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Reactivity of Iron-Sulfur Proteins with Nitric Oxide

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REACTIVITY OF IRON-SULFUR PROTEINS WITH NITRIC OXIDE

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in

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by
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B.S., University of Dhaka November, 2010
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AcnB   aconitase B

ASKA   ASKA library (A complete Set of *E.coli* K-12 ORF Archive)

DNIC   dinitrosyl iron complex

DTT   Dithiothreitol

EPR   electron paramagnetic resonance

[Fe-S]   Iron-Sulfur

Ilvd   dihydroxyacid dehydratase

NO   nitric oxide

NOS   nitric oxide synthase

ROS   reactive oxygen species

RNS   reactive nitrogen species

TrxA   thioredoxin

TrxB   thioredoxin reductase

ABSTRACT

Iron-sulfur ([Fe-S]) proteins are widely distributed in the biological systems. These proteins are involved in diverse fundamental life processes such as photosynthesis, respiration, ribosome biogenesis, DNA synthesis, and repair. Dysfunction of these proteins or their biogenesis has been implicated in causing cardiovascular diseases, neurodegenerative diseases, and various cancers. Iron-sulfur proteins are highly sensitive to nitric oxide (NO) and readily form dinitrosyl iron complexes (DNICs) upon exposure. Restoration of the iron-sulfur cluster in the NO-modified protein requires a two-step repair process. The first step is to remove DNICs from proteins. The second step is to re-assemble the iron-sulfur cluster in proteins. In this work, we report that protein-bound DNICs can be transferred to thioredoxin (TrxA) under reducing conditions in the presence of a reducing mediator. DNICs formed in two [4Fe-4S] proteins, aconitase B (AcnB) and dihydroxyacid dehydratase (IlvD) were used as DNIC donors to explore DNICs transfer to TrxA. AcnB is an enzyme involved in the citric acid cycle in *Escherichia coli*. IlvD is required for the branched-chain amino acid biosynthesis. To be functional, both proteins require an intact [4Fe-4S] cluster. In the first experiment, IlvD-DNICs or AcnB-DNICs and TrxA were incubated in the presence of dithiothreitol (DTT) to monitor the DNICs transfer from these protein-bound DNICs to TrxA. Experiment results suggest that DNICs may be partially transferred from IlvD-DNICs or AcnB-DNICs to TrxA under reducing conditions. We also utilized the TrxAB/NADPH system which is responsible for maintaining reducing conditions in cells to mediate the DNIC transfer, and found that the TrxAB/NADPH system can also promote the DNICs transfer from AcnB-DNICs to TrxA. The results from this study may provide a new pathway of DNIC removal for the repair of the NO-modified iron-sulfur proteins.

CHAPTER 1
INTRODUCTION

1.1 Diversity and functions of iron-sulfur proteins

Iron-sulfur proteins are a group of proteins that contain iron-sulfur clusters. There are over 500 unique iron-sulfur proteins present in three domains of life (1). Many iron-sulfur proteins are highly conserved. These proteins are critical for important biological processes such as photosynthesis and respiration, nitrogen fixation, DNA synthesis and repair, ribosome biogenesis, lipoic acid and heme biosynthesis, and regulation of gene expression (2). Iron-sulfur clusters are considered one of the most ancient prosthetic groups in proteins (3). Although iron and sulfur can exist in various combinations to form an iron-sulfur cluster, the [2Fe-2S] and [4Fe-4S] clusters are the most common iron-sulfur clusters found in nature (Figure 1.1) (4). The iron-sulfur clusters are usually coordinated by cysteine or histidine residues. In most cases, iron-sulfur clusters are attached to the protein by cysteine residues via the sulfhydryl group of the cysteine(s), which coordinates the iron atoms. However, some other amino acids such as histidine and aspartic acid can also be used as a ligand (5,6).

Iron-sulfur proteins participate in diverse functions, including electron transfer, enzyme catalytic activity, and regulation of gene expression. In mitochondrial electron transport chain complexes I-III, up to 12 different iron-sulfur cluster proteins have been found (7). In nature most iron-sulfur clusters transfer one electron, but the double-cubane type iron-sulfur cluster in nitrogenase has the potential to transfer two electrons at a time (8).
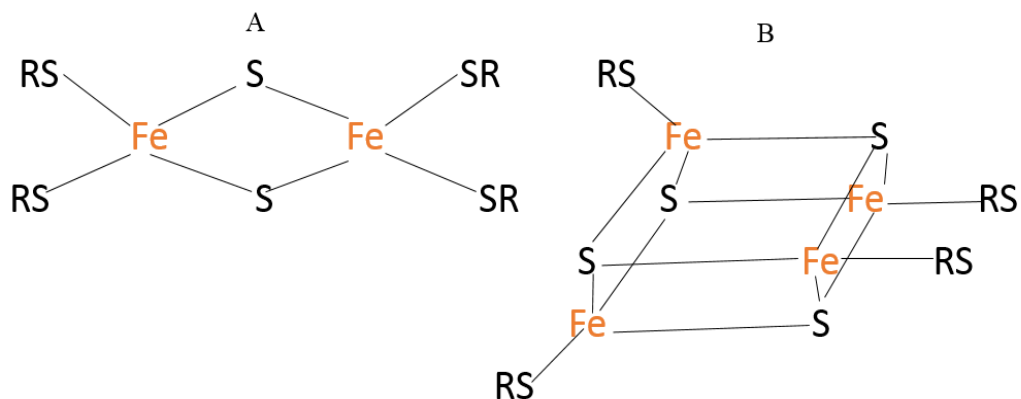
Figure 1.1 Iron-sulfur clusters. A, a [2Fe-2S] cluster and B, a [4Fe-4S] cluster. Iron-sulfur clusters attached through the sulfhydryl-R groups.

Besides transferring electrons, iron-sulfur clusters may have an active role in catalytic center in proteins. For example, in the citric acid cycle, aconitase binds hydroxyl group of isocitrate/citrate in a unique iron in the [4Fe-4S] cluster of aconitase (9).

Iron-sulfur clusters are highly sensitive to free radicals such as nitric oxide (NO) and reactive oxygen species. Recently, a [2Fe-2S] protein NsrR was characterized as a sensor specific to NO in bacteria (10). NsrR exerts its function as a transcription repressor for NO responses (11). When the NsrR [2Fe-2S] cluster is modified by NO, the protein is released from operator DNA to allow expression of genes responsible for NO response which include NO detoxification genes (12).

Another example is the transcription factor SoxR that has a [2Fe-2S] cluster and possesses the oxidative stress sensing capability. When the [2Fe-2S] cluster of SoxR is oxidized or NO modified, it initiates the transcription of over 30 oxidative stress response and detoxification genes (13,14).

