Binding of ATP and of 1,N^6-Ethenoadenosine Triphosphate to Rabbit Muscle Phosphofructokinase†

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ABSTRACT: The binding of 1,N^6-ethenoadenosine triphosphate (ϵATP) to rabbit muscle phosphofructokinase has been studied by fluorescence and circular dichroism, and compared with that of its counterpart, ATP. Muscle phosphofructokinase binds 11.3 ± 1.2 mol of ϵATP per tetramer with an average dissociation constant of 60 μM. This is in agreement with the report of 3 ATP binding sites per phosphofructokinase protomer (Kemp & Krebs, 1967). The binding of ϵATP is relatively homogeneous in comparison with the biphasic binding of ATP. Saturating concentrations of ATP, GTP, and ADP displace about 80% of the bound ϵATP from the enzyme, whereas $FruP_2$ and AMP displace only 27%. Citrate, on the other hand, enhances the affinity of phosphofructokinase for ϵATP. The effects of the binding of ATP and ϵATP on the conformation of enzyme have also been compared. Binding of ATP results in increases in both the local rigidity and the ellipticity of the tryptophanyl side chains, whereas binding of ϵATP causes a slight decrease in the local rigidity and has virtually no effect on the ellipticity.

Phosphofructokinase (ATP:fructose-6-phosphate 1-phosphotransferase) has been purified from a number of sources. Its kinetics and molecular characteristics have been studied extensively (Bloxham & Lardy, 1973; Mansour, 1972). These studies have shown that the enzyme is activated and inhibited by its substrates, products, and several other metabolites. The binding of these effectors to phosphofructokinase isolated from rabbit skeletal muscle (Kemp & Krebs, 1967; Colombo et al., 1975; Hill & Hammes, 1975) and from sheep heart (Lorenson & Mansour, 1969; Setlow & Mansour, 1972) has also been studied recently. Of particular interest is the stoichiometry of binding for ATP, which is both substrate and inhibitor of the enzyme. Studies on rabbit muscle phosphofructokinase indicate that each enzyme protomer (90 000 daltons) binds 3 mol of ATP (Kemp & Krebs, 1967). Results from the sheep heart enzyme reveal that 3.6 mol of ATP are bound per 100 000 g of enzyme.

1,N^6-Ethenoadenosine 5′-triphosphate (ϵATP)[1] is a fluorescent analogue of ATP. Its characteristic fluorescence properties include a high quantum yield (about 50%), moderately long lifetime (20 ns), and broad emission band whose shape and position (415 nm maximum) are relatively insensitive to changes in solvent. In addition, Secrist et al. (1972) have reported that ϵATP can replace ATP in its function as both substrate and allosteric inhibitor of rabbit muscle phosphofructokinase.

We believed that a detailed study of the binding of ϵp to rabbit muscle phosphofructokinase could be of interest. This report summarizes the results of fluorescence polarization and circular dichroism studies of the system. The experiments were designed to provide information on the stoichiometry and binding equilibrium, to delineate the influence of substrates and effectors on ϵATP binding, and to probe the effects of ATP and ϵATP on the conformation of the enzyme.

Experimental Procedure

Reagents. ATP, fructose 6-phosphate, and fructose 1,6-bisphosphate were obtained from Sigma Chemical Co.; GTP, ADP, AMP, aldolase and triosephosphate isomerase were from P-L Biochemicals, Inc.; α-glycerophosphate and dithiothreitol were from Calbiochem. These materials were used without further purification.

1,N^6-Ethenoadenosine 5′-triphosphate (ϵATP) was prepared according to the method of Secrist et al. (1972). The product, which contained small amounts of contaminating ϵADP after prolonged storage, was further purified by passing through a DEAE-cellulose (DE-52) column (1.0 × 5.0 cm) which had been equilibrated with 0.09 M sodium acetate buffer (pH 4.4). The column was first washed with 0.09 M sodium acetate (pH 4.4). After the contaminating materials had been eluted, the column was then washed with 0.02 N HCl. The ϵATP fractions were pooled and neutralized immediately with 1 N NaOH. It was then lyophilized to dryness and stored at 0 °C. For experiments, it was redissolved in 25 mM glycylglycine (pH 7.0). The concentration of ϵATP was measured using the extinction coefficient of 5.6×10^3 L mol^{-1} cm^{-1} at 275 nm (Secrist et al., 1972).

