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Studies on Phosphofructokinase. Effect of Sodium-Dodecyl-Sulfate and the Specificity of D-Fructose 6-Phosphate Site.

Abdel-latif Elsayed Ashour
Louisiana State University and Agricultural & Mechanical College

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Effect of Sodium Dodecyl Sulfate and the Specificity of D-Fructose 6-phosphate Site

A Dissertation

Submitted to the 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

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ABSTRACT

A study of the influence of the anionic detergent sodium dodecyl sulfate (SDS), on the enzyme phosphofructokinase (PFK) has provided information concerning the relationship between protein conformation and its catalytic activity.

The binding of SDS to the enzyme depends on the detergent concentration. It appears that at high SDS concentration, the detergent binds at two stages, fast (within the first 10 min.), and slow (which lasts for approximately 2 hrs.).

Treatment of the enzyme with increasing concentrations of SDS brought about a sigmoidal kind of inhibition. This inhibition was a result of the detergent binding at different sites on the enzyme, causing changes in the protein conformation. The number of moles of SDS bound was found to be approximately 7±1 moles/mole of PFK.

Several of the known effectors of PFK proved to be efficient protectors against inhibition of this enzyme by SDS.

The kinetic data obtained suggested that the adenine nucleotides compete for the same regulatory site, as suggested by data obtained by other approaches. On the other hand, citrate binds at a different site from that of
the adenine nucleotides. D-Arabinose 5-phosphate competes with D-fructose 6-phosphate (F6P) for the same site. 2,5-Anhydro-D-mannitol 1-phosphate binds the F6P site. Fructose 1,6-diphosphate did not protect PFK against inhibition by SDS, possibly due to its low affinity for the enzyme.

It has been found from the difference spectroscopic measurements that the tyrosyl and tryptophyl residues change their environment upon the binding of SDS. Also, the enzyme might have a nucleotide or oligonucleotide associated with its structure.

Circular dichroism studies revealed characteristic conformational changes in the enzyme which varied with the concentration of SDS used.

The specificity of the F6P site of PFK was studied through testing many analogues of the substrate (F6P). The data obtained showed that: (a) PFK binds only the furanose tautomer of F6P, (b) the enzyme has a restrictive anomeric specificity for the β-form in the phosphorylation process, (c) the C-3 hydroxyl of F6P must be present and must have an L-configuration for significant activity, (d) the configuration of C-5 must have the D-type, (e) C-4 hydroxyl is not essential for binding or catalysis, (f) either C-1 or C-2 hydroxyl must be present, and (g) the C-6 hydroxyl must have an anionic
group, with at least one charge for binding. The compounds that acted as alternate substrate were: 2,5-anhydro-D-mannitol 1-phosphate, (α+β) methyl-D-fructofuranoside 6-phosphate, D-tagatose 6-phosphate, D-tagatose 6-sulfate (poor substrate), and L-sorbose 6-phosphate (poor substrate), and D-psicose 6-phosphate (poor substrate). The analogues that acted as competitive inhibitors were: D-arabinose 5-phosphate and 2,5-anhydro-D-glucitol 6-phosphate.
SECTION ONE: Sodium Dodecyl Sulfate-Induced Inactivation of Phosphofructokinase
I. INTRODUCTION AND OBJECTIVES

The enzyme, phosphofructokinase (ATP:D-Fructose-6-phosphore 1-phosphotransferase, EC 2.7.1.11), is one of the key regulatory enzymes of the glycolytic pathway. It catalyzes the reaction:

\[
\text{D-F6P} + \text{ATP} \xrightarrow{\text{Mg}^{++}} \text{FDP} + \text{ADP} + \text{H}^+ 
\]

Two of the good comprehensive reviews of PFK have been published by Bloxham and Lardy (1973), and Mansour (1972). Both reviews describe its structural features, principal kinetic properties and the proposed mechanisms of action. Following are some of the PFK history pertaining to my work.

The two most important characteristics of PFK are:
(a) its existence in several molecular forms which appear to be a function of pH, enzyme concentration and modifier concentration, and (b) the modulation of its activity by a large number of modifying ligands. The inhibition of PFK by citrate and excess ATP, the activation by FDP, ADP, c-AMP and NH$_4^+$ ions, might be a necessity for its crucial position within the cell's metabolic machinery.

Recently, there has been several reports concerning the binding of different metabolites by PFK. The results obtained by Kemp (1967, 1969) have indicated that the binding of any one of its metabolites could cause
structural changes leading to changes in the affinity of the enzyme for the others. ADP, AMP and c-AMP were bound competitively to the same binding site and act as de-inhibitors by reducing the amount of ATP bound at one or the two regulatory sites of ATP. The inhibition by citrate is either through direct inhibition of F6P binding or by increasing the affinity for ATP which in turn decreases the affinity for F6P. All these modifiers were found to be bound in 1 mole/90,000 g except ATP which binds in the ratio of 3 moles. Lorenson and Mansour (1969) in their binding study with sheep heart PFK confirmed the observations of Kemp and added that both F6P and FDP were shown to have two binding sites for each per 100,000 m wt. of the enzyme. Also, he indicated that citrate may act by decreasing the affinity for F6P and does not enhance the binding for ATP, but may even compete with it for its binding site.

Recently, a new character of PFK has been reported by a Russian group (Volodina et al., 1971, 1972; Pechenova et al., 1973). They indicated the presence of a nucleotide containing phosphorus and pentose firmly bound to PFK, and has an absorption maximum at 257 nm.

The aim of this work is to study the conformation and the binding sites of PFK by investigating the effect of SDS on the enzymatic activity, ultraviolet difference
spectrum and circular dichroism. The binding of different ligands by PFK is discussed.

If, as has frequently been postulated, the enzyme activity is a function of the protein conformation, then changes in the solvent environment which affect activity should also affect protein conformation and vice versa. Many reports by different authors have demonstrated that SDS is one of the most effective and convenient ways to study the relationship between activity and conformation.

The effect of SDS and other n-alkyl sulfate esters on the activity of glutamate dehydrogenase by Maxwell (1962) and by Rogers and Musko (1969,1972) showed that the reaction was stimulated at low concentration, inhibited at higher concentration and that hydrophobic bonding was of major importance in the affinity of these compounds for their binding sites. Applying a modified Hill-type equation to the data, SDS was found to bind cooperatively with the highest relative inhibitory potency. In line with that, Pomeranz (1963) found that the inactivation of crystalline α-amylase is time and pH dependent. The same effect was observed by Torchiniskii and Shipkiter (1963) working with aspartate-glutamate transaminase and Mircevova and Siminova (1966) with asparatate transcarbamylase.

Koike et al. (1969) compared the effect of SDS on tryptophan oxygenase activity, in presence and in absence of some modulators. He concluded that the binding
of these effectors to allosteric sites mediates conformational alterations in the quaternary structure, and strengthens the subunits interaction. This causes a higher concentration of SDS to be a requirement to produce an equivalent effect as in absence of the effectors. Additional evidence that enhancement in the activity of α-chymotrypsin was noted at higher SDS concentration was made by Gaudin and Viswanatha (1972).

The character and the mechanism by which SDS displays its effect on protein structure, was started way back in 1939. Anson (1939) first demonstrated that a commercial detergent, largely composed of SDS, was a more potent protein denaturant than urea or guanidine (very effective at low concentration). Since then, many works have been published describing concepts and speculations regarding this matter. Different techniques have been used, such as, ultraviolet difference spectroscopy, hydrodynamic measurements, CD and ORD.

SDS is well known to protect at very low concentration some proteins against denaturation through either stabilization of ordered structures or organizing the disordered ones of the native protein. It is concluded by Markus et al. (1964) and Barre et al. (1965) that the protection is, in part, based on the formation of detergent bridges extending between a group of nonpolar residues and
positively charged residues (Arg, His and ε-NH₂ group of Lys), located on different loops of the folded structure.

Apparently, proteins either bind large amounts of the detergent or none at all (all or none). Probably once a small amount is bound, it causes disruption of the protein and more is subsequently added (Lundgren et al. (1943); Nelson (1971)).

A great deal of work by many investigators (Hill and Briggs (1956); Biglow and Sonnenberg (1962); Jirgenson (1962, 1966); Pitt-Rivers (1968); Tanford (1968); Fish et al. (1970); Visser and Blout (1971); Nelson (1971); Steinhardt and Reynolds (1969) led to the following conclusions: (a) Aromatic side chains (e.g. Try and Tyr) acquire an altered environment as a result of SDS binding, mostly by their exposure to the solvent. (b) SDS binds very tightly and maximum binding depends on its ability to unfold the protein and the absence of disulfide bonds. (c) Increasing SDS concentration at a given ionic strength will not result in an increase in monomer concentration above a specific critical value (critical micellar concentration), above which each monomer will be incorporated into a micelle. (d) A wide variety of reduced proteins (e.g. BSA, Lysozyme, ovalbumin, Ribonuclease, cyt c, LDH, Mb, Hb, chymotrypsinogen, β-lactoglobulin, F₁ and F₂ histones, etc.) bind identical amounts of SDS on gm to gm bases, (0.4 gm SDS/gm protein for one group and 1.4 gm SDS/gm protein for the other). PFK was found to be of the
second group by Igou (1973). This binding is nonspecific and produces a cooperative conformational change, which leads to a uniform structure (rode-like shape) in the complexed state, at higher SDS concentration. (c) The hydrophobic interior and crevices are good attractive sites in which nonpolar tails can be buried. This leads to conformational changes and creates new helical regions in many cases. Original helices have poor affinity for the detergent tail.

The work described in this dissertation includes a study of the effect of SDS on PFK activity and of the conformational change induced by its binding.

Two of the most useful analytical methods in detecting conformational changes are spectropolarimetry (ORD and CD) and ultraviolet difference spectroscopy.

Circular dichroism (CD) results when an asymmetric chromophore absorbs right hand and left hand circularly polarized light unequally. The CD spectrum is a result of measuring the absorbed light over a spectrum of wavelengths. Many authors (Dratz, 1966), (Freisheim and D'souza, 1971), (Hirata et al., 1971), (Igou et al. 1974) have utilized this technique successfully in observing conformational changes for several proteins and peptide chains.

Ultraviolet difference spectroscopy of proteins is based on the UV absorption in the region 250-310 nm due to
the electronic excitation of their aromatic side chains, tyr, try, and to some extent phe. The perturbations in the absorption spectra of these side chains give information concerning their environment and location, which is brought about by a change in the pH, temperature, solvent composition, etc. As a result of chromophore perturbation, the spectrum will be altered and shifted along the wavelength axis, to a longer (red shift) or shorter (blue shift) wavelength. This is usually accompanied by an increase or decrease in intensity of the spectrum. The red shift of a chromophore might be interpreted as an increase in the polarizability in the immediate environment, such as that produced by absorbed hydrophobic tails on these chromophores. The blue shift might be explained as due to the exposure of buried chromophores to the aqueous solvent. Difference spectroscopy is characterized by its wide applicability to many problems and has been used to determine: the thermodynamic parameters for thermal denaturation; specific interactions between enzymes and their substrates; binding of small molecules and large ions; enzyme actions; and reporter groups. For more detailed information reference should be made to several recent reviews, (Scheraga, 1961), (Wetlauffer, 1962), (Herskovits and Laskowski, 1967), (Donovan, 1969) and (Steinhardt and Reynold, 1969).
The research reported in this dissertation has a dual purpose: (a) to study the structure function relationship of PFK; and (b) to provide information on the nature of SDS binding to PFK.
II. MATERIALS AND METHODS

Reagents

Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine 3',5'-cyclic monophosphate (c-AMP), β-nicotinamide adenine dinucleotide reduced form (NADH), D-fructose 6-phosphate (F6P), D-fructose 1,6-diphosphate (FDP), phosphoenol pyruvate tricyclohexylamine salt (PEP), dithiothreitol (DTT), bovine serum albumin (BSA), aldolase, α-glycerophosphate dehydrogenase-triosephosphate isomerase (α-GDH-TPI), lactic dehydrogenase (LDH), pyruvate kinase (PK), and ethylenediamine tetraacetate (EDTA) were purchased from Sigma Chemical Company. Ammonium sulfate (enzyme grade), potassium chloride, magnesium sulfate and citrate were purchased from "Baker Analyzed" Reagent; imidazole from Aldrich Chemical Company, Inc.; 2,5-anhydro D-mannitol 1-phosphate synthesized by Koerner et al. (1974); tris (hydroxylmethyl) aminomethane (tris-ultra pure) and lauryl sodium sulfate (SDS-ultra pure) from Schwarz/Mann Company.