1.2 Properties of Nitric Oxide

Nitric Oxide (NO) is a lipid and water soluble gas that can diffuse readily across biological membranes (15). As a free radical, NO has an unpaired electron that is responsible for its reactive properties (15). NO is able to rapidly react with metalloproteins and with other free radicals. NO is involved in diverse physiological processes such as regulating the immune system, neurotransmission, regulating blood pressure, and smooth muscle movement (15).

1.3 The sources and production of Nitric Oxide in the biological system

In the mammalian cells there are three types of NO synthases present: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) (16-20). The activity of nNOS and eNOS depends on the intracellular Ca^{2+} and are thus often referred together as cNOS (21). The expression of cNOS is constitutive. On the other hand, the iNOS expression is inducible and independent of the intracellular Ca^{2+} level. This type of expression is found in the activated macrophages (22,23). Due to the difference in the net NO production of cNOS and iNOS, NO concentration can fluctuate significantly in human body (23). NO is usually produced at nanomolar concentrations by cNOS for signaling. On the other hand, inducible NO production by iNOS generates micromolar levels of NO, and is involved in infection and inflammation (23,24).

1.4 Nitric Oxide as a signaling and cytotoxic molecule

In 1980, two scientists, Furchgott and Zawadzki reported endothelium-derived relaxing factor, characterized by a half-life of few seconds (25). Later, in 1986, Furchgott identified this factor as NO, and Ignarro confirmed this discovery (26,27). Once guanylate cyclase is activated by NO, it produces cGMP from GTP. NO is able to trigger guanylate cyclase activation in neighboring cells and eventually in the whole tissue by its rapid diffusion ability through the

membrane. In a cascade of reactions cGMP activates cGMP-dependent protein kinase by regulating intracellular Ca^{2+} concentration to control many functions in harboring tissue (28).

Two properties of NO are attributed to its characteristic signaling properties. The first, its rapid diffusion ability for being small and uncharged. The second, a very short half-life time. NO molecule is significantly small compared to the majority of the signaling molecules. However, the local concentration of NO molecule can vary significantly because of net production and a rapid diffusion ability. It is estimated that NO can diffuse and cross a cell within one second (29). Another factor is the half-life time for the NO molecule is significantly lower compared to other signaling molecules participating in the physiological processes. Therefore, only nearby cell or tissue is affected. For this very reason NO produced in the gut is unable to produce any effect in the brain (24). However, the high reactivity and rapid diffusion capability will allow NO to change rapid local physiological processes (30).

As NO is a highly reactive molecule, the mammalian circulatory system is organized in a way that the effect of NO can be self-regulated. For example, the packaging of hemoglobin in red blood cell prevents re-entry of NO molecule into cells by absorbing a high amount of NO (31). Moreover, hemoglobin from lysed red blood cells is more efficient in absorbing NO. This mechanism slows down guanylate cyclase significantly and thus is responsible for vasoconstriction when a blood vessel is cut (30). In 1996, NO was proposed as the key molecule responsible for signal averaging mechanism, a sensing process in the brain that allows the synaptic plasticity (32). An additional signal sequence present in nNOS to bind with N-methyl-D-aspartate (NMDA) receptor is important for the signal averaging mechanism.

It is important to note that based on concentration NO can function as a signaling molecule or a cytotoxic molecule. The normal NO level in the human body ranges from 10 nM

4

to 1 μM, which is enough to work as a signal transduction molecule in the antiapoptotic pathway. However, a concentration higher than 1 μM will be responsible for necrotic death of the cell by inactivating ATPase (33). Therefore, NO is a signaling molecule at nanomolar concentration, but it will act as a cytotoxic molecule at micromolar concentrations (21). Experiments revealed that high level of NO works as a defense against pathogenic bacteria or tumor cells (34,35). NO can cause cellular damage in various ways, such as irreversible oxidation of thiol groups in the proteins, and reaction with superoxide to produce peroxynitrite ($ONOO^-$), a molecule able to destroy proteins and lipids by oxidation. Excessive production of NO that is beyond the ability of the body to neutralize or eliminate is known as nitrosative stress. This stress is responsible for modification of cellular macromolecules and thus has been implicated in conditions including neurodegenerative diseases, cardiovascular diseases, and cancer (15).

1.5 Iron-sulfur proteins are one of the primary targets for NO-mediated modification in *E. coli*

When iron-sulfur proteins are exposed to NO they readily form dinitrosyl-iron complexes (DNICs), which are composed of two NO molecules bound to a single iron atom (Figure 1.2) (36). Electron paramagnetic resonance (EPR) spectroscopy is a common method to detect the spin of unpaired electrons in free radicals complexed with transition metals, such as those found in DNICs (37). The DNICs can be detected by a characteristic EPR signal at $g = 2.04$ (37).
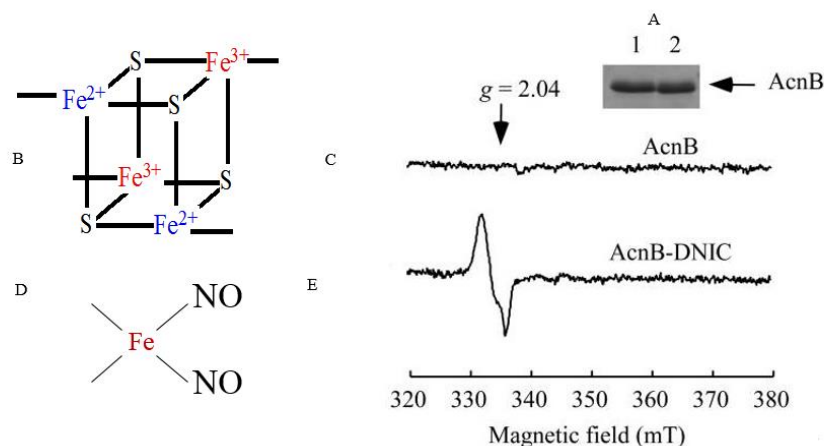
Figure 1.2. Iron-sulfur cluster forms DNICs upon NO exposure. A. Purified untreated AcnB (1), and NO-treated AcnB (2). B. A [4Fe-4S] cluster. C. Iron-sulfur clusters in the oxidized state are EPR silent. D. dinitrosyl iron complex (DNICs). E. Characteristic EPR signal for protein-bound DNICs.

Studies have shown that iron-sulfur proteins such as aconitase [4Fe-4S] cluster, dihydroxyacid dehydratase [4Fe-4S] cluster, ferredoxin [2Fe-2S] cluster, and SoxR [2Fe-2S] cluster can be modified to form dinitrosyl-iron complexes (37-43). DNICs have been detected and identified in diseases responsible for excessive NO production such as Parkinson disease (44) and in various cancers (45). This evidence indicated that iron-sulfur proteins may be converted to protein-bound DNICs when they are exposed to pathophysiological NO concentration. Additionally, research from the gene microarray experiment reveals that when bacterial cells are exposed to NO, the genes responsible for iron-sulfur cluster assembly and repair are upregulated (46-48). This evidence further suggests that iron-sulfur proteins are one of the primary targets for NO-mediated cytotoxicity (49,50).