All solutions were prepared using deionized glass distilled water. Other chemicals were reagent grade.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was prepared by the method of Ling et al. (1966). The final ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate (pH 8.0), containing 1 mM EDTA and 1 mM dithiothreitol, and dialyzed against the same buffer to give a stock enzyme solution of 10 to 15 mg/mL. The enzyme had a specific activity of 100 to 120 units/mg.

Before experiments the enzyme was dialyzed against the buffer consisting of 25 mM glycylglycine, 25 mM sodium glycerophosphate, 1 mM EDTA, and 5 mM 2-mercaptoethanol, pH 7.0, for 16 h. This buffer provides a degree of stability to the enzyme without interfering with the various kinetic parameters (Kemp & Krebs, 1967; Colombo et al., 1975). The dialysate was then treated with about 1 mg per mL of Norit A for 30 min to remove excess nucleotides (Cross & Fisher, 1970). The suspension was clarified by centrifuging at 12 000*g*. Phosphofructokinase concentrations were determined by using

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[1] Abbreviations used: ϵATP, 1,N^6-ethenoadenosine triphosphate; $FruP_2$, fructose 1,6-bisphosphate.

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$E_{283}^{1\%}$ = 10.9 in 0.1 M NaOH. Molar concentrations of phosphofructokinase were based on a molecular weight of 380 000 (Paetkau et al., 1968). Except when otherwise indicated, all experiments were carried out in the presence of 6 mM $MgCl_2$ dissolved in the buffer described above.

Fluorescence Anisotropy Measurements. Fluorescence anisotropy measurements were made using the Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer equipped with polarization accessory as well as using the instrument described by Isenberg and co-workers (Evett & Isenberg, 1969; Evett et al., 1970; Ayres et al., 1974). Excitation and emission wavelengths were set at 310 and 440 nm, respectively, for ϵATP fluorescence. Constant temperature was maintained by circulating water from a Forma constant temperature bath.

Emission anisotropy is defined by the equation (Jablonski, 1960)

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ is the intensity of the component vibrating in the direction of propagation of the exciting light and $I_{\perp}$, the intensity of the component vibrating normally to the plane defined by the direction of excitation observation.

The anisotropy values were used to estimate the fraction of ϵATP bound to phosphofructokinase from the relation

$$f_b = \frac{r_E - r_2}{r_1 - r_2} \quad (1)$$

where r_E is the observed anisotropy of the equilibrium mixture; r_1 and r_2, both constants, are the respective anisotropies of bound and free ϵATP. This equation is obtained directly from the principle of additivity of anisotropies (Weber, 1952).

The anisotropy of bound ligand is usually obtained by extrapolation to infinite protein concentration. Since the binding of ϵATP to phosphofructokinase is relatively weak, we adopted a second approach based on the fact that the anisotropy of ϵATP dissolved in glycerol at infinite viscosity should be the same as that of ϵATP rigidly bound to a high molecular weight protein. The anisotropy extrapolated to infinite protein concentration was 0.12 ± 0.02 while that extrapolated to infinite viscosity in glycerol was 0.128 ± 0.004. Because of its greater precision, the value of r_1 obtained from measurements on the glycerol solution was used in the calculations. The error in r_E and r_2 was estimated to be 2%. The use of eq 1 assumes that there are two fluorescent species, free and bound ϵATP, with identical extinction coefficients and quantum yields. Absorption and intensity measurements vindicated the latter two assumptions. The observed anisotropies were corrected for the small contribution of protein "blank" by use of the addition law (Weber, 1952).

Whether the anisotropy of the complex is independent of the number of ligand molecules bound is difficult to verify directly in this case. We carried out several independent binding measurements using the ultracentrifuge equipped with scanner (Schachman, 1963). General agreement between the results obtained by ultracentrifugation and fluorescence anisotropy supports the applicability of eq 1 to the binding of ϵATP to phosphofructokinase.[2]

Fluorescence Titration. The procedure for the titration was adopted from that described by Anderson & Weber (1965). A solution of phosphofructokinase (2 mL) was placed in a 1-cm cuvette. The ϵATP solution was added to the cuvette from a Hamilton microsyringe. The contents were mixed gently with a polyethylene rod and the fluorescence anisotropy of ϵATP was measured after each addition. The total dilution never exceeded 10% and was usually less than 5%.

Measurement of the Intrinsic Protein Fluorescence. In the measurement of the intrinsic fluorescence of phosphofructokinase, the excitation and emission wavelengths were set at 298 and 340 nm, respectively. Contribution of ϵATP to the fluorescence anisotropy observed in the mixture containing phosphofructokinase and ϵATP was always subtracted according to the addition law.

The apparent decrease in protein fluorescence intensity, due to the competition of ϵATP and the aromatic amino acid residues for the exciting light, was corrected using a method similar to that used in studies on heme proteins (Seery & Muller-Eberhard, 1973). The fluorescence intensity of a tryptophan solution, with optical density at 298 nm equivalent to that of the enzyme, was measured in the presence of varying concentrations of ϵATP. The small linear decrease in tryptophan fluorescence (23% "screening" was observed at 100 μM ϵATP) was used to correct corresponding points in the titration of phosphofructokinase. This method assumes that ϵATP and tryptophan do not interact directly in this concentration range.

Circular Dichroism. Circular dichroism spectra were measured on a Jasco J-41A spectropolarimeter under nitrogen flush at 22 °C. The absorbancy of all samples was kept near 1.5. Cuvettes with light paths of 1–10 mm were used.

Results

Binding of ϵATP. Titrations of rabbit muscle phosphofructokinase with ϵATP, both in the presence (6 mM) and in the absence of $MgCl_2$, are shown as plots of $1/\bar{n}$ vs. $1/[X]$ in Figure 1. The values of $\bar{n}$, the ratio of the concentration of bound ϵATP to enzyme concentration, were calculated in terms of enzyme tetramer, assuming a molecular weight of 380 000 (Paetkau et al., 1968); [X] is the free ϵATP concentration.

Our results, covering a 200-fold range of ϵATP concentration, show that saturation was never reached. Further increases in ϵATP concentration (to more than 150 μM) result in a large increase in the proportion of free ligand. Correspondingly, the error in the determination of the fraction of bound ligand becomes large as the observed anisotropy approaches that of free ϵATP.

In order to estimate stoichiometry, we prepared double-reciprocal plots of $1/\bar{n}$ vs. $1/[X]$ based on the following equation for simple, homogeneous binding (Klotz, 1946).

$$\frac{1}{\bar{n}} = \frac{1}{NK_d}\frac{1}{[X]} + \frac{1}{N}$$

In this equation K_d is the dissociation constant and N is the number of binding sites occupied by the ligand when it is present at saturating concentrations. Least-squares analysis of the nearly linear plots obtained in the presence of magnesium gives values for N and K_d of 11.30 ± 1.20 and 6.0 ± 2.2 × 10^{-5} M, respectively. Since this method of graphical treatment does not consider possible occupation of low affinity sites at much higher ligand concentrations, the stoichiometry obtained must be regarded as a minimum value. Hill plots, based on N = 12, give a Hill coefficient of 0.93 ± 0.06. The very in-

[2] The binding was also examined in the ultracentrifuge equipped with scanner (Schachman, 1963). A solution containing 240 μM ϵATP and 2.13 mg/mL of phosphofructokinase was sedimented at 36 000 rpm at 22 °C until the boundary was well separated from the meniscus. The absorbancies of the supernatant liquid and of the plateau region measured at 305 nm yielded the proportion of free and bound ϵATP. The value of $\bar{n}$ obtained under this condition was 5.3. Similar experiments carried out in the presence of 0.47 mM and 0.94 mM citrate gave $\bar{n}$ values of 5.8 and 6.2, respectively. These values were similar to an average value of 5.5 ± 0.4 calculated from the fluorescence measurement under similar conditions.

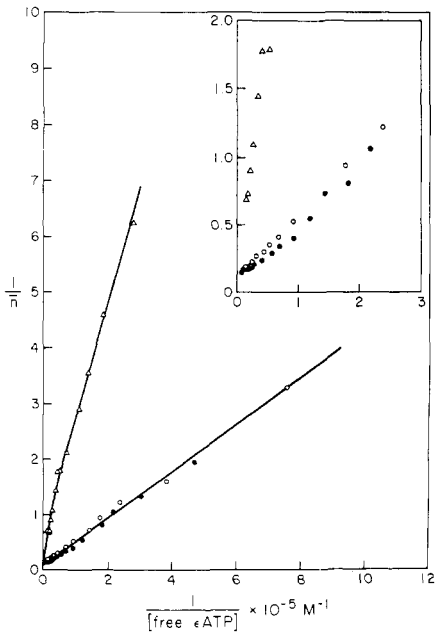

FIGURE 1: Double-reciprocal plot for binding of ϵATP by phosphofructokinase. $\bar{n}$ is the average number of mol of ϵATP bound per 380 000 g of protein. The phosphofructokinase concentrations were 1.52 mg/mL (△), no $MgCl_2$ added; 1.52 mg/mL (○) and 1.0 mg/mL (●) with 6 mM $MgCl_2$ added. The buffer contained 25 mM glycylglycine, 25 mM glycerophosphate, 1 mM EDTA, and 5 mM β-mercaptoethanol (pH 7.0). The temperature was 20 °C. The portion of the curve in the lower left-hand corner is enlarged in the insert.

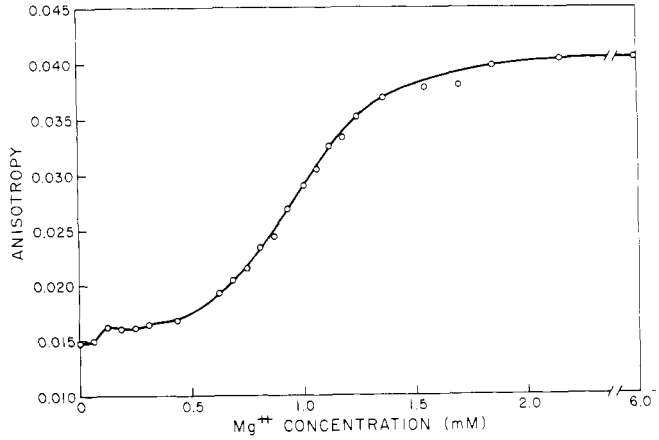

FIGURE 2: Influence of magnesium ion on the fluorescence anisotropy of a solution containing ϵATP and phosphofructokinase. A mixture of 0.75 mg/mL of rabbit muscle phosphofructokinase and 1.78×10^{-6} M ϵATP was titrated with increments of 0.15 M $MgCl_2$. Excitation was at 310 nm and emission at 440 nm. Bandwidth for both excitation and emission was 8 nm. Refer to Figure 1 for other conditions.

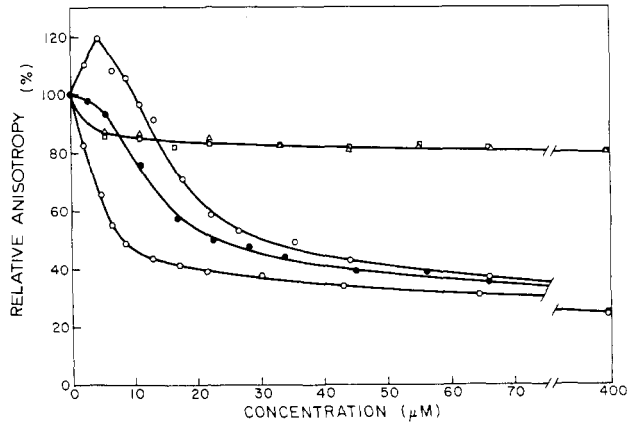

FIGURE 3: Influence of substrate, ATP (○), products, ADP (○), $FruP_2$ (△), and other effectors, GTP (●), AMP (□), on the fluorescence anisotropy of a solution containing phosphofructokinase (0.73 mg/mL) and ϵATP (1.6×10^{-6} M) at 22 °C. Excitation and emission wavelengths were 310 and 440 nm, respectively. Bandwidth for both excitation and emission was 8 nm.

complete data obtained in the absence of magnesium ion indicate that the average affinity of the enzyme for ϵATP is an order of magnitude lower.

Influence of Magnesium Ion on the Binding of ϵATP. Figure 2 shows the effect of increasing magnesium ion concentration on the fluorescence anisotropy of a mixture containing phosphofructokinase and ϵATP. The experiment was performed at a low molar ratio of ϵATP to phosphofructokinase, where a significant enhancement of anisotropy is obtained. The fractional change in anisotropy is directly related to the fractional change in the amount of ϵATP bound. Differentiation of eq 1 gives $df_b = dr_E/(r_1 - r_2)$. It follows that

$$\frac{f_b'' - f_b'}{f_b'} = \frac{r_E'' - r_E'}{r_E' - r_2} \qquad (2)$$

where the primes designate corresponding values of f_b and r_E. Since r_2 is small (0.0032), the relative change in the amount of ϵATP bound approximates the fractional change in anisotropy. The data in Figure 2 show that saturating magnesium ion concentrations produce a 3.3-fold increase in the amount of ϵATP bound. Half-maximum binding of ϵATP to the enzyme occurs at a magnesium concentration of 1 mM.

