spectra demonstrated that sea anemone P450 was concentrated in the same PEG pellet that concentrated the immunoreactive 50-60 KDa proteins.

In contrast to the 50-60 KDa immunoreactive proteins, the unique 40 KDa protein that is strongly recognized by the CYP2K antibody has characteristics that are atypical of cytochrome P450. For instance, the N-terminal region of the 40 KDa protein has no sequence identity to any portion of the trout CYP2K protein, the N-terminal region of the spiny lobster CYP2L1, or the N-terminal region of the clam CYP30 (Table 4-2) (Brown et al., 1998; James et al., 1996; Buhler et al., 1994). Further, the protein appears to be a cytosolic protein as opposed to a membrane protein. In addition to being a lower molecular weight protein than expected, it also has a higher pI than that expected for P450. Finally, the purification of this protein was relatively atypical for P450 and the later steps in the purification lacked the characteristic P450 spectral properties.

Immunoreactive 50-60 KDa Proteins

The recognition of several sea anemone microsomal proteins between 50-60 KDa by various vertebrate P450 antibodies indicates that the sea anemone contains multiple P450 isoforms. All of the antibodies used were prepared from a different vertebrate P450 isoform and their specificity has been examined in that vertebrate (Bandiera et al., 1995; Miranda et al., 1990; Park, 1986); however, their specificity has not been well established in the marine invertebrates. Until the specificity of these antibodies has been established, recognition by a
particular antibody indicates sequence similarity with that subfamily, but does not classify that protein as a member of that subfamily. Thus, the recognition in each of the sea anemone samples of the same molecular weight protein by several of the P450 antibodies (i.e., anti-CYP1A, -CYP2E, -CYP3A) could result from one (or both) of two possibilities: (1) one P450 isoform that contains epitope regions to several antibodies; or, (2) multiple P450 isoforms of the same molecular weight that each contain an epitope to one of these antibodies. Based on these possibilities, the CYP1A, 2E, and 3A antibodies could be recognizing a minimum of the same three isoforms or a maximum of nine isoforms in the sea anemone microsomes.

The literature supports the concept that these CYP1A, 2E, 3A antibodies recognize multiple isoforms of the same molecular weight in the sea anemone. For instance, in a mussel (M. edulis; Peters et al., 1998a) and a sea star (A. rubens; den Besten, 1998), two P450 antibodies (polyclonal rabbit anti-perch CYP1A and anti-trout CYP3A from Dr. Lars Forlin) each recognized more than one protein (i.e., proteins with different molecular weights). In contrast, these CYP1A and CYP3A antibodies recognized proteins of the same molecular weight in a different species of mussel (M. galloprovincialis; Peters et al., 1998b); however, the CYP1A antibody had a greater difference in the intensity of the bands recognized between M. galloprovincialis collected from two sites with different levels of pollutants. An increase in the intensity of recognition by only one of the two antibodies suggests that they are recognizing different P450 isoforms of the same molecular weight in this mussel. The polyclonal goat anti-rat CYP2E (from Oxford Biomedical) and the rabbit anti-trout CYP3A (from Dr. Lars Forlin) antibodies recognized different molecular weight proteins in M. galloprovincialis (Peters et al., 1998b), while they recognized the same molecular weight proteins in M. edulis (Peters et al., 1998a) (Table 4-3). It is important to note that these antibodies are not from the same source as those used in this study; however, they set a precedent in some invertebrate species for the
recognition of multiple isoforms of the same molecular weight with each containing an epitope region to one of the antibodies.

The recognition pattern detected by the CYP2B, 2K and 2C11* antibodies was quite different than the CYP1A, 2E, and 3A antibodies, indicating that the anti-CYP2B, -CYP2K, and -CYP2C11* are recognizing different P450 isoforms (Table 4-1; Figs 4-2 to 4-8). These results potentially indicate the presence of six more P450 isoforms; however, it is possible that some isoforms that were immunodetected by anti-CYP2K and -2C11* are identical to isoforms recognized by the CYP1A, 2E, and 3A antibodies.

The detection of a doublet in the Alaskan A. elegantissima as compared to the singlet in A. xanthogrammica and B. cavernata was not a species-specific difference because the doublet was not also detected in the Californian A. elegantissima. However, it may have been the result of differential expression. Multiple factors will alter the expression of certain P450 isoforms, such as season (i.e., season collected), diet, and exposure to inducing agents (e.g., Kirchin et al., 1992; Quattrochi & Lee, 1984; Snyder, 1998a; Sole, 1995; Stegeman & Hahn, 1994; Weinstein, 1995). The A. elegantissima (from Alaska) also contains a different symbiont than either of the other two Anthopleura. The more northern Anthopleura tend to contain a diatomaceous symbiont, while the more southern Anthopleura tend to contain an algal symbiont. Although the symbiont is not included in the microsomal preparation, any microsomal proteins produced as a result of the symbiotic relationship would not have been removed.

Differential expression of the P450 isoforms between the sea anemone species could also account for why the 50-60 KDa protein was detected in most A. elegantissima and A. xanthogrammica microsomal preparations, but tended to be less consistent in B. cavernata microsomes and often was not observed in C. gigantea microsomes. A difference in P450 expression was observed in the blue crab through Western blot analysis (Obserdorster et al., 1996). When blue crabs (C. sapidus) were exposed to tributyl tin, the polyclonal anti-scup
Table 4-3. Molecular weight of putative P450 isoforms detected by P450 antibodies.