1.6 Repair of NO-modified proteins

As iron-sulfur proteins are involved in various cellular processes, their modification to DNICs will inactivate protein functions (49). Thus the protein-DNICs must be repaired for the cells to survive. Experiments revealed that protein-DNICs repair is a two-step process. The first

6

step is DNICs removal, and the second step is reassembly of new iron-sulfur clusters in proteins. Previously, it has been reported that NO-modified iron-sulfur proteins can be quickly repaired in *E. coli* cells under the aerobic growth condition (14,46).

In *E. coli*, two major gene clusters are responsible for iron-sulfur cluster assembly, *iscRSUA-hscBA-fdx* (51) and *sufABCDE* (52). If *E. coli* cells are exposed to NO or NO derivatives, the expression of these gene clusters are upregulated (48). Previously, it was reported that in the presence of oxygen protein-DNICs can be decomposed by L-cysteine (53,54). This decomposition process uses the thiol ligand exchange mechanism, where an equilibrium is established between L-cysteine and protein-bound DNICs (55). At the very beginning, L-cysteine will extrude $Fe(NO)_2$ from protein-DNICs via the thiol ligand exchange and become L-cysteine-bound DNICs. Because this L-cysteine DNICs is unstable in the presence of oxygen, it will be rapidly degraded into nitrite and ferrous ion (Figure 1.3) (56). However, this DNICs removal process requires L-cysteine in excess of physiological concentrations (57,58).
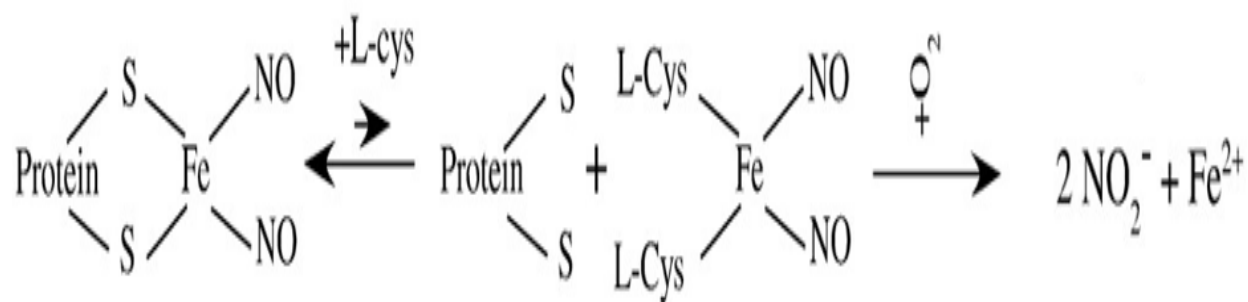
Figure 1.3. In the presence of L-cysteine and oxygen protein-DNICs can be broken down. At the very beginning NO bound iron is transferred to L-cysteine by the thiol ligand exchange mechanism. L-cysteine-DNICs is unstable and breaks down in the presence of oxygen.

1.7 Statement of research objectives

The goal of this study is to explore DNICs exchange between protein-DNICs and cellular proteins involved in maintaining redox regulation, which may provide a new DNIC removal mechanism. The TrxAB/NADPH system is one of the major sources for intracellular thiols in cells (59). It is known that TrxAB/NADPH system plays an important role to maintain reducing conditions inside the cell. Thus, a reducing agent such as dithiothreitol (DTT) or TrxAB/NADPH reduction system may perform two functions. The first, they can help to create a mimic of physiologically reduced condition for the DNICs transfer reaction. The second, transfer from protein-DNICS to TrxA can be more efficient using such a reductant. Both DTT and TrxB possess two thiols (-SH) groups and are able to form a disulfide bond. However, DTT is a stronger reducing agent *in vitro* than the TrxAB/NADPH system. Anaerobic conditions were used for this reaction for several reasons. The proposed overall reaction for the exchange of DNIC is shown in Figure 1.4.
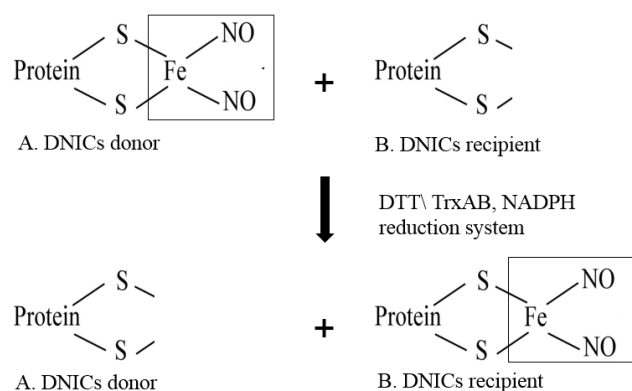
Figure 1.4. Protein-DNICs exchange scheme. In the figure, A is a protein-DNIC, which will act as DNICs donor and B is a part of cellular reduction system, which will work as DNICs recipient. This exchange of DNICs between proteins will be mediated by reducing agents such as DTT or a biological reduction system such as TrxAB/NADPH. The dinitrosyl iron complex of protein-DNICs is highlighted.

CHAPTER 2
DYNAMIC EXCHANGE OF DINITROSYL IRON COMPLEXES BETWEEN IRON-SULFUR PROTEINS

2.1 Introduction

Nitric Oxide (NO) can be synthesized in biological systems either by NO synthases (60), chemical reduction processes such as from the nitrate, or the nitrite reductase reaction (61,62). NO plays diverse physiological roles in different fundamental life processes (1,63). The physiological effect of NO is dependent on its local concentration. At nanomolar concentration, NO acts as a neurotransmitter and signaling molecule, whereas, in micromolar concentration NO is a powerful weapon against pathogenic bacteria and tumor cells (21,64). Unlike reversible NO binding with heme in proteins, the binding of NO with iron-sulfur proteins disrupts the iron-sulfur cluster and thus dramatically reduces protein function (46-48,50,65). Experiments have revealed that these protein-bound DNICs are abundant in the *E. coli* cells even upon a transient micromolar level NO exposure, and these DNICs are stable *in vitro* under both aerobic and anaerobic conditions (36,37,41,43,56,66-69). As iron-sulfur proteins are involved in multiple cellular processes, modifying this group of proteins will inhibit multiple cellular functions. Therefore, it is suggested that iron-sulfur clusters are one of the primary targets for NO-mediated cytotoxicity (46).