Influence of Substrate, Products, and Other Effectors on the Binding of ϵATP. Figure 3 shows the influence of substrate (ATP), products (ADP, $FruP_2$), and other effectors of the enzyme (AMP, GTP) on the fluorescence anisotropy of the ϵATP–phosphofructokinase mixtures. All of these compounds cause a decrease in the amount of ϵATP bound to phosphofructokinase. Saturating concentrations of ADP, GTP, and ATP displace about 80% of the bound analogue. The concentrations required to produce a half-maximal decrease in anisotropy are 5, 15, and 20 μM for ADP, GTP, and ATP, respectively. This concentration of ATP is in accord with the Michaelis constant (20 μM) reported for muscle phosphofructokinase (Hanson, 1970; Hanson et al., 1973). ADP, on the other hand, is more effective in displacing bound ϵATP than it is in reversing the ATP inhibition of catalysis, which often requires concentrations greater than 100 μM (Passoneau & Lardy, 1962).

The action of ATP, however, differs from that of ADP or GTP in that there is an enhancement in ϵATP binding at concentrations lower than 10 μM, with maximum enhancement occurring at 4.5 μM.[3] This observation was repeatable; the maximum enhancement ranged between 10 and 20% in several measurements. The precise reason for this enhancement is unknown. We believe that it could be the result of a conformational change in the enzyme accompanying the binding of ATP. Since the molar ratio of ϵATP to PFK is very low, about 1, most of the binding sites are vacant. The initial binding of ATP at low concentrations does not necessarily involve competition for the sites already occupied by ϵATP. A conforma-

[3] An increase in the average anisotropy of the bound ϵATP, due to increased rigidity of the binding sites, could also lead to this effect. However, the fact that most (72%) of the ϵATP is free would require the anisotropy of the complex to exceed the limiting anisotropy of ϵATP (0.156 vs. 0.128).

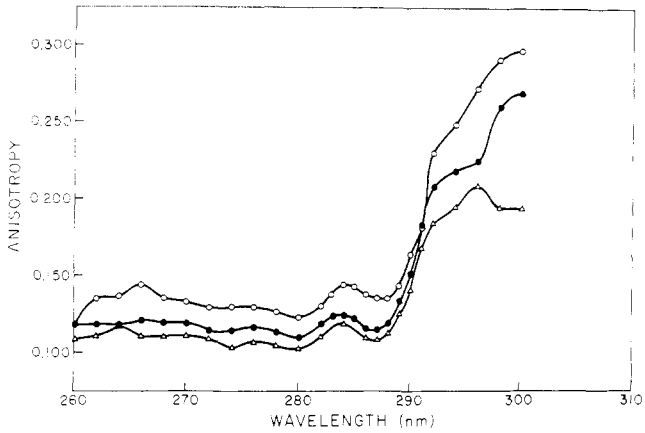

FIGURE 4: Polarization spectra of the intrinsic protein fluorescence of native phosphofructokinase (0.81 mg/mL) (●) and its complexes with ATP (1.40×10^{-4} M) (○), and ϵATP (1.04×10^{-4} M) (△) at 22 °C. Emission was at 340 nm. The direct contribution of ϵATP to the spectrum was subtracted according to Weber's addition law.

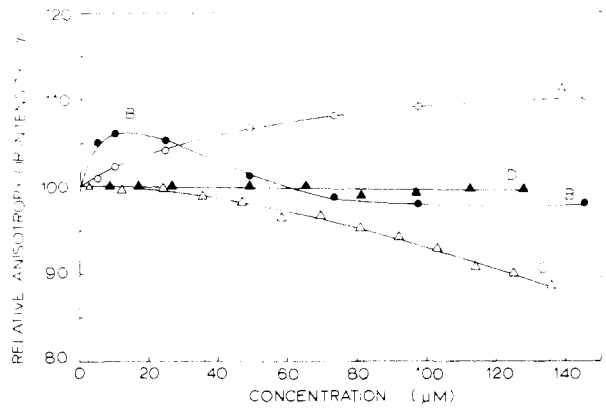

FIGURE 5: Influence of ATP and ϵATP on the intrinsic protein fluorescence of phosphofructokinase. (A) Influence of ATP on the anisotropy and (B) on the intensity. (C) Influence of ϵATP on the anisotropy and (D) on the intensity. The excitation and emission wavelengths were fixed at 298 and 340 nm, respectively. Enzyme concentration: 0.8 mg/mL, 22 °C. Other conditions are listed under Figure 1.

tional change induced by ATP binding may facilitate the binding of ϵATP to remaining unoccupied binding sites.

Saturating levels of AMP and $FruP_2$, both activators of the enzyme, displace about 27% of the bound ϵATP. The fact that these two effectors show similar concentration dependence agrees with kinetic data on the activation of ATP-inhibited phosphofructokinase (Mansour, 1972). Citrate causes a dramatic enhancement in the anisotropy, corresponding to 2.6-fold increase in the amount of ϵATP bound. Citrate has been shown to enhance the affinity of the enzyme for ATP (Kemp & Krebs, 1967).

Influence of ATP and ϵATP on the Intrinsic Protein Fluorescence of Phosphofructokinase. Polarization spectra, obtained by measuring the polarization of fluorescence produced by excitation with light of varying wavelengths, have been used to detect conformational changes affecting the local freedom of rotation of the tryptophanyl and tyrosyl side chains of proteins (Anderson & Weber, 1966). Since these aromatic amino acids have short fluorescence lifetimes, the polarization spectra of proteins as large as phosphofructokinase are not affected by the rotational diffusion of the macromolecule as a whole.

Figure 4 shows the fluorescence polarization spectra of 2.13 μM rabbit muscle phosphofructokinase and its complexes with 140 μM ATP and 104 μM ϵATP recorded at a fixed emission wavelength of 340 nm. The binding of ATP results in a uniform increase in anisotropy over the entire range of wavelengths. This effect is usually explained by increased local rigidity of the tryptophanyl side chains (Anderson & Weber, 1966). An alternate explanation, that the average anisotropy is increased by the selective enhancement of the quantum yield of a single rigidly bound residue, is less likely since the observed changes in fluorescence intensity are small and the anisotropy is initially close to the limiting value. The binding of ϵATP causes a small decrease in anisotropy. Since ϵATP itself emits at 340 nm, the spectrum was corrected for a small contribution (7–12%) from ϵATP by using the addition law (Weber, 1952).

Titrations of the enzyme with either ATP or ϵATP were carried out measuring both the anisotropy and the total emission intensity obtained at fixed excitation and emission wavelengths of 298 and 340 nm, respectively. The results (Figure 5) show that the anisotropy increases monotonically with increasing concentrations of ATP. The intensity, on the other hand, undergoes a biphasic change with a maximum effect occurring in the same ATP concentration range producing enhancement of ϵATP binding (Figure 3).

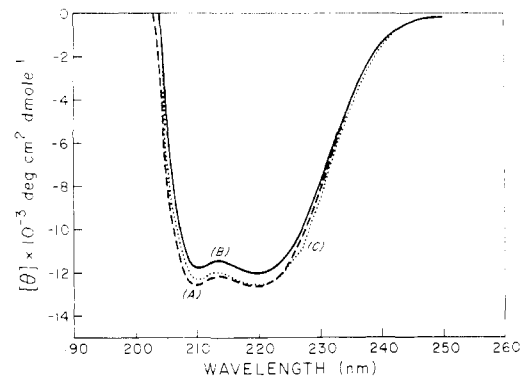

FIGURE 6: The circular dichroism spectra of solutions containing 0.21 mg/mL native phosphofructokinase and (A) no additions; (B) 1.1×10^{-4} M ATP; (C) 1.04×10^{-4} M ϵATP. The spectra were expressed in terms of mean residue ellipticity. Path length: 1 mm. Refer to Figure 1 for conditions.

There is little or no energy transfer from the tryptophanyl side chains to the bound ϵATP, evidenced by the fact that the protein fluorescence intensity is virtually unaffected by the binding. The spectral overlap integral (Forster, 1947) indicates that the characteristic energy transfer distance, at which the probability of transfer between optimally oriented molecules is 50%, is ca. 19 Å for tryptophan and ϵATP. The absence of transfer in phosphofructokinase suggests that the distances and/or orientations between donor and acceptor are unfavorable.

Circular Dichroism Studies. Figures 6 and 7 show the circular dichroism spectra of rabbit muscle phosphofructokinase and its complexes with ATP and ϵATP. Both nucleotides cause barely detectable changes in ellipticity in the wavelength range 205–240 nm, suggesting that the binding of either causes little change in the secondary structure of the protein.