Phosphofructokinase (PFK) was prepared from young rabbit muscle by the method of Ling et al. (1965). The crystallization step with ATP was omitted to avoid the effect of this nucleotide on the kinetic characteristics of the enzyme. Instead, PFK was passed through G-200
sephadex column to insure highly purified sample, particularly for structural work.

I stored PFK as a concentrated solution (12.0 mg/ml) in 0.1 M KH$_2$PO$_4$ buffer, pH 8.0, containing 0.2 mM EDTA at 4°C, and I noticed that the yield and the specific activity were usually high, so long as the rabbit was young (≈ 3 months old).

In one of the experiments, the auxiliary enzymes, namely aldolase, α-glycerophosphate dehydrogenase-triosephosphate isomerase were dialyzed to eliminate all NH$_4^+$, in which it is usually suspended. The dialysis was for 36 hrs. against three changes of 50 mM Tris-$\text{Cl}$, pH 8.0, containing 20 mM EDTA.

Enzyme Assay

In most of my experiments, I assayed the enzyme activity by the coupled procedure depicted in Figure 1A, which depends on the rate of ADP appearance. The procedure is a modification of glutamine synthetase assay by Wellner et al. (1966). The assay mixture contained 50 mM Tris-$\text{Cl}$, pH 7.8, 4 mM MgSO$_4$, 50 mM KCl, 0.2 mM EDTA, 3 mM DTT, 3 mM PEP, 1 mM ATP, 1 mM F6P, 0.16 mM NADH. The auxiliary enzymes pyruvate kinase and lactic dehydrogenase were present in large excess to avoid being limiting (≈ 30 units each). An appropriate amount of PFK (0.06 μg/ml) was added to initiate the reaction.
In few experiments which will be indicated later on, I used the coupled procedure described by Ling et al. (1966), which depends on FDP assay as shown in Figure 1B.

To test the effect of SDS on PFK, this enzyme was incubated at 4°C for 2 hrs. in 0.05 M Tris-phosphate, pH 8.0, 1 mM EDTA, 3 mM DTT, and the predetermined concentrations of SDS, in presence or in absence of different effectors. In the case of using an effector in the incubation mixture, it was added before adding SDS. I noticed the formation of micelle aggregate for most of the SDS concentrations I have used when plain water was the solvent. Therefore, I prepared SDS solutions in the same buffer described above to avoid micellar formation. PFK concentration in the incubation mixture was 2.4x10⁻³ mg/ml. Final concentrations of SDS in the incubation mixtures ranged from 1x10⁻⁶ M to a concentration that caused complete inhibition of PFK activity.

The enzymatic activity was assayed by monitoring the change in absorbance of NADH at 340 nm, using a Gilford recording spectrophotometer. I set the full chart width for one inch per minute. An enzyme unit was defined as the amount which catalyzes the conversion of 1 µmole of F6P to FDP per minute at pH 7.8 and 28°C.

In most of the experiments I have done in presence and absence of different effectors, enzyme reaction velocities were computed as the change in OD per minute.
and converted to percentage of original activity (control) by the equation:

\[
\frac{v_i}{v} \times 100 = \% \text{ of original activity} \tag{1}
\]

where,

\[v_i = \text{velocity of the enzyme in presence of SDS (OD/min.)},\]

and

\[v = \text{velocity of the native enzyme in absence of SDS (OD/min.)}.
\]

Difference Spectroscopy Measurements

Experimental considerations:

1. The difference spectral studies were carried out with the aid of a Cary Model 14 Spectrophotometer, and the very small difference spectrum was recorded using an expanded scale (0.0 - 0.1 absorbance unit) slide wire.

2. The instrument was allowed time to warm up and stabilized to avoid amplifier drift.

3. Scanning speed was slow enough to prevent "over-ride" on the absorption of the peaks.

4. A flat baseline in the region 350-310 nm is an indication of absence of turbidity, since nonconjugated
Figure 1

This figure indicates the principle and the difference between the two coupled assay procedures. Figure 1A depends on the coupled assay of ADP by the two auxiliary enzymes pyruvate kinase (PK) and lactic dehydrogenase (LDH). While Figure 1B shows the principle of the method of Ling et al. (1966), in which the product FDP was subjected to the auxiliary enzymes namely aldolase, triosephosphate isomerase and α-glycerolphosphate dehydrogenase. In both cases the decrease in NADH absorbance was measured at 340 nm.
proteins are transparent in this region (the tyrosyl residue starts to absorb at 310 nm).

5. A suitable protein concentration may be chosen by plotting the height of the difference spectrum of a series of sample and reference solutions having different protein concentrations against the protein concentration to get a straight line passing through the origin (Beer's Law). The working protein concentration is any one on the linear portion of the curve.

6. An air-air and/or buffer-buffer baseline (zero line) is obtained for the instrument and/or instrument plus cells. This baseline is adjusted to zero throughout the wavelength range of interest, by using a set of potentiometers called "multipots". Their function is to adjust the baseline as a function of wavelength.

7. Well-matched cells should not produce deviations from the baseline in the experimental absorption region of the spectrum of more than ±0.002 absorbance unit, otherwise, the baseline must be readjusted.

8. Exact volumetric manipulations are of great importance. Therefore, errors inherent in pipetting, volumetric adjustment, and transfer of solutions must be reduced to a minimum.

9. Difference spectral measurements should be made soon after the solutions are prepared (10-30 min.), since standing might cause slow aggregation and turbidity.
Absorption cells

It was very convenient to use the double compartment "tandem cells" of Herskovits and Laskowski (1962), (Figure 2). The cells were designed to subtract the solvent and the protein contribution to the difference spectra, which usually occurs by using one compartment cells.

The front part of the cell compartment containing the protein plus the perturbant (SDS) is filled with buffer. This is balanced by placing the perturbant with buffer in the "blank" compartment of the reference cell. The solution compartment of this cell is reserved for the protein solution. The positioning of the filled cells in the instrument should be such that the blank compartments face the source of radiation, so that the light beams enter the blank-containing sections of the cells first (Figure 2).

The amount of buffer, SDS, and protein should be the same in both cells. Any minor difference, particularly in the protein concentration, causes positive or negative absorption relative to the baseline. To correct for that, the solutions should be matched either by patient dilution of the more concentrated solution or by adding more protein to the more dilute one.

Difference spectral measurements

The baseline was first adjusted using the "multipot" knobs, to get a satisfactory zero line in absence and in
Figure 2

The tandem double-compartment cells.
Protein + SDS
Buffer

REFERENCE CELL

Incident light
Buffer + SDS
R1

SAMPLE CELL

Incident light
Buffer

Protein + SDS
S1
S2
presence of the cell and the buffer. The instrument adjustments were at 5 period selector to lower the noise without losing sensitivity. The chart speed was set at x10 (which is considerably slow), the marker at 100 A°, and the scanning was carried out in the region from 330-220 nm.

Final concentration of the solutions in the 3 ml tandem cell compartment was 50 mM Tris-phosphate, pH 8.0, 1 mM EDTA, 0.1% PFK as one equivalent. The solutions were mixed by inversion with parafilm "M" over the top of the cell windows. The baseline was established again by equalizing the protein concentration in both cells. Aliquots of SDS were then added to each cell in equivalents, the largest volume added did not exceed 1/100 of the total cell volume. Equal volumes of the buffer were then added to the corresponding compartments. The spectrum was then recorded after each detergent addition. SDS aliquots used were 4, 6, 10, 20, and 50 equivalents prepared in the same previously described buffer to avoid micellar formation (Figure 3).

A second part was to study the difference spectrum induced by binding the substrate F6P to the enzyme at 1, 3, and 5 mM. The protocol of the experiment was exactly similar to the one described above. The only difference was using F6P instead of SDS. Following the recording of the spectrum of the three substrate concentrations, 50 equivalents of SDS were added and the spectrum was recorded.
Figure 3

A diagram to show the tandem double-compartment cell and the composition of the solutions used. The cells contained 50 mM Tris-phosphate buffer, pH 8.0, 0.1 mM EDTA, 1 equivalent of PFK, and 4, 6, 10, 20, and 50 equivalents of SDS.
Reference Cell (R)

Reference Beam

Reference Cell (R)

Equivalents of SDS +
50 mM Tris-P
1.0 mM EDTA

1.0 Equivalent of PFK +
50 mM Tris-P
1.0 mM EDTA

R₁

PFK & SDS are separated

R₂

Sample Cell (S)

Sample Beam

Sample Cell (S)

50 mM Tris-P
1.0 mM EDTA

PFK & SDS are in the same compartment

S₁

Equivalents of SDS +
50 mM Tris-P
1.0 mM EDTA

1.0 Equivalent of PFK +
50 mM Tris-P
1.0 mM EDTA

S₂
Chymotrypsin Digestion of PFK

Purified PFK was reduced, alkylated, and dialyzed against 0.5% $(\text{NH}_4)_2\text{HCO}_3$, according to Hirs Method (1967). Digestion of PFK suspension by chymotrypsin (2% by wt. of PFK used) was carried out at pH 8.0 for 4 hrs. The reaction was then stopped by lowering the pH to pH 2.0 with formic acid. The digested enzyme was applied to an ion exchange column (Dowex 50-x8), column was developed with gradient of 0.2 M pyridine, pH 2.0 to 2.0 M pyridine, pH 5.0. The pH of the gradient was adjusted with acetic acid.

This experiment was done with the help of Dr. E. Blakeney.

Circular Dichroism

CD was carried out on a Durrum-Jasco J-20 recording spectropolarimeter, calibrated on the basis of $[\theta] = 7260 \text{deg cm}^2/\text{dmole}$ for d-10-camphorsulfonic acid in water (Cassim and Yang, 1970). The calibration factor $F$ was used in Equation (2), for calculating the mean residue ellipticity.

$$[\theta] = \frac{FS(E-B)m \text{res}}{cL} \quad (2)$$
where,

\[ \theta \] = mean residue ellipticity in deg cm\(^2\)/dmole

F = calibration factor for CD

E = experimental chart reading in cm

B = blank or baseline value in cm

S = sensitivity in m°/cm

\( \bar{m}_{\text{res}} \) = mean residue molecular weight in g/mole

c = concentration of solute in mg/ml

L = length of cell path in mm

The mean residue molecular weight used for PFK was 110. Circular silica cells of 1.0 and 10 mm were used depending on the protein concentration. Solutions were made up to test the effect of varying SDS concentrations on the CD of PFK under the following conditions: (a) At 1 mg/ml PFK, 50 mM Tris-phosphate, pH 8.0, 1 mM EDTA. SDS solution was prepared in high concentration in the same buffer and aliquots of 10, 20, 30, 50 and 100 equivalents (in microliter volumes) were taken and introduced into the circular cells using Hamilton microliter syringe to attain the predetermined SDS concentrations. (b) At 0.2 mg/ml PFK, 50 mM Tris-phosphate, pH 8.0, 1 mM EDTA. SDS solution was added in 20, 30, 50, 100 and 150 equivalents relative to PFK. (c) At 0.02 mg/ml PFK, 50 mM Tris-phosphate, pH
8.0, 1 mM EDTA. SDS were taken in 4, 6, 10, 20, 30, 50, and 100 equivalents.

The volume of SDS did not exceed 1/50 of the total sample volume. A control was made at zero SDS concentration. Sample scannings were obtained at 218 nm at which the change in α-, β-, and random coil structures are clear, and also in the aromatic region of the spectrum. Blanks were scanned periodically in the same wavelength regions.
III. RESULTS

Effect of Time of Incubation of SDS with PFK

A preliminary experiment to test the effect of increasing concentrations of SDS on PFK activity indicated that the loss of the catalytic activity is time dependent. Figure 4 demonstrates this time effect at $2 \times 10^{-6}$, $2.5 \times 10^{-5}$, and $4 \times 10^{-5}$ M SDS for 2 hrs. incubation period. The experimental conditions were as described under "Materials and Methods". The activity was measured immediately after incubation, then after 10, 20, 30, 60 and 120 minutes incubation periods. It is clear that more than 50% of the activity was lost within the first 10 minutes at $2.5 \times 10^{-5}$ M and $4 \times 10^{-5}$ M SDS. Complete loss of the activity was observed within 2 hrs. at $4 \times 10^{-5}$ M SDS, while at $2.5 \times 10^{-5}$ M SDS the loss was 60% of the original activity. On the other hand, there was slight enhancement of the activity at $2 \times 10^{-6}$ M SDS relative to the control in absence of SDS. The conclusion of this experiment is that the inactivation occurs on two stages: the first one involves rapid loss of the activity within the first 10 minutes and the second is relatively slow and continues until the reaction reaches equilibrium within 2 hrs. The extent of inactivation is dependent on SDS concentrations at a fixed time.
Figure 4

Activity of PFK using three different SDS concentrations $2 \times 10^{-6}$, $2.5 \times 10^{-5}$, and $4 \times 10^{-5}$ M as a function of the incubation time. Each incubation mixture contained 50 mM Tris-phosphate, pH 8.0, 3 mM DTT, 1 mM EDTA, $2.4 \times 10^{-3}$ mg/ml PFK, and the corresponding SDS concentrations. The incubation was carried out in ice at 4°C. The assay mixture (1 ml) contained 50 mM Tris-Cl, pH 7.8, 4 mM MgSO$_4$, 50 mM KCl, 0.2 mM EDTA, 3 mM DTT, 1 mM ATP, 1 mM F6P, 3 mM PEP, 20 units of the auxiliary enzymes pyruvate kinase plus lactic dehydrogenase, 0.06 μg PFK from the treated SDS incubation mixtures, and 0.16 mM NADH.
Inactivation of PFK as a Function of Increasing SDS Concentrations

Figure 5 indicates the inactivation of PFK as a function of SDS; PFK was preincubated for two hours before assaying its activity. Incubation and assay mixtures were as described under Materials and Methods and in Figure 4. It can be seen that the percentage of the reaction velocity in the presence of SDS in the reaction mixture to that in its absence ($v_i/v \times 100$) is slightly increasing at low SDS concentrations. At SDS concentrations greater than $5 \times 10^{-6}$ M, the activity decreases progressively until complete inhibition is reached at $4 \times 10^{-5}$ M. An SDS concentration of $2.25 \times 10^{-5}$ M is required to effect a 50% reduction of the enzymatic activity. The observed loss of enzymatic activity seems to be a sigmoidal inhibition under this condition. This tends to indicate that the activity exhibited by PFK in the presence of SDS is due to an oligomeric form of the enzyme and that PFK is not disrupted to the monomeric form by SDS concentration up to $4 \times 10^{-5}$ M.

PFK Inactivation as a Function of SDS Concentration in the Presence of Bovine Serum Albumin

Bovine serum albumin used to be one of the constituents of the dilution mixture (stock solution), to stabilize
Figure 5

Inactivation of PFK as a function of SDS concentration. PFK was preincubated for 2 hrs. prior to the assay. Reactants and conditions of incubation and assay were as described under "Materials and Methods" and Figure 4.
CONTROL (NO EFFECTORS)

% ACTIVITY

[SDS], μM

0 10 20 30 40

0 20 40 60 80 100
the enzyme during its assay. I was faced with the problem of whether to include it as usual or not in the incubation mixture with SDS. I carried out the experiment shown in Figure 6 in the presence and in the absence of 0.01% BSA in the incubation mixtures as described in Figure 3 to resolve this problem. From Figure 6 it is clear that bovine serum albumin interferes with PFK inactivation and acts as a protector against SDS inactivation for PFK. Complete inhibition occurred at about 70 μM SDS compared to 40 μM in its absence. This indicates a protecting effect of bovine serum albumin on PFK against the action of SDS. This is probably brought about by the binding of part of the added SDS by the albumin or binding the albumin by PFK. Because of this complicating effect, I excluded the bovine serum albumin from all the experiments with SDS. Reactants and conditions were as described in Figure 5. It is noticed that under these experimental conditions, the incubation time has very little effect on the inactivation after the first 10 minutes and could be neglected.

Inactivation of PFK as a Function of SDS Concentration in Presence of Some Nucleotide Effectors

Figure 7 illustrates the effect of the nucleotides, AMP, ADP, c-AMP, ATP, and MgATP on the SDS-induced inactivation of the native enzyme. All nucleotides were
Figure 6

PFK inactivation as a function of SDS concentration in presence of 0.01% BSA in the incubation mixture. BSA was added to PFK, then SDS, and incubated for 2 hrs. prior to the assay. Incubation and assay condition as in Figure 5.
0.01% Bovine serum albumin (BSA)

CONTROL (No effector)

% ACTIVITY

[SDS], μM

0 20 40 60 80
Figure 7

The protection of PFK against SDS-induced inactivation by incubating the native enzyme with 1 mM of ATP, c-AMP, MgATP (1:4), and ADP separately. Incubation time was 2 hrs. The protector was added to the enzyme before adding SDS. Experimental conditions were as described in Figure 6.
1 mM ADP

Mg ATP (4:1 mM)

1 mM AMP

1 mM ATP

1 mM c-AMP

CONTROL (No effect)

[SDS], µM

% ACTIVITY

0 20 40 60 80 100 120

0 20 40 60 80 100
present in the incubation mixture at 1 mM concentration. It is clear that all of them protect PFK against the action of SDS, i.e., a higher concentration is required to bring about a 50% inhibition equivalent to that observed in the absence of these nucleotides. From some preliminary experiments the sequence of addition and the time effect were not significant. The incubation time was 2 hrs. The Figure shows that the percentage of activity and the protection by AMP and ATP separately are identical. The same thing can be said for c-AMP and MgATP, except that they offer much more protection than AMP or ATP. ADP offers the highest protection of all of them.

A 50% inactivation for the control with no effectors and in presence of AMP or ATP, c-AMP or MgATP, and ADP as protectors was obtained at 25, 41, 55, and 83 μM SDS, respectively. A complete inactivation for the same consecutive group was shown at 40, 60, 71, and 110 μM SDS. It should be noted that all curves exhibited a sigmoidal profile.

Of particular significance is the control point at which there is complete inhibition (4×10^{-5} M). This same concentration of SDS in presence of ATP or AMP, c-AMP or MgATP, and ADP produces 55, 93 and 116% activity, respectively.

The conclusion from this experiment is that AMP, MgATP, and c-AMP exert a protective effect against SDS
action although to varying extents. ADP offers the highest protection relative to other nucleotides.

**Protection Against SDS-Induced Inactivation of PFK by Its Modulators and a Substrate Analogue**

Figure 8 provides a comparison of the protection against SDS-induced inactivation affected by incubating the native enzyme with 1 mM F6P, 1 mM FDP, and 1 or 10 mM 2,5-anhydro-D-mannitol 1-phosphate, separately. It is clear that FDP and 2,5-anhydro-D-mannitol 1-phosphate, at 1 mM, each has largely no effect on protection compared to the control experiment in absence of any effector. Increasing 2,5-anhydro-D-mannitol 1-phosphate concentration to 10 mM induces some protection, although it is still low compared to the protective effect of F6P.

A 50% inactivation was observed at the following concentrations of SDS and of effectors: the control, FDP (1 mM), and 2,5-anhydro-D-mannitol 1-phosphate (1 mM) at about 27 μM SDS; for 2,5-anhydro-D-mannitol 1-phosphate (10 mM) at 39 μM SDS; and for F6P (1 mM) at 64 μM SDS. Comparing the inhibitory concentrations of SDS in the presence and in the absence of F6P, it is obvious that approximately double the inhibitory concentration of the control was required to inhibit PFK in presence of F6P (1 mM).
Figure 8

The inactivation of PFK by SDS in presence of the product (FDP), the substrate (F6P), and the substrate analogue 2,5-anhydro-D-mannitol 1-phosphate, separately. Reactants and conditions were as described in Figure 7.
The Effect of D-Arabinose 5-phosphate on the SDS-Induced Inactivation of PFK

It has previously been shown that D-arabinose 5-phosphate competitively inhibits the catalytic activity of PFK (Koener et al. (1974). It can be seen that increasing D-arabinose 5-phosphate concentrations inhibit the catalytic activity in absence of SDS (Figure 9). In its presence, increasing D-arabinose 5-phosphate concentrations protect against the induced inactivation by SDS. If there is no protection, the two curves would appear to be parallel with the one in presence of both SDS and D-arabinose 5-phosphate being of lower activity due to their combined inhibitory effects. As the concentration of D-arabinose 5-phosphate reaches 17 mM protection begins to appear against SDS-induced inactivation. The incubation time was one hour prior to the assay. The F6P concentration in the assay mixture was relatively low, namely 0.02 mM, so as to cause the D-arabinose 5-phosphate inhibition to be more pronounced. The $2.5 \times 10^{-5}$ M SDS concentration used represents about 40% activity of the control curve in Figure 5. Reactants and conditions were as described in Figure 8.
The protection against SDS-induced inactivation by increasing D-arabinose 5-P (competitive inhibitor) concentration in the incubation mixture. Incubation time was 1 hour and the F6P concentration was 0.02 mM. Other reactants and conditions were as previously described in Figure 8.
$v(OD./min.)$ vs. $[A5P]$, mM

- **2.5 \times 10^{-5} \text{M SDS}**
- **CONTROL (NO SDS)**
The Lack of Protective Effect of Citrate Upon the SDS-Induced Inactivation of PFK

Citrate is very well known as a potent inhibitor for PFK (Abrahams and Younathan, 1973). It can be seen from Figure 10 that citrate has no protective effect as observed with D-arabinose 5-phosphate (Figure 9). Increasing concentrations of citrate inhibit the catalytic activity in the absence and in the presence of SDS in a parallel manner. This indicates that citrate affords no protection against the inactivating effect of SDS, and their effects are additive. The incubation mixture here is the same as previously described in Figure 9, while the assay procedure is completely different. The method of Ling et al. (1966) was used and the auxiliary enzymes were dialyzed. The assay mixture contains 50 mM imidazole-HCl buffer (pH 7.0), 0.5 mM F6P, 1 mM ATP, 1 mM NH₄Cl, 4 mM MgSO₄, 0.2 mM EDTA, 3 mM DTT, 0.16 mM NADH, auxiliary enzymes in large excess, and PFK in an appropriate amount. The reason for using this assay method and pH 7.0 is to observe the inhibitory effect of citrate more accurately. Incubation time was 1 hour prior to the assay, and citrate was added before SDS. 2.5x10⁻⁵ M of SDS was used, which gives about 40% activity on the control curve (Figure 4).
Effect of citrate on the SDS-induced inactivation of PFK. The assay method of Ling et al. (1966) was used. The assay mixture contains 50 mM Imidazole-HCl buffer (pH 7.0), 0.5 mM F6P, 1 mM ATP, 1 mM NH\textsubscript{4}Cl, 4 mM MgSO\textsubscript{4}, 0.2 mM EDTA, 3 mM DTT, 0.16 mM NADH, auxiliary enzymes (aldolase, triosphosphate isomerase, \(\alpha\)-glycerolphosphate dehydrogenase) in excess, and PFK in an appropriate amount. The enzyme was incubated for 1 hour with increasing concentrations of citrate and a fixed amount of SDS (2.5x10\textsuperscript{-5} M).
CONTROL (NO SDS)

25 × 10^{-5} M SDS

\[ v(\text{OD./min.}) \]

\[ [\text{CITRATE}], \text{ mM} \]
The Effect of SDS on the Sigmoidal Kinetics of PFK

It is known that an inhibitory concentration of ATP will increase the sigmoidicity of the PFK curve with respect to F6P while activators will decrease it. To produce the sigmoidal curve for the enzyme, it is necessary to know this inhibitory level of ATP. As shown in Figure 11, PFK inhibition by ATP is also a function of the NH$_4^+$ concentration in the assay mixture. This experiment was carried out under the same experimental conditions of Figure 10 except for the use of 0.1 mM F6P, and three different NH$_4^+$ concentrations (0.1, 1, and 2 mM). A concentration of 0.5 mM ATP was found to be sufficient to produce the sigmoidal curve as shown in Figure 12.