Previously, when *E. coli* cells was used to study the effect of pathophysiological NO exposure, it was revealed that at least bacterial growth is inhibited in the minimal media, a phenomenon known as bacteriostasis (53). However, exposure to air negates the bactericidal effect and the NO-treated cells grow normally (56). In addition to that, it was reported that this protein-DNICs is able to from an equilibrium with L-cysteine possibly by the thiol ligand exchange (56,70). However, to make a feasible thiol ligand exchange, the L-cysteine

concentration used was significantly higher than the amount present in *E. coli* cells under normal physiological conditions (56). It was also reported that the cells can efficiently remove DNICs, and repair the [Fe-S] clusters when starved cells are supplemented with glucose under aerobic conditions, so a repair process may exist that does not involve small thiols.

In this current study, we investigated the transfer of protein-DNICs to the TrxA by the thiol ligand exchange mechanism. We generated DNICs-bound recombinant *E. coli* iron-sulfur proteins by NO modification to serve as DNIC donors (36). TrxA was selected because it has thiol groups that can accept the DNICs. This TrxAB/NADPH system is a widely distributed cellular redox control system. In preliminary studies, DNICs transfer was observed when protein-DNICs is mixed with TrxA and subsequently separated. In current experiment we have explored whether thiol ligand exchange mechanism is able to transfer DNICs from protein-DNICs to TrxA.

2.2 Materials and Methods

2.2.1 NO exposure of *E. coli* cells and purification of His-tagged IlvD-DNICs and AcnB-DNICs

Overnight grown recombinant *E. coli* cells containing plasmid IlvD-pCA24N carrying the chloramphenicol resistance gene were diluted 1:50 in LB medium with 25 µg/mL chloramphenicol and grown for three hours at 37˚C at 250 rpm to reach an OD_{600}~ 0.6. . Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) and incubated for 6 h at 25˚C, at 180 rpm. The cells were then harvested and suspended in 20 mM Tris 500 mM NaCl pH 8.0 (71). Oxygen was removed from the cell suspension by purging with pure argon through a silastic tube for 15 min with constantly stirring. NO gas was passed through a soda-lime column to remove N_2O and higher nitrogen oxides before being delivered to the cell suspension. The cells were bubbled with NO for 5 minutes, followed by a second purge with argon to remove excess NO. The NO-treated cells were pelleted and re-

suspended in 20 mM Tris 500 mM NaCl pH 8.0. The cells were lysed by the French press, and the lysate was clarified by centrifugation. The lysate was loaded onto a fast-flow Ni-NTA column attached to a ÄKTA Fast Protein Liquid Chromatography system (FPLC), which was pre-equilibrated with a running buffer containing 20 mM Tris 500 mM NaCl pH 8.0. IlvD-DNICs was eluted with an elution buffer containing 20 mM Tris 500 mM NaCl pH 8.0 and 250 mM imidazole. The purified IlvD-DNICs was passed through a Hi-Trap desalting column to remove imidazole. The purified protein was more than 95% pure based on SDS-PAGE gel analysis. The same procedure was used to purify AcnB-DNICs.

2.2.2 Native TrxA purification

Overnight grown *E. coli* stock culture containing plasmid TrxA-pDL59 was diluted 1:500 in terrific broth. After overnight growth at 37°C, the cells were induced for protein expression at 42°C for 10 hours, reaching an OD_{600}~ 10. The cells were dissolved in 20 mM Tris pH 7.6 and lysed by passing through the French press. Streptomycin sulfate (2% w/v) was added while stirring for 1 h, and the lysate was then centrifuged for 1 h at 16,000 rpm. The resulting supernatant was dialyzed exhaustively overnight against 20 mM Tris pH 7.6. Then the cell extract was centrifuged for 1 hour at 16,000 rpm, filtered through a 0.45 μm filter, and purified using Q-sepharose column equilibrated with 20 mM Tris pH 7.6. TrxA was eluted with the elution buffer containing 20 mM Tris and 140 mM NaCl. Fractions containing TrxA were pooled based on SDS-PAGE gel analysis and concentrated by 80% ammonium sulfate precipitation. The precipitate was pelleted by centrifugation and dissolved in 1.8 mL of 20 mM Tris pH 7.6. TrxA from protein mixture was purified by gel filtration. Fractions that have TrxA were pooled together for dialysis against double distilled water. The purified TrxA was over 95% pure as judged by SDS-PAGE gel, Figure 2.1, A. The concentration of native TrxA was measured by its

absorbance at OD_{280} (extinction coefficient, $\varepsilon = 13,700$ M^{-1} cm^{-1}), Figure 2.1, B.

2.2.3 Native TrxB purification

Native TrxB was purified according to the protocol published by Dr. Mulrooney (72). Overnight grown *E. coli* strain harboring pTrR301 was diluted in 2 ×YT media supplemented with 100 µg/mL ampicillin, 20 mM glucose, and 50 mM potassium phosphate, pH 7.0 and grown for 21 hours at 37˚C at 250 rpm. Before harvesting cultures were chilled on ice and centrifuged at 10,000 g at 4˚C for 10 minutes. The cell pellet was kept at -20˚C for 2 hours. Then cells were thawed and suspended in 18 mL running buffer, containing 10 mM sodium phosphate buffer, 0.3 mM EDTA, pH 7.6, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 µM FAD. The cells were lysed by French press and 2% (w/v) streptomycin sulfate was added in cell extract while stirring. When streptomycin sulfate dissolved completely, the cell extract was centrifuged at 27,000 *g* for 30 min at 4˚C. The supernatant was collected and precipitated with 56% ammonium sulfate while stirring. Then cell extract-ammonium sulfate solution was centrifuged at 12,000 *g* for 10 minutes at 4˚C. The pellet had a yellow color indicative of FAD, which was dissolved in 25 mL of running buffer. This solution was dialyzed against 2 L running buffer, total three times. The dialyzed sample was filtered through a 0.45 µm syringe filter before FPLC purification with ADP sepharose column. The ADP sepharose column (2 cm × 19 cm) was pre-equilibrated with running buffer. The column was washed with a 5 column volume of running buffer. 20mM Tris and 124 mM NaCl was used as an elution buffer. Then fractions were collected and checked to measure the ratio of absorbance between 280nm/456nm in a UV-Vis spectrophotometer. Fractions were checked on an SDS-PAGE gel for purity of TrxB, Figure 2.1, A. The protein concentration was measured by a UV-Vis spectrophotometer using the extinction coefficient of TrxB at 280 nm ($\varepsilon = 17,700$ M^{-1} cm^{-1}), Figure 2.1, C. The purified TrxB was reconstituted with