<table>
<thead>
<tr>
<th>Species</th>
<th>Anti-P450</th>
<th>KDa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Invertebrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sea star, <em>A. rubens</em></td>
<td>scup CYP1A*</td>
<td>54</td>
<td>den Besten, 1998</td>
</tr>
<tr>
<td>Worm, <em>E.f. fetida</em> e</td>
<td>perch CYP1A</td>
<td>54, 65, 71</td>
<td>Achazi et al., 1998</td>
</tr>
<tr>
<td>Mussel, <em>M. edulis</em></td>
<td>perch CYP1A</td>
<td></td>
<td>Sole et al., 1996</td>
</tr>
<tr>
<td>Mussel, <em>M. edulis</em> e</td>
<td>perch CYP1A</td>
<td>42.5, 48.1</td>
<td>Peters et al., 1998a</td>
</tr>
<tr>
<td>Mussel, <em>M. galloprovincialis</em></td>
<td>perch CYP1A</td>
<td>54</td>
<td>Porte et al., 1995</td>
</tr>
<tr>
<td>Mussel, <em>M. galloprovincialis</em></td>
<td>perch CYP1A</td>
<td>48</td>
<td>Peters et al., 1998b</td>
</tr>
<tr>
<td>Chiton, <em>Cryptochiton stelleri</em></td>
<td>trout CYP1A</td>
<td>54</td>
<td>Schlenk &amp; Buhler, 1989</td>
</tr>
<tr>
<td>Bivalve, <em>Donax trunculus</em></td>
<td>scup CYP1A</td>
<td>n.d.</td>
<td>Yawetz et al., 1992</td>
</tr>
<tr>
<td>Sea star, <em>A. rubens</em></td>
<td>scup CYP2B</td>
<td>46</td>
<td>den Besten, 1998</td>
</tr>
<tr>
<td>Mussel, <em>M. edulis</em> e</td>
<td>rat CYP2B</td>
<td>52.8, 48.1, 43.9</td>
<td>Peters et al., 1998a</td>
</tr>
<tr>
<td>Mussel, <em>M. galloprovincialis</em></td>
<td>rat CYP2B</td>
<td>52</td>
<td>Peters et al., 1998b</td>
</tr>
<tr>
<td><em>Euglena gracilis</em></td>
<td>rat CYP2B*</td>
<td>50</td>
<td>Briand et al., 1993</td>
</tr>
<tr>
<td><em>Euglena gracilis</em></td>
<td>rat CYP2C11</td>
<td>50</td>
<td>Briand et al., 1993</td>
</tr>
<tr>
<td>Mussel, <em>M. edulis</em> e</td>
<td>rat CYP2E</td>
<td>52.7, 47.2</td>
<td>Peters et al., 1998a</td>
</tr>
<tr>
<td>Mussel, <em>M. galloprovincialis</em></td>
<td>rat CYP2E</td>
<td>52</td>
<td>Peters et al., 1998b</td>
</tr>
<tr>
<td><em>Euglena gracilis</em></td>
<td>rat CYP2E</td>
<td>52</td>
<td>Briand et al., 1993</td>
</tr>
<tr>
<td>Florida spiny lobster, <em>P. argus</em></td>
<td>lobster CYP2L</td>
<td>30, 52</td>
<td>James &amp; Boyle, 1998; Boyle &amp; James, 1996</td>
</tr>
<tr>
<td>Slipper lobster, <em>Scyllarides nodifer</em></td>
<td>lobster CYP2L</td>
<td>52, 55</td>
<td>James &amp; Boyle, 1998</td>
</tr>
<tr>
<td>Blue crab, <em>C. sapidus</em></td>
<td>lobster CYP2L</td>
<td>50</td>
<td>Obserdorster et al., 1998</td>
</tr>
<tr>
<td>Sea star, <em>A. rubens</em></td>
<td>scup CYP3A</td>
<td>substantially &gt; 53</td>
<td>den Besten, 1998</td>
</tr>
<tr>
<td>Mussel, <em>M. edulis</em> e</td>
<td>trout CYP3A</td>
<td>67.4, 52.8, 44.5</td>
<td>Peters et al., 1998a</td>
</tr>
<tr>
<td>Mussel, <em>M. galloprovincialis</em></td>
<td>trout CYP3A</td>
<td>47</td>
<td>Peters et al., 1998b</td>
</tr>
<tr>
<td>Mussel, <em>M. edulis</em> e</td>
<td>rat CYP4A</td>
<td>50.9, 44.1</td>
<td>Peters et al., 1998a</td>
</tr>
<tr>
<td>Mussel, <em>M. galloprovincialis</em></td>
<td>rat CYP4A</td>
<td>50.9, 44.1</td>
<td>Peters et al., 1998b</td>
</tr>
<tr>
<td>Scallop</td>
<td>aromatase</td>
<td>n.d.</td>
<td>Matsumota et al., 1997</td>
</tr>
</tbody>
</table>

* monoclonal Ab; b no blot shown or MW given; c partially purified; n.d., Not detected
CYP3A antibody cross-reacted with the hepatopancreas microsomal fraction, while there was no cross-reactivity with this antibody to unexposed individuals. Consistent with this finding, exposure of sea anemone to low concentrations of cadmium resulted in the induction of a 50-60 KDa protein recognized by several P450 antibodies (Heffernan et al., 1996). The inducibility of these 50-60 KDa sea anemone proteins is consistent with these proteins being cytochrome P450.

The detection of multiple 50-60 KDa proteins by the same P450 antibody in A. elegantissima was consistent with results observed in other invertebrates (e.g., Peters et al., 1998a; 1998b; James & Boyle, 1998; Boyle & James, 1996; Boyle et al., 1998b). Such detection suggests either different evolutionary pathways or simply less divergence between P450 isoforms in invertebrates (versus vertebrates). Further studies towards isolation and characterization of invertebrate P450 isoforms may lead to a better understanding of these issues.

The recognition of a 50-60 KDa protein in the sea anemone by CYP1A, 2B, 2C11*, 2E, and 3A antibodies is consistent with observations in other marine invertebrates (e.g., den Besten, 1998; Peters et al., 1998a; 1998b; James & Boyle, 1998). The estimated molecular weights of these sea anemone immunoreactive proteins were typically within the same range of values as observed in other marine invertebrates (Tables 4-1 and 4-3). The CYP2B antibody was the only antibody that recognized a protein (~59 KDa) larger than reported in other marine invertebrates, 44-53 KDa (Briand et al., 1993; den Besten, 1998; Peters et al., 1998a; Sole et al., 1996).

While organisms from additional phyla were examined with the CYP2K antibody in this study, the CYP2K blots were optimized for the detection of a 40 KDa protein. Thus, the shorter staining time may have resulted in the protein concentration having been too low to detect the 50-60 KDa protein in most of the samples (Fig. 4-7).

The lack of recognition by the rat CYP4A antibody was unexpected. CYP4A is thought to be an ancient cytochrome P450 originating over 800 million years ago (Nebert & Gonzalez, 1987; Nelson & Strobel, 1987). This isoform has been shown to be involved in endogenous
metabolism of fatty acids in the rat liver (e.g., Lewis, 1996), and its presence has been clearly established in several marine invertebrates (Livingstone et al., 1989; Peters et al., 1998a; 1998b; Snyder, 1998a; 1998b; Wootton et al., 1995). In the mussel digestive gland microsomes, the Western blots that indicated the presence of CYP4A were probed with a different polyclonal rat CYP4A antibody than the one used in this study. This difference in antibody used may account for the lack of detection of a CYP4A isoform in the sea anemone microsomes.

While immunodetection alone is not sufficient to demonstrate the presence of a particular protein in an organism, the presence of cytochrome P450 in the sea anemone has been clearly established (Heffernan & Winston, 1998; previous chapters). Further, the results from steps taken towards purifying the 50-60 KDa proteins suggest that these 50-60 KDa proteins are the sea anemone cytochromes P450. First, a 50-60 KDa was recognized in the 14-20% PEG pellet by several P450 antibodies (i.e., anti-CYP1A, -CYP2B1/2 (from Oxford Biomedical), -CYP2E, and -CYP3A) (Fig. 4-9). Further, spectral analyses indicated that P450 was present in the PEG pellet that contained these 50-60 KDa immunoreactive bands. Interestingly, the spectral properties also indicated that P450 precipitated between 8-12% PEG, but the immunoreactive bands were not detected in the 0-14% pellet. Similar to this finding, the rabbit liver microsomal P450 precipitated in PEG over a broad range, but specific isoforms precipitated within a relatively narrow PEG range (LM-1, 6-10%; LM-7, 10-13%) (Guengerich, 1977). The precipitation of the sea anemone P450 between 8-18% PEG was similar to studies reported on several vertebrates (e.g., 10-16% Elshourbagy & Guzelian, 1982; 8-12% Koop et al., 1982; 6-13% Miki et al., 1987; 9-15% Ryan et al., 1982; 10-16% West et al., 1979) and marine invertebrates (e.g., 0-5% Batel et al., 1986; 4-15% Kirchin, 1988; 0-16% Quattrochi & Lee, 1984a; 0-10% Quattrochi & Lee, 1984b).

The n-octylamine column is a general hydrophobic column that binds numerous CYP enzymes. This column has been used to partially purify several invertebrate P450 isoforms (e.g.,
Batel et al., 1986; Conner & Lee, 1981; 1982; Kirchin, 1988; Peters et al., 1998a; 1998b; Porte et al., 1995). In the sea anemone (*A. xanthogrammica*), the n-octylamine column retained one or more 50-60 KDa proteins that were weakly recognized by anti-CYP2E and anti-CYP2B1/2 (from Oxford Biomedical) in column eluate (data not shown). However, there was no indication of a 417 nm absorbance in any of the fractions. Thus, the CO-difference binding spectra was not examined. The lack of detection of the 417 nm absorbance may have been the result of the protein having been too dilute or having lost its heme-center. The heme may have been lost during the process of removing the detergents and concentrating the proteins through an Amicon Centriprep 30. Finally, DEAE chromatography is typically used in P450 purification protocols to separate the P450 isoforms. The sea anemone P450 isoforms may also be retained by DEAE; coomassie blue stained gels indicated that there was a 50-60 KDa protein retained by the DEAE resin (Fig. 4-10). Further studies (Western blots with the various P450 antibodies) may demonstrate that these 50-60 KDa proteins that bound to the DEAE are P450 isoforms.

These data indicate that the 50-60 KDa sea anemone microsomal proteins recognized by the various vertebrate P450 antibodies are cytochrome P450 and that multiple P450 isoforms exist within sea anemones. These findings are based on the detection of several 50-60 KDa proteins that contain physical properties similar to those of known P450 isoforms. Isolation and characterization of these proteins may identify the number of isoforms that are recognized by these antibodies, their diversity, and their endogenous as well as exogenous role(s) in these organisms. The steps examined towards purification of these proteins indicate that more typical P450 purification methods might isolate these CYP-immunoreactive 50-60 KDa proteins.