Figure 12 depicts the sigmoidal kinetics of PFK as a function of F6P concentration at 2.0x10$^{-6}$ and 2.5x10$^{-5}$ M SDS concentrations. The incubation time was 1 hour. The assay mixture contained 0.1 mM NH$_4$Cl, 0.5 mM ATP and imidazole-HCl buffer (pH 7.0). It is clear that the incubation of PFK with SDS at low concentration (2.0x10$^{-6}$ M) or higher concentration (2.5x10$^{-5}$ M) did not affect the sigmoidicity relative to the control curve in the absence of SDS.
Activity of PFK as a function of ATP concentration, at 0.1 mM F6P, 0.1, 1, and 2 mM NH$_4^+$.

Each reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.0), 4 mM MgSO$_4$, 0.2 mM EDTA, 3 mM DTT, 0.16 mM NADH, excess dialyzed auxiliary enzymes, and an appropriate amount of PFK. Other reactants and conditions were as described under "Materials and Methods" and Figure 10.
v (OD./min.)

2.0 mM NH₄Cl
1.0 mM NH₄Cl
0.1 mM NH₄Cl

V (OD./min.)

[ATP], mM

0 0.2 0.4 0.6 0.8

49
Figure 12

PFK activity as a function of F6P concentration in the presence of $2.0 \times 10^{-6}$ and $2.5 \times 10^{-5}$ M SDS in the incubation mixture. The enzyme was incubated for one hour. The assay mixture contained 0.1 mM NH$_4$Cl, 0.5 mM ATP and imidazole-HCl buffer (pH 7.0). Experimental conditions were as described previously in Figure 11.
Treatment of Some of the Previous Data in Terms of the Hill Equation

The previous sigmoidal curves (Figure 12) in the presence and in the absence of SDS were replotted in terms of the Hill equation (Figure 13).

\[ \log \frac{v}{V_{\text{max}}} = n \log [F6P] - \log K_m \]  

where,

\( v \) = velocity of PFK at a particular FGP concentration  
\( V_{\text{max}} \) = maximum velocity, obtained experimentally  
\( K_m \) = Michaelis constant, obtained from the interception with the \( \log \frac{v}{V_{\text{max}}-v} \) axis  
\( n \) = Hill number (the slope of the line) a measure of the cooperativity between the subunits

Figure 13 confirmed the previously reached conclusion of Figure 12. The Hill coefficient was about the same for all of them, i.e., 2.2, 2.1, and 2.2 for control, 2.0x10^-6 M SDS and 2.5x10^-5 M SDS, respectively. This indicates that the degree of cooperativity is the same in the presence and in the absence of SDS.

The values of the Hill coefficient obtained may seem to be low, but they usually vary from 2.0 to 5.4 depending
Figure 13

Replot of PFK activity as a function of [F6P] (data from Figure 10) in terms of the Hill equation.
on the concentration and ratio of ATP to other activators present in the system.

Replotting the Inactivation Curve (Figure 5) of PFK as a Function of Increasing SDS Concentration in the Presence and in the Absence of the Natural Substrate (F6P) in a Modified Form of Hill Equation

It has been shown mathematically that some of the characterized types of enzyme inhibition are amenable to a Hill-type treatment. Thus the logarithm of a function of the degree of inhibition bears a linear relationship to the logarithm of the inhibitor concentration over a wide range. In most of the cases that have been studied, the slope of such a line has an integral value indicating that exactly one or more inhibitor molecules react cooperatively to totally inactivate the enzyme. In other cases, the slope does not have an integral value. This is mostly due to incomplete allosteric interaction or incomplete inhibition (Atkinson, 1965 and 1966).

A modified Hill-type equation was derived and suggested by Dr. W. L. Mattice to fit the inactivation curve of PFK by SDS in the presence and in the absence of effectors. This equation was applied to the data obtained in presence and in absence of F6P. The suggested equation was the following:
\[ \log \left( \frac{v}{v_i} - 1 \right) = \alpha \log[SDS] + \log c \quad (4) \]

where,

\[ v \] = the velocity in the absence of SDS
\[ v_i \] = the velocity in the presence of SDS
\[ \alpha \] = the interaction coefficient (the slope, or the number of moles per mole of PFK)
\[ c \] = constant which can be determined from the interception of the line with the ordinate

Figure 14 and 15 are replots of the data of Figure 5 and the F6P curve of Figure 8 according to Equation (4).

The conclusion from these two plots is that the binding of SDS to PFK in the presence and in the absence of 1 mM F6P in the incubation mixture is cooperative.

Also, 7.3±1.0 moles and 7.1±0.6 moles of SDS are bound per mole of PFK in the presence and in the absence of the substrate, respectively. These values for \( \alpha \) were calculated by using unweighted least-squares according to Fraser and Suzuki (1973).

The Difference Spectra of PFK as a Function of Increasing SDS Concentration

Figures 3 and 4 show the tandem double-compartment cells, the composition of the different solutions, and
Replot of the inactivation curve of PFK as a function of SDS concentration (Figure 5), in terms of a modified Hill-type equation.
$a = 7.1 \pm 0.6$
Figure 15

Replot of the inactivation curve of PFK in presence of 1 mM F6P as a function of SDS concentration (Figure 8), in terms of a modified Hill-type equation.
\[ \text{ImM F6P} \]

\[ \alpha = 7.3 \pm 1.0 \]
The difference spectra of PFK as a function of increasing SDS concentration. Reactants and conditions were as previously described under "Materials and Methods".
the positioning in the Cary 14 cell compartments. The difference spectral experiments were carried out at room temperature by titrating the enzyme with increasing aliquots of SDS. The difference spectra obtained are shown in Figure 16, at 4, 6, 10, 20, and 50 equivalents of SDS. There are characteristic changes in the shape, the location, and the intensity of the spectra at low SDS equivalents (less than 10) and at higher equivalents (greater than 10). At low SDS equivalents there exist a broad trough between 295 nm and 310 nm, and two peaks with maxima at 282.5 nm and 288.7 nm. A broad trough extends from 277.5 nm to 236.3 nm, with increasing negative intensity along this wavelength range and reaches its minimum around 238.7 nm. At higher SDS equivalents (10, 20, and 50), there is progressive increase in the negative intensity and two troughs appear centered at 295 nm and 286 nm. The negative intensity of the trough near 240 nm increased as a function of SDS concentration, with the appearance of a shoulder near 260 nm at 20 equivalents of SDS.

**The Difference Spectra of PFK as a Function of Increasing F6P Concentration**

Figure 17 depicts the difference spectra of PFK at 1, 3, 5 mM F6P, and 5 mM F6P plus 50 equivalents of SDS. First noted is that the general shape of the spectra was
Figure 17

The difference spectra of PFK as a function of increasing F6P concentration and in the presence of 50 equivalents of SDS. Experimental conditions were as described under "Materials and Methods".
similar in the presence of varying F6P concentration but quite different after adding the 50 equivalents of SDS plus the 5 mM F6P. Secondly, there was an increase in the intensity of the spectrum from 1 to 3 mM F6P and then a decrease at 5 mM. Thirdly, there was a peak at 250 nm, troughs at 280 nm and 238 nm, and a shoulder around 260 nm which was completely abolished after addition of SDS. Also, the 50 equivalents of SDS produce two troughs at 286 nm and 295 nm. Reactants and conditions were as described in Figure 16 and under "Materials and Methods".

The Ultraviolet Absorption Spectra of the Nucleotide Containing Fraction Isolated after Digestion with Chymotrypsin

In Figure 16 a trough of an unusual shape and intensity appeared in the short wavelength region between 230 and 260 nm. Such an absorption in this region is unexpected from the usual protein components known to absorb here. This and other evidence which will be mentioned in the discussion led me to a search for a nucleotide or oligonucleotide in association with PFK. Consequently, a chymotrypsin digestion was carried out on PFK, and the product subjected to an ion exchange chromatography. Fractions of the effluent were monitored in the ultraviolet light to locate the fractions containing the nucleotide.
Figure 18 depicts the ultraviolet spectra of the fraction containing the nucleotide (or oligonucleotide) in neutral, alkaline (pH 11.0), and acidic (pH 1.0) solutions. The spectrum is greatly affected by acidic pH, while it is approximately the same at neutral and alkaline pH's.

Circular Dichroic Changes of PFK as a Function of Increasing SDS Concentration

As previously indicated under "Materials and Methods", the CD spectrum of PFK, at 0.02, 0.2, and 1 mg of protein/ml was scanned throughout the aromatic and the peptide chain absorption region. The only positive data obtained was in the peptide region (240-200 nm). Consequently, a wavelength of 218 nm was used to monitor the change in molar ellipticity, [θ], as a function of increasing SDS equivalents.

Figure 19 shows the change in [θ] of PFK as a result of its titration with increasing SDS concentration. Experimentally three PFK concentrations and 2, 4, 10, 20, 30, 50, and 100 equivalents of SDS were used. It is clear that the change in the [θ] is dependent on the protein concentration. This also might indicate that the change in the conformation is quite different in each case of protein concentration examined. It is difficult to tell exactly what kind of conformational changes occur when
Figure 18

Ultraviolet spectrum of the nucleopeptide isolated from the chymotrypsin digestion of PFK. The spectrum was carried out under acidic, neutral, and alkaline conditions. Cary 14 adjusted at 0.5 A°/sec (x10), 100 A° marker and recorder selector at 1.
Figure 19

Plot of changing ellipticity at 218 nm of PFK (0.02, 0.2, and 1 mg/ml) in 50 mM tris-phosphate buffer (pH 8.0), and 1 mM EDTA versus increasing SDS equivalents. The baseline signal is subtracted from the experimental signal to obtain the net signal from which the ellipticities [\( \theta \)] are calculated according to Equation (2). The spread shown at each point indicates the degree of noise (error in the signal).
The diagram shows the change in $-\theta_{218} \times 10^{-3}$ deg cm$^2$ dmole$^{-1}$ with the concentration of SDS (Equivalents) for different concentrations of PFK. The concentrations are 0.02 mg PFK/ml, 1.0 mg PFK/ml, and 0.2 mg PFK/ml.
comparing them to model systems of α-, β-, and random coil structures.