FAD by incubation with 0.1 mM FAD for 4 h at room temperature, followed by desalting and analysis of the protein-bound FAD at 450 nm (ε = 11,300 M^{-1} cm^{-1}). The FAD
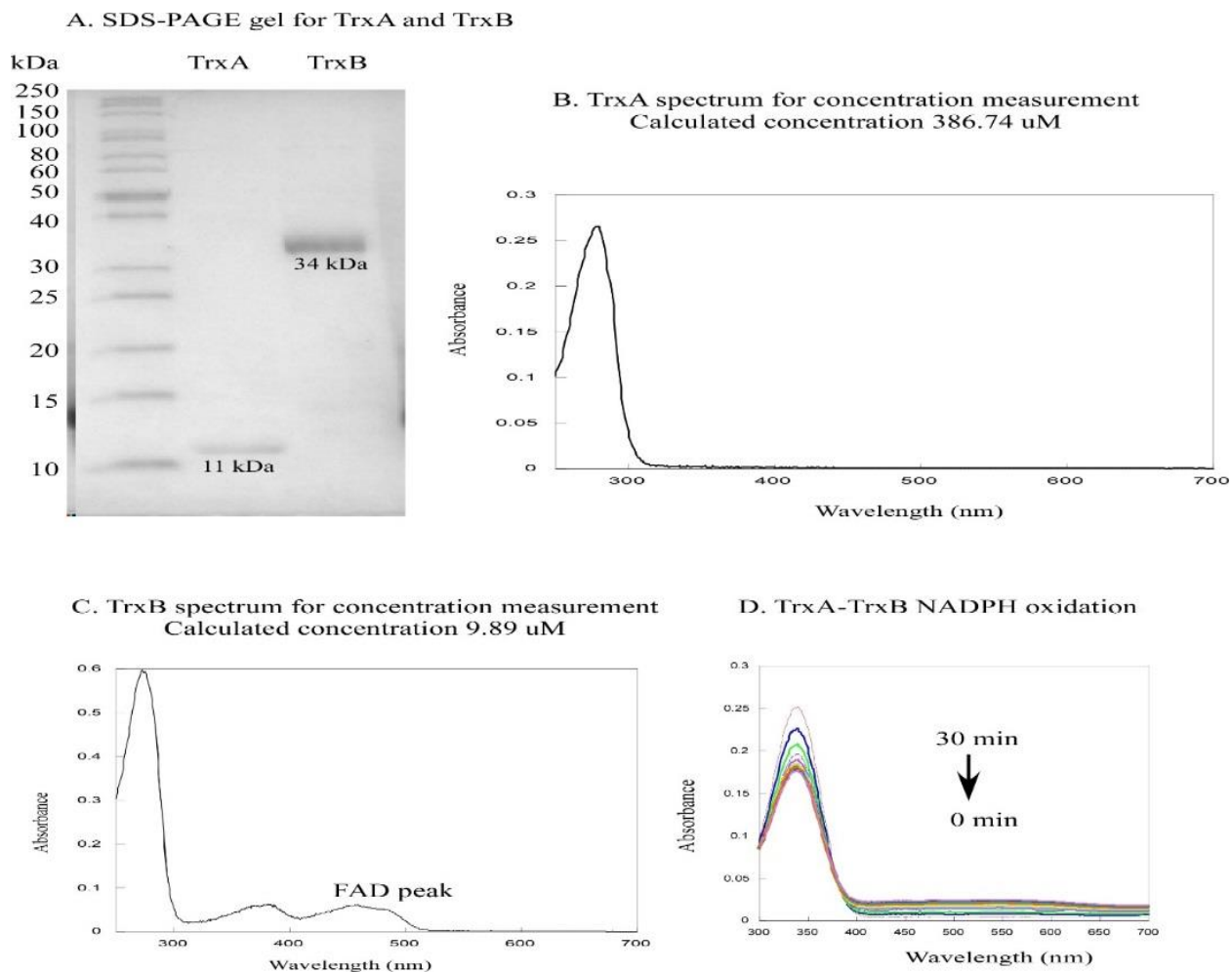
Figure 2.1. TrxA and TrxB purification and activity check. A. TrxA and TrxB were purified and checked on SDS gel for purity. B. Spectrum for TrxA C. Spectrum for TrxB. D. NADPH oxidation curves for TrxB.

concentration, reflecting active TrxB, was 9.89 μM after reconstitution. To check if both TrxA and TrxB were in active form, TrxA 100 μM, TrxB 0.5 μM and NADPH 0.5 mM were used in a spectrophotometer to detect NADPH oxidation. In the control the same amount of TrxA and TrxB were added and spectrum was checked without adding any NADPH. NADPH oxidation confirmed that both the purified TrxA and TrxB are in active form and ready to use for the subsequent experiments.

2.2.4 EPR measurements

Tubes which may contain protein-DNICs were subjected to EPR experiments. Samples were loaded into quartz EPR tubes and were frozen immediately in liquid nitrogen. The EPR spectra were recorded at X-band on a Bruker ESR-30 spectrophotometer using an Oxford instruments ESR-9 flow cryostat (Chemistry Department, Louisiana State University). Routine EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 20 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 2.0 mT; sample temperature, 4.5 K; receive gain, 1.0×10^5.

2.3 Results

2.3.1 TrxA and TrxB are purified in native form and active

Before experimenting with the native form of TrxA and TrxB, his-tagged TrxA, TrxB were purified and used to check NADPH oxidation. If TrxB is able to oxidize TrxA in the presence of NADPH then both TrxA and TrxB are biologically active. However, no NADPH oxidation was observed for his-tagged TrxA and TrxB. The histag may interfere with TrxA and TrxB interaction to prevent the reaction. Then both TrxA and TrxB were purified in native form. The native form of TrxA and TrxB were able to oxidize NADPH, Figure 2.1, D. Therefore the purified native form of TrxA and TrxB are biologically active and these proteins are ready to use for the next sets of experiments. All the experiments done in this thesis were done using the native form of TrxA and TrxB.

2.3.2 Protein-DNICs and TrxA can be separated by the ion-exchange Chromatography

Before the actual DNICs transfer experiments, we adjusted protein separation conditions. For this experiment, TrxA (60 µM to 100 µM) and IlvD-DNICs (40 µM) were mixed together up to a final volume of 1 mL and it was checked if MonoQ sepharose column is able to separate two

proteins in a given set of conditions. The purification process was adjusted by using 20 mM Tris

pH 8.0 as the running buffer and 20 mM Tris 1M NaCl pH 8.0 as the elution buffer. The proteins

content in eluted fractions was checked in SDS-PAGE gel to check separation of IlvD-DNICs

and TrxA and their distribution. For all experiments TrxA eluted first compared to IlvD-DNICs.