**Immunoreactive 28 and 40 KDa Proteins**

Most cytochromes P450 reported for invertebrates (non-insect) are between 46-56 KDa (e.g., Batel et al., 1986; Berger & Fairlamb, 1993; Conner & Singer, 1981; Kirchin et al., 1992; Lee, 1986; Livingstone et al., 1989; Quattrochi & Lee, 1984a; 1984b; additional references in
Table 4-3). Nevertheless, in several species of sea anemone, a lower molecular weight protein was strongly recognized by both anti-CYP3A1 (~28 KDa) and by anti-CYP2K1 (~40 KDa) in addition to the 50-60 KDa microsomal proteins (Figs. 4-4, 4-7, and 4-8). The recognition of these proteins does not appear to be from non-specific binding because neither protein was recognized by the other P450 antibodies, which were also produced in the rabbit. The detection of these lower molecular weight proteins in the sea anemone is consistent with several other invertebrate studies which reported detecting 30-44 KDa proteins by a P450 antibody (Boyle & James, 1996; James & Boyle, 1998; Peters et al., 1998a; 1998b).

The identity of the sea anemone 28 KDa protein is unknown; however, a similar molecular weight protein (~30 KDa) was recognized in the spiny lobster by the polyclonal rabbit anti-lobster CYP2L antibody (Boyle and James, 1996). These authors noted that they had evidence for the presence of a sliced (truncated) variant of CYP2L; the 30 KDa protein could be the translated product of that spliced variant or a breakdown product of P450. This explanation may also account for the identity of the lower molecular weight proteins recognized by the trout CYP3A in several species of sea anemone (~28 KDa), the blue crab (~32 KDa), and a sea star (~24 KDa). Such low molecular weight proteins (i.e., 30-40 KDa) were not detected in trout liver microsomes by this CYP3A antibody.

The 40 KDa protein was detected by the polyclonal anti-trout CYP2K in most of the marine invertebrates examined (Fig. 4-8), but not in the four vertebrate species (representatives of four vertebrate classes) (Fig. 4-7). In the rainbow trout, Western blot and ELISA analyses on purified proteins have demonstrated the high specificity of this antibody for P450 members of the 2 family (i.e., CYP2K isoform, ~54 KDa; CYP2M, ~50 KDa; CYP2B, MW not given), and anti-CYP2K does not recognize purified cytochromes P450 outside of its family (i.e., CYP1A or CYP3A) (Buhler & Wang-Buhler, 1998; Miranda et al., 1990). Nevertheless, despite the strong
recognition of the 40 KDa protein by the CYP2K antibody, there is evidence that the sea anemone 40 KDa protein may not be a cytochrome P450.

Due to the particularly strong recognition of the 40 KDa protein by this antibody and its uniqueness to the marine invertebrates, efforts were focused on purifying and identifying this protein. The early steps examined for a protein purification protocol (i.e., solubilization and PEG precipitation) were relatively typical of a P450. Similar to several vertebrate P450 isoforms (e.g., 10-16% Elshourbagy and Guzelian, 1982; 9-15% Ryan et al., 1982; 10-16% West et al., 1979), the highest concentration of the 40 KDa protein was present in the 12-18% PEG 6000 pellet. Also similar to the invertebrate P450, a large percentage of the 40 KDa protein precipitated over a wide range of PEG (e.g., 0-5% Batel et al., 1986; 4-15% Kirchin, 1988; 0-16% Quattrochi & Lee, 1984a; 0-10% Quattrochi & Lee, 1984b). However, adjusting the percentage of PEG used to remove the contaminating proteins resulted in too great of a loss of the 40 KDa protein. While several marine invertebrate studies have used PEG precipitation in their P450 purification procedures, they typically used a very broad PEG range (0-5% Batel et al., 1986; 4-15% Kirchin, 1988; 0-16% Quattrochi & Lee, 1984a; 0-10% 1984b), suggesting that they suffered from the same problem. Due to the relatively low concentrations of P450 in invertebrates as compared to vertebrates, these studies were probably more focused on obtaining the maximum amount of P450 at the expense of purity.

The results from the remaining purification procedures were atypical for P450. For instance, mammalian studies have classically used DEAE chromatography to separate different P450 isoforms. While DEAE chromatography has been less useful for separating isoforms, the invertebrate P450 isoforms are also retained by a DEAE column. In this case, the 40 KDa protein was not retained by the DEAE sephacel at pH 7.8 (Figs. 4-10 to 4-12). Further, the 40 KDa protein did not bind to either n-octylamine or chloramphenicol column resin, both of which are commonly used to bind P450 isoforms. An n-octylamine column has been used to partially
purify several invertebrate P450 isoforms (e.g., Batel et al., 1986; Conner & Lee, 1981; 1982; Kirchin, 1988; Peters et al., 1998a; 1998b; Porte et al., 1995). These results suggest that the 40 KDa protein might not be a P450, although an alteration in the hydrophobicity or active site of the enzyme could have prevented it from binding to the n-octylamine or chloramphenicol columns.

The N-terminal sequence data were unambiguous and closely matched several published sequences for GAPD (which can have a molecular weight of 31 to 36 KDa; see Pasquali et al., 1996 and Sanchez et al., 1996). Thus, 2D gels were performed to determine if the immunoreactive protein band were composed of a GAPD and a putative P450 isoform that had comigrated on the 1-dimensional gel. The 2D gel electrophoresis demonstrated that the 40 KDa band from the 1D gel was composed of multiple proteins and that several of these proteins were recognized by the CYP2K antibody, suggesting that they might be different isoforms (Figs. 4-15 and 4-16). However, the N-terminal sequence obtained from two spots (pI ~ 8.7, 15 residues; pI ~ 8.5, 10 residues) in the 2D gel were identical to the 15 residue sequence obtained from the 40 KDa band of the 1D gel (Table 4-2). Further, these N-terminal sequences were completely different from any portion of the trout CYP2K protein, the N-terminal region of a clam CYP30, and the N-terminal region of the lobster CYP2L (Brown et al., 1998; Buhler et al., 1994; James et al., 1996).