ATP and ϵATP are remarkably dissimilar in their effects on the ellipticities recorded between 250 and 310 nm. The dramatic effect observed on the binding of ATP to the enzyme is emphasized by the circular dichroism difference spectrum (C) shown in Figure 8. This difference spectrum comprises a strong positive band corresponding to the 260-nm absorption maximum of ATP and a negative band in the wavelength region corresponding almost exclusively to absorption by tryptophan. The strong positive CD band centered at 260 nm is due

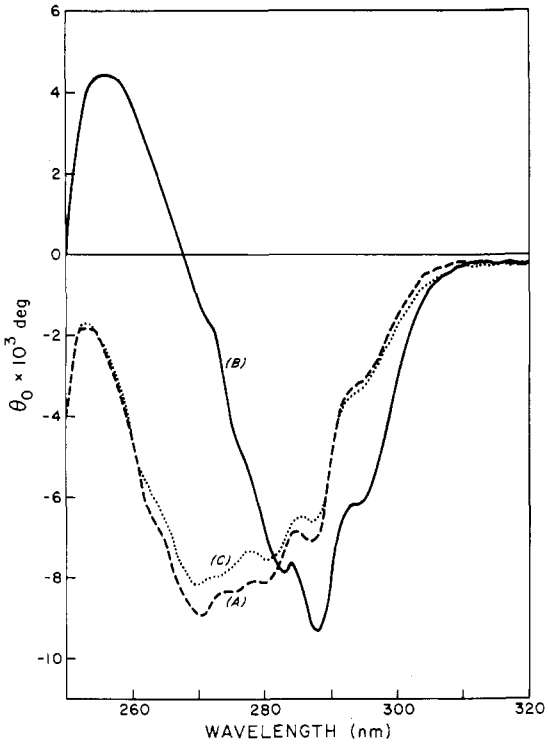

FIGURE 7: The circular dichroism spectra of solutions containing 1.4 mg/mL native phosphofructokinase and (A) no additions, (B) 8.2×10^{-5} M ATP, (C) 9.6×10^{-5} M ϵATP. The spectra were expressed in terms of observed ellipticity. Refer to Figure 1 for other conditions.

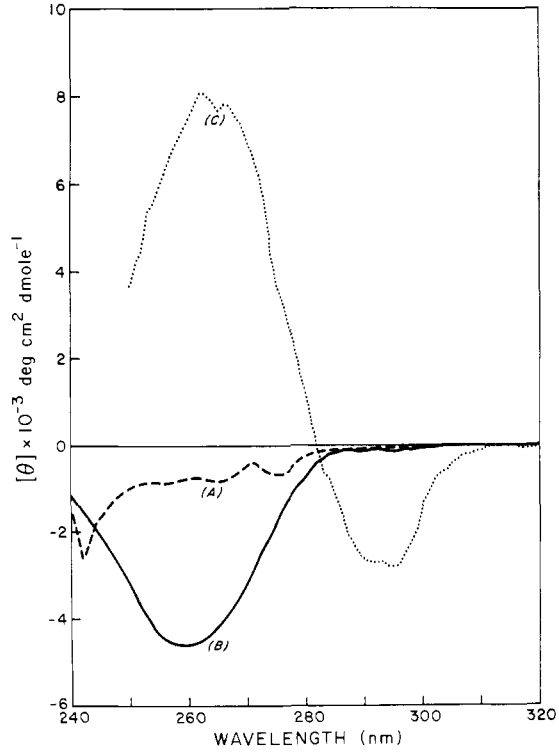

FIGURE 8: The circular dichroism difference spectrum of solutions containing native phosphofructokinase and its complex with ATP (curve C). This difference spectrum was obtained by subtracting the CD spectrum of native phosphofructokinase (curve A, Figure 7) from the CD spectrum of the ATP-phosphofructokinase complex (curve B, Figure 7), and is expressed in terms of ATP molecular ellipticity. Also shown are the circular dichroism spectra of free ϵATP (curve A) and free ATP (curve B). Conditions: 22 °C, path length 10 mm.

to the induced asymmetry of the adenine ring, reflecting the asymmetric environment of the binding site. The circular dichroism spectra of both free ATP (B) and ϵATP (A) are included here for comparative purposes. Titration of the enzyme with these two nucleotides shows that the ellipticity of the protein at 288 nm undergoes a maximum change of 50% upon the addition of ATP and virtually no change when ϵATP is added. This striking observation is not simply due to a difference in the amount of the coenzyme bound. Calculations show that at a ligand concentration of 100 μM, more than 6.6 mol of ATP (Kemp & Krebs, 1967) or 5.7 mol of ϵATP will bind per mol of enzyme.