The same condition was found effective to separate AcnB-DNICs and TrxA mixture. Presence or

absence of reducing agents, either TrxB or DTT did not affect separation, Figure 2.2.
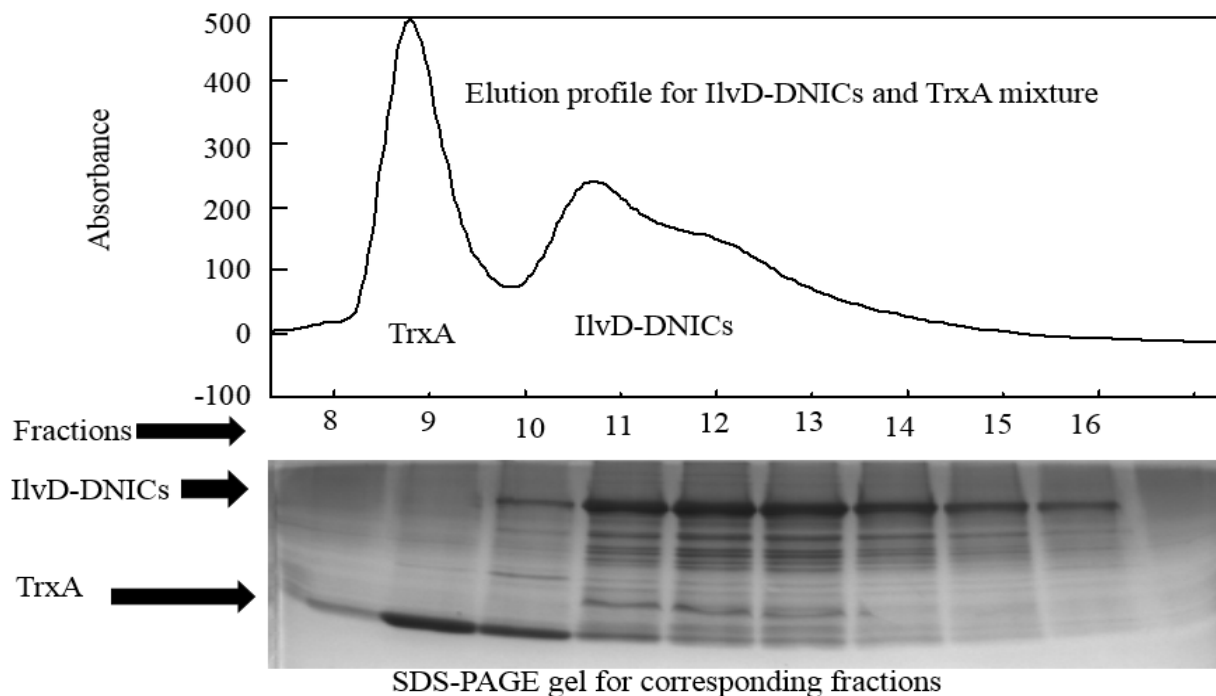
Figure 2.2. TrxA and IlvD-DNICs mixture can be separated by FPLC using MonoQ column. During elution two proteins from two different elution peaks in FPLC. TrxA elutes before the IlvD-DNICs. Each point in the horizontal line shows different elution fractions. After separation the fractions were run on SDS-PAGE gel to check the proteins present in a particular fraction.

From the elution profile and SDS-PAGE gel it was found that after mixing two proteins,

IlvD-DNICs and TrxA were separated by MonoQ. We did the same experiment with DTT or

TrxB, and found that the elution profiles were similar.

2.3.3 Comparing similar fractions between experiment and control

When we were able to separate IlvD-DNICs and TrxA by MonoQ, the next step was to check if a comparison can be established between TrxA fractions eluted from control and experiment reaction based on the amount of TrxA present in SDS-PAGE gel, Figure 2.3.
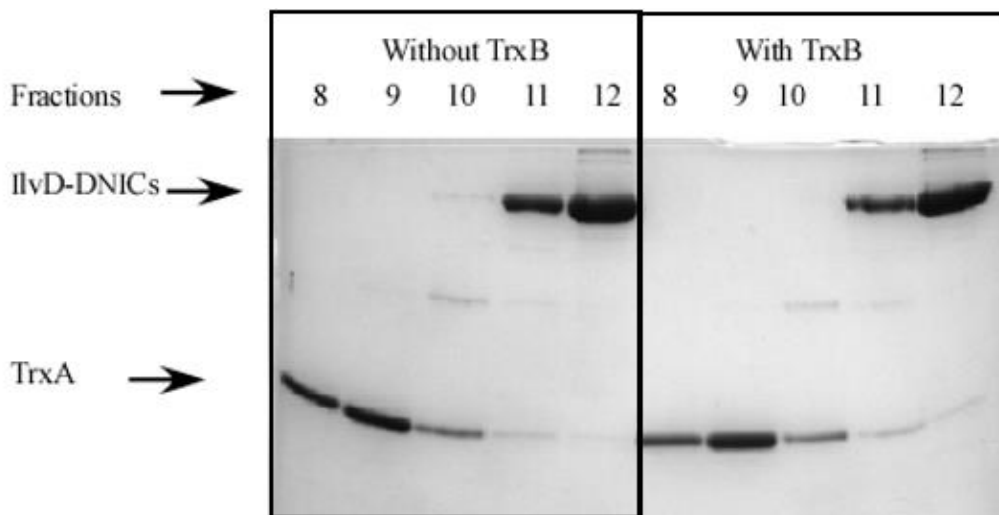
Figure 2.3. Comparing protein distributions with and without reducing conditions on SDS-PAGE gel. Protein mixture of IlvD-DNICs and TrxA with or without TrxB eluted from FPLC, and run on SDS-PAGE gel mainly to compare between control and experimental TrxA fractions. By checking SDS-PAGE gel, fractions that predominantly contain one type of protein were used to check in EPR for the presence of DNICs signal.

From running side by side fractions from experiment and control it can be concluded from Figure 2.3 that fraction 8 in both sides is comparable, as both fractions contain similar amounts of TrxA. This is also true for fractions 9 in both experimental and control. So, EPR signal for fractions similar to 8 or 9 will be used to check the reducing agent mediated DNICs transfer. If DNICs signal is detected in fractions 8 and 9 from experiment but not in the control set of 8 and 9 (TrxA) fractions, that will indicate that DNICs transfer is reducing agent mediated. IlvD-DNICs signal is also compared between control and experimental fractions, for example comparing between fractions 11 or fractions 12.