In addition to the N-terminal sequence, other data argue against the 40 KDa protein being a P450. First, while recent studies have reported recognition of lower molecular weight proteins by P450 antibodies (Boyle & James, 1996; James & Boyle, 1998; Peters et al., 1998a; 1998b); the typical molecular weight for P450 is between 50-60 KDa (Table 4-3). Second, the recognition of the 40 KDa protein was at least five times more intense in the cytosol of the sea anemone than in the microsomes. While this would be an unusual finding for a protein that is normally membrane-bound, it would explain the unusual behavior of the 40 KDa protein with
respect to the purification procedures used. Third, in addition to the atypical purification
scheme, the purification could not be monitored by the CO-difference binding spectra (although
it could be monitored by the 417 nm absorbance). Finally, the estimated pIs for these 40 KDa
sea anemone isoforms are higher than the typical range (5.0 to 6.5) seen for cytochromes P450,
although some P450 isoforms have pIs as high as 7.0 or 8.1 (Guengerich, 1982).

With the exception of the N-terminal sequence data, none of the above discrepancies
would by itself eliminate the possibility of the 40 KDa protein being a P450. For instance, there
are two examples of cytosolic P450 isoforms found in bacteria (Lewis, 1996; Ortiz de
Montellano, 1995). Further, the lack of any P450 binding spectra associated with the partially
purified 40 KDa protein may have been due to loss of the heme-center of the protein,
denaturation of the protein during the purification procedure, or too little of P450 in the solution
to demonstrate the spectral properties. Finally, it is difficult to evaluate the importance of the pI
values because there was no information on the running conditions for the referenced P450
proteins. However, when the discrepancies are considered in total, they suggest that the sea
anemone 40 KDa protein might not be a cytochrome P450.

In regards to the presence of a cytosolic protein in the sea anemone microsomes, the
large amount of connective tissue in the sea anemone microsomal preparation may have resulted
in poor separation of the cytosol and microsomes. The presence of connective tissue has been
shown to interfere with the production of the microsomes in mammalian lung (Schenkman &
Kupfer, 1982). Based on TEM images, the sea anemone microsomes did not appear to contain
any mitochondria (data not shown), while the cytosolic fraction still contained microsomes.

Despite the high specificity in the trout of the polyclonal rabbit anti-trout CYP2K
antibody for P450 members of the 2 family (Buhler & Wang-Buhler, 1998; Miranda et al.,
1990), polyclonal antibodies have occasionally been shown to recognize specific, charged regions
of non-target proteins in addition to the epitope regions of the proteins to which the antibodies
were made. Thus, given the findings discussed above, the CYP2K antibody appears to be recognizing a cytosolic protein in several of the marine invertebrates. Based on the N-terminal sequence, these proteins have a high sequence identity with several cytosolic and non-cytosolic proteins: GAPD, thymidine phosphorylase, ubiquinol cytochrome c reductase, 20 α-hydroxysteroid dehydrogenase, and lactate dehydrogenase (Table 4-2).

The N-terminal sequence most closely matched published GAPD sequences. Thus, a GAPD antibody was examined for its ability to recognize the partially purified 40 KDa protein which was recognized by the polyclonal CYP2K antibody (Fig. 4-13). The monoclonal rabbit GAPD antibody recognized the control sample of purified rabbit GAPD, but it did not detect the 40 KDa protein (Fig. 4-14). Although this GAPD antibody apparently recognizes a snail heart muscle GAPD (personal communication with Biogenesis), the monoclonal nature of this GAPD antibody might have prevented recognition of the 40 KDa protein. Nevertheless, due to the lack of recognition by the GAPD antibody of a 30-40 KDa protein in the cytosolic fraction of either the rainbow trout or sea anemone (B. cavernata), the GAPD antibody results are inconclusive.

If the 40 KDa proteins are GAPD, typically a highly conserved protein, then they have some unusual properties as compared to other GAPD proteins. For instance, the CYP2K antibody recognized the protein only in the invertebrate species. Further, the estimated pIs for these spots were high for GAPD; pIs were 6.7 and 6.5 for GAPD in E. coli (Pasquali et al., 1996) and were between 6.5 to 7.0 for GAPD in yeast (Sanchez et al., 1996), as estimated with IPG Strips under the same denaturing conditions used in this study. Finally, the purification of the 40 KDa protein could be monitored based on a positive absorbance at 417 nm, suggesting that it has a heme-center (although the 40 KDa protein might have co-purified with a heme-centered protein).

Based on the available evidence, the 40 KDa is presumed to be a GAPD, but that has not been proven. Both GAPD and P450 can migrate as multiple spots on a 2D gel (e.g.,
Guengerich, 1982; Pasquali et al., 1996; Sanchez et al., 1996), and it is conceivable that both of the spots on the 2D gel contained GAPD and P450. This seems unlikely, given that for both spots they would have to have the same pIs as well as molecular weights to comigrate in the two dimensions. There is also still a possibility that the two proteins that were sequenced are GAPD, while the other proteins may be various P450 isoforms. Nevertheless, that would still mean that the CYP2K antibody recognizes a GAPD in invertebrates. These results indicate that caution needs to be exercised when using vertebrate P450 antibodies in marine invertebrates, particularly when considering unique molecular weight proteins.

Given the above issues and that only the first 15 residues of the N-terminal sequence for 40 KDa protein have been identified, the high sequence identity of the 40 KDa protein with several other candidate proteins should not be ignored (Table 4-2). Studies examining the presence of GAPD activity in the partially purified sample would assist in identification of this 40 KDa protein. These additional studies should also examine the cross-reactivity of the monoclonal CYP2K antibody with the 40 KDa protein in the partially purified sample, as well as in the various invertebrate microsomal and cytosolic fractions.