Discussion

The results reported above on the stoichiometry of ϵATP binding to rabbit muscle phosphofructokinase show that the enzyme binds 11.3 ± 1.2 mol of ϵATP per mol of active enzyme. This value corresponds to approximately 3 mol of ϵATP bound per enzyme protomer and is in accord with the results of ATP binding by the same enzyme (Kemp & Krebs, 1967). However, the binding of ϵATP differs from that of ATP in several ways. The binding of ϵATP to rabbit muscle phosphofructokinase appears to be more homogeneous; the double-reciprocal plots of $1/\bar{n}$ vs. $1/[X]$ are nearly linear and the Hill coefficient of binding is close to one. This is in contrast to the biphasic binding of ATP. In addition, the affinity of the enzyme for ϵATP is 4- to 12-fold smaller than that for ATP, as is revealed by the dissociation constants of 60 μM and 5 to 15 μM for ϵATP and ATP, respectively.

The most remarkable difference between the two nucleotides lies in subtle effects on the conformation of the enzyme. The binding of ATP to phosphofructokinase has been shown to cause a change in the conformation of the enzyme leading to a decrease in the local mobility (Jones et al., 1972, 1973) as well as in the reactivity (Kemp, 1969; Mathias & Kemp, 1972) of the "class I" thiol group.[4] Our results on the fluorescence polarization and circular dichroism spectra of the ATP-phosphofructokinase complex add to the complexity of the nature of ATP binding by showing that the binding of ATP also leads to increases in both the local rigidity and the ellipticity of the tryptophanyl side chains of the enzyme. The binding of ϵATP, on the other hand, causes a slight increase in the local freedom of rotation of the tryptophanyl side chains and has virtually no effect on the ellipticity. One may ask whether these pronounced local effects are linked to extensive alterations in the overall three-dimensional folding of phosphofructokinase. Circular dichroism measurements show that there is little effect on the long-range secondary structure. Sedimentation velocity studies carried out by us show that the tetramer predominates under the conditions of our experiments. Thus changes in aggregation, such as the dissociation obtained at very high (10 mM) ATP concentrations (Uyeda, 1970), are not involved.

Nonetheless, the two nucleotides exhibit many similarities in their binding to phosphofructokinase. The binding of ϵATP is highly dependent on the magnesium ion concentration. The depletion of magnesium ion results in a rather incomplete binding, and the affinity of the enzyme for ϵATP diminishes about eightfold. A similar observation has also been noted on the binding of ATP to spin-labeled muscle phosphofructokinase (Jones et al., 1973). In that instance, MgATP binds sigmoidally to the enzyme labeled with 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinyloxy at the class I thiol group,

[4] Designation of the "class I" thiol group of phosphofructokinase is taken from the classification of Kemp & Forest (1968).

with a half-saturation point of 400 μM at pH 7.5. The binding of free ATP is hyperbolic and considerably weaker (K_s = 1.3 mM). Moreover, saturating concentrations of ATP displace most of the bound ϵATP from the enzyme, with the half-saturation point comparable to the Michaelis constant. This suggests that ϵATP is bound to phosphofructokinase at sites very similar, if not identical, to those for ATP. The observation that activators such as ADP, AMP, and fructose 1,6-bisphosphate are also able to displace the bound ϵATP, with ADP being most effective, is of interest. MgATP has been shown to bind to phosphofructokinase at specific inhibitory sites (Kemp, 1969; Mathias & Kemp, 1972). Because of the similarities between the binding of ϵATP and ATP to muscle phosphofructokinase, both in stoichiometry and sites, we believe that ϵATP binds to the inhibitory sites under the conditions of our experiments. The demonstration that ADP, AMP, and $FruP_2$ partially displace the bound ϵATP from the enzyme is consistent with this interpretation.

It is also interesting to note the effect of citrate on the binding of ϵATP. Kemp and his co-workers have suggested that citrate inhibits phosphofructokinase by decreasing the affinity of the enzyme for fructose 6-phosphate and increasing the affinity for ATP (Kemp & Krebs, 1967; Kemp, 1969; Mathias & Kemp, 1972). This enhancement in ATP affinity is achieved by the binding of citrate to the enzyme at sites distinct from the ATP binding sites (Colombo et al., 1975). In a study of the binding of ATP to sheep heart phosphofructokinase, Lorenson & Mansour (1969) observed that citrate did not enhance the binding of ATP; instead they found that high concentrations (1 mM) of citrate inhibit ATP binding. Our observations on the effect of citrate on ϵATP binding indicate that citrate enhances the affinity of the enzyme for ϵATP. This suggests that citrate may as well enhance the affinity of phosphofructokinase for ATP and thus lends further support to the observation of Kemp and his co-workers.

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