Figure 20 depicts the effect of SDS on the CD spectrum of PFK (0.2 mg/ml) in the presence and in the absence of 1 mM F6P. Quite obvious here, is the difference in the CD spectrum in the presence and in the absence of the substrate. In the absence of F6P the [θ] decreases to a minimum at 30 equivalents of SDS, then increases to a maximum at 40 equivalents of SDS followed by another decrease. In the presence of F6P the spectrum, as a whole, moves in the opposite direction with the sharp minimum at 30 equivalents becoming a shallow valley. This might indicate that the conformational changes in presence of the substrate are completely different and in the opposite direction from these in its absence.
Figure 20

Plot of changing ellipticity at 218 nm of PFK (0.2 mg/ml), in the presence and in the absence of 1 mM F6P versus increasing SDS concentrations. Experimental conditions were as described under "Materials and Methods" and as in Figure 19.
IV. DISCUSSION

In this study, the anionic detergent SDS has been used to gain an insight into the dependence of the activity of PFK on its state of aggregation and the nature of the forces that hold its subunits together in the native oligomeric form. Moreover, the techniques of UV difference spectroscopy and circular dichroism were applied to investigate the conformational changes induced by SDS in presence and in absence of the natural substrate (F6P) of this enzyme.

The data reported here shed some light on the nature of SDS binding to PFK. It appears that some of the SDS molecules bind at a fast rate, within the first ten minutes, and the remainder at a slower rate, until the reaction reaches equilibrium within approximately two hours. Treatment of the results of Figure 5 and 8 in the presence and in the absence of 1 mM F6P with a Hill-type equation indicated that the inactivation of PFK was caused by a sigmoidal type inhibition and approximately 7 moles of SDS were bound per mole of PFK. Also, treatment of the sigmoidal curves of Figure 12 with the Hill equation confirmed the same kind of sigmoidal inhibition. The SDS binding to PFK appears to be cooperative. From these data, the binding of the first few molecules of SDS induces a slight conformational change, which then
facilitates the binding of subsequent ligands. This kind of binding was observed by many investigators (Ray et al., 1966, Reynolds et al., 1967).

There was no effect by SDS binding at low or high SDS concentration, on the sigmoidal kinetics of PFK and the cooperativity between its subunits was apparently retained.

While these results show that SDS at low concentration activates PFK, a loss of activity begins to appear at higher concentrations until complete inhibition is reached, in the presence or in the absence of effectors. This kind of behavior has been observed with many other enzymes as indicated in the "Introduction". Of particular interest is the activation of dihydrofolate reductase up to twice the control value by increasing urea concentrations (Reyes and Huennekens, 1967). Also, the slight inhibition followed by a large increase in the activity of chymotrypsin (using n-acetyl-L-tyrosine ethyl ester as substrate) by increasing SDS concentrations (Gaudin and Viswanatha, 1972) provides a similar example. The observed effect of SDS on PFK is in general agreement with these and other reports. It seems that a low concentration of SDS brings about a conformational change indicative of an increase in enzymatic activity. At higher concentration, however, inhibition results.

Recently, the kinetic characteristics of PFK have been studied extensively. One of these allostERIC
features of PFK is the effect of the adenine nucleotides on its activity. The ATP inhibition and activation of the enzyme was shown by Parmeggiani and Bowman (1963), and Uyeda and Racker (1965) to depend on its concentration as well as on the pH. Another important characteristic of PFK is its sigmoidal response to increasing F6P concentration at relatively low pH (Mansour and Mansour, 1962). The binding studies on rabbit skeletal muscle and sheep heart PFK by Kemp and Krebs (1967), and Lorenson and Mansour (1969) indicated the presence of a binding site that is highly specific for the adenine nucleotides, namely, AMP, ADP, ATP, and c-AMP. The number of binding sites per protomer for AMP, ADP, c-AMP, ATP, F6P, and FDP was found to be 1, 1, 1, 3, 2, and 2, respectively. The three binding sites of ATP include one catalytic and two regulatory sites. The two binding sites each of F6P and FDP are two different classes of binding, one of low affinity and one of high affinity.

The results presented in Figure 7 and 8 might provide a clue as to the nature of the binding sites of these different modulators and substrates. It can be seen from Figure 7 that all four adenine nucleotides examined induce about the same relative conformational change upon binding to the regulatory site. They bring about the same relative protection against SDS inactivation. Also, this kind of behavior seems to be in accordance with results reported
from other laboratories (Mansour, 1972), which indicate that all these nucleotides activate PFK and compete for the same regulatory site. It appears that ATP and AMP provide about the same protection. c-AMP or MgATP offer slightly better protection than either ATP or AMP. ADP offers the greatest protective effect against SDS-induced inactivation. These relative protective effects of the three groups might be explained on the basis of their structures and their fit at the regulatory site. ADP appears to induce the highest protection as a result of having highest affinity for the regulatory site. ATP has one phosphate group more and AMP one less than ADP. Thus, their affinity for the regulatory site is probably lower than that of ADP. Adding Mg$^{++}$ to ATP or using c-AMP as protectors afforded more protection than in the case of ATP alone or AMP, but still less than ADP. Therefore, their binding at the regulatory site is probably tighter than AMP or ATP alone.

The protection against SDS-induced inactivation by F6P, FDP, and 2,5-anhydro-D-mannitol 1-phosphate, as shown in Figure 8, is highest with F6P. This is probably due to the fact that F6P has the highest affinity, i.e., lowest dissociation constant, for PFK as compared to the other 2 sugar phosphates. It appears that the existence of the hydroxyl group at C-2 of the natural substrate makes a difference in the fit at the catalytic site.
However, increasing 2,5-anhydro-D-mannitol 1-phosphate to 10 times the original concentration affords more protection, but still less than for F6P. This indicates that the hydroxyl group at C-2 is not critical for activity. FDP at the same concentration as F6P offers no protection against the inactivation of PFK. This is probably due to the low affinity of PFK for FDP or because of its binding at a specific site different from that of the adenine nucleotide or the F6P sites. The possibility that FDP binds at a site different from that of F6P was shown to exist by Setlow and Mansour (1972) in their binding studies with PFK.

As mentioned in the "Introduction", citrate is a potent competitive inhibitor for PFK. It is assumed by Kemp and Krebs (1967), and Lorenson and Mansour (1969) that it exerts its effect either by binding the same regulatory site as ATP or nearby this site. The results of Figure 10 indicated that citrate did not afford the kind of protection expected from binding to the adenine nucleotide regulatory site. This suggests that citrate binds a site different from the nucleotide site. Arabinose 5-phosphate apparently competes with F6P for the catalytic site, since it provided the same kind of protection as in the case of F6P. Besides this, there is a similarity in their structures; the difference being the lack of a hydroxymethylene group in the arabinose.
5-phosphate molecule corresponding to the C-1 in F6P (Figure 26, Section Two).

Difference spectroscopy and CD measurements

An interesting piece of information was obtained from the difference spectral studies of Figure 16 and 17. This information may help in revealing the nature of the binding sites of SDS and the changes in the environment of some of the aromatic residues of PFK.

The absorption of light between 270 and 310 nm by proteins is almost all due to tyrosyl and tryptophyl residues (Wetlaufer, 1962). In the short wavelength region near 230 nm, several components such as aromatic amino acid residues, histidine, cysteine, and peptide groups contribute to the absorption. This renders this region less meaningful than the aromatic region.

The results in Figure 16 indicate that the detergent produces characteristic difference spectra even at low concentrations (4 and 6 equivalents). This detergent effect is characterized by a slight depression between 295 and 310 nm, which becomes deeper as the number of equivalents increase. This depression may be attributed to an alteration in the environment of the tryptophan and tyrosine residues, mostly caused by increased solvation (Donovan, 1969). The two peaks at 282.5 and 288.7 nm are characteristic of tyrosine red shift, with some contribution
by tryptophan. This red shift might be a result of shielding of the chromophore by the hydrophobic tail of the detergent (Steinhardt et al. 1972). The trough at 295 nm is very characteristic of the blue shift of the tryptophan residue incident to transfer from apolar to more polar environment. The blue shifted trough at 286.5 nm contains components due to both tryptophan and tyrosine.

The region from 260 to 275 nm contains elements contributed mostly by phenylalanine. This contribution was shown by Herskovits (1967) to be slight and can be neglected. Thus it seems feasible that there is another unknown component different from phenylalanine absorbs in this region.

The trough near 240 nm increases sharply in intensity and broadens over a wide range of wavelength as the number of SDS equivalents increases. The change of absorption of the peptide bonds, either because of a change in their geometrical arrangement or because of an environmental change, would not produce such an unusual trough. Their absorption was shown by Polet and Steinhardt (1968) to be sharp, narrow, and near 230 nm. The CD experiment (Figure 19) proved the presence of a conformational change in the peptide backbone as a result of SDS binding under the same experimental conditions as the difference spectroscopy experiment. Consequently, if we assume that all the components are contributing to the absorption in this
region, they are still not enough to explain this unusual trough. The amino acid analysis performed in three different laboratories (Parmeggiani et al. 1966; Paetkau et al. 1968; Tarui et al. 1972) did not account for the total weight of PFK. This discrepancy led Bloxham and Lardy (1973) to suggest that the presence of other components contribute to the total structure of PFK. This suggestion was supported by a series of reports by a Russian group (Volodina et al., 1971, 1972; Pechenova et al., 1973) who provided evidence for the existence of a nucleotide tightly bound to PFK. All these observations lead me to believe that some kind of nucleotide or oligonucleotide is contributing to this trough (230-260 nm) together with the other components. Further evidence for the presence of a nucleotide was obtained by carrying out the chymotrypsin digestion, applying the product to ion exchange column and monitoring the effluent fractions in the UV region as shown in Figure 18. At the time this dissertation was being written, detailed information and characterization of this nucleotide were still being carried out by a group under the direction of Dr. E. S. Younathan.

The protection against SDS-induced inactivation of PFK by different effectors may be interpreted in the light of the results obtained by the difference spectra (Figure 17) and the CD spectra (Figure 20). The binding of the substrate by PFK induces changes in the environment of
some of the chromophores (Figure 17). The spectral changes observed in the presence of the substrate were different from those observed in its absence. In addition, the CD spectrum (Figure 20) indicates a change in the conformation of PFK in the presence of F6P opposite to the direction of the change induced by SDS alone. Thus, the changes in the chromophores environment and in the conformation of the enzyme, as a result of the substrate binding, might explain its protective effect against SDS action. It appears from Figure 19 that the effect of SDS on PFK conformation might depend on the protein concentration and its state of aggregation. Consequently, it would be difficult to correlate these data with the corresponding data in the kinetic work. This is because the amount of protein used in the kinetic work is much less than that used in the CD and the difference spectroscopy experiments. However, the CD and the difference spectroscopy studies gave some information concerning the conformational changes brought about in PFK upon binding of different modulators and SDS to the enzyme.
SECTION TWO: The Specificity of Fructose 6-phosphate Site
I. INTRODUCTION AND OBJECTIVES

The interaction between an enzyme and its substrate can best be understood if the three-dimensional structures of both are known. In the case of carbohydrate substrates, they usually exist in solution as equilibrium mixtures of several forms. Determining the configuration of the substrate that is accepted by the enzyme is necessary to reveal the structural relationship between them.

The role of phosphofructokinase in the regulation of glycolysis is well documented (Bloxham and Lardy, 1973). The structural requirements for the substrate seem to be much more rigid than those for an activator, an inhibitor or a deinhibitor (Mansour, 1972). Only D-fructose 6-phosphate acts as a substrate very efficiently, and little is known of the exact structural specificity of this site of PFK. A detailed knowledge of the substrate specificity is an important primary step in the delineation of a stereochemical model of the active site and the mechanism of action of the enzyme.

In aqueous solution aldo- and ketohexoses and their derivatives exist as cyclic configurations in rapid equilibrium via acyclic ones (Pigman and Isbell, 1968). A knowledge of the equilibrium distribution of these tautomeric forms is a prerequisite of the tautomeric and anomeric specificity of the enzymatic system.
Several inconclusive reports by Avigad et al. (1970), Gray and Barker (1970), and Sewenson and Barker (1971) have failed to assess quantitatively the contributions of the D-fructose 6-phosphate tautomeric forms using different techniques, namely, UV, ORD, CD, IR, and NMR.

Koerner et al. (1973) using Fourier transform $^{13}\text{C}$ magnetic resonance spectroscopy succeeded in determining quantitatively the percentage of each forms of the substrate (D-fructose 6-phosphate) and the product (D-fructose 1,6-diphosphate). They also suggested that the $\beta$-anomer might be the form involved in the enzyme-substrate complex. The same results they obtained ($\geq 20\%\alpha$, $\leq 80\%\beta$, and less than 1.5% keto or hydrated keto form) were confirmed by Benkovic et al. (1973), and Wurster and Hess (1974) using other independent techniques (NMR and stopped flow kinetic measurements).

The objective of this study was to investigate the tautomeric, the anomeric, and some other related aspects of the structural specificity of the D-fructose 6-phosphate site of PFK.

As mentioned previously little was known about the substrate (D-fructose 6-phosphate) specificity, and little work has been done to investigate this point. As with the case of determining the tautomeric forms of D-fructose 6-phosphate, our laboratory was the first to determine unequivocally the substrate specificity of PFK. A
preliminary report (Koerner et al., 1973-A) and a paper (Koerner et al., 1974), which include some of the work presented here, have been published.

The first report investigating the phosphoryl acceptor specificity was by Totten and Lardy (1949). They indicated that D-tagatose 6-phosphate was phosphorylated by PFK at about 50% the rate of D-fructose 6-phosphate. Sedoheptulose 7-phosphate was also used slowly at reasonable concentration.

Later Lardy (1962) reported that L-sorbose 1-phosphate, L-sorbose 6-phosphate, and D-ribose 5-phosphate were not detectably phosphorylated by PFK.

D-glucose 1-phosphate was shown by Eyer et al. (1971) to act as an alternate substrate for PFK. The phosphorylation rate was found to be 0.7% that of D-fructose 6-phosphate.

More recently Uyeda (1972) has shown that rabbit muscle PFK catalyzed the phosphorylation of D-fructose 1-phosphate at 5% of the rate of D-fructose 6-phosphate. Also, D-fructose 1-phosphate was found to be a competitive inhibitor of the phosphorylation of D-fructose 6-phosphate indicating that both compete for the same active site.

Concerning the anomeric specificity of PFK, Benkovic et al. (1972) reported that the β-D-fructose 6-phosphate was rapidly used by PFK. Whether the α-anomer was utilized directly or only after mutarotation to the β-form, was not
established. Bloxham and Lardy (1973) using space-filling models of α-D-fructose 1-phosphate as an analogue of β-D-fructose 6-phosphate and L-sorbose 1-phosphate as an analogue of α-D-fructose 6-phosphate confirmed Benkovic et al.'s report.

It was suggested in two consecutive reports by Bar-Tana and Cleland (1974) that PFK is specific both for the β-anomers of D-fructose 6-phosphate and D-fructose 1,6-diphosphate and for the D-configuration at C-5. This suggestion was based on the high activity of the 2,5-anhydro-D-mannitol 1-phosphate and the failure of L-sorbose 6-phosphate or 2,5-anhydro D-glucitol 6-phosphate to act as substrates. They also showed that the affinity for 1-deoxyfructose 6-phosphate is the same as D-fructose 6-phosphate in presence of MgATP, while it is 30-fold less in absence of MgATP. This indicated that the C-1 hydroxyl group is important for binding.

Replacement of the phosphoryl moiety of D-fructose 6-phosphate by a sulfuryl group was found by Martensen and Mansour (1974) to serve as a substrate for the heart PFK. The apparent $K_m$ and $V_{max}$ were found to be of approximately 100 fold and 0.03 that of D-fructose 6-phosphate, respectively.

It would be helpful to make some terms used in this section clear, such as the following:
1) **Absolute configuration (D,L):** All optically active compounds may be related stereochemically to a single parent compound that has been arbitrarily chosen to serve as a standard of reference for stereoisomers, which is the 3-carbon sugar glyceraldehyde.

2) **Tautomers:** The cyclic-acyclic isomers of a reducing sugar.

3) **Epimers:** Two sugars that differ in the configuration around one specific asymmetric carbon atom only.

4) **Anomers:** The two distinguishable isomers, namely, the α- and β-forms, which result from the creation of a new asymmetric carbon upon the formation of the hemiacetal linkage (i.e., in the cyclization process) of aldoses and ketoses. This new asymmetric carbon is C-1 in glucopyranose and C-2 in fructofuranose forms.
II. MATERIALS AND METHODS

Materials

D-Mannitol, dihydroxyacetone, D-xylulose 5-phosphate, D-mannitol 1-phosphate, D-glucitol 6-phosphate, D-ribose 5-phosphate, 2-deoxy-D-ribose 5-phosphate, D-arabinose 5-phosphate, D-fructose 1-phosphate, and D-fructose were purchased from Sigma Chemical Company.

1,6-Hexanediol monophosphate, 1,5-pentanediol monophosphate, diethylene glycol monophosphate, (2-tetrahydrofuryl)-methanol phosphate, 2,5-anhydro-D-mannonate 6-phosphate, D-psicose 6-phosphate, L-sorbose 6-phosphate, 2,5-anhydro-D-mannose 6-phosphate, (α+β)-methyl-D-fructofuranoside 6-phosphate, 1,4-anhydro-D-arabinitol 5-phosphate, 2,5-anhydro-D-mannitol 1-phosphate, 2,5-anhydro-D-glucitol 6-phosphate, 6-bromo-6-deoxy-2,5-anhydro-D-mannitol 1-phosphate, D-tagatose 6-phosphate, and D-tagatose 6-sulfate were synthesized in our laboratory by Mr. T. A. W. Koerner, Jr. and Mr. R. J. Voll under the direction of Dr. E. S. Younathan.

Preparation of the enzyme and all other reagents used were the same as mentioned in Section One.

PFK Assay

The assay procedure was the same as described in Section One under "Materials and Methods" (Figure 1A).
The procedure depends on assay of the ADP produce by coupling to NADH oxidation via pyruvate kinase and lactic dehydrogenase. This procedure allows us to test many analogues, without concern about the acceptance of the product by aldolase in the method of Ling et al. (1966). The reaction was initiated by the addition of F6P or its analogues at the concentrations indicated in the Figures under "Results". Bovine serum albumin (0.01%) was added to the dilution mixture of PFK to increase the enzyme stability.

Data Processing

Kinetic data were depicted as a Lineweaver and Burk (1934) plots for the substrate analogues and as a Dixon (1953) plots for the inhibitors. The kinetic constants were calculated from the best fitting regression lines generated through an iterative least squares analysis of the data. This analysis was programmed in Fortran using a slight modification of the program recommended by Cleland (1967) and performed on an IBM 360 computer. The programming was carried out by Mr. R. J. Voll.
III. RESULTS

For some time, little was known about the relative amounts of the various forms of D-fructose 6-phosphate in solution and under the experimental conditions of PFK assay. These isomers are two cyclic furanose forms (α- and β-anomers) and acyclic keto and hydrated keto forms as shown in Figure 21. Our laboratory made a large contribution in determining the percentage of each of these forms (Koerner et al., 1973). The percentage of these isomers was found to be approximately 20%, 80%, and less than 1.5% for the α-anomer, the β-anomer, and the keto or hydrated keto forms, respectively.

Figure 21 shows the two readily interconvertible ring-chain tautomers available to PFK to act upon. To determine the tautomeric specificity of PFK, several analogues of the ring and the open chains were tested as an alternate substrate for PFK (Figure 22). These analogues are locked in one conformation to eliminate the possibility of tautomerism. (α+β) Methyl-D-fructofuranoside 6-phosphate and (2-tetrahydrofuryl) methanol phosphate were tested as analogues of the cyclic furanose tautomer. D-mannitol, D-mannitol 1-phosphate, D-glucitol 6-phosphate, D-xylulose 5-phosphate, dihydroxyacetone, diethylene glycol monophosphate, 1,5-pentanediol monophosphate, and 1,6-hexanediol monophosphate were tested enzymatically as
Figure 21

The tautomeric and anomeric forms of D-fructose 6-phosphate in solution were determined by Koerner et al. (1973). The percentage of each was found to be 20%, 80%, and less than 1.5% for the α-, β-, and keto or hydrated keto form of D-fructose 6-phosphate, respectively.
KETO-D-FRUCTOSE 6-P  HYDRATED KETO FORM

$\lessapprox$ Less than 1.5%

$\alpha$-D-FRUCTOFURANOSE 6-P  $\beta$-D-FRUCTOFURANOSE 6-P

$\lessapprox$ 20%  $\lessapprox$ 80%
Analogues used to determine the tautomeric specificity of PFK. All these compounds were tested at a maximum concentration of 5 mM relative to 1 mM for D-F6P in 1 ml assay mixture. (α+β) Methyl-D-fructose 6-phosphate was the only compound that acted as an alternate substrate for PFK. The $K_m$ and $V_{max}$ were 3.8 mM (D-F6P $K_m = 0.043$ mM) and 40% of that of D-F6P, respectively. The assay conditions were as described under "Materials and Methods".
analogues of the acyclic keto and hydrated keto tautomers. Only (α + β) methyl-D-fructofuranoside 6-phosphate displayed enzymatic activity as an alternate substrate of D-fructose 6-phosphate. Its kinetic constants were $K_m = 3.8 \text{ mM}$ and $V_{max} = 40\%$ that of D-fructose 6-phosphate. All other analogues were tested at a maximum concentration of 5 mM and were inactive. This tends to indicate that PFK has a tautomeric specificity for the furanose form of D-fructose 6-phosphate. The assay procedure was as described under "Materials and Methods".

Because of the thermodynamic predominance of the β-form in solution of both D-fructose 6-phosphate and D-fructose 1,6-diphosphate (Koerner et al., 1973), it is suggested that β-D-fructose 6-phosphate is the form of D-fructose 6-phosphate acted upon by PFK. To assess this point, we tested the two compounds shown in Figure 23 as alternate substrates or inhibitors of PFK. The first analogue (2,5-anhydro-D-mannitol 1-phosphate) was tested as an analogue of the β-anomer. The second one (2,5-anhydro-D-glucitol 6-phosphate) was tested as an analogue of the α-anomer. The only difference between the first and the second one is the configuration around the one carbon that corresponds to C-2 of D-fructose 6-phosphate. The β-anomer analogue was found to be a good alternate substrate for PFK with a $K_m = 0.41 \text{ mM}$ and a $V_{max} = 87\%$ of that of D-fructose 6-phosphate as depicted in Figure 24.
Figure 23

Analogues used to determine the anomeric (C-2) specificity of PFK. 2,5-anhydro-D-mannitol 1-phosphate and 2,5-anhydro-D-glucitol 6-phosphate were tested as analogues of the β-anomer and the α-anomer, respectively. The first acted as an alternate substrate (Figure 24), while the second as a competitive inhibitor (Figure 25).
\[ \begin{align*}
\text{α-D-Fructofuranose 6-P} & \quad \text{β-D-Fructofuranose 6-P} \\
\text{2,5-Anhydro-D-glucitol 6-P} & \quad \text{2,5-Anhydro-D-mannitol 1-P (Inhibitor)}
\end{align*} \]
Figure 24

Lineweaver-Burk plot of the initial rates of phosphorylation of D-fructose 6-phosphate and 2,5-anhydro-D-mannitol 1-phosphate as a function of substrate concentration. The kinetic constants ($K_m$ and $V_{max}$) are as shown in the Figure together with that of D-fructose 6-phosphate. Assay conditions are as described under "Materials and Methods".
FRUCTOSE 6-P
K = 0.043 ± 0.008 mM
V = 217 ± 15 μmoles/min/mg protein
max

2,5-ANHYDRO-MANNITOL 1-P
K = 0.41 ± 0.08 mM
V = 190 ± 15 μmoles/min/mg protein
max
However, the α-anomer analogue was a competitive inhibitor of PFK with a $k_i = 0.34$ mM as shown in Figure 25. These results strongly suggest that PFK binds both the α- and β-anomers of D-fructose 6-phosphate, but phosphorylates only the β-form.

Having determined the tautomeric and the anomeric (C-2) specificity of D-fructose 6-phosphate, a set of analogues that represent the systematic configurational permutations of the C-1, C-3, C-4, and C-5 positions of D-fructose 6-phosphate were tested. This study was intended to gain information about the configurational requirements at these carbons of the substrate.

Figure 26 shows a series of analogues in which functionality was progressively removed from β-D-fructose 6-phosphate. Removing the C-1 hydroxymethylene functional group resulted in a compound (D-arabinose 5-phosphate) that acted as an inhibitor and therefore was bound to the enzyme. Removing the C-1 hydroxyl group only resulted in a compound (l-deoxy-β-D-fructose 6-phosphate) that acted as a competitive inhibitor or a very poor substrate. However, removing both the C-1 hydroxymethylene, and the C-2 hydroxyl resulted in a compound (1,4-anhydro-D-arabinitol 5-phosphate) that had no activity. Another series of compounds at the bottom of Figure 26 represents the transformation of the C-1 hydroxyl of 2,5-anhydro-D-mannitol 1-phosphate to an aldehyde group, a carboxylic
Figure 25

Dixon plot of the competitive inhibition of phosphorylation of D-fructose 6-phosphate by 2,5-anhydro-D-glucitol 6-phosphate. Two fixed concentrations (0.023 mM and 0.046 mM) of D-fructose 6-phosphate were used in the assay mixture. The $K_i$ for 2,5-anhydro-D-glucitol 6-phosphate was found to be $0.34 \pm 0.07$ mM. The assay conditions are as described under "Materials and Methods".
FRUCTOSE 6-P

K_i = 0.34 ± 0.07 mM

0.023 mM

0.046 mM

[2,5-ANHYDRO-GLUCITOL 6-P], mM
Figure 26

The three analogues shown on the middle line of this figure were used to determine the C-1 specificity of PFK. D-arabinose 5-phosphate was found to act as a good competitive inhibitor ($K_i = 0.93 \pm 0.05$ mM), whereas, 1,4-anhydro-D-arabinitol 5-phosphate was inactive. The bottom row of the Figure depicts analogues that resulted from transforming the C-1 hydroxyl of 2,5-anhydro-D-mannitol 1-phosphate to a carboxylic group, aldehyde group, and a bromine group. These three analogues were inactive. Maximum concentration for any of these analogues in the assay mixture was 20 mM. Assay conditions are as described under "Materials and Methods".
\[ \beta\text{-D-Fructofuranose 6-P} \]

- I-Deoxy-\(\beta\text{-D-Fructofuranose 6-P} \) (inhibitor)
- 1,4-Anhydro-D-Arabinitol 5-P
- \(\beta\text{-D-Arabinofuranose 5-P} \) (inhibitor)
- 6-Bromo-6-deoxy-\(2\text{-D-Mannitol} \) 1-P
- 2,5-Anhydro-\(D\text{-Mannitol} \) Mannonate 6-P
- 2,5-Anhydro-\(D\text{-Mannose} 6-P \)
group or bromine group. These substitutions at the C-1 hydroxyl led to the formation of completely inactive compounds. It should be noted that the analogue 1-deoxy-\(\beta\)-D-fructose 6-phosphate was shown to be a competitive inhibitor by Bar-Tana and Cleland (1974). The maximum concentration used in the assay mixture for any of these compounds was 20 mM.

As demonstrated in Figure 26, D-arabinose 5-phosphate was found to be a competitive inhibitor of PFK. Figure 27 shows the Dixon plot of the competitive inhibition of PFK by D-arabinose 5-phosphate. Assay conditions were as described under "Materials and Methods". D-fructose 6-phosphate concentrations used were 0.023 mM, 0.046 mM, and 0.092 mM. The \(K_i\) for this analogue was found to be 0.93 ± 0.05 mM.

The analogues shown in Figure 28 were used to determine the importance of the C-3 hydroxyl for the binding of sugar phosphate to the D-fructose 6-phosphate site. D-Psicofuranose 6-phosphate is an analogue identical with D-fructose 6-phosphate except for the reversal of the configuration around C-3. This analogue was found to be a substrate for PFK (Figure 29). However, it had a \(K_m\) two orders of magnitude higher than the \(K_m\) for D-fructose 6-phosphate. This indicates that PFK has a poor affinity for D-psicose 6-phosphate. The other two analogues, namely, D-ribofuranose 5-phosphate and 2-deoxy-D-ribofuranose
Figure 27

Dixon plot for the competitive inhibition of phosphorylation of D-fructose 6-phosphate by D-arabinose 5-phosphate. Three lines are shown corresponding to three fixed concentrations (0.023 mM, 0.046 mM, and 0.092 mM) of D-fructose 6-phosphate in the assay mixture. A $K_i = 0.93 \pm 0.05$ mM was found for the inhibitor. Assay conditions are as described under "Materials and Methods".
$K_i = 0.93 \pm 0.05 \text{ mM}$
Analogues used to determine the C-3 specificity of PFK. β-D-Psicose 6-phosphate was found to act as a substrate. Its kinetic constants are shown in Figure 29. The only difference between this analogue and D-fructose 6-phosphate is that the configuration around C-3 is reversed. The other two analogues shown in this Figure resulted from either removal or changing the configuration of C-2 hydroxyl of D-arabinose 5-phosphate. Both compounds were inactive. Maximum concentration used in the assay of these analogues was 10 mM in 1 ml assay mixture. Assay conditions were as described previously under "Materials and Methods".
β-D-FRUCTOFURANOSE 6-P

β-D-PSICOFURANOSE 6-P (SUBSTRATE)

β-D-RIBOFURANOSE 5-P

2-DEOXY-β-D-RIBOFURANOSE 5-P
5-P are analogues of D-arabinose 5-phosphate, a competitive inhibitor of PFK (Figure 27). Removing the C-2 hydroxyl of D-arabinose 5-phosphate or reversing its configuration from L to D results in two inactive compounds, namely, 2-deoxy-D-ribofuranose 6-phosphate or β-D-ribofuranose 5-phosphate. These changes at C-2 of these analogues corresponds to changes at C-3 of D-fructose 6-phosphate. This indicates that both the presence and the configuration of the C-3 hydroxyl group is essential for binding and appreciable substrate phosphorylation.

Figure 29 depicts the Lineweaver-Burk plot of the initial rates of phosphorylation of D-fructose 6-phosphate and D-psicofuranose 6-phosphate as a function of substrate concentration. D-Psicofuranose 6-phosphate acted as a substrate with $k_m = 3.0 \pm 1.0$ mM (D-fructose 6-phosphate $K_m = 0.032 \pm 0.02$ mM) and $V_{max}$ approximately 45% that of D-fructose 6-phosphate. Assay conditions were as described under "Materials and Methods".

The two analogues represented in Figure 30 were used to study the effect of reversing the configuration (epimerisation) around C-4 and C-5 of D-fructose 6-phosphate. D-Tagatose 6-phosphate, the C-4 epimer of D-fructose 6-phosphate, was found to be a good substrate for PFK. Its kinetic constants $K_m$, $V_{max}$ were found to be approximately equal to these of D-fructose 6-phosphate within the experimental error. On the other
Figure 29

Lineweaver-Burk plot of the initial rates of phosphorylation of D-fructose 6-phosphate and D-psicose 6-phosphate as a function of substrate concentration. The kinetic constants for this alternate substrate and D-fructose 6-phosphate are shown in the Figure. Assay conditions are as described under "Materials and Methods".
PSICOSE 6–P

$K_m = 3.0 \pm 1.0 \text{ mM}$

$V_{max} = 50.4 \pm 6.0 \mu\text{moles/min/mg protein}$

FRUCTOSE 6–P

$K_m = 0.03 \pm 0.01 \text{ mM}$

$V_{max} = 10.6 \pm 9.0 \mu\text{moles/min/mg protein}$
Figure 30

Analogues used to determine the C-4 and C-5 specificity of PFK. D-Tagatose 6-phosphate acted as a good substrate (Figure 31) for PFK, while α-L-sorbose 6-phosphate acted as a poor substrate. The configuration of the functional groups around C-4 and C-5 of these two analogues are reversed with respect to the corresponding ones of D-fructose 6-phosphate. Maximum concentration used of α-L-sorbose 6-phosphate in the assay mixture was 25 mM. Assay conditions are as described in Figure 29.
\[ \beta-D-FRUCTOFURANOSE\ 6-P \]

\[ \alpha-L-SORBOFURANOSE\ 6-P \quad \text{(POOR SUBSTRATE)} \]

\[ \beta-D-TAGATOFURANOSE\ 6-P \quad \text{(SUBSTRATE)} \]
hand, L-sorbose 6-phosphate, the C-5 epimer was found to be a poor substrate. It has a $K_m = 11.0$ mM and $V_{\text{max}} = 15\%$ that of D-fructose 6-phosphate. Maximum concentration used in its assay was 25 mM.

Figure 31 illustrates the Lineweaver-Burk plot of the initial rates of D-fructose 6-phosphate and D-tagatose 6-phosphate as a function of substrate concentration. $K_m$ and $V_{\text{max}}$ for D-fructose 6-phosphate were $0.043 \pm 0.009$ mM and $138 \pm 7 \mu$ moles/min/mg. protein, respectively; while those of D-tagatose 6-phosphate were $0.054 \pm 0.009$ mM and $144 \pm 7 \mu$ moles/min/mg. protein, respectively. Assay conditions were as described under "Materials and Methods".

Figure 32 depicts 5 compounds used to determine the effect of the net charge and the importance of the phosphate group of the substrate on PFK activity. Four of these analogues were tested as alternate substrates or inhibitors for PFK. The fifth one (D-fructose 6-sulfate) was found by Martensen and Mansour (1974) to act as an alternate substrate. The apparent $K_m$ and $V_{\text{max}}$ for the latter compound were of approximately 100 fold and 0.03 that of D-fructose 6-phosphate, respectively. D-Tagatose 6-sulfate was found to act as a poor substrate at a maximum concentration of 10 mM. These results indicate that the replacement of the phosphoryl moiety by a sulfuryl group does not abolish their abilities to serve as alternate substrates for PFK. Removal of the phosphate
Figure 31

Lineweaver-Burk plot of the initial rates of phosphorylation of D-fructose 6-phosphate and D-tagatose 6-phosphate as a function of substrate concentration. The kinetic constants for this alternate substrate and D-fructose 6-phosphate are shown in the Figure. Assay conditions are as described under "Material and Methods."
$$K_m = 0.054 \pm 0.009 \text{mM}$$

$$V_{\text{max}} = 1.44 \pm 0.7 \text{µmoles/min/mg protein}$$

$$K = 0.043 \pm 0.009 \text{mM}$$

$$V_{\text{max}} = 1.38 \pm 0.7 \text{µmoles/min/mg protein}$$
Figure 32

Analogues used to determine the effect of the net charge of the substrate on PFK activity. All these compounds were tested in our laboratory, except D-fructose 6-sulfate. This compound was shown in the Figure for comparison. It was found to be an alternate substrate by Nartensen and Mansour (1974). 2,5-Anhydro-D-mannitol and D-fructose were found to be inactive, while the other two acted as poor substrates. Maximum concentration used for these analogues in the assay mixture was about 20 mM. Assay conditions are as described under "Materials and Methods."