In summary, while the 40 KDa protein does not appear to be a cytochrome P450, the overall evidence confirms that the 50-60 KDa proteins detected by the CYP antibodies are cytochromes P450. First, sea anemone clearly contains a cytochrome P450-dependent mixed-function oxidase system. Second, the 50-60 KDa proteins had molecular weights typical of P450 isoforms. Finally, these proteins conformed to standard purification procedures for cytochromes P450 and their purification could be tracked through appropriate spectral properties.
Chapter 5: Summary

Several studies have demonstrated rapid uptake and slow elimination rates of xenobiotics in anthozoans as compared to other marine invertebrates, but they did not examine the ability of anthozoans to metabolize these compounds to more hydrophilic substances that can be eliminated. The addition of a reactive functional group (e.g., -OH, -NH₂, -SH, -COOH) by P450 is often a prerequisite to phase II conjugation reactions, which render lipophilic molecules more hydrophilic by addition of highly polar groups (e.g., glutathione, sulfate, glucuronate) at the oxidized sites. The initial oxidation of many hydrophobic exogenous compounds is performed by the cytochrome P450-dependent mixed-function oxidase (MFO) system (Chapter 1).

The presence of a functional cytochrome P450-dependent MFO system has been clearly demonstrated in several marine invertebrate phyla; however, its presence had been far less rigorously documented in cnidarians. Early cnidarian studies failed to detect either cytochrome P450 activity or its characteristic spectral properties, probably as a result of low P450 contents, endogenous inhibitors to P450, and low reductase activities. In contrast, several recent studies indicated the presence of a functional cytochrome P450-dependent MFO system in two cnidarian classes — Hydrozoa and Anthozoa. The studies reported here indicate that a functional cytochrome P450-dependent MFO system is present in the microsomal fraction of the sea anemones Anthopleura xanthogrammica, A. elegantissima, and Bunodosoma cavernata.

Although it was not possible to quantitate the P450 specific content from A. elegantissima, A. xanthogrammica, or many of the B. cavernata microsomal preparations because of interfering absorbance at 490 nm, a characteristic 450 nm peak was consistently observed in each of these sea anemone species under reduced conditions in the presence of CO. In the few individuals for which P450 could be quantitated, the spectral properties of the CO-liganded, DTN-reduced microsomes indicated that B. cavernata contains approximately 52 pmol P450/mg protein. The P450 content observed in B. cavernata microsomes was comparable to
values typically observed in other marine invertebrates, in which values ranged from 20 to 140 pmol P450/mg microsomal protein (Chapter 2).

Consistent with observations in the mollusc and other marine invertebrates, sea anemone microsomal DTN-reduced, CO-difference spectra had a large 418 nm peak. The actual identity of the 418 nm peak is currently unknown; however, the evidence suggests that it is not entirely reflective of denatured cytochrome P450. The presence of additional heme-centered proteins, such as a peroxidase, could account for the large 418 nm chromophore and the spectral characteristics within the 500-700 nm region (Chapters 2 and 3).

A functional P450-dependent MFO system requires the presence of a reductase to transfer the electrons to P450 either directly (i.e., NADPH-cytochrome P450 reductase) or indirectly (i.e., NADH-cytochrome b5 reductase and cytochrome b5). Each species of sea anemone contained NAD(P)H-cytochrome c and NADH-ferricyanide (b5) reductase activities. The NADPH-cytochrome c (P450) reductase activity was at the lower end of the range observed in other marine invertebrates and much lower than in the earthworm (L. terrestis), the barnacle (B. eburneus), and several molluscs. The NADH-cytochrome c reductase and NADH-ferricyanide (b5) reductase activities were similar to activities observed in the sea urchin (E. esculinitus), but significantly lower than that observed in other marine invertebrates (Chapter 2).

Despite the relatively low reductase activities as compared to many other marine invertebrates, the sea anemone microsomes were capable of metabolizing the P450-dependent monooxygenase reactions, aldrin epoxidation and ethoxyresorufin O-dealkylation, in the presence of NAD(P)H. Both of these reactions were linear with respect to time and protein concentration. Further, they both required the presence of either NADH or NADPH; there was no NAD(P)H-independent activity as observed in mollusc (Chapter 2).

NADPH- and NADH-dependent EROD activity was consistently observed in A. xanthogrammica and A. elegantissima; however, it was below the detection limit of the assay in
Despite the much higher NADH-cytochrome c and ferricyanide reductase activities, the sea anemone monooxygenase reactions consistently preferred NADPH as a cofactor for ethoxyresorufin O-dealkylation. The EROD activities detected were similar to values found in many other marine invertebrates.

In contrast to the EROD activity, NADPH- and NADH-dependent aldrin epoxidation activity was consistently observed in *B. cavernata* and *A. elegantissima*. It was also observed in most of the *A. xanthogrammica* microsomal preparations; however, the values detected in this species were often very close to the detection limit of the assay. In most of the microsomal preparations, the aldrin epoxidation activity tended to be slightly higher with NADPH as opposed to NADH, while the rest of the microsomal preparations strongly preferred either NADPH (~30%) or NADH (~5%) as an electron donor for aldrin epoxidation. In each species of sea anemone, the aldrin epoxidation activities were much lower than values observed in other marine invertebrates. While these sea anemone species might not be very efficient at aldrin epoxidation, further analysis would need to be performed to ensure that the low activities are not an artifact of the presence of endogenous inhibitors or the result of a narrow pH range in these sea anemones that is different than seen for several other marine invertebrates (Chapter 2).

This varying electron-donor preference for aldrin metabolism in the sea anemone could result from differential expression (in batches of microsomes) of multiple P450 isoforms that are able to catalyze aldrin epoxidation. The presence of multiple isoforms that are differentially expressed would also explain the higher variability in rate of aldrin epoxidation between different microsomal preparations as compared to the EROD activity. The existence of more P450 isoforms capable of metabolizing aldrin as compared to ethoxyresorufin would be consistent with results observed in vertebrates.

Further evidence for the presence of multiple microsomal P450 isoforms in the sea anemone is indicated by immunodetection studies. Several antibodies raised against rat or fish
P450 isoforms of the CYP 1, 2, and 3 families recognized protein(s) between 50-60 KDa in the microsomal fraction of *A. elegantissima*, *A. xanthogrammica*, and *B. cavernata*. Determination of the actual number of putative P450 isoforms detected by these antibodies is complicated by several of these antibodies (i.e., anti-CYP1A, -CYP2E, and -CYP3A) recognizing a 50-60 KDa protein of the same molecular weight. Recognition of the same molecular weight proteins could result from these antibodies detecting either one P450 isoform with multiple epitope sites or multiple P450 isoforms of the same molecular weight that each contain an epitope to one of these antibodies. However, the recognition patterns detected by anti-CYP2B, 2C11*, and 2K were each quite different than the pattern detected by the CYP1A, 2E, and 3A antibodies. These results indicate that multiple P450 isoforms exist within the sea anemone (Chapter 4).

The presence of differential expression of the putative P450 isoforms could explain the detection of a doublet band in the Alaskan *A. elegantissima* as compared to the singlet in *A. xanthogrammica*, *B. cavernata*, and *C. gigantea*. The doublet was not the result of a species-specific difference because the doublet was not also detected in the Californian *A. elegantissima*. Many factors will alter the expression of certain P450 isoforms, such as season (i.e., season collected), diet, and exposure to inducing agents. The presence of a diatomaceous symbiont in the Alaskan *Anthopleura* as compared to an algal symbiont in the two Californian *Anthopleura* may also influence the expression of these P450 isoforms. Differential expression would also explain why the 50-60 KDa protein was detected in most *A. elegantissima* and *A. xanthogrammica* microsomal preparations, but tended to be less consistent in *B. cavernata* microsomes and often was not observed in *C. gigantea*.

In addition to the standard 50-60 KDa proteins, there are proteins at unique molecular weights that are strongly recognized by anti-trout CYP2K (~40 KDa) and anti-trout CYP3A (~27 KDa). The detection of these lower molecular weight proteins in the sea anemone is consistent with several other invertebrate studies that reported the detection of 30-44 KDa
proteins by a P450 antibody. Currently, the identity of these proteins is unknown, although there is evidence to suggest that the 40 KDa protein, which is so strongly recognized by the polyclonal rabbit anti-trout 2K antibody, is not a P450. Given the findings discussed in Chapter 4, the CYP2K antibody appears to recognize in several of the marine invertebrates a cytosolic protein, which has high N-terminal sequence identity with a GAPD. However, the 40 KDa protein also seems to have some characteristics that are atypical of a GAPD. Further, the N-terminal sequence also has good sequence identity with several additional proteins (i.e., thymidine phosphorylase, ubiquinol cytochrome c reductase, 20 α-hydroxysteroid dehydrogenase, and lactate dehydrogenase).

While these results indicate a need to be cautious when assigning an identity to these immunoreactive proteins, other evidence indicated that the 50-60 KDa proteins are P450. First, Western blots probed with several P450 antibodies demonstrated that the sea anemone microsomes contained proteins between 50-60 KDa with epitope regions (i.e., similar amino acid sequences) common to multiple vertebrate P450 isoforms (i.e., CYP1A, 2B, 2C11+, 2E, 2K, and 3A). Second, these proteins are within the expected molecular weight range (i.e., 50-60 KDa) for P450. Third, the procedures examined towards purifying the 50-60 KDa proteins revealed proteins with additional physical characteristics similar to known P450 isoforms; for instance, they are precipitated by PEG and they bind to an n-octylamine column. Finally, the P450 binding spectra demonstrated that sea anemone P450 was concentrated in the same PEG pellet that concentrated the immunoreactive 50-60 KDa proteins.

Most of the sea anemone P450 studies were performed on microsomal preparations composed of the entire columnar region rather than on microsomes from specific tissues that might concentrate P450. However, the preliminary studies herein indicated that cytochrome P450 is relatively evenly distributed throughout the sea anemone. The lack of any tissue-specific concentration of P450 in the sea anemone is in agreement with findings in the sea star (den
den Besten (1990) suggested that organisms without a circulatory system may not concentrate P450 because no particular region plays a more dominant role in processing of food or pollutants. While marked differences in P450 content should have been detectable in the experimental design used in this study, subtle differences in P450 content may have been missed due to dissections not completely separating tissues of different P450 contents, the contribution of the algal symbiont, or potential interference of other heme-proteins in the detection of P450. Future studies should focus on additional individuals, symbiont-free sea anemones, and the examination of additional MFO properties, as well as other members of the phylum (Chapter 3).