2.3.4 DTT may reduce both Ilvd-DNICs and AcnB-DNIC to transfer DNICs to TrxA

To investigate DNICs transfer from IlvD-DNICs to TrxA, the protein-bound DNICs (40 µM) were incubated with TrxA (60 µM) in the absence or presence of DTT (2mM final concentration) at 37°C for 1 h under anaerobic conditions. After the incubation, the reaction was stopped by diluting reaction 1:20 in 20 mM Tris 500 mM NaCl pH 8.0. The IlvD-DNICs was then separated from TrxA by MonoQ column. Fractions containing either TrxA or IlvD were checked in EPR for DNICs detection. DNICs signal was compared between control and experimental TrxA fractions and between IlvD fractions, Figures 2.4 and 2.5.
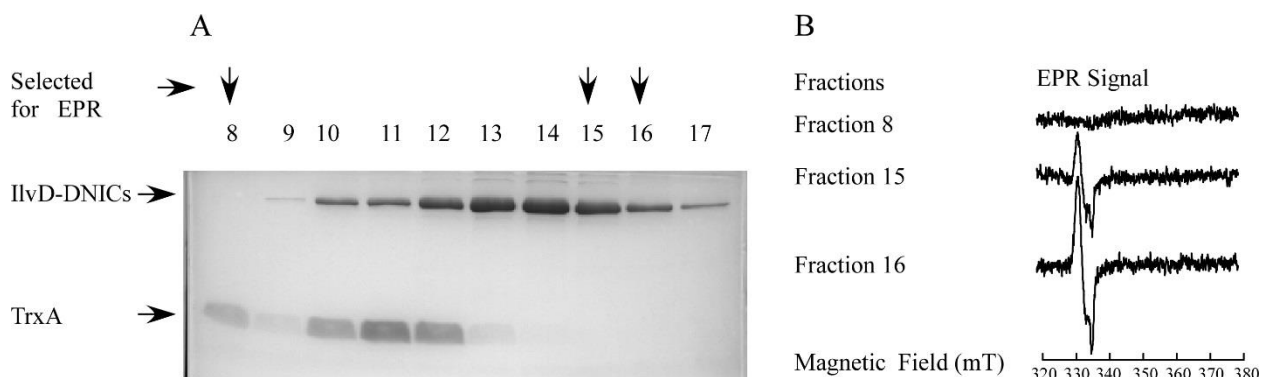
Figure 2.4. DNICs are unable to transfer to oxidized TrxA. A. Fractions eluted from FPLC were checked on SDS gel to identify protein distribution. Fraction 8 contains TrxA and fractions 15 and 16 contained IlvD-DNICs. The arrow mark on the top of lane indicates that these tubes were selected for EPR. B. EPR spectrum of selected fractions. This figure is representative of 1 experiment.

From this experiment, fraction 8 which is a fraction from control experiment that contains TrxA did not show any signal whereas IlvD-DNICs containing fractions 15 and 16 did show DNICs signal as usual. The result indicates that without a reducing agent DNICs are probably not being transferred.

17
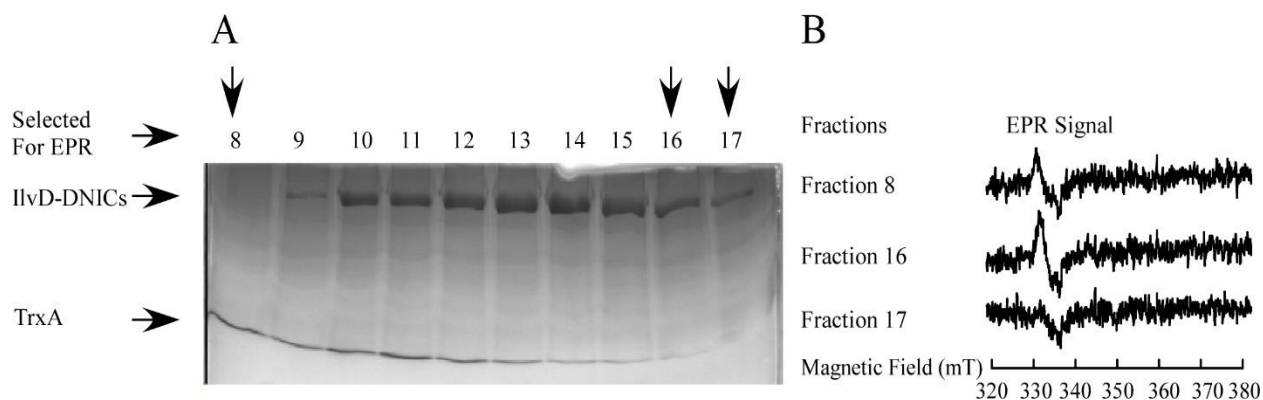
Figure 2.5. DNICs are partially transferred to TrxA under reducing conditions. A. Fractions eluted from FPLC used to check on an SDS gel for the distribution of proteins. Tube 8 contains TrxA and tube 16 and 17 contain IlvD-DNICs. B. EPR results for selected fractions. This figure is representative of 1 experiment.

Figure 2.5 shows that there is DNIC EPR signal in fraction 8, which contains TrxA, and in fractions 15 and 16, which contain IlvD-DNICs. So, both proteins contain DNICs. It may be concluded that reducing reagents may help mediate DNICs transfer. TrxAB/NADPH is one of the predominant reduction systems in the cell. Therefore, the logical next step will be if TrxB and NADPH instead of DTT has the similar activity in promoting the DNIC transfer.

2.3.5 Cellular TrxAB/NADPH reduction system may mediate DNICs transfer from AcnB-DNICs to TrxA

To mimic the biological system, the TrxAB/NADPH system was used. To investigate DNICs transfer from AcnB-DNICs to TrxA, the AcnB-DNICs (40 μM) were incubated with TrxA (60 μM) in the absence or presence of TrxB (2μM final concentration) at 37°C for 1 h under anaerobic conditions. NADPH was degassed and injected in both vials at a final concentration of 0.5 mM. After the incubation, the reaction was stopped by diluting 1:20 in 20 mM Tris 500 mM NaCl pH 8.0. The AcnB-DNICs was then separated from TrxA by using MonoQ column in FPLC. Fractions containing either TrxA or AcnB were checked with EPR, Figures 2.6 and 2.7.
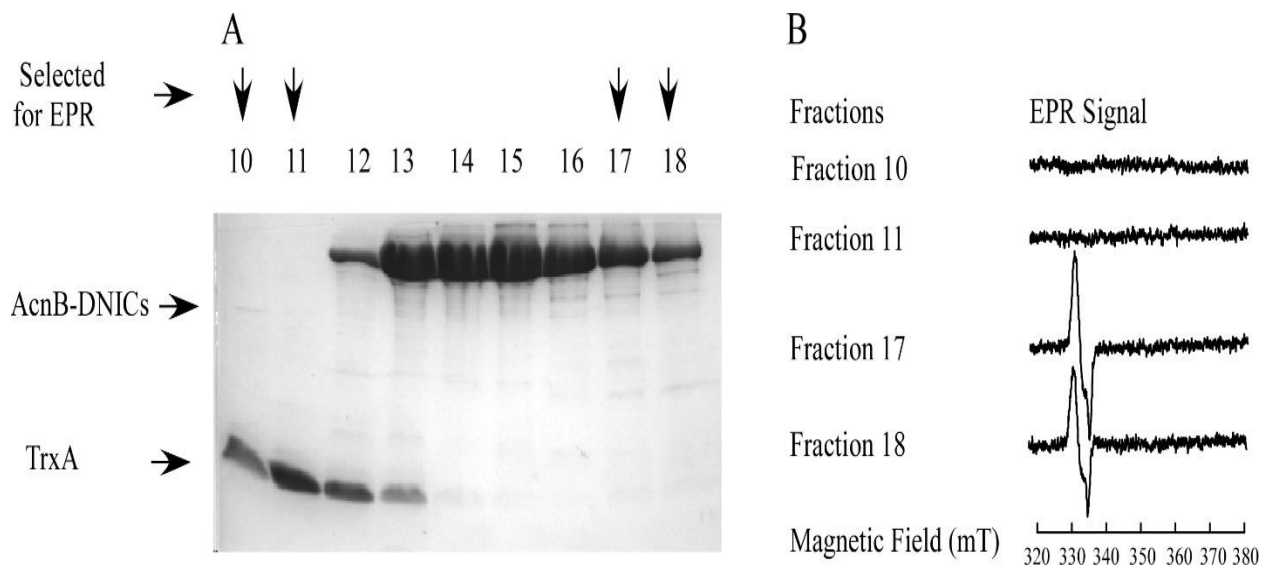
Figure 2.6. In the absence of TrxB, DNICs are not being transferred to TrxA. A. Fractions eluted from FPLC were checked on SDS-PAGE gel for the distribution of proteins. Fractions 10 and 11 contain only TrxA and fractions 17 and 18 contain only AcnB-DNICs. B. EPR spectrum of selected fractions. This figure is representative of one experiment.

In this experiment, fraction 10 and 11, which contain TrxA did not have any DNIC EPR signal whereas fractions 17 and 18 containing AcnB-DNICs did have DNIC EPR signal. The results also supports that protein-bound DNICs cannot be transferred to oxidized TrxA.

From this TrxB mediated experiment, for fraction 9, 10, and 11, which contain TrxA as well as fractions 15, 16, and 17, DNIC EPR signal is detected, indicating that TrxB may be able to mediate DNICs transfer to TrxA. However, the experiment was done only once. Additional experiments are required to confirm the results reported in this study.
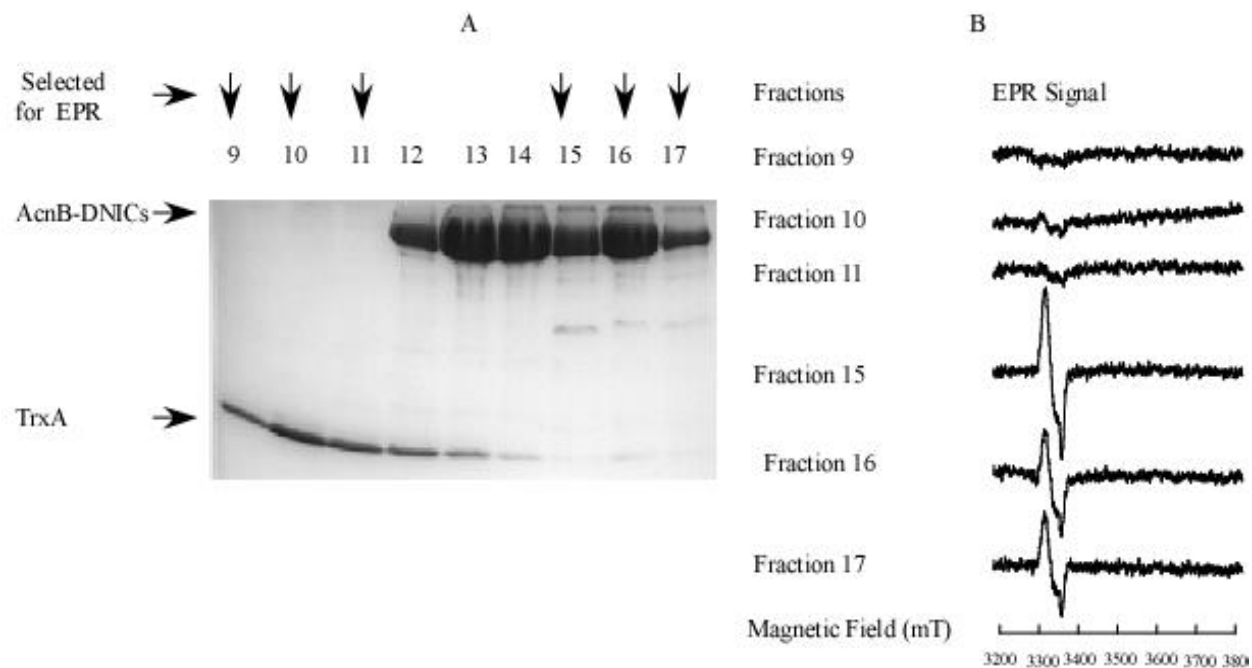
Figure 2.7. TrxAB/NADPH reduction system may also be able to transfer DNICs to TrxA. A. Eluted fractions from FPLC used to check on SDS gel for the distribution of proteins. Fractions 9, 10, and 11 contain TrxA and fractions 15, 16, and 17 contain AcnB-DNICs. B. EPR results for the presence of DNICs for selected fractions. This figure is representative of one experiment.

CHAPTER 3
CONCLUSIONS AND FUTURE DIRECTIONS

From the current study, our preliminary results indicate that DNICs may be transferred from protein-bound DNICs to TrxA, which could be considered a new mechanism to remove DNICs. Previously, it was found that in the presence of oxygen and L-cysteine, protein-DNICs can be broken down into ferrous ion and nitrite (56). So far there are no reports of DNIC decomposition under anaerobic conditions. Therefore, we have used anaerobic conditions to determine if DNIC transfer is possible. Although we did the experiment using *E. coli*, it is expected that for homologous human proteins, we will find the same type of result under similar experimental conditions. DTT may be able to mediate DNICs removal from both IlvD-DNICs and AcnB-DNICs to TrxA because of its strong reducing capacity *in vitro*. Nevertheless, the experimental results reported in this thesis need to be further confirmed and verified.

For future experiments, it is of interest to investigate the kinetics of DNICs transfer from protein-DNICs to TrxA or other protein recipients. Different factors such as concentration, temperature, and equilibrium for the reaction may contribute to the transfer process. We may also explore the specificity of DNICs transfer in different proteins. Also, additional experiments may be carried out with other reduction systems in the cells.

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