Circular Dichroism studies on the aromatic residues of fructose 1, 6-Bisphosphatase from Turkey liver

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CIRCULAR DICHROISM STUDIES ON THE AROMATIC RESIDUES OF FRUCTOSE 1,6-BISPHOSPHATASE FROM TURKEY LIVER

A THESIS
SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY
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DEPARTMENT OF CHEMISTRY

ATLANTA, GEORGIA
AUGUST 1976
This thesis presents a detailed description and analysis of the circular dichroism studies performed on the aromatic residues of fructose 1,6-bisphosphatase (FBPase) from turkey liver under various experimental conditions.

Circular dichroism studies performed on the aromatic residues of FBPase indicate that the presence of the substrate, fructose 1,6-bisphosphate (FBP) and/or the allosteric inhibitor, adenosine monophosphate (AMP) as well as changes in the pH of the medium produce significant effects on the conformation of the enzyme. The effect of the inhibitor, AMP, on the conformation of the enzyme is more pronounced than that of the substrate, FBP.
ACKNOWLEDGEMENT

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INTRODUCTION

Fructose 1,6-bisphosphatase (FbPase)(EC 3.1.3.11), also known as D-fructose 1,6-bisphosphate 1-phosphorylase, catalyzes the conversion of fructose 1,6-bisphosphate (FbP) to fructose 6-phosphate and inorganic phosphate in gluconeogenesis (1)(Fig. 1).

\[ \text{FbP} \xrightarrow{\text{FbPase}} \text{Fructose-6-Phosphate} + \text{HPO}_4^{2-} \]

**Fig. 1.** Catalytic conversion of Fructose 1,6-bisphosphate to fructose-6-phosphate plus phosphate.

The Embden-Meyerhof pathway, gluconeogenesis, and the role of fructose 1,6-bisphosphatase in this pathway were suggested by McGilvery and his colleagues (2). The above named phenomenon was later confirmed by Leuthardt and his colleagues (3). Fructose 1,6-bisphosphatase requires a free divalent cation such as Mg\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\) for catalytic activity (4).

The enzyme is active at neutral pH (5), and it is allosterically inhibited by adenosine monophosphate (AMP) (6,7,8). It has a high degree of specificity to the substrate, fructose 1,6-bisphosphate (FbP). It possesses
four binding sites for the substrate (FbP), and an equal number of binding sites for the allosteric inhibitor (AMP) (9,10,11).

Furthermore, the enzyme has four identical subunits, and each subunit has a molecular weight of about 36,000. Therefore, the total molecular weight of the enzyme is about 144,000 (12). There are fifty-two tyrosine, thirty-nine phenylalanine, and four tryptophan residues per mole of the enzyme. The overall amino acid composition of the enzyme from turkey, chicken, and rabbit livers is shown in the table on the next page.

Circular dichroism (CD) studies have been performed on the aromatic residues of rabbit liver FbPase by Tamburro et al. (13). The aim of this research was to perform similar CD studies on the aromatic residues of turkey liver FbPase in the native form.
Amino Acid Composition of Native Chick, Turkey, and Rabbit Liver FbPase

<table>
<thead>
<tr>
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<th>Chick</th>
<th>Turkey</th>
<th>Rabbit</th>
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<tbody>
<tr>
<td>Lysine</td>
<td>92.8</td>
<td>98</td>
<td>119.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>10.3</td>
<td>16</td>
<td>23.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>52.9</td>
<td>57</td>
<td>53.7</td>
</tr>
<tr>
<td>Cysteine</td>
<td>26.4</td>
<td>16</td>
<td>21.6</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>133.4</td>
<td>139</td>
<td>134.7</td>
</tr>
<tr>
<td>Threonine</td>
<td>62.2</td>
<td>75</td>
<td>71.5</td>
</tr>
<tr>
<td>Serine</td>
<td>71.4</td>
<td>97</td>
<td>76.0</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>75.4</td>
<td>92</td>
<td>103.8</td>
</tr>
<tr>
<td>Proline</td>
<td>46.4</td>
<td>55</td>
<td>58.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>114.1</td>
<td>128</td>
<td>102.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>100.8</td>
<td>111</td>
<td>117.1</td>
</tr>
<tr>
<td>Valine</td>
<td>98.9</td>
<td>84</td>
<td>99.7</td>
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<tr>
<td>Methionine</td>
<td>24.5</td>
<td>28</td>
<td>33.3</td>
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<tr>
<td>Isoleucine</td>
<td>91.9</td>
<td>76</td>
<td>75.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>97.2</td>
<td>103</td>
<td>111.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>45.2</td>
<td>52</td>
<td>45.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>31.8</td>
<td>39</td>
<td>35.9</td>
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<tr>
<td><em>Tryptophan</em></td>
<td>4*</td>
<td>4*</td>
<td>3.9</td>
</tr>
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*Tryptophan is found in the N-terminal end of each subunit of FbPase. Assuming four identical subunits, comprising the enzyme, then there should be a minimum of four tryptophans.
LITERATURE REVIEW

Properties of Fructose 1,6-Bisphosphatase (FbPase)

It has been discovered that an important site of the regulation of gluconeogenesis and glycolysis is at the step where fructose 1,6-bisphosphate is either synthesized or hydrolyzed (14)(Fig. 2).

\[ \begin{align*}
\text{F-6-Pi} & \xrightarrow{\text{FbPase}} \text{F-6-Pi} + \text{HPO}_4^- \\
\text{F-6-Pi} & \xrightarrow{\text{ATP}} \text{F-6-Pi} + \text{HPO}_4^- \\
\text{F-6-Pi} & \xrightarrow{\text{PFK}} \text{F-6-Pi} + \text{HPO}_4^- \\
\text{F-6-Pi} & \xrightarrow{\text{Mg}^{2+}, \text{H}_2\text{O}} \text{F-6-Pi} + \text{HPO}_4^- \\
\end{align*} \]

**Fig. 2.** Fructose 1,6-Bisphosphate synthesis and hydrolysis. (PFK = phosphofructokinase, ATP = Adenosine triphosphate, FbPase = fructose 1,6-bisphosphatase)

In gluconeogenesis, FbPase regulates the rate of formation of glucose from pyruvate and phosphoenolpyruvate (Fig. 3). In the direction of glycolysis, the enzymatic activity of phosphofructokinase is inhibited by citrate and adenosine triphosphate (ATP). This inhibition is reversed by adenosine monophosphate (AMP)(15).

A number of mechanisms including allosteric control by AMP have been proposed for the regulation of FbPase. The inhibition of FbPase by AMP has been investigated using enzymes isolated from several biological sources. The results indicate that the direction of flow in gluconeogenesis is
Fig. 3. Schematic diagram of gluconeogenesis and glycolysis. (PGI = phosphoglucoisomerase, ATP = Adenosine triphosphate, PFK = phosphofructokinase)
being controlled by the ratio of AMP to ATP (16). For example, a high ratio of AMP to ATP favors the direction of glycolysis which prevents gluconeogenesis from occurring. Nevertheless, the inhibition of FbPase by AMP protects the cells against any wasteful dephosphorylation of fructose 1,6-bisphosphate during glycolysis.

In human beings, FbPase has been associated with certain physiological disorders. These disorders have been attributed to the deficiency of FbPase. For example, a deficiency of FbPase is believed to be responsible for Von Gierke's disease which is nothing but glucose-6-phosphate's absence. It should be noted that glucose-6-phosphate is a precursor of glucose. Therefore, absence of glucose-6-phosphate leads to hypoglycemia, hyperinsulinism and severe metabolic acidosis (17,18).

Mammalian Fructose 1,6-Bisphosphatase

During the past two decades FbPase has been isolated from many biological sources such as rabbit liver (19), rabbit muscle (12), rat liver (20), and swine kidney (21). Of particular interest is the FbPase isolated from rabbit liver (19). This enzyme was first isolated by Gomori in 1943 (4). Since then, a large amount of information has been accumulated on the functions and properties of this enzyme. Rabbit liver FbPase has been found to exist in two homogeneous forms that differ from each other with respect to functional and molecular parameters (22). For example, the neutral FbPase is found to have a significantly higher molecular weight than
the alkaline enzyme.

Furthermore, rabbit liver FbPase in the native state has a molecular weight of about 130,000 (13). It requires Mg\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\) for catalytic activity (4). It is active at neutral and alkaline pH (5), and it is allosterically inhibited by adenosine monophosphate (AMP)(11). It has also been discovered that the rabbit liver FbPase binds four equivalents of Mn\(^{2+}\) at neutral pH and four additional equivalents above pH 8.5 (11).

Avian Fructose 1,6-Bisphosphatase

There has been little work done on FbPase from avian species as compared with the amount of work performed on mammalian species (23). Of particular interest is the FbPase isolated from chicken and turkey livers in our laboratory. The isolated enzyme can be stored up to three months in the presence of Mg\(^{2+}\) or Mn\(^{2+}\) in Tris-HCl buffer at pH 7.5 without any significant loss in catalytic activity (23,24). It has also been shown in our laboratory that FbPase from two different avian species are very closely related (23). For example, the two enzymes from chicken and turkey livers have similar molecular weights, amino acid composition and electrophoretic mobility.

Turkey liver FbPase purified in our laboratory is homogeneous (23). It has a molecular weight of 144,000 and four identical subunits. It is active at both neutral and alkaline pH. Like all FbPase reported so far, the purified FbPase from turkey liver requires divalent cations (Mg\(^{2+}\), Co\(^{2+}\) or
Mn$^{2+}$) for catalytic activity. The values of $K_m$ as estimated from double reciprocal plots using the noninhibitory cation concentrations are 0.42 x $10^{-3}$M for Mg$^{2+}$ and 0.028 x $10^{-3}$M for Mn$^{2+}$. The maximal activity with Mg$^{2+}$ as the metal cofactor is 60% of the maximal activity observed with Mn$^{2+}$ (23).

Turkey liver FbPase shows a high affinity for the substrate, fructose 1,6-bisphosphate (FbP). The enzyme's affinity for FbP is decreased with an increase in pH. The values of $K_m$ as estimated from double reciprocal plots using the noninhibitory FbP concentrations are found to be approximately 5.3 x $10^{-6}$M at pH 7.5 and 1.43 x $10^{-5}$M at pH 9.2 (23). As is characteristic of most FbPases studied, the enzyme from turkey liver is strongly inhibited by adenosine monophosphate (AMP). The degree of inhibition by AMP decreases as the temperature is increased. It has been reported that the $K_{50}$ at 42°C is approximately 50 μm as compared with 12 μm at 30°C (23). Furthermore, the sensitivity of turkey liver FbPase towards AMP inhibition decreases markedly with an increase in pH. For example, with 20 μm AMP, the enzyme activity is inhibited approximately 70% at pH 7.0 whereas no inhibition is observed at pH 9.4 (23).

Finally, the isolated turkey liver FbPase shows a single band in gel electrophoresis in either the presence or absence of sodium dodecyl sulfate (SDS). Since SDS causes subunits to dissociate, this suggests that the enzyme may be composed of subunits of equal size (23).
Choice of Species for Study

It is generally recognized that a critical step in gluconeogenesis is the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate, catalyzed by the enzyme, FbPase (1). This gluconeogenic enzyme has been obtained from a large number of biological species. Most of the well characterized FbPases are from the mammalian species (4,7,11). Since not enough work has been performed on the enzyme from avian species (23,24), this study was undertaken so as to analyze and characterize the enzyme from avian species.

Preliminary work with turkey liver FbPase in our laboratory suggested that turkey tissues would be a reasonable source of the enzyme (23). Moreover, extensive work with the enzyme performed by Rumph (25) and Burkes (26) confirmed that turkey tissues would really be a good source of the enzyme for this investigation. Above all, turkey tissues are commercially available in sufficient quantities and are not too expensive for large scale purification.

Circular Dichroism Theory

In the continuing search for relationships between structure of biological macromolecules and their functions, the study of conformation in solution must play an important role. It is only within the last two decades that investigators have succeeded in establishing the circular dichroism (CD) technique to study the conformation of biological macromolecules in solution. This technique is extremely useful to detect changes in the periodic arrangement of amino acid
residues, changes in the local environment of amino acid residues, and changes at certain specific sites subsequent to a particular interaction (28).

A detailed discussion of the application of CD to the study of biological macromolecules has been published by Beychok (28). Other methods used in the application of CD for biological macromolecules have been provided by Bayley (27), Greenfield and Fasman (29), and Chen, Yong and Martinez (30). Analysis of the conformation states of FbPase by the CD technique has been reported by Tamburro et al. (rabbit liver FbPase)(13), Davis (chicken liver FbPase)(31), and Burkes and Rumph (turkey liver FbPase)(25,26).

There are two broad approaches to the analysis of CD. They are the phenomenological approach and the theoretical approach. In the phenomenological approach, the CD spectra of complex molecules are analyzed in terms of the sum of the contribution from specific structures such as alpha helices, beta sheets, and random coils. This multicomponent kind of analysis depends upon the comparison of experimental CD spectra with those of the well-characterized structures taken alone or in simple combinations (30). This procedure, even though highly complicated, is successful, but it is limited by the variability of the reference states, and the degree of correspondence of these with the actual conformations present (27). In the theoretical approach, optical rotation theory is used to predict the optical activity of the proposed structure, and it is the total conformation which determines
the optical activity. It should be noted that optical activity is shown by molecules which do not possess either a center of symmetry or a mirror plane. Also, the term conformation is used to cover the full three-dimensional organization of macromolecules, particularly in proteins.

CD is an absorptive phenomenon and it is observable only in the frequency interval where the absorption occurs. CD involves the study of differential absorption of light that is circularly polarized in opposite directions. Therefore, CD at a given frequency or wavelength (\( \lambda \)) is defined as the difference between the extinction coefficients of the left- and the right-handed circularly polarized components of plane polarized light. In order for CD to occur the biological molecule involved should be in an asymmetric environment. Hence, it should be re-emphasized that CD can be observed only within the frequency or wavelength region where the asymmetric molecule absorbs light.

The absorptive phenomenon of CD is described thusly: light, or electromagnetic radiation, consists of oscillating electric and magnetic fields. In linearly polarized light, the sinusoidally varying electric field is confined to a plane, as is the magnetic field. The electric and magnetic planes are perpendicular to one another. When the electromagnetic radiation or light impinges on a molecule, the electrons are perturbed, and they are displaced from the unexcited ground-state configurations, by the electric field of the light, to the excited upper-state configurations.
This electronic transition is characterized by the frequency or wavelength at which it occurs, and the magnitude of the electronic displacement involved (28).

Below is the mechanistic diagram of CD:

**PLANE POLARIZED LIGHT, \( \mathbf{E}_R \)**

\[ \mathbf{E} = \text{ELECTRICAL VECTOR} \]

When the plane polarized light, \( \mathbf{E}_R \), passes through an asymmetric molecule, there is a differential absorption. The CD effect is given by \( \Delta \varepsilon = \varepsilon_L - \varepsilon_R \), the difference in extinction coefficients between the left and the right circularly polarized components of plane polarized light. For example, if the left-handed circularly polarized component absorbs stronger than the right-handed circularly polarized component, then the result is elliptically polarized light, as shown in the diagram. Ellipticity is another way of expressing CD which is given by the angle \( \theta \), which is equal to the arc tangent of the minor axis (a) over the major axis (b) of the ellipse. The molar ellipticity, \([\theta]\) is related to \( \Delta \varepsilon \) as follows:

\[ [\theta] = 3300 \cdot \Delta \varepsilon \]

The units for molar ellipticity, \([\theta]\), are degrees cm\(^2\)/decimole.
In order for a CD band to be observed, it is necessary that the displacement of the electronic charge has a circular component as well as linear component in the direction of the applied field. This will induce a magnetic transition moment as well as an electric transition moment (28). For example, if an electron moves along a helical path, the electric field of the light induces an oscillating dipole on the helical path; the result is a magnetic moment in the direction of the electric field of the light and an electric moment with components in the same direction as the magnetic moment.

Circular dichroism is characterized by the rotational strength of the electronic transition which could be positive or negative. Rotational strength ($R_K$) is defined as the integrated intensity beneath a single band in circular dichroism spectra. $R_K$ measures the interaction of a chromophore, K, with its dissymmetric environment. It also measures the asymmetry induced in the electron distribution within a chromophore (Figs. 4 and 5). Optical activity is, therefore, characterized by the rotational strength, $R_K$, of a particular electronic transition. Hence, CD spectra are represented by the $R_K$ which is the sum of series of transitions. The $R_K$ of a particular transition is given by the Rosenfield equation shown below:

$$R_K = I_M (\vec{\mu}_e \cdot \vec{\mu}_m)$$

The above equation states that the rotational strength of the Kth transition is the imaginary ($I_M$) portion of the dot
For the Peptide Chromophore, The $\pi \rightarrow \pi^*$ Transition (190nm) and the $n \rightarrow \pi^*$ Transition (210nm) Correspond to Transitions From Ground State $|0>$ to Excited States $|\alpha>$ and $|\beta>$. 

Fig. 4. The Mixing of Transitions Within a Chromophore.
1) \[ |0 \rangle \begin{array}{c} G \cdot S \\ E \cdot S \end{array} \phi = C_1 \ |\alpha \rangle + C_2 \ |\beta \rangle \] where \( C_1 \gg C_2 \)
\[ \vec{\mu}_e \quad \vec{\mu}_m \]
This is mainly \( \alpha \) at 190nm

2) \[ |0 \rangle \begin{array}{c} G \cdot S \\ E \cdot S \end{array} \phi = -C_2 \ |\alpha \rangle + C_1 \ |\beta \rangle \] This is mainly \( \beta \) at 210nm
\[ \vec{\mu}_e \quad \vec{\mu}_m \]

\( R_K \) for equation 1:
\[ R_1 = C_1 \vec{\mu}_e \times C_2 \vec{\mu}_m = C_1 C_2 (\vec{\mu}_e \times \vec{\mu}_m) \] Mainly \( \alpha \) at 190nm

\( R_K \) for equation 2:
\[ R_2 = -C_2 \vec{\mu}_e \times C_1 \vec{\mu}_m = -C_2 C_1 (\vec{\mu}_e \times \vec{\mu}_m) \] Mainly \( \beta \) at 210nm

\[ \therefore R_1 = + \text{ at 190nm} \]

\[ R_2 = - \text{ at 210nm} \]

\textbf{Fig. 5.} How To Calculate \( R_K \), The Rotational Strength, From the Rosenfeld Equation \( R_K = l_M (\vec{\mu}_e \cdot \vec{\mu}_m) \).
product of the electric and magnetic transition dipoles (Fig. 5)(27).

Hence, to obtain optical activity in CD, the transition must have both electric and magnetic transition dipole properties. Since these quantities are vectors, they must not be orthogonal.

Biological macromolecules have the characteristic ability to induce asymmetry in small bound molecules which are symmetric in the unbound condition. The nature of the induced asymmetry depends on the asymmetry of the site in the macromolecule and it can thus serve as a probe in the study of the conformational states using the phenomenon of CD. For example, using a peptide bond as a model (Fig. 4), it could be shown that CD can be generated in biological macromolecules with neither helical structures nor asymmetric molecules, by the interaction of the excited states of chromophores.

There are two methods of chromophoric excitations in CD. They are the mixing of transitions within a chromophore and the coupling of transitions between chromophores. The mixing of transitions within a chromophore occurs in the peptide bond region (200 - 250 nm). These transitions are: \( n \rightarrow \pi^* \) and \( \pi \rightarrow \pi^* \) transitions (Fig. 4).

In the \( n \rightarrow \pi^* \) transition, an electron goes from a non-bonding (n) orbital to an antibonding orbital (\( \pi^* \)) of the carbonyl group (Fig. 4). This transition is accompanied by electron rotation which has a non-zero magnetic transition
moment dipole. The above mentioned transition occurs at approximately 210nm (Fig. 4). In the \( \pi \rightarrow \pi^* \) transition an electron is displaced from a \( \pi \) orbital of the nitrogen to a \( \pi^* \) orbital of the carbonyl group. This transition has a non-zero electric transition moment dipole, and it occurs at approximately 190nm (Fig. 4).

In order to observe CD in the peptide bond region (200 - 250nm) as described above, there must be transitions satisfying the aforementioned Rosenfeld equation (Fig. 5):

\[
R_K = I_M (\vec{\mu}_e \cdot \vec{\mu}_m)
\]

where \( I_M (\vec{\mu}_m \cdot \vec{\mu}_e \neq 0) \). The above phenomenon demonstrates CD as a mixing of transitions within a chromophore. These transitions are the type which are largely responsible for the existence of optical activity in proteins within the 200 - 250nm wavelength region. Similarly, the transitions described above are also responsible for the existence of optical activity in aromatic residues. For example, the absorption of tyrosine residues in proteins near 280nm is due to the promotion of one of the electrons in a \( \pi \) bonding orbital to an antibonding \( \pi^* \) orbital of the benzene ring.
EXPERIMENTAL

Materials

Frozen turkey livers were obtained from Pel-Freeze Biologicals, Inc., Rogers, Arkansas. D-fructose 1,6-bisphosphate (FbP) and adenosine monophosphate (AMP) were procured from Sigma Biochemicals, St. Louis, Missouri. Chelex 100 was obtained from Bio Rad Laboratories, Richmond, California. All other chemicals were reagent grade products.

Methods

Absorption Measurements: Spectrophotometric absorption measurements were performed using a Cary 17 recording spectrophotometer, a Coleman Model 124 double beam grating U. V.-Visible Spectrophotometer, and a Hitachi 191 digital U. V.-Visible Spectrophotometer. Hellma Suprasil 1cm quartz cells (1cm rectangular) were used. CD measurements were performed using a Jasco Durrum Model SS-20 Spectropolarimeter flushed with nitrogen gas. Appropriate attention to the effects of scanning speed, slit width, and instrument time constants insured optimal spectral resolution. All the experiments were done at room temperature. Hellma Suprasil 5cm quartz cells were used in performing all the CD experiments.

The glassware utilized for the experiments were thoroughly cleaned by washing first with Sparkleen detergent, followed by rinsing with deionized water, and finally with Chelex-treated distilled water. The 4cm quartz cells were also thoroughly cleaned with diluted hydrochloric acid,
and rinsed with deionized water followed by rinsing with Chelex-treated distilled water.

Treatment of buffers with Chelex 100: Chelex 100 (100 - 200 mesh) was washed several times with distilled water and a suspension of the resin was then titrated to the pH value of the buffer solution with either hydrochloric acid (HCl) or Sodium hydroxide (NaOH) as appropriate. The buffer (Tris HCl) was then mixed with the settled resin, and the mixture was stirred for about thirty minutes at room temperature. The Chelex-treated buffer was carefully decanted and its pH was checked.

It has been reported that the treatment of buffers with Chelex 100 helps to remove heavy-metal ions and impurities which might be present in the enzyme solution.

Purification of Turkey Liver FbPase: The turkey liver fructose 1,6-bisphosphatase was provided by Han et al. (23).

The purified enzyme for the circular dichroism studies was treated with Chelex 100 by dialyzing overnight against 1 liter of 50mm Chelex-treated tris-hydrochloric acid buffer at pH 7.5.

Determination of FbPase Concentration: FbPase concentration was measured by the 215nm - 225nm method of Murphy and Kies (32). The formula used is shown below:

\[ \Delta \text{Abs } 225,215 \times 154/1000 = \text{mg/ml} \]

The absorption spectra were taken with a Cary 17 Model Spectrophotometer using Chelex-treated tris-hydrochloric
acid as the blank. Wavelength range was from 210 to 250 (U. V. region). Concentrations of the enzyme were from 0.40 to 0.55 mg/ml.

**Circular Dichroism Studies:** The circular dichroism spectra of the Chelex-treated fructose 1,6-bisphosphatase (FbPase) were recorded under various experimental conditions:

(A) The CD spectra of the native FbPase alone in Chelex-treated tris-HCl buffer were recorded at pH 7.0, 8.0, and 9.0. The baselines were first recorded using 50mM Chelex-treated tris-HCl buffer.

(B) The CD spectra of the native FbPase plus 0.1mM fructose 1,6-bisphosphate (FbP) in solution were recorded at pH 7.0, 8.0 and 9.0. The baselines were first recorded using 50mM Chelex-treated tris-HCl buffer plus 0.1mM FbP.

(C) The CD spectra of the native FbPase plus 0.1mM adenosine monophosphate (AMP) in solution were recorded at pH 7.0, 8.0 and 9.0. The baselines were first recorded using 50mM Chelex-treated tris-HCl buffer plus 0.1mM AMP.

(D) The CD spectra of the native FbPase in the presence of both FbP and AMP were recorded. First, 0.1mM FbP was added to the enzyme followed by the addition of 0.1mM AMP at the aforementioned pH's. The baselines were first recorded by the addition of 0.1mM FbP and 0.1mM AMP, respectively, to 50mM Chelex-treated tris-HCl buffer.

(E) Finally, the CD spectra of the native FbPase in the presence of AMP and FbP, respectively, were recorded. First, 0.1 mM AMP was added to the enzyme followed by 0.1mM FbP at
the same aforementioned pH's. The baselines were first of all recorded by the addition of 0.1mM AMP and 0.1mM FbP, respectively, to 50mM Chelex-treated tris-HCl buffer.

The CD data obtained from the above experiments were analyzed by a PDP 8/E computer. First, the experimental CD spectra, known as the ellipticity values, were fed into the computer. The molar ellipticity values were then calculated by the computer using the equation below:

$$[\theta] = \frac{\theta}{L \times C \times N \cdot R}$$

where $[\theta]$ is the molar ellipticity per residue, $\theta$ is the observed ellipticity value from the experimental CD spectra, $L$ is the cell length, $C$ is the concentration of the enzyme, and $N \cdot R$ is the number of residues of the enzyme, which is 1244 for FbPase. The units for the $[\theta]$ were in degrees cm$^2$ per decimole. The CD data for all the experiments were reported in terms of $[\theta]$ versus $\lambda$ (wavelength) in the region studied (250 - 300nm). All the CD spectra were taken in triplicates for signal averaging purposes, and the experiments were reproduced at different concentrations. The average deviation was about 2%.
RESULTS AND DISCUSSION

The results of the CD studies performed on the aromatic residues of FbPase from turkey liver in the presence of the substrate, FbP, and the inhibitor, AMP, under various experimental conditions are discussed below.

(A) Effect of pH on CD of FbPase: In Fig. 6 is reported the CD spectra in the near ultraviolet region (250 - 300nm) of the native FbPase at pH 7.0, 8.0 and 9.0. The CD spectra show that the enzyme conformation has substantial dependence on pH. It can be seen that the magnitude of the CD spectrum is reduced as the pH is increased. This observation indicates that as the pH of the enzyme solution is increased; perhaps the rotational mobility of the optically active aromatic residues is increased. As a result, the electrostatic field about the aromatic chromophores becomes less asymmetrical due to rotational averaging; thereby decreasing the CD of the aromatic chromophores in the enzyme.

(B) Effect of Substrate, FbP, on CD of FbPase: Fig. 7 shows the effect of the substrate, FbP, on the CD spectra of FbPase at pH 7.0, 8.0 and 9.0. The CD spectra in the near ultraviolet region (250 - 300nm) show that the binding of FbP to the enzyme induces a slight conformational change. It can be observed that in Fig. 7, the ellipticity values at pH 7.0, 8.0 and 9.0 are slightly reduced as compared to those of the native enzyme by itself (Fig. 6). Furthermore,
at pH 8.0 and 9.0 in Fig. 7, there exist negative CD at lower wavelength (below 270nm) which does not occur on the CD spectra of the native enzyme in the absence of FbP (Fig. 6). These observations show that the enzyme is in two different conformational states either in the presence or absence of the substrate, FbP.

(C) Effect of the Inhibitor, AMP, on CD of FbPase:
The CD spectra of the enzyme in the presence of the inhibitor, AMP, are shown in Fig. 8. The CD spectra show a strong influence of the inhibitor on the CD of the enzyme at pH 7.0, 8.0 and 9.0. The enzyme-inhibitor complex shows a large negative CD at the above mentioned pH's below 280nm wavelength. These large negative dichroic spectra are not observed in the CD spectra of the native enzyme either in the presence or absence of the substrate. As a matter of fact, the CD spectra of the enzyme in the presence of AMP are different from those obtained from the enzyme and/or substrate (Fig, 7,8). The above circumstances indicate that the addition of AMP to the enzyme changes very significantly the conformation of the enzyme.

(D) Effects of the Substrate, FbP, Plus the Inhibitor, AMP, Respectively on CD of FbPase: Analytical observations of the CD curves of FbPase upon the addition of substrate and the allosteric inhibitor (FbP is added to the enzyme first, followed by AMP) reveal that the effect of AMP is far greater than that of FbP. This is because the CD spectra
Fig. 8. Near the Ultraviolet (250 - 300nm) Circular Dichroism Spectra of FbPase in the presence of 0.1mM inhibitor (AMP) at pH 7.0, 8.0 and 9.0 in 50mM Chelex-treated tris-HCl buffer.
of the enzyme in the presence of both FbP and AMP are very similar to those obtained in the presence of AMP alone (Figs. 8 and 9).

(E) Effects of the Inhibitor, AMP, Plus the Substrate, FbP, Respectively on CD of FbPase: Thorough analytical observations of the CD curves of FbPase in the presence of AMP and FbP (AMP is added first to the enzyme solution followed by FbP) confirm that the effect of the allosteric inhibitor is far greater on the CD of the enzyme than that of the substrate. Furthermore, the shapes of the CD spectra of the enzyme in the presence of AMP and FbP and vice versa, are very similar to those obtained in the presence of AMP alone (Figs. 8, 9 and 10).

In summary, Fig. 11 shows the CD spectra of the native enzyme alone, the enzyme in the presence of FbP, and the enzyme in the presence of AMP, all at pH 7.0. In Fig. 11 it can be seen that the CD spectra of the enzyme either in the presence or absence of FbP have essentially the same shape except that there is a slight decrease in optical activity. On the contrary, the CD spectrum of the enzyme plus AMP is different from the spectrum of the enzyme alone.

The data obtained in this investigation suggest that the presence of the substrate, and/or the allosteric inhibitor as well as changes in the pH of the medium do produce some significant changes in the conformation of the enzyme. However, the effect of the inhibitor is far more pronounced than that of the substrate.
Fig. 9. Near Ultraviolet (250 - 300nm) Circular Dichroism Spectra of FbPase in the presence of 0.0mM substrate (FbP) plus 0.1mM inhibitor (AMP) at pH 7.0, 8.0 and 9.0 in 50mM Chelex-treated tris-HCl buffer.
Fig. 10. Near Ultraviolet (250 - 300nm) Circular Dichroism Spectra of FbPase in the presence of 0.1mM inhibitor (AMP) plus 0.1mM substrate (FbP) at pH 7.0, 8.0 and 9.0 in 50mM Chelex-treated tris-HCl buffer.
Fig. 11. Near Ultraviolet (250 - 300nm) Circular Dichroism spectra of FbPase at pH 7.0, FbPase in the presence of 0.1mM substrate (FbP) at pH 7.0, and FbPase in the presence of 0.1mM inhibitor (AMP) at pH 7.0 in 50mM Chelex-treated tris-HCl buffer.
CD studies on the secondary structures of FbPase performed by Burkes (25) and Rumph (26) show that FbPase undergoes significant secondary conformational changes in the presence of FbP and AMP as well as changes in the pH of the medium. The CD data obtained in their studies show that the addition of AMP to the enzyme solution in the presence of FbP yields further changes in the secondary structures of the enzyme, but the addition of FbP to the enzyme solution in the presence of AMP does not further change the secondary conformations. This indicates that AMP has a more significant effect on the secondary structures of the enzyme than FbP. Similarly, AMP has been observed to have more effect on the aromatic residues of FbPase than FbP.

The amino acid composition of FbPase indicates that there are about fifty-two tyrosine, thirty-nine phenylalanine and four tryptophan residues per mole of the enzyme. This finding suggests that tyrosine chromophores are the major contributors to the optical activity of the aromatic residues in the enzyme.

When FbP is added to the enzyme solution, there is a reduction in optical activity (Fig. 7). This can be explained on the assumption that FbP, on interacting with the enzyme, can increase the rotational mobility of the optically active tyrosine residues, making them more symmetrical and thereby reducing the CD of these aromatic chromophores. An alternative explanation could be that the binding of FbP to the enzyme could render optically inactive some
tyrosine or phenyalanine residues previously optically active. The fact that the addition of the substrate, FbP, does not influence the shape of the CD spectra, but only the ellipticity values suggests that the effect of substrate on CD of FbPase is related to the modification of the environment of the aromatic residues contributing to the optical activity of the native enzyme in the absence of substrate, FbP (13).

The addition of the allosteric inhibitor, as in the case of the substrate, affects the optical activity of the aromatic residues by a conformational change, but this conformational change has to be restricted to very few tyrosine residues because of the large negative dichroism at the lower wavelength (Fig. 8). It is apparently phenyalanine and tryptophan residues which are contributing to the optical activity of the aromatic chromophores.

Due to the fact that the effects of the substrate and the allosteric inhibitor on the enzyme greatly differ (Fig. 11), it can be postulated that different aromatic residues are affected by the binding of the substrate, FbP, and the inhibitor, AMP, to the enzyme.
CONCLUSION

The data obtained including all the observations, results and discussion suggest that turkey liver FbPase undergoes conformational changes under all the experimental conditions studied in this report.

From the circular dichroism (CD) studies on the aromatic residues of turkey liver FbPase, it is apparent that the catalytic and regulatory functioning of the enzyme, in relation to the pH, or to the presence of the substrate (FbP) and the allosteric inhibitor (AMP) are accompanied by significant changes in the conformation of the enzyme.
REFERENCES