In conclusion, despite the lack of MFO activity reported in anthozoans and scyphozoans by several cnidarian studies, other studies indicate that anthozoans and hydrozoans do contain a functional cytochrome P450-dependent MFO system. In particular, this study further demonstrates the presence of P450 in several species of sea anemone (Phylum: Cnidarian). The evidence reported in this dissertation is based on the presence of [1] a characteristic cytochrome P450 spectra, [2] active MFO components, [3] metabolism of several cytochrome P450 substrates, and [4] immunoreactivity with cytochrome P450 antibodies.
References Cited


James, M.O. (1990). Isolation of cytochrome P450 from hepatopancreas microsomes of spiny lobster, *Panulirus argus*, and determination of catalytic activity with NADPH cytochrome P450 reductase from vertebrate liver. *Arch. Biochem. Biophys.* 282:8-17.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


129

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
### Table A-1. NAD(P)H aldrin epoxidation activity (30 °C) of each microsomal preparation as determined from the HP-608 column.

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>pmol/hr/ mg protein</th>
<th>pmol/hr/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADH</td>
<td>NADPH</td>
</tr>
<tr>
<td>AE 1</td>
<td>1.87 ± 0.22</td>
<td>2.19 ± 0.86</td>
</tr>
<tr>
<td>AE 2</td>
<td>0.51 ± 0.10</td>
<td>0.73 ± 0.42</td>
</tr>
<tr>
<td>AE 3</td>
<td>2.65 ± 0.22</td>
<td>4.19 ± 0.61</td>
</tr>
<tr>
<td>AE 4</td>
<td>0.52 ± 0.16</td>
<td>0.95 ± 0.11</td>
</tr>
<tr>
<td>BC 1</td>
<td>2.26 ± 0.29</td>
<td>2.71 ± 0.28</td>
</tr>
<tr>
<td>BC 2</td>
<td>1.48 ± 0.47</td>
<td>0.44 ± 0.09</td>
</tr>
<tr>
<td>BC 3</td>
<td>1.11 ± 0.11</td>
<td>1.45 ± 0.04</td>
</tr>
<tr>
<td>BC 4</td>
<td>1.60 ± 0.06</td>
<td>1.52 ± 0.21</td>
</tr>
<tr>
<td>BC 5</td>
<td>1.60 ± 0.32</td>
<td>1.72 ± 0.19</td>
</tr>
<tr>
<td>AX 1</td>
<td>0.65 b</td>
<td>0.55 ± 0.14 a</td>
</tr>
<tr>
<td>AX 2</td>
<td>0.85 ± 0.03 a</td>
<td>1.45 ± 0.27</td>
</tr>
<tr>
<td>AX 3</td>
<td>0.17 ± 0.05 b</td>
<td>0.92 b</td>
</tr>
<tr>
<td>AX 4</td>
<td>1.27 ± 0.03 a</td>
<td>1.03 ± 0.14</td>
</tr>
<tr>
<td>AX 5</td>
<td>0.25 ± 0.05</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>AX 6</td>
<td>0.20 ± 0.05 a</td>
<td>0.72 ± 0.0 a</td>
</tr>
<tr>
<td>AX 7</td>
<td>0.40 b</td>
<td>0.47 ± 0.07 a</td>
</tr>
<tr>
<td>AX 8</td>
<td>no activity</td>
<td>0.88 ± 0.21</td>
</tr>
</tbody>
</table>

Due to *A. xanthogrammica* being at the detection limit of the assay, any variation in extraction efficiency strongly affected the amount of dieldrin detected in that sample. Thus, samples that contained negative values or were much different than others after subtracting the control (reaction without cofactor) were removed from calculation of mean ± 1 std error.

* removed 1 of the triplicates from mean ± std error.

b removed 2 of the triplicates from mean ± std error.

c g wet tissue per mL were not known.
Appendix B: Letter of Permission

FCR/JP/154
23 February 1999

Ms L M Heffeman
320 Choppin Hall
Louisiana State University
Baton Rouge
Louisiana LA 70803
USA

Dear Ms Heffeman


As per your letter dated 19th February 1999, we hereby grant you permission to reprint the aforementioned material in your thesis at no charge subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made as follows:

   FOR JOURNALS: "Reprinted from Journal title, Volume number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier Science".

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world English rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely,

Helen Wilson
Frances Rothwell (Mrs)
Subsidiary Rights Manager

The processing of permission requests for all Elsevier Science (including Pergamon imprint) journals has been centralised in Oxford, UK. Your future requests will be handled more quickly if you write directly to: Subsidiary Rights Department, Elsevier Science, PO Box 800, Oxford OX5 1DX, UK. Fax: 44-1865 853333; e-mail: permissions@elsevier.co.uk

132
Vita

Linda Marie Heffernan was born in Delmar, New York, on 19 February 1965. After graduating from Bethlehem Central High School in 1983, she attended Russell Sage in Albany, New York, where she completed an associates of science degree. In 1983, she attended the University of Maine in Orono, Maine, where she completed her bachelor of science degree with a double major in biochemistry and biology. In 1985, she accepted a research position with Doctor Pamela DenBesten at Forsyth Dental Center. For the next five years, she examined the effect of fluoride on enamel development. On 16 May 1992, she married Scott William Herke after a courtship of nearly six years. The summer before going back to school, she volunteered as a park ranger for Acadia Nation Park in Mount Desert Island, Maine. In 1993, she and her husband moved to Baton Rouge, Louisiana, to attend graduate school at Louisiana State University. With her marriage still intact, she graduated with a degree of Doctor of Philosophy in the biochemistry in December 1999.
Candidate:  Linda Marie Heffernan

Major Field:  Biochemistry

Title of Dissertation:  Characterization of the Microsomal Mixed-Function Oxidase System of Several Species of Sea Anemones (Phylum: Cnidaria)

Approved:  

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

William B. Stickle Jr.

Daniel P. Burda

Barbara Smith

Patrick J. DiMaio

Date of Examination:

19 July 1999