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groups from D-fructose 6-phosphate and 2,5-anhydro-D-mannitol 1-phosphate produces two inactive compounds, namely, D-fructose and 2,5-anhydro-D-mannitol, respectively. This indicates that the presence of a phosphate or sulfate group on C-6 of D-fructofuranose is essential. Transfering the phosphate group from C-6 to C-1 produces a compound (D-fructose 1-phosphate) which acts as a poor substrate. All these compounds were tested at a maximum concentration of 20 mM. The assay conditions were as described previously under "Materials and Methods".
IV. DISCUSSION

Phosphofructokinase is considered as the key regulatory enzyme of glycolysis (Bloxham and Lardy, 1973). Our research team has sought cooperatively to delineate the mechanism of action of this important enzyme. As a prerequisite to elucidate this mechanism, the specificity of the D-fructose 6-phosphate site was determined. One of the chosen means to approach this aim, was to test the activity of D-fructose 6-phosphate analogues as alternate substrates or inhibitors for PFK. Many of these analogues are locked in a cyclic or acyclic form i.e., incapable of tautomerization. In addition, most of the cyclic forms, according to its acceptance by PFK, probably exist to a large extent in solution in the β-furanose form. Determining the exact percentages of these cyclic analogue forms is still going on in our laboratory.

Having determined the percentage of D-fructose 6-phosphate forms in solution by Koerner et al. (1973), the thermodynamic predominance of the β-form of the substrate and the product suggested that the β-form was the one acted upon by PFK. This suggestion was strongly supported by testing the cyclic analogues, namely, (α+β) methyl-D-fructofuranoside 6-phosphate and (2-tetrahydrofuryl) methanol phosphate as analogues of the cyclic furanose tautomer. Also, the different acyclic forms shown in
Figure 22 as analogues of the open keto and hydrated keto forms. It is noticeable that the acyclic analogues have most of the structural features of the acyclic open chain tautomers of D-fructose 6-phosphate. For example, dihydroxyacetone was chosen as a good analogue, by assuming that the carbonyl group of the open chain is essential for binding. Also, D-mannitol 1-phosphate and D-glucitol 6-phosphate were used as good analogues if we assume that the configuration of the hydroxyl groups and the negative charges of the phosphate residue in the open forms are essential for binding. All these analogues were inactive as alternate substrates or inhibitors of PFK. On the other hand, (α+β) methyl-D-fructofuranoside 6-phosphate acted as an alternate substrate. This indicates that PFK has a tautomeric specificity for the furanose form of D-fructose 6-phosphate. The next step was to determine if the enzyme possessed a specificity for either the α- or the β-anomer of this furanose form.

Two analogues of the α- and the β-anomers of the furanose form of D-fructose 6-phosphate were tested (Figure 23). 2,5-Anhydro-D-mannitol 1-phosphate, an analogue of the β-form was found to be a good alternate substrate for PFK with a $K_m = 0.41$ mM and a $V_{max} = 87\%$ of that of D-fructose 6-phosphate. 2,5-Anhydro-D-glucitol 6-phosphate, an analogue of the α-form, was found to be an effective competitive inhibitor of PFK with a $k_i = 0.34$ mM.
The close similarity of $K_m$ and $K_i$ indicates that the enzyme has about equal affinity for both compounds. The only difference between the two analogues is the configuration around C-2 that corresponds to the anomeric carbon of D-fructose 6-phosphate. Apparently the shift from the $\beta$-form to the $\alpha$-form puts the $-\text{CH}_2\text{OH}$ group in a position unfavorable for its phosphorylation. These results strongly suggest that the enzyme binds both $\alpha$- and $\beta$-forms, phosphorylates the $\beta$-form only, and the C-2 hydroxyl of the furanose form is not essential for activity. The acceptance of the $\beta$-form by the enzyme has recently been substantiated by the fast-flow kinetic work of Wurster and Hess (1974).

To explore further the specificity of the $\beta$-D-fructose 6-phosphate site, we set out first to determine the C-1 specificity. The analogues represented in Figure 26 were used for this purpose. These compounds differ from $\beta$-D-fructose 6-phosphate or 2,5-anhydro-D-mannitol 1-phosphate (alternate substrate) in the lack or change in configuration of the one carbon that corresponds to C-1 of $\beta$-D-fructose 6-phosphate. One of these analogues namely, 1-deoxy-$\beta$-D-fructofuranose 6-phosphate was shown to be a good inhibitor by Bar-Tana and Cleland (1974). The fact that $\beta$-D-arabinose 5-phosphate (which lacks the $-\text{CH}_2\text{OH}$ relative to C-1 of D-fructose 6-phosphate) and 1-deoxy-$\beta$-D-fructofuranose 6-phosphate are good
competitive inhibitors of PFK indicates that the C-1 hydroxy1 or hydroxymethylene is not essential for binding although obviously essential for enzymatic activity, i.e., for phosphorylation. 1,4-Anhydro-D-arabinitol 5-phosphate (has neither -CH$_2$OH nor C-2 hydroxyl relative to $\beta$-D-1 fructose 6-phosphate) was inactive as an alternate substrate or inhibitor. This indicates that C-2 hydroxyl is important in binding. This conclusion appears to contradict our finding that 2,5-anhydro-D-mannitol 1-phosphate (which lacks the OH group on the carbon that corresponds to C-2 on D-fructose 6-phosphate) is a good substrate for PFK. However, this apparent contradiction can be resolved by concluding that an -OH, either on C-1 or on C-2 has to be present in the sugar phosphate for binding PFK. The series of compounds at the bottom of Figure 26 were enzymatically inactive. This suggests that either C-1 or C-2 hydroxyl most probably is involved in hydrogen-bond formation with the enzyme.

In determining the importance of the C-3 hydroxyl in binding, three compounds were used as demonstrated in Figure 28. D-Ribose 5-phosphate (has a reversed configuration of C-3 hydroxyl and no -CH$_2$OH relative to D-fructose 1 6-phosphate), and 2-deoxy-$\beta$-D-ribose 5-phosphate (lacks
C-3 hydroxyl and \(-\text{CH}_2\text{OH}\) relative to D-fructose 6-phosphate, are analogues of D-arabinose 5-phosphate. D-Psicose 6-phosphate is an epimer of \(\beta\)-D-fructose 6-phosphate (has a reversed configuration of C-3 hydroxyl relative to D-fructose 6-phosphate). D-Psicose 6-phosphate was the only sugar phosphate to act as an alternate substrate with \(K_m = 3.0 \pm 1.0\) mM and \(V_{\text{max}} \approx 46\%\) that of D-fructose 6-phosphate. It seems feasible to conclude that reversal of the configuration around C-3 resulted in retention of some activity. This would suggest that the presence and the configuration of the C-3 hydroxyl is essential for tight substrate binding and maximal catalytic activity.

D-Tagatose 6-phosphate and \(\alpha\)-L-sorbose 6-phosphate are two epimeric isomers of D-fructose 6-phosphate. Their structures compared to \(\beta\)-D-fructose 6-phosphate are given in Figure 30. The configuration in these two epimers is reversed around carbons number 4 and 5, respectively. These two analogues were chosen to study the effect of such epimerization on their affinity for PFK. D-Tagatose 6-phosphate, the C-4 epimer, was found to act as an exceedingly good alternate substrate for PFK; whereas, L-sorbose 6-phosphate, the C-5 epimer, was found to be a very poor alternate substrate. The kinetic constants of D-tagatose 6-phosphate were found to be almost equal to those of \(\beta\)-D-fructose 6-phosphate (Figure 31). It seems that the configuration around C-4 is of no
importance; whereas that around C-5 is important for binding and catalysis of the reaction. It should be noted that D-tagatose 6-phosphate was shown to act as an alternate substrate by Totten and Lardy (1949), who did not determine its kinetic constants, however.

The charge specificity and its importance were investigated by testing the analogues given in Figure 32, as alternate substrates or inhibitors. D-Fructose 1-phosphate was shown by us and by Uyeda (1972) to act as a poor substrate. The β-furanose form of this analogue has a structure that is almost superimposed on β-D-fructose 6-phosphate when rotated 180°. This is why it acts as a poor substrate. Replacement of the phosphoryl moiety of β-D-fructose 6-phosphate (Martensen and Mansour, 1974) and that of β-D-tagatose 6-phosphate by a sulfuryl group does not abolish their abilities to act as an alternate substrates. In addition, the inactivity of D-fructose and 2,5-anhydro-D-mannitol would suggest that at least one charge has to be present for binding. Moreover, it seems that one charge is not enough for maximal activity.

From the above described findings, we can sum up the specificity of the D-fructose 6-phosphate site of PFK as follows:

1) Rabbit muscle phosphofructokinase binds only the furanose tautomer of D-fructose 6-phosphate.
2) Only the β-anomer of D-fructose 6-phosphate is phosphorylated.

3) Neither the C-1 nor the C-2 hydroxyl of D-fructose 6-phosphate is essential for binding. However, one of the two must be present and probably forms a hydrogen bond with the enzyme.

4) The C-3 hydroxyl must be present and must have an L-configuration for significant activity.

5) Neither the presence nor the configuration of the C-4 hydroxyl is essential for binding or catalysis.

6) The configuration of C-5 must have the D-type.

7) The C-6 hydroxyl must bear an anionic group, with at least one charge for binding.
REFERENCES


APPENDIX I

TABLE OF ABBREVIATIONS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AMP</td>
<td>Adenine monophosphate</td>
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<tr>
<td>ADP</td>
<td>Adenine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine triphosphate</td>
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<tr>
<td>2,5-ADM 1-P</td>
<td>2,5-Anhydro-D-mannitol 1-phosphate</td>
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<tr>
<td>A5P</td>
<td>Arabinose 5-phosphate</td>
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<td>Arg</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C.A.</td>
<td>Chemical Abstract</td>
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<td>C</td>
<td>Centigrade</td>
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<tr>
<td>CD</td>
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<td>c-AMP</td>
<td>Cyclic 3',5'-adenosine monophosphate</td>
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<td>cyt c</td>
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<td>Dithiothritol</td>
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<td>d mole</td>
<td>Decimole</td>
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<td>degree(s)</td>
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<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
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<td>Ethylenediamine tetra acetate</td>
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<td>F6P</td>
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<td>FDP</td>
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<tr>
<td>gm</td>
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<td>G3P</td>
<td>Glyceraldehyde 3-phosphate</td>
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137
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OD.</td>
<td>Optical density</td>
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<tr>
<td>ORD</td>
<td>Optical rotatory dispersion</td>
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<td>P</td>
<td>Phosphate</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PEP</td>
<td>Phosphoenol pyruvate</td>
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<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PYR</td>
<td>Pyruvate</td>
</tr>
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<td>PK</td>
<td>Pyruvate kinase</td>
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<td>phe</td>
<td>Phenylalanine</td>
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<td>SDS</td>
<td>Lauryl sodium sulfate (sodium dodecyl sulfate)</td>
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VITA

Born: November 18, 1939, at Mahala Kobra, Egypt (A.R.E).

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Major Field: Biochemistry

Title of Thesis: Studies on Phosphofructokinase. Effect of Sodium Dodecyl Sulfate and the Specificity of the D-Fructose 6-Phosphate Site

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

January 16, 1975