Influence of Substrates and Effectors on the Fluorescent Complex between Phosphofructokinase and 8-Anilino-1-naphthalenesulfonate†

David P. Bloxham

ABSTRACT: Phosphofructokinase enhances the fluorescence of 8-anilino-1-naphthalenesulfonate in aqueous solution. The emission maximum of the phosphofructokinase–sulfonate complex is 480 nm compared to 530 nm for the free dye. There are 40 binding sites for the sulfonate per 380,000 daltons of phosphofructokinase with a statistical binding constant of 10 μM for the sulfonate. AMP, ADP, ATP, and fructose 6-phosphate decrease the fluorescence of the phosphofructokinase–sulfonate complex. The effects of ATP and fructose 6-phosphate are additive and equal to the maximal effect of AMP alone. If the enzyme is denatured in 6 M urea or inactivated by succinylation, the various effectors do not decrease the fluorescence of the phosphofructokinase–sulfonate complex. The binding sites for nucleotides were distinguished on the basis of their ability to decrease fluorescence. One site, which may be the catalytic site, has a wide specificity for nucleoside triphosphates; the other site is highly specific for AMP. Carboxymethylation of phosphofructokinase modifies the ability of ATP to decrease fluorescence whereas the action of AMP is unaffected.

The fluorescent dye 8-anilino-1-naphthalenesulfonate has been used successfully as a probe in the investigation of hydrophobic regions in proteins, particularly in the case of bovine serum albumin (Weber and Young, 1964; Daniel and Weber, 1966; Weber and Daniel, 1966). In some instances the binding of the sulfonate occurs at specific sites on the protein. Thus, for bovine serum albumin, the sulfonate may bind at the fatty acid binding site (Santos and Spector, 1972), for apohemoglobin and apomyoglobin it may bind at the hemin site (Stryer, 1965), and for transaldolase it may bind at the fructose 6-phosphate site (Brand, 1970). An alternative use of the dye is to investigate interactions between effectors and proteins which may be associated with changes in the fluorescence of the protein–sulfonate complex (Thompson and Yielding, 1968; Harris, 1971; Seery and Anderson, 1972; Barrett-Bee and Radda, 1972).

In the present work, the fluorescence of the phosphofructokinase–sulfonate complex was studied. Phosphofructokinase contains a large number of binding sites for the sulfonate and it seems unlikely that it is bound to specific regions on the protein. However, the fluorescence of the complex appears to be a useful indicator of conformational changes that may be induced in the protein by effectors of the enzyme.

Experimental Procedures

Rabbit muscle phosphofructokinase was prepared by the method of Ling *et al.* (1965) and crystallized by the procedure of Parmeggiani *et al.* (1966). The enzyme had a specific activity of 100–120 U/mg. Before use, it was dialyzed against 0.1 M potassium phosphate, pH 8, containing 1 mM EDTA. On standing over a period of several days a slight precipitate may form and this should be removed by centrifugation. Phosphofructokinase concentrations were determined by using $E_{283}^{1\%} = 10.9$ in 0.1 M NaOH. Molar concentrations of phosphofructokinase are based on a molecular weight of 380,000 (Paetkau *et al.*, 1968).

sRMP[1] was obtained from P-L Biochemicals, Milwaukee, Wis. This was converted to the triphosphate (sRTP) by the method of Murphy *et al.* (1970). nRMP and nRTP were prepared by the method of Faerber and Scheit (1971) and were a gift from Dr. K. H. Scheit, Gottingen, Germany. Magnesium 8-anilino-1-naphthalenesulfonate was obtained from Eastman Kodak. Magnesium was removed from solutions of the sulfonate by passage over a column of Dowex 50 (H^+) and the effluent was neutralized with NH_4OH.

Fluorescence Measurements. An Aminco-Bowman spectrophotofluorimeter was used with an exciting wavelength of 406 nm and an emission wavelength of 480 nm. The correction for protein absorption or fluorescence under these conditions was minimal; however, a small correction for the fluorescence of free dye was required. Titrations with the sulfonate were performed by the manual additions of small volumes of a concentrated sulfonate solution to a standard solution of phosphofructokinase in 0.1 M potassium phosphate, pH 7, in a 1-cm quartz cuvet. The dilution never exceeded 5% of the starting volume. The binding constants for phosphofructokinase with the sulfonate were determined from the equation developed by Klotz (1947)

$$\frac{[\text{Phosphofructokinase}]}{xD} = \frac{1}{n} + \frac{K_D}{n} \frac{1}{(1 - x)D}$$

where x is the fraction of dye bound, n is the number of binding sites, K_D is the statistical binding constant, and D is the sulfonate concentration. All data were fitted to straight

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[1] Abbreviations used are: nRMP, 2-amino-9-β-D-ribofuranosylpurine 5′-monophosphate; nRTP, 2-amino-9-β-D-ribofuranosylpurine 5′-triphosphate; sRMP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-monophosphate; sRTP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate; DTNB, 5′,5′-dithiobis(2-nitrobenzoic acid).

lines by the least-squares fit method. The dye concentration was calculated from the molar absorption coefficient of 4.95×10^8 (Weber and Young, 1964). For each concentration of the sulfonate, x was determined from the relation: $x = (F_{obsd} - F_{dye})/(F_{max} - F_{dye})$ (Laurence, 1952; Thompson and Yielding, 1968) where F_{obsd} is the observed fluorescence, F_{max} is the fluorescence at infinite phosphofructokinase concentration, and F_{dye} is the fluorescence of the dye in free solution. F_{max} was obtained for a given sulfonate concentration by measuring the fluorescence at numerous concentrations of phosphofructokinase. Reciprocal plots of F_{obsd} *vs.* phosphofructokinase concentration gave F_{max} at infinite enzyme concentration.

Succinylation of Phosphofructokinase. Freshly dialyzed phosphofructokinase (15 mg/ml) in 0.1 M potassium phosphate, pH 8, containing 1 mM EDTA was diluted with 3.5 vol of 50 mM $NaHCO_3$ containing 0.2 mM EDTA. Succinic anhydride in acetone was added to give a final concentration of 2 mM (4% acetone final). After 45 min at 4° the reaction was terminated with excess cysteine hydrochloride and the enzyme was dialyzed against 0.1 M potassium phosphate, pