CIRCULAR DICHROISM STUDIES OF FRUCTOSE 1,6-DIPHOSPHATASE

A THESIS

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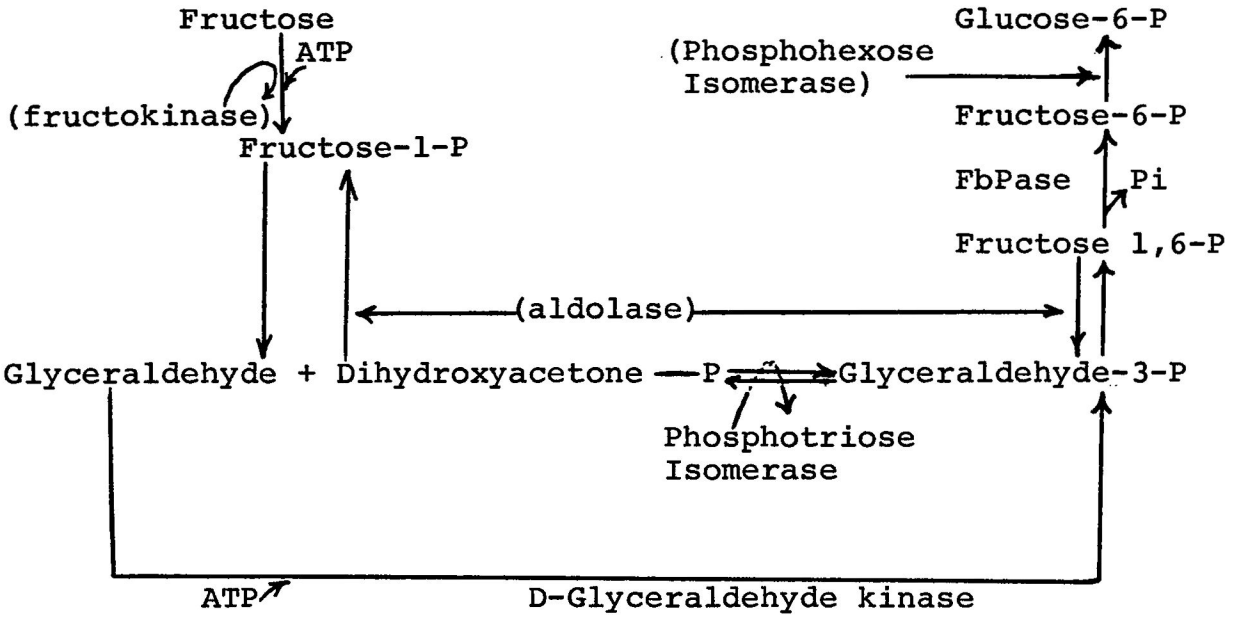
INTRODUCTION

A systematic study of the circular dichroism of Fructose 1,6-Bisphosphatase (FbPase) yields important information about the enzyme's secondary structure. Fructose 1,6-Bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase (E.C. 3.1.3.11)) catalyses the reaction: D-fructose 1,6-bisphosphate + $H_2O \rightarrow$ D-fructose—6—phosphate + Pi. Fructose 1,6-bisphosphatase was first discovered in rabbit liver by Gomori in 1943 (1).

It was first thought that fructose 1,6-bisphosphatase was inactive at acidic or neutral pH. (However, this was the alkaline form of the enzyme.) Years later the role of specific FbPase in carbohydrate metabolism was recognized. FbPase is shown to specifically hydrolyze the 1-phosphate group of FbP to yield fructose-6-phosphate (2,3). A specific role of FbPase was suggested by McGilvery et al., relating an increase in the level of FbPase concentration whenever there is an occurence of favorable conditions for gluconeogenesis (4).

The role of FbPase in gluconeogenesis is illustrated in Fig. 1. This pathway was devised by Hers and Kusaka (5). It is now recognized that FbPase is a key factor in the regulation of gluconeogenesis. In observing the catalytic and regulatory functions of the enzyme, questions arise as

Fig. 1. Pathway of Fructose Metabloism in Liver.



to whether any changes occur at or near the active site in catalysis and/or regulation.

The study of circular dichroism of FbPase may yield useful information as to whether changes in the secondary structure of FbPase accompany activation and/or deactivation. In studying circular dichroism spectra of FbPase, this report will use the phenomenological approach of analysis. The phenomenological method is one where the spectra of complex molecules are analyzed in terms of the sum of contributions from specific structures. These structures are helices, coils and sheets. This analysis of multicomponents depends upon comparison of experimental spectra with those of the well-characterized structures, taken alone and/or in combinations at known percentages (6). This report will

quantitate the percentages of alpha helix, Beta-pleated sheet, and random coil structures in different conformations of FbPase from avian species, using the above method. Observations on the effects of temperature, pH, and/or other enzyme modifiers are also reported.

LITERATURE REVIEW

Properties of Mammalian Fructose 1,6-bisphosphatase

Fructose 1,6-bisphosphatase from a large number and variety of sources has been shown to be an allosteric enzyme subject to inhibition by adenosine-5' monophosphate (AMP) and excess amounts of fructose 1,6-bisphosphate (substrate FbP). Several studies have shown the existence of four interacting sites of AMP binding and also four independent sites for substrate binding per molecule of enzyme (7). The purified enzyme is found to exhibit distinct chemical and physical properties. Some of the chemical properties are an absolute requirement for divalent cation Mg^{+2} or Mn^{+2}, EDTA stimulation, neutral pH optimum, a high degree of substrate specificity, and allosteric inhibition by high substrate concentration.

In humans, FbPase has been associated with certain physiological disorders. These disorders have been attributed to FbPase deficiency. A deficiency of FbPase is thought to be chiefly responsible for Von Gierke's disease (absence of glucose-6-phosphate, chronic lactic acidosis), and which also leads to hypoglycemia and severe metabolic acidosis (8-9).

Properties of Avian Fructose 1,6-Bisphosphatase

There has been very little work done with FbPase from avian species as compared with the amount of work done on mammalian species. Marquodt and Olson report the temperature of thermal inactivation of chicken liver and muscle FbPase (10, 11). They concluded that the stabilizing effect of the compounds (FbP and AMP) would induce changes in unheated avian FbPase. These compounds also stabilized FbPase following heat treatment. The latter effect is thought to be a reflection of the abilities of the compounds to prevent irreversible changes in the secondary or tertiary structures of the avian enzyme (10,11). In a second work by Olson and Marquodt, a significant difference in the isoelectric point between avian and mammalian FbPase was noted. The isoelectric point of avian FbPase was found to be 8.1 for the liver enzyme and 8.6 for the muscle enzyme. This is considerably higher than for mammalian FbPase, eg., swine kidney is 5.9, rabbit liver is 6.5, rabbit kidney and muscle are slightly higher than 6.5. The enzyme from different sources, however, has the same metabolic role. Han and co-workers have shown that the enzyme FbPase from two different avian species are very closely related (12, 13). The two enzymes from chicken and turkey have similar molecular weights, amino acid compositions, and electrophoretic mobility. Turkey liver FbPase purified by the method of Han et al. yields a homogeneous FbPase (12). The enzyme has a molecular weight of 144,000 and may exist

as four identical subunits.

Circular Dichroism and the Phenomenological Approach

Circular dichroism has become an important and useful analytical technique in determining the conformation of polypeptides and biological macromolecules. This technique is very useful in detecting changes in orderly or periodic arrangement of amino acids in a polypeptide chain, changes induced in the local environment of the amino acid residues, and/or changes at certain specific sites caused by certain interactions. Methods used in the application of circular dichroism to biological macromolecules have been published by Bayley (14), Beychok (15), Greenfield and Fasman (16), and Chen, Yang, and Martinez (17). The effect of temperature on the circular dichroism of polypetides has been reported by Tiffany and Krimm (18). The only previous report on circular dichroism spectra of FbPase is that of Tamburro et al., (rabbit liver FbPase) (19), and Davis (chicken liver FbPase) (20).

Circular dichroic properties of molecules are determined by their conformation. The circular dichroism spectra are a function of light absorption. In the visible and ultraviolet regions of the electromagnetic spectrum (600-180 nm), the interaction of radiation with the polarizable electrons of the molecules cause a redistribution of the electron density. At certain frequencies (ν) the energy of the incident radiation will satisfy the condition $\Delta E=h\nu$, where ΔE is the energy difference

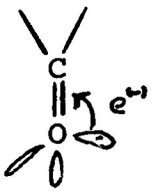
between the ground state and an electronic excited state, and the electron will undergo a transition into the excited state. Optical activity is a phenomena of circular dichroism which derives from the unequal interaction of the right and left handed circularly polarized components of plane polarized light by assymetric molecules. Optical activity is characterized by the rotational strength of a transition which may be positive or negative. Circular dichroism spectra may therefore be represented as a sum of a series of transitions (14). In order for a transition to occur, the electron transition moment $(\vec{\mu}e) \neq 0$ (represents a displacement of charge). In addition to the electronic transisiton, one may also have magnetically allowed transition (μ_m). These are transitions due to charge rotation.

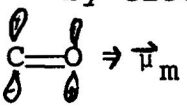
In circular dichroism, transitions due to $(\vec{\mu}e \neq 0)$, and $(\vec{\mu}_m \neq 0)$ occur only with molecules which do not possess either a center of symmetry or a mirror plane. The simplest example of this is the helix.

Circular dichroism spectra are generated in molecules that are without helical structures by an interaction of excited states of chromophores: (a) mixing of transitions within a chromophore, and (b) coupling of transitions between chromophores.

The transitions which occur in the region studied in this report (200 - 250 nm peptide bond region), are the $N \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions.

(a) $N_P \rightarrow \pi^*$ electron leaves nonbonding P_Y orbital of oxygen and enters antibonding orbital of carbonyl group. This is accompanied by electron rotation which induces $\vec{\mu}_m$.



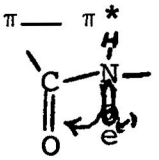


$\vec{\mu}_e = 0$

$\vec{\mu}_m \neq 0$

The transition occurs at approximately 210 nm.

(b) $\pi \rightarrow \pi^*$ electron is displaced from π electron density of nitrogen to π^* orbital of carbonyl group.



$\vec{\mu}_m = 0$

$\vec{\mu}_e \neq 0$

The transition occurs at approximately 190 nm.

In order for one to observe circular dichroism in the peptide bond region there must be transitions satisfying one condition:

$$\text{im}\ (\vec{\mu}_m \cdot \vec{\mu}_e \neq 0)$$

This demonstrates circular dichroism as a mixing of the transitions in a and b above.

The aforementioned transitions are the type which are largely responsible for the existence of optical activity in proteins. The optical activity observed here was expressed as: $X = F_h \times {}_h + F_B \times {}_B + F_{R.C.} \times {}_{R.C.}$ with $F_h + F_B + F_{R.C.} = 1$ and all F's ≥ 0. The F's are the fraction of the helix, beta sheet, and random forms in a protein molecule. The X_h, X_B, and $X_{R.C.}$ are the ellipticity values that are obtained

if a protein consists of segments of pure alpha helix (h), beta sheet (B), and random coil (R.C.) (This is for each wavelength.) The X would be the molar ellipticity. Techniques of expressing optical activity as percentages of alpha helix, beta sheet, and random coil, coupled with the methods of analysis of circular dichroism spectra (see methods), yield the experimental results.

EXPERIMENTAL

Materials

Frozen turkey livers were obtained from Pel-Freeze Biologicals Inc., Rogers, Arkansas. D-fructose 1,6-bisphosphate (FbP), nicotiamide adenine dinucleotide phosphate (NADPH), ethylene-diamine-tetra-acetate (EDTA), adenosine-5' monophosphate (AMP), sodium dodecyl sulfate (SDS), sodium acetate, bovine serum albumin, glucose-6-phosphate dehydrogenase, and phosphoglucose isomerase were obtained from Sigma Biochemicals, St. Louis, Missouri. Glucose-6-phosphate dehydrogenase, phosphoglucoisomerase, myokinase, pyruvate kinase, and adenosine triphosphate (ATP) were also purchased from the Sigma Biochemicals. Triethanolamine, diethanolamine, triflouracetic anhydride, sephadex G-200, Tris-HCL, cobalt chloride, and sodium chloride (analytical grade) were obtained from Fisher and/or Eastman Organic Chemicals.

Whatman cellulose phosphate P11 was purchased from H. Reeve Angel Inc., of New Jersey. Before use, the powder was washed alternately with alkali and acid, then with distilled water until neutral pH was obtained. The cellulose was then stored at $5^{o}C$ in 0.1 M sodium acetate buffer (pH 5.6), containing 1 mM EDTA. Immediately before use, the powder was filtered. All other chemicals used were of reagent grade.

Methods

Absorption measurements. Spectrophotometric absorption measurements were obtained using a Colemen Model 124 double beam grating U. V. visible spectrophotometer, a Hitachi 191 digital U. V. visible spectrophotometer, and a Cary 17 recording spectrophotometer. Matched Hellma Suprasil quartz cells were used (1 cm rectangular). Circular dichroism spectra were recorded using a Jasco Durrum Model SS-20 spectropolarimeter flushed with nitrogen gas. Variable temperature measurements were made using 1.0 cm Durrum quartz variable temperature cells. The temperature of samples in the cell was varied using a water bath and a Brinkman Lauda K-2/R refrigeration device. The water traversed approximately 150 cm of Tygon tubing (from refrigerant to the cell). The temperature of the water varied by approximately 0.2° C over this length at 42° C and by 0.1° C at 22° C (20).

Protein determination. The protein content in the initial stages of purification of the enzyme was measured by the method of Lowry (21) using bovine serum albumin as the standard and/or by the 260/230 method of Bernlor (22). Protein concentration in mg/ml=184B - 76.1C where B and C are absorbances at 230 and 260 nm respectively using a 1 cm cuvette. For solutions of purified enzyme, the concentration was determined from the absorbance at 280 nm and/or by the (215nm-225nm) (154) method of Murphy and Kies (23). This latter method is based on the peptide bond absorption in the region 195-225 nm.

Enzymatic assays. Fructose 1,6-bisphosphatase activity

is assayed by observing spectrophotometrically at 23° C the rate of reduction of NADP at 340 nm. The standard reaction mixture existed at pH 7.0 and pH 9.2, the pH of optimum activity of the neutral and alkaline forms of FbPase respectively. The standard reaction mixture (1.0 ml) contained 0.005 M glycine-NaOH pH (9.5) or 0.05 M Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM MgCl, 1 unit each of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase, and an approiate aliquot of fructose 1,6-bisphosphatase (usually 10 λ). The reaction mixture containing every component except the FbP is incubated inside the spectrophotometer for a period of four min. The reaction was then iniated by the addition of the FbP. One unit of enzymatic activity is defined as the amount required to catalyze the hydrolysis of 1 μm of FbP under the assay conditions. Specific activity is defined as units per mg of protein.

$$\text{Sp. Ac.} = \frac{A_{340}\ \text{nm/min (vol ml) (1000)}}{6.22\ (1\ \text{cm})\ (\text{mg protein})}$$

Choice of Species

The enzyme fructose 1,6-bisphosphatase has been extensively studied. Nost of the studies, however, have been on mammlian FbPase. FbPase has been shown to play a leading role in gluconeogenesis. Little is known about avian FbPase. This study will allow one to have a broader view of FbPase from avian species, and to fully compare FbPase from different species when studying the functions regulated by the enzyme Turkey liver was chosen for study because FbPase in

gluconeogenesis conditions is found largely in the liver (4). Turkey livers are large compared to some other avian species and are commercially available.

Purification of Turkey Liver FbPase

During the entire procedure, all operations were performed at 0-4° C unless otherwise stated. The characteristic results of the procedure are summarized in Table 1.

Preparation of Crude Extract

500 g (dry wt.) of frozen livers were suspended (1/4, w/v) in 0.075 M Tris-HCL containing 0.01 mM EDTA and 0.01 M sodium bicarbonate. This mixture was homogenized for 2 min in a Waring blender and then centrifuged at 32,000 x g for 60 min. The supernatant was filtered through glass wool and pH adjusted to 7.5 with 2 N NaOH.

Heat fraction

Portions of the supernatant were heated with constant stirring in a water bath at 85° C. When the temperature of the enzyme reached 60° C, it was removed and cooled to below 10° C in an ice bath. Then the portions were pooled and the precipitate was removed by centrifugation for 30 min. at 26,000 x g. The supernatant fraction was filtered through glass wool and dialyzed for 8 hr. against 20 vol of 0.10 M sodium acetate buffer (pH 6.0) containing 1 mM EDTA (three changes of buffer).

Phosphocellulose chromatography

The dialyzed fraction was treated with phosphocellulose P11 with constant stirring. During this addition, the pH was

maintained at 6.3 with 2 N NaOH. The phosphocellulose slurry (deep red) was removed by light vacuum filtration. The clear filtrate retained 90% of the total activity present in the heat fraction. The filtrate was diluted with an equal volume of cold distilled water containing 0.1 mM EDTA. The pH was adjusted to 5.7 by addition of 2 N acetic acid, and the phosphocellulose suspension was again added, this time until all the enzymatic activity had been absorbed. The suspension was filtered on a Buchner funnel and the filtrate discarded. The phosphocellulose paste was washed on the funnel with 3 l of 0.15 m sodium acetate buffer (pH 5.6) containing 0.1 mM EDTA. The moist paste was transferred to a glass column (4.5 cm in diameter and 60 cm in height) and washed with 0.20 M sodium acetate buffer (pH 5.8) containing .1 mM EDTA until the abosrbance at 280 nm of the washing was near or equal to 0. The enzyme was eluted with a solution of 0.08 mM FbP and 0.04 M AMP in 0.24 M sodium acetate buffer (pH 6.3) containing 0.1 mM EDTA. During the elution the activity emerged as a sharp peak, and those fractions with specific activity approaching 19 units per mg were combined.

Disc Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed at room temperature in the standard 7.5% gel at pH 7.0 according to Davis (24), or in 10% SDS polyacrylamide gel at pH 7.5 as described by Webner and Osborn (25). The stain used and polypeptide chain mol wt were: Bovine serum albumin (68,000),

aldolase (40,000), ribonuclease A (13,700), and chymotrypsinogen A (25,000). In all cases, 50 to 200 µg of protein were applied to the columns.

Amino Acid Composition Determination

The amino acid chromatogram was recorded using a Beckman amino acid analyzer by Travis and co-workers at the University of Georgia, Biochemistry Department. The identity of each amino acid was established on the basis of the area under each curve. This method is accurate to within 2%.

COOH Terminal Analyses

Purified turkey liver FbPase, which was stored in Tris-HCl-NaCl buffer (pH 7.0) containing 0.1 mM EDTA was dialyzed for 24 hr against 500 vol of glass distilled water and lyophilized to complete dryness. The dry powder (5 mg) was then dissolved in 1.0 ml of 0.2 M NH_4HCO_3 buffer (pH 8.0). This solution (0.2 ml) was then taken and incubated with 100 µl of carboxypeptidase B-DNP.

Samples were taken out after 5 hr, 6 hr and 20 hr of incubation and applied to Whatman No. 3 chromatography paper. The amino acids were allowed to descend chromatographically in a butanol: acetic acid: water (4:1:5,v/v/v) mixture for 22 hr. The chromatogram was dried and sprayed with ninhydrin, $CuNO_3$ reagent. The amino acids were identified by comparison with authentic standards.

NH_2 Terminal Analysis

The solution (100µl) containing 5 mg/ml of enzyme in

0.2 M NH_4HCO_3 (pH 8.0) was incubated with 50 µl of leucine aminopeptidase while a 150 µl fraction was incubated with 75 µl carboxypeptidase M. The incubation was 21 hr for both preparations. A 100 µl fraction of each preparation was then applied to Whatman No. 3 chromatographic paper and chromatographed in the organic phase of a butanol: acetic acid: water (4:1:5, v/v/v) mixture for 22 hr. The chromatogram was then dried and sprayed with ninhydrin $CuNO_3$ reagent and the NH_2 terminal amino acid was determined as previously described.

Sedimentation Equilibrium

A portion of the stock enzyme that was routinely stored in Tris-HCL-NaCl buffer was dialyzed exhaustively against 500 vol of glass distilled water for 24 hr and lyopholized to complete dryness. A 5 mg fraction was dissolved in 1 ml of 0.1 M NaCl. This preparation was frozen in an acetone dry ice bath and taken to the University of Georgia. The enzyme preparation was tested for homogeneity, and the mol wt was determined.

A Beckman Model E. analytical ultracentrifuge was employed in the mol wt analysis.

Circular Dichroism Studies

The circular dichroism spectra of the purified homogeneous FbPase was obtained under several conditions: (a) the spectra of the native enzyme alone in solution was recorded at pH 7.0, 8.0, and 9.0 and in the range from 22° C to 42° C at 10° C intervals; (b) the enzyme plus 0.1 mM AMP in solution at the

prementioned pH's and temp; (3) the enzyme plus 0.1 mM FbP at pH 7.0 and 9.0 at the indicated temp; and (d) the enzyme plus 0.1 mM FbP plus 0.1 mM AMP at ph 7.0 and the indicated temp. The instruments used were as reported in materials. The method used in interpreting the spectra was derived from techniques developed by Greenfield and Fasman (16), and Chen (17). In this method a first approximation of the % alpha helix, and approximation of the % beta sheet and random coil are calculated. These approximations involve a comparison of experimentally determined ellipticity values with a set of reference values. The reference values are those which would be obtained if a protein consisted of 100% of a particular type of structure. The reference ellipticity values are those reported by Chen (17). These values were obtained by recording the circular dichroism spectra of five model proteins. The proteins used by Chen are ones whose percentage composition of alpha helix, beta sheet, and random coil structures had previously been determined by x-ray crystallographic data.

The data obtained in our experiments were interpreted by a computer program, "Magic". The circular dichroism data determined experimentally is reported in terms of θ (molar ellipticity) at each wavelength in the region studied (212 nm - 250 nm). The units of $[\theta]$ are deg cm^2/decimole. The data of the experimental spectra of each wavelength is fed into the computer to be analyzed by the "Magic". The first approxi-

mation of the % alpha helical structure is first calculated by the program. This approaimation is the % alpha helix derived from the experimental value at 222 nm. From Greenfield and Fasman both the beta sheet form and the random coil have a low ellipticity equal to approximately 5,450 deg cm^2 / decimole at 222 nm (16). The alpha helix has an extremum at this point equal to - 31,500 deg cm^2 / decimole. The values here are those of the reference values derived by Chen (17). Therefore, to a first approximation, the % alpha helix may be calculated by:

$$\% \text{ Alpha Helix} = \frac{(\theta)_{222 \text{ nm}} - 5,450}{31,500 - 5,450}$$

This value is only an estimate. The program sets it as the initial alpha and then calculates a theoretical curve assigning arbitrary percentages to beta sheet and random coil. The calculated curve is compared at each wavelength with the experimental curve. The difference between the calculated and the experimental curve at each wavelength is squared, and the total is summed. the program then resets the theoretical % of alpha helix, beta sheet and random coil by 10 %, and repeats the process. This process is repeated until a curve is calculated which is virtually superimposable with the experimental curve. The % of alpha helix, beta sheet, and random coil which yield the superimposable curve are reported as the structural percentages of the experimentally obtained curve.

The CD spectrum of a protein whose secondary structure is well known from x-ray diffraction studies was analyzed with "Magic" to determine the program's accuracy. The conformation of the protein selected, lysozyme, as determined by "Magic" agrees with the results of x-ray diffraction studies of lysozyme. X-ray diffraction shows that lysozyme contains 29% alpha-helix, 11% beta sheet structure and 60% random conformation. The "Magic" analysis of lysozyme's CD spectrum shows that lysozyme contains 33% alpha-helis, 12% beta sheet structure and 55% random conformation. The agreement between the results of x-ray diffraction and those of the MAGIC analysis is good (20).

RESULTS AND DISCUSSION

Purification and Molecular Weight Determination

Fructose 1,6-bisphosphatase was purified from turkey livers as described in Methods. The description of the fold of purification of the enzyme is given in Table 1. The pH profile of the purified enzyme demonstrates maximal catalytic activity at pH 7.5 $\pm$ 0.2. This pH profile is relatively identical to that which existed in the crude and heated extracts. Fig. 2 shows the specific activity as the enzyme was eluted from phosphocellulose column. Fractions of specific activity ranging 18-20 units-mg were pooled and used for analysis in the study.

The native FbPase migrated as a single species on polyacrylamide gels in the presence of S. D. S. Electrophoresis performed without S. D. S. is a much slower process. The enzyme without S. D. S. migrated as a complete protein rather than as a single subunit. The FbPase mobility on the gels was compared to the mobilities of proteins of known molecular weight in order to estimate the molecular weight of FbPase (Fig. 3.). The proteins of known molecular weight are as reported in Methods. Using the formula of Webner and Osborn (25) for calculating mobility, and comparing with the standards

TABLE 1

Purification of Turkey Liver FbPase

Fraction	Total protein (mg)	Total units * pH 7.5 (umole/min)	Total units * pH 9.2 (umole/min)	Specific activity pH 7.5 (umole/min/mg)	Specific activity pH 9.2 (umole/min/mg)	Activity ratio of pH 7.5/9.2
Crude extract	38,584	1,957	753	0.071	0.029	2.6
Heat fraction	17,872	1,867	691	0.138	0.051	2.7
Phosphocellulose eluate	43	730	270	19.800	7.333	2.7

* The enzyme activities at pH 7.5 and pH 9.2 were assayed under the standard conditions described in Experimental.

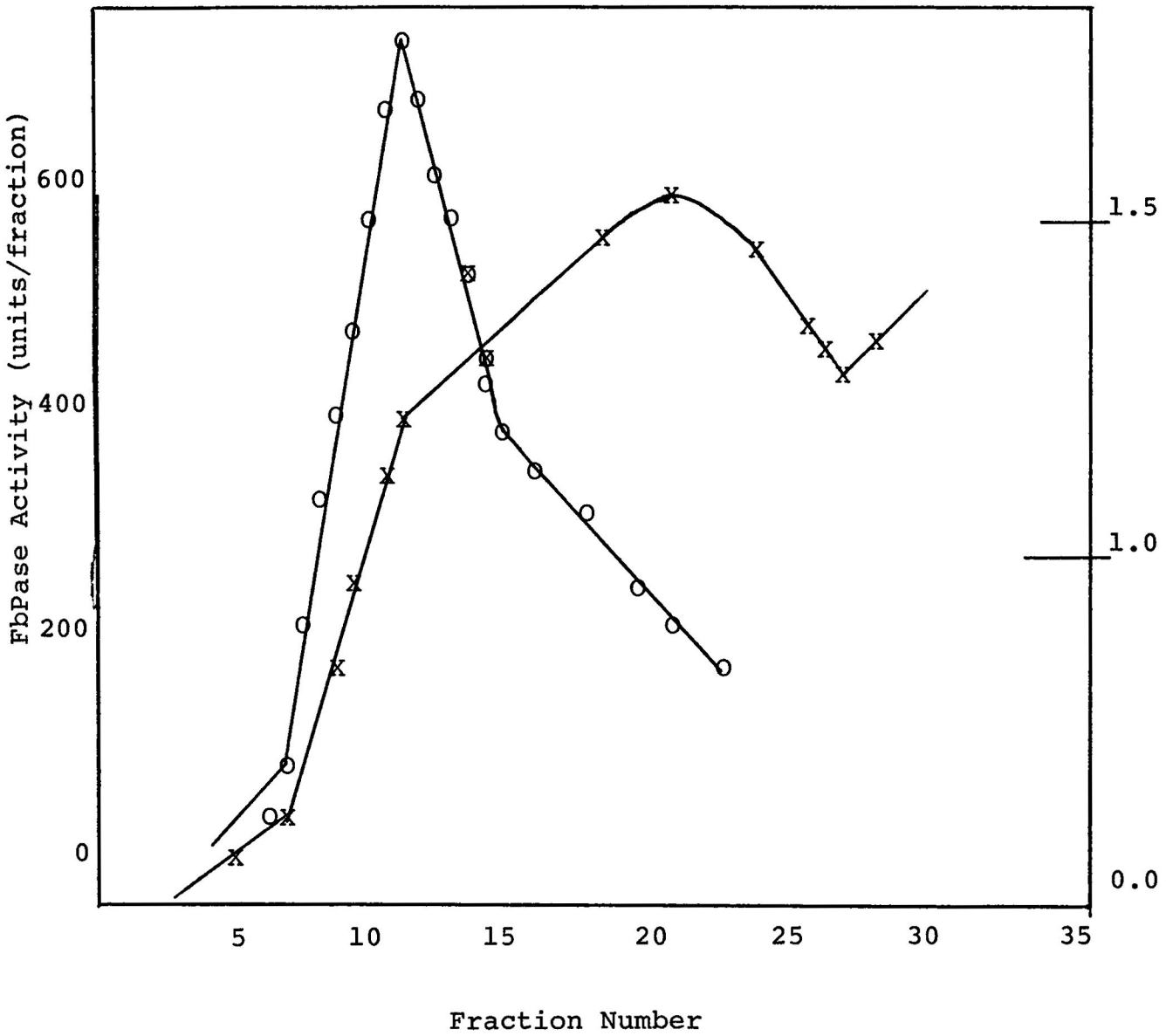


Fig. 2 Elution of FbPase*

* Selective substrate-inhibitor elution of FbPase from phosphocillulose column. O——O activity: X——X A_{280}
Fraction = 4 ml.

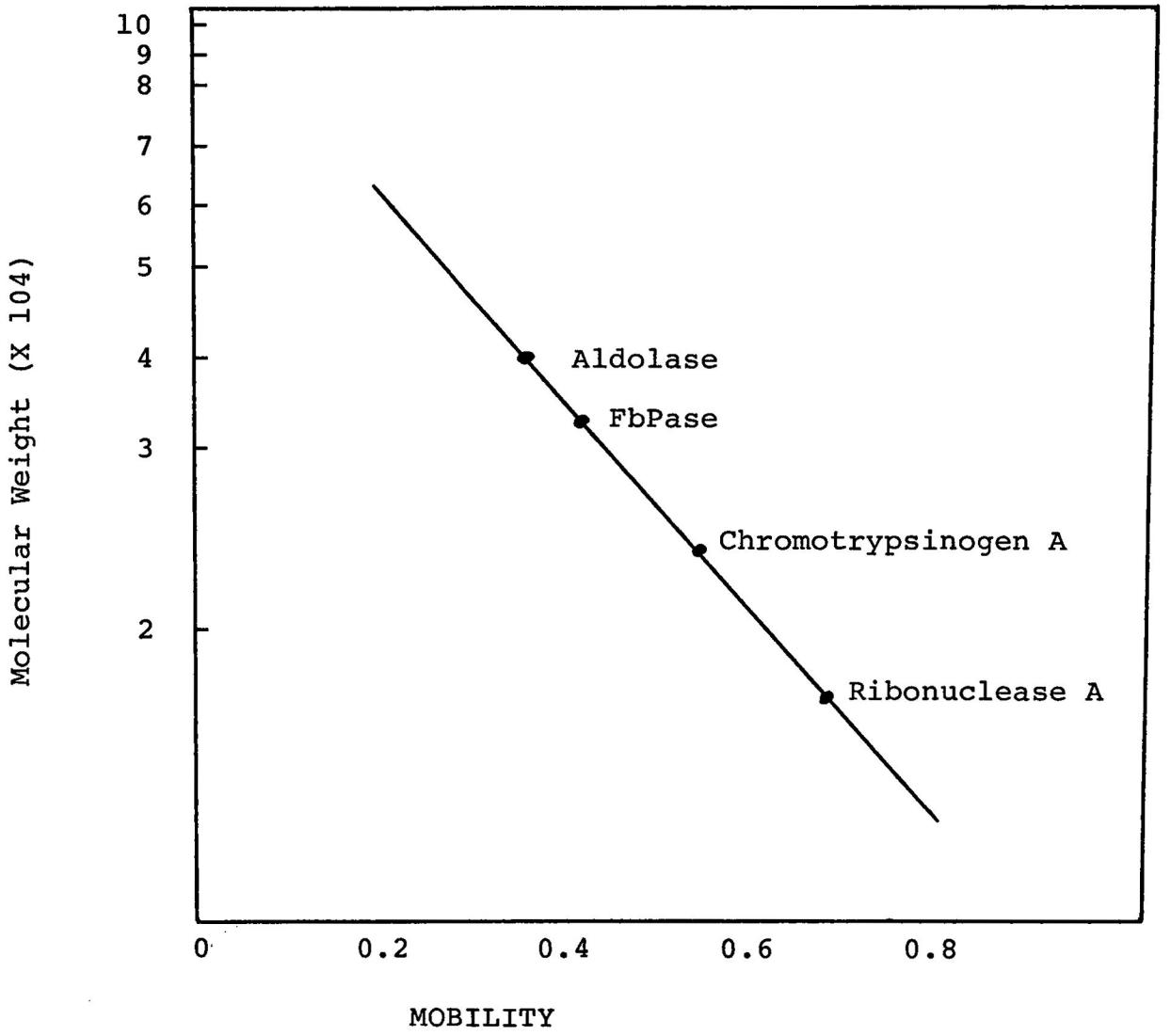


Fig. 3. Molecular Weight Determination*

* Estimation of the subunit molecular weight of FbPase from turkey liver. The comparison of the electrophoretic mobility of the subunits of turkey liver FbPase with the mobilities of proteins of known molecular weights on SDS gels. The mobilities are calculated from the formula of Webner and Osborn (25).

the molecular weight of the FbPase subunit was estimated to be 36,000. FbPase has been shown to be a tetramer protein with 4 subunits (3). This suggests that the total molecular weight of FbPase is approximately 144,000. The appearance of a single band on the gel indicates the preparation was homogeneous, and free of other proteins.

Having obtained an approximate molecular weight of 144,000 for FbPase by gel electrophoresis, a sample was analyzed with a Beckman amino acid analyzer. The analysis was performed as indicated in the methods. Table 2 shows the percentage composition of the amino acids in turkey liver FbPase; also shown is a comparison with the amino acids of chick and rabbit liver FbPase.

The number of the amino acids tends to give approximately the same molecular weight as that obtained by gel electrophoresis. A final check was the determination of the molecular weight by sedimentation equilibrum (see Methods). The result here was as previously predicted which leads us to believe that the purified native homogeneous turkey liver FbPase is a tetramer having a molecular weight of approximately 144,000.

Optical Activity and the Effect of pH Temperature, AMP, and FbP on the Conformation of Fructose 1,6-Biophosphatase.

In these tables, (3-6) the computed conformational changes of fructose 1,6-bisphosphatase from turkey liver are reported. The changes are reported over a pH range of 7.0 to 9.0 at 1.0 unit increments. At each pH, three spectral measurements were

TABLE 2

Amino Acid Composition of Native Chick, Turkey and Rabbit Liver FbPase

NO. OF RESIDUES PER MOLE OF ENZYME

AMINO ACID	CHICK	TURKEY	RABBIT
LYSINE	93	98	120
HISTIDINE	10	17	24
ARGININE	53	57	54
CYSTEINE	26	17	22
ASPARTIC	133	139	135
THREONINE	62	75	72
SERINE	71	95	76
GLUTAMIC	75	92	104
PROLINE	46	55	59
GLYCINE	114	128	102
ALANINE	101	111	117
VALINE	99	85	100
METHIONINE	25	28	33
ISOLEUCINE	92	76	75
LEUCINE	97	103	111
TYROSINE	45	52	46
PHENYALANINE	32	39	36
TRYPTOPHAN	4*	4*	4

*MINIMUM ESTIMATE

TABLE 3

Conformational Percentages in Turkey Liver FbPase, a Function of Temperature and pH.

pH	Temp.	% alpha helix	% beta sheet	% random coil
7.0	22°C	27.0	11.0	62.0
7.0	32°C	24.0	11.0	65.0
7.0	42°C	18.0	10.0	72.0
8.0	22°C	10.0	4.0	86.0
8.0	32°C	9.0	3.0	88.0
8.0	44°C	7.0	3.0	90.0
9.0	22°C	0.0	9.0	91.0
9.0	32°C	0.0	9.0	91.0
9.0	42°C	0.0	8.0	92.0

TABLE 4

Conformational Percentages in Turkey Liver FbPase When Bound to Allosteric Inhibitor AMP as a Function of Temperature and pH Concentration of Inhibitor is 0.1 mM.

pH	Temp	% Alpha Helix	% Beta Sheet	%Random Coil
7.0	22°C	10.0	11.0	79.0
7.0	32°C	10.0	11.0	79.0
7.0	42°C	9.0	11.0	80.0
8.0	22°C	3.0	3.0	94.0
8.0	32°C	3.0	3.0	94.0
8.0	42°C	3.0	3.0	94.0
9.0	22°C	0.0	10.0	90.0

TABLE 5

Conformational Percentages of Turkey Liver FbPase When Bound to its Substrate (FbP) as a Function of Temperature. The pH is Constant. Substrate Concentration is 0.1 mM.

ph	Temp	% Alpha Helix	% Beta Sheet	% Random Coil
7.0	22°C	8.0	5.0	87.0
7.0	32°C	10.0	8.0	82.0
7.0	42°C	9.0	9.0	82.0

TABLE 6

Conformational Percentages of Turkey Liver FbPase when Bound to Both Substrate and Inhibitor as a Function of Temperature. The pH is Constant, both Substrate and Inhibitor Concentrations are 0.1 mM.

ph	Temp	% Alpha Helix	% Beta Sheet	%Random Coil
7.0	22°C	9.0	10.0	81.0
7.0	42°C	11.0	8.0	81.0

obtained corresponding to three different temperatures, 22° C → 42° C at 10° C increments. (Three temperatures are studied except where otherwise indicated.) Large amounts of ordered forms of structure (alpha helix, and beta sheet) appear in the enzyme at or near neutral pH, (see Table 3). Increasing temperature from 22° C to 32° C at pH 7.0 gives an 11.4% decrease in alph helical structure, 0.0% increase in beta sheet structure, and a 4.8% increase in random coil structure. Increasing the temperature to 42° C yields a 33.3% decrease in alpha helix, a 9.0% decrease in beta sheet, and a 16.1% increase in random coil conformations.

For example, under conditions of varying pH at 22° C, changing the pH from 7.0 to 8.0 gives a 64% decrease in alpha helix structure, a 63.3% decrease in beta sheet structure, and a 38.5% increase in random coil structure (Fig. 4-8). This change in alpha helix and random coil structure is also observed when the pH of the medium is increased to 9.0. Other factors that yield decreased optical activity, from which decreases in alpha helix and beta sheet structures are observed, are the additions of the substrate (FbP) or the inhibitor (AMP). The effect which temperature exhibits on the enzyme is reduced markedly when either the substrate or the inhibitor is bound to FbPase (Fig. 9, 10). Fig. 8 shows the effect of AMP bound to FbPase. However, a similar set of curves are obtained when FbP is bound to FbPase. The addition of FbP to FbPase produces a change in the conformation, and reduced optical

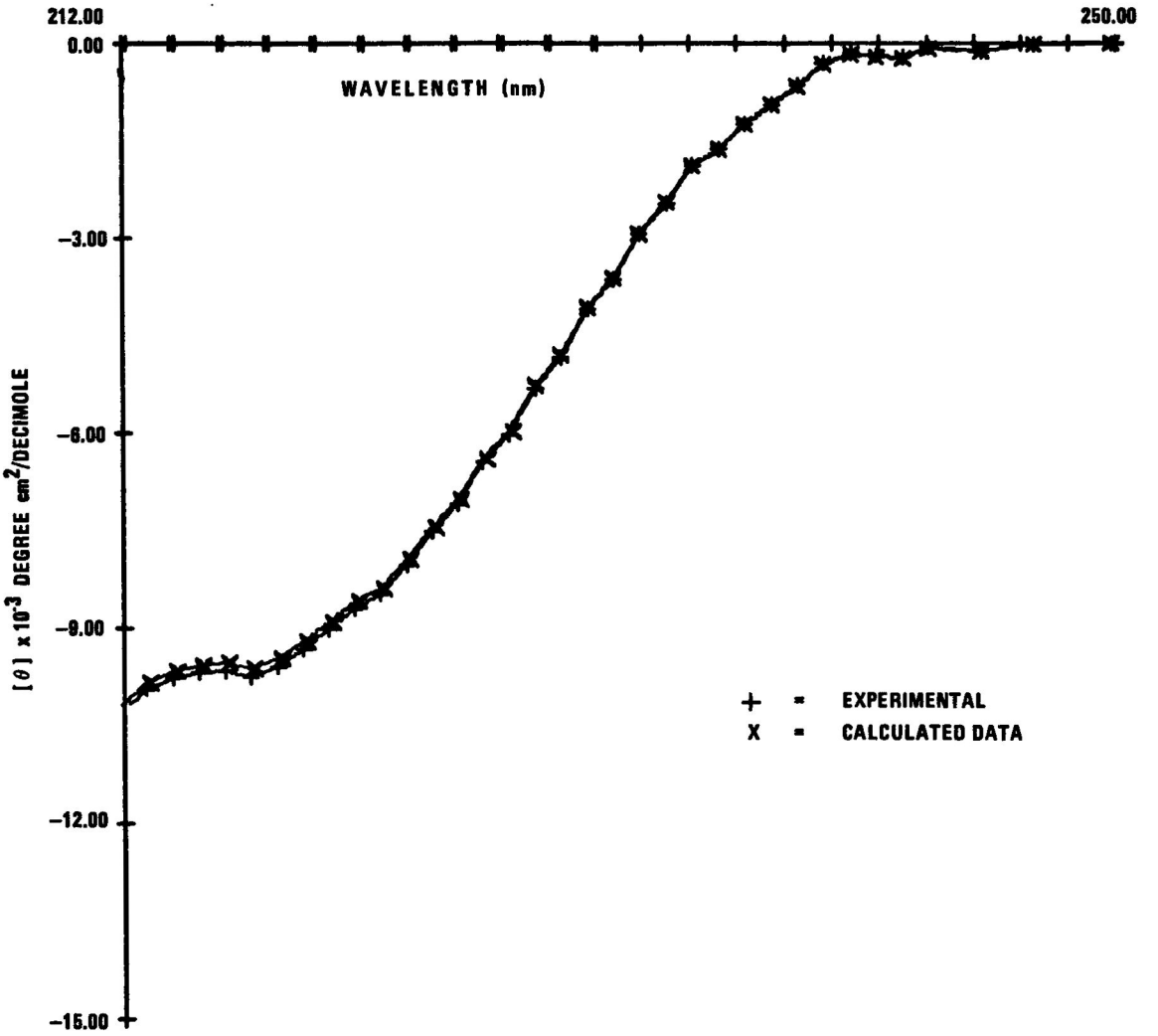


Fig. 4. Calculated Reference values versus computed % calculated curve, with values of 20% helix.

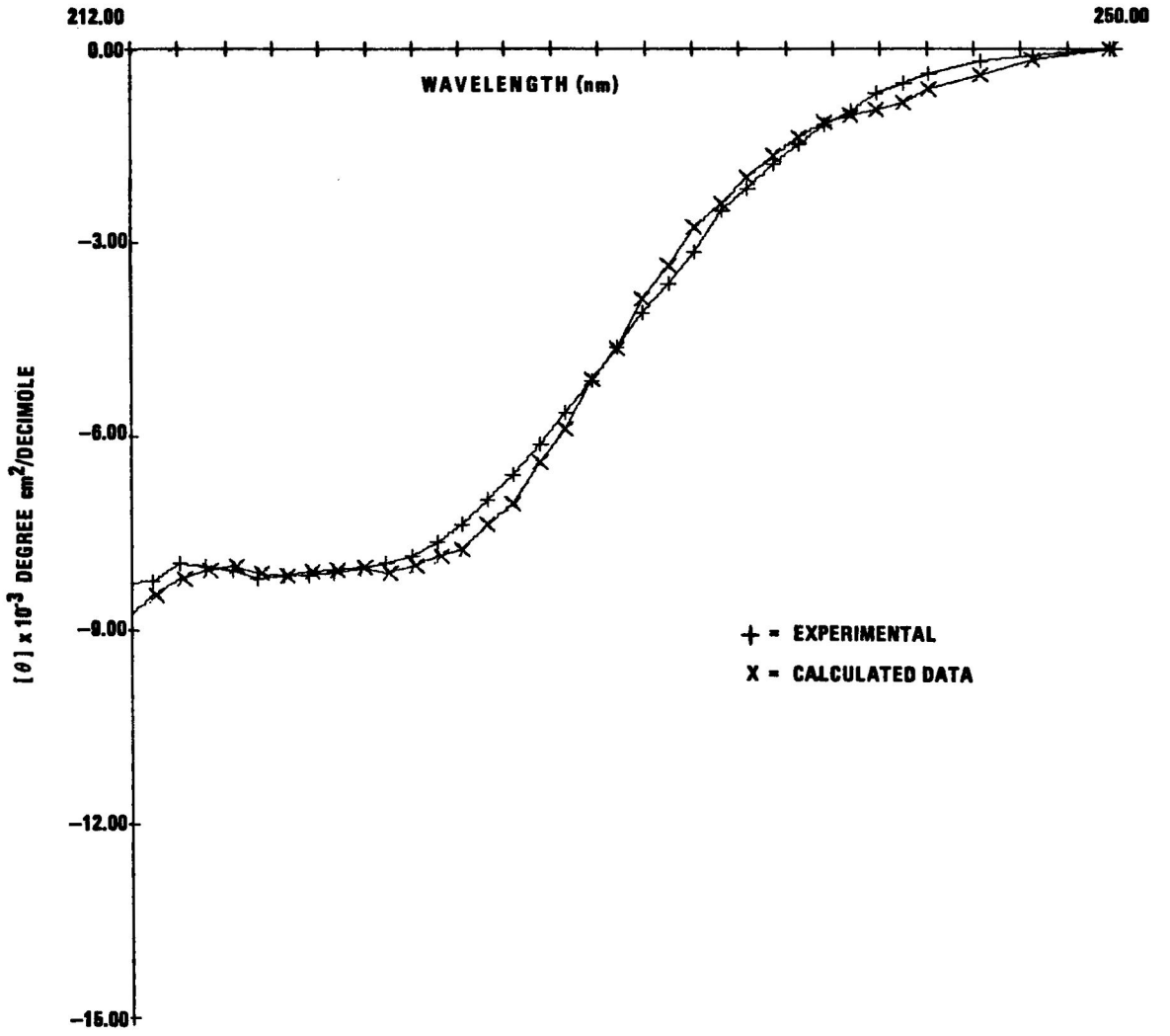


Fig. 5. Experimental spectra versus computed calculated curve pH 7.0 at 42°C.

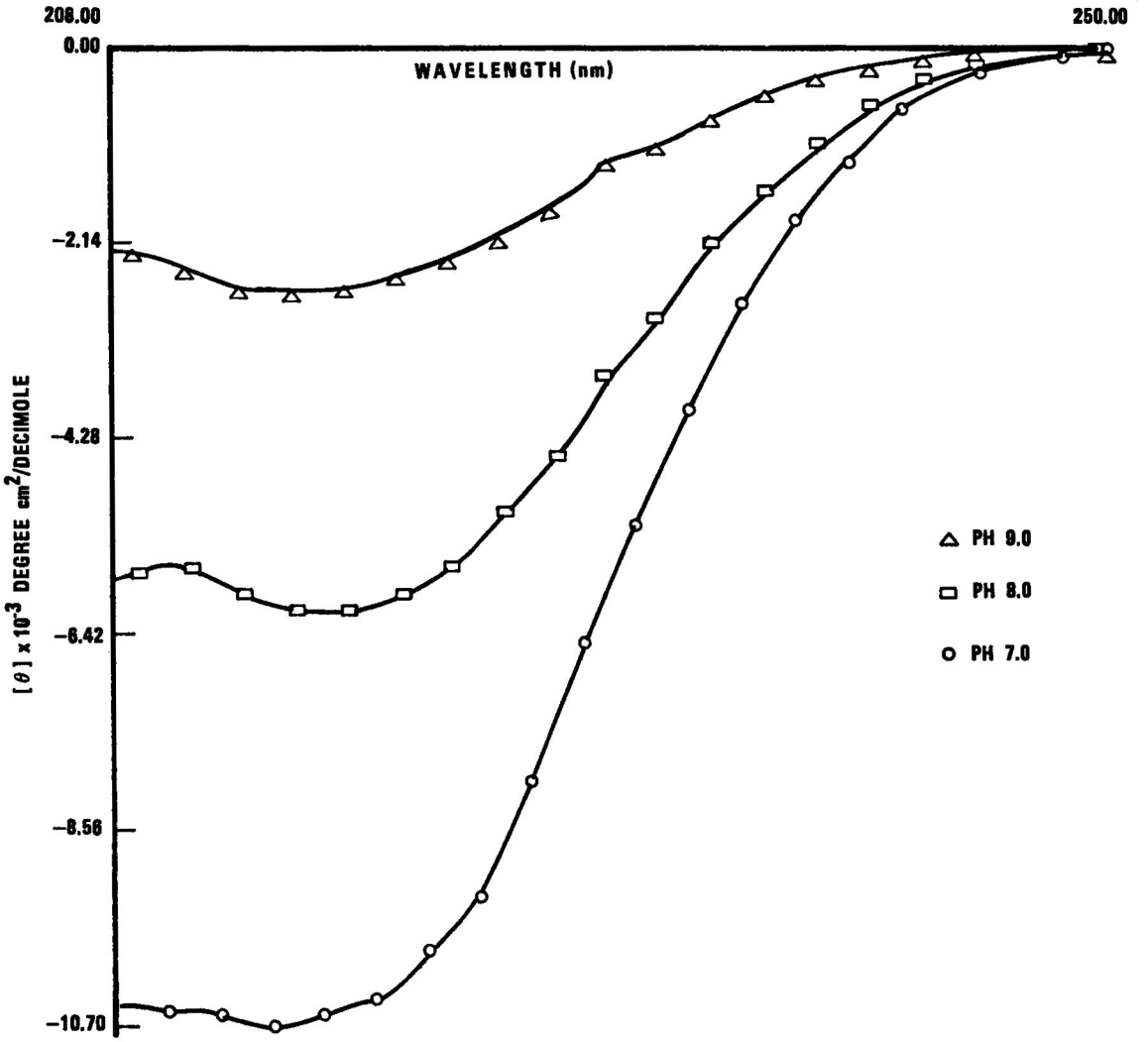


Fig. 6. FbPase experimental curves at pH's 7.0, 8.0, and 9.0 and temperature of 42°C.

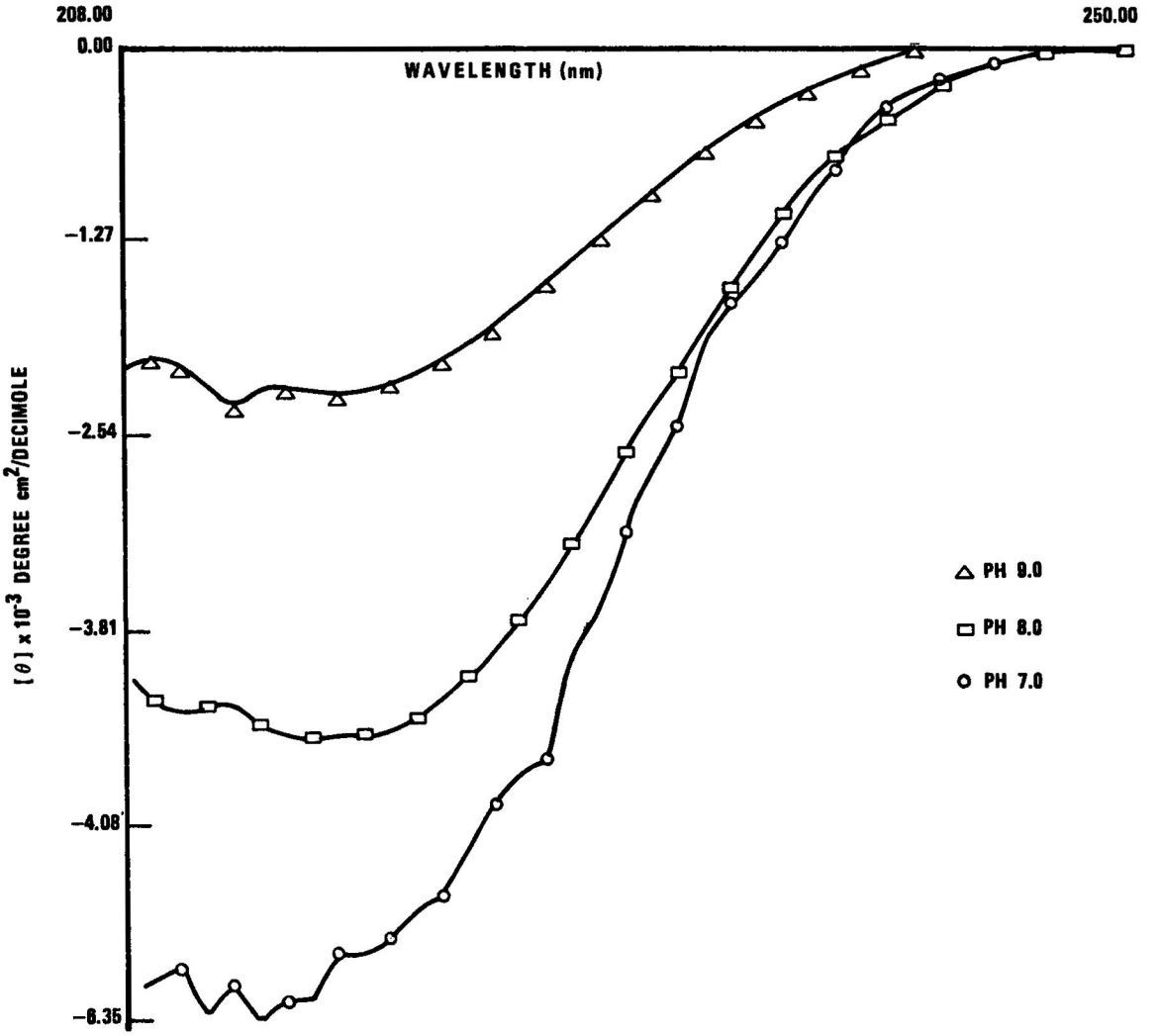


Fig. 7. FbPase bound by Amp, experimental curves at pH's 7.0, 8.0, and 9.0 temperature of 42°C.

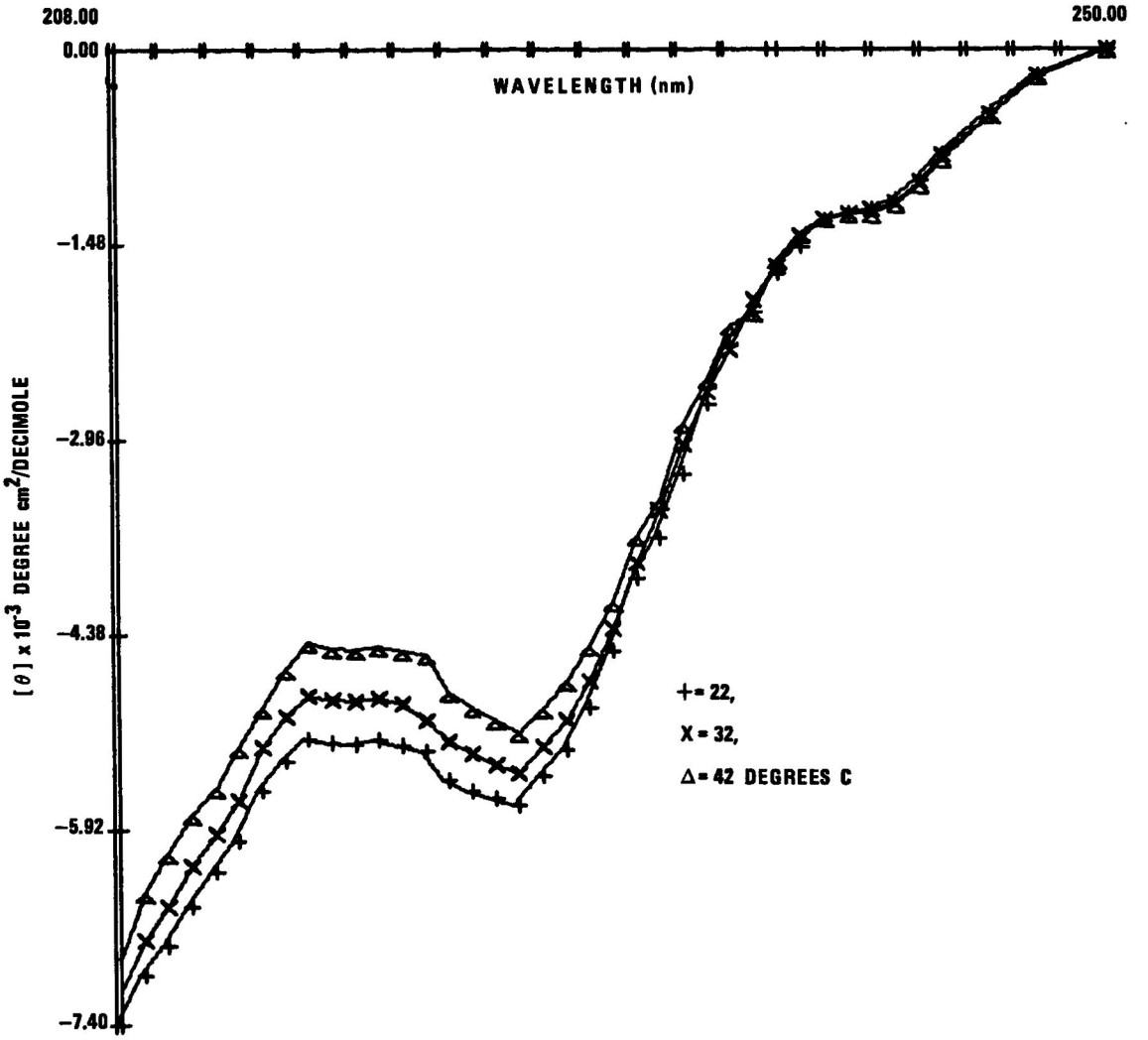


Fig. 8. FbPase at pH 8.0 and temperatures of 22, 32, and 42°C experimental curves.

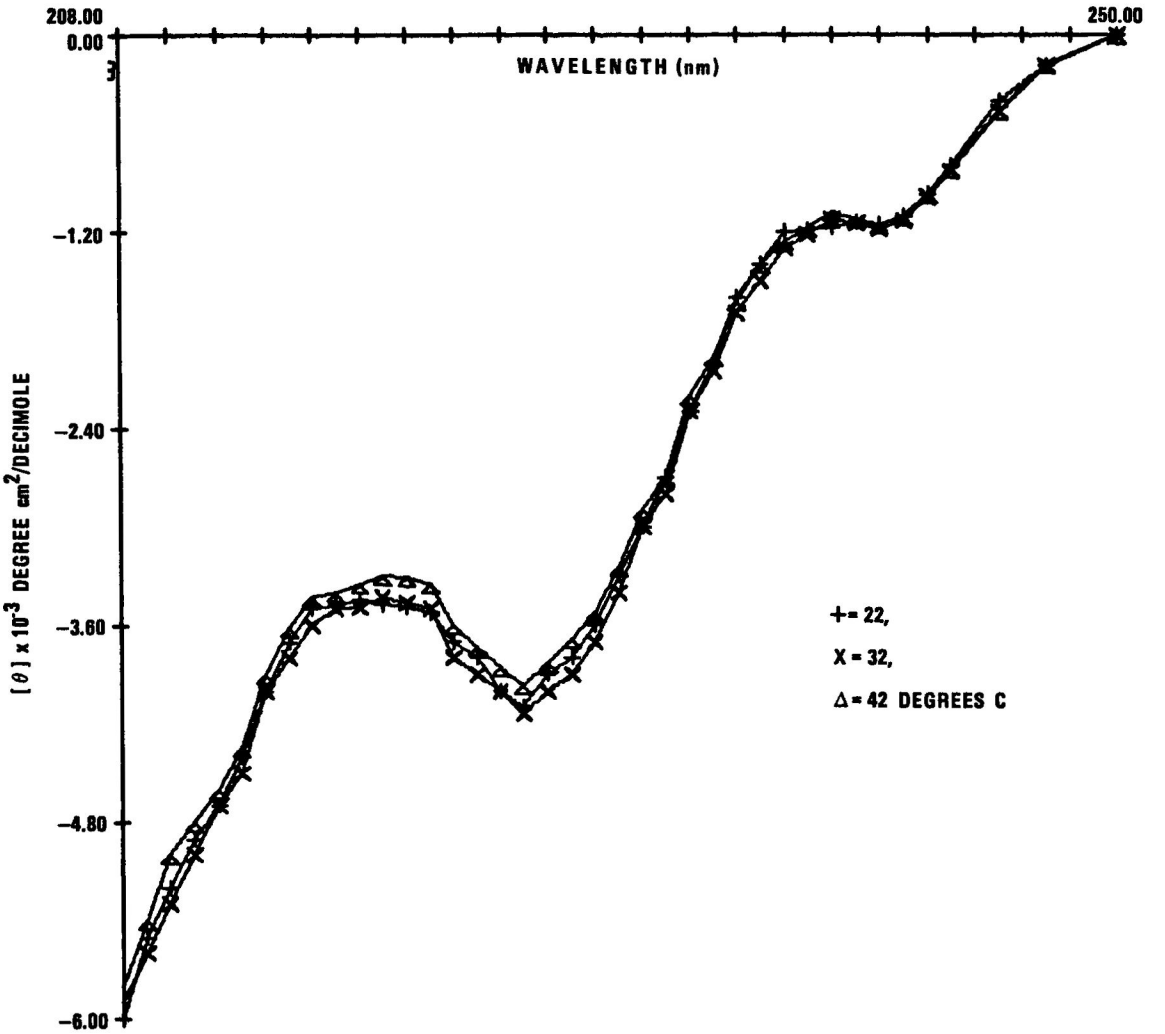


Fig. 9. FbPase bound by AMP at pH 8.0 and temperatures of 22, 32, and 42°C all are experimental curves.

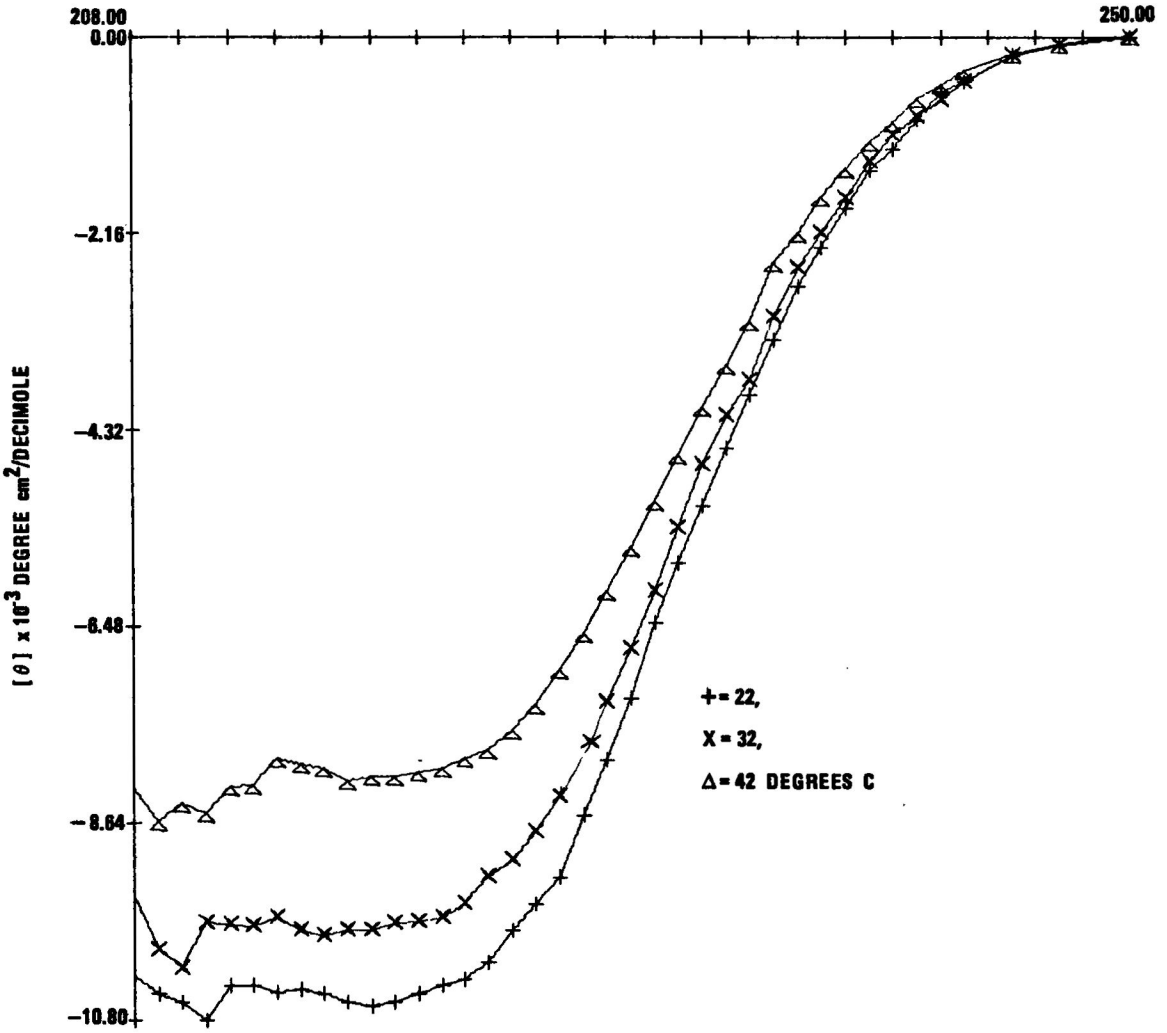


Fig. 10. FbPase pH 7.0 experimental curves at temperatures of 22, 32, and 42°C.

activity. However, the addition of AMP to FbPase bound with FbP yields further changes in the conformation (Table 5, and Fig. 9).

The conformations referred to in the Tables are approximate. The figures, however, show the actual curve obtained experimentally. In the experiments, the following assumptions were made:

(1) The wavelength region studied (212 - 250 nm) is the best region to study.

(2) Chromophores other than amides have minimal effect in this region (16).

Supportive evidence for the assumptions are: (a) the region below 200 nm is not used in the estimate because the beta form of polypeptides is greatly solvent dependent, generating peaks in this region (26), (b) the alpha helix exhibits the greatest chain length dependence in this region (27), (c) the high extinction coefficients of aromatic amino acids below 200 nm with possibility of exhibiting circular dichroism (28), and (d) the magnitude and position of random coils may vary in the region below 200 nm (29).

The proteins used in the model to generate the reference data were chosen for the following reasons:

(1) The rotational strengths of the $N \longrightarrow \pi^*$ and $\pi \longrightarrow \pi^*$ transitions which are observed are chain length dependent in the region studied.

(2) Chen determined his data from five proteins in solution, (x-ray diffraction data shows globular proteins

as having short helical, and beta sheet structures, with compact and rigid random structures) (17).

(a) This is in contrasts to synthetic polypeptides which form long chain alpha helices, and beta sheets, with extended random coil structures (synthetic polypeptides in the pure form of helices, beta sheet, and random coil, have been suggested for use as model proteins).

In statement (2) above, one must assume that the structure of a protein is essentially identical in solid state and in aqueous solution. Chen points out that wet crystals of protein containing considerable amounts of salt and solvent were used for x-ray diffraction studies. It would therefore seem reasonable to assume that the structure of the protein molecules (unlike flexible polymers which are sensitive to solvent composition and salt concentrations) are compact and rigid. Therefore, it seems improbable that the structure of protein molecules would undergo drastic changes between crystalline and solution form. This does not rule out local fluctuation of conformation at certain sites of the protein molecule. Our method of analysis, however, remains valid as long as the secondary structures of the protein molecule are not affected by transitions from crystal to solution and vice versa.

CONCLUSION

The data obtained, and all spectral observations suggest that FbPase undergoes structural changes under all the conditions studied in this report. Binding of FbPase to FbP and AMP markedly reduces the effect of structural change due to temperature and pH. Also the binding of FbP to the enzyme seems to enhance the affinity of FbPase for AMP. Observations of the circular dichroism of FbPase bound to FbP exhibits increased optical activity when AMP is added to the solution.

FbPase has been reported to have a very rigid conformation. A primary reason may be:

(a) X-ray diffraction studies indicate that random coil structures in proteins are compact and rigid.

(b) Our analysis demonstrates FbPase as not having less than 62 % random coil structure at any time.

From the data of this study it is apparent that the catalytic and regulatory functioning of FbPase, in relation to pH, temperature, or the bindings of substrate or inhibitor is accompanied by significant alterations in the enzyme's secondary structure.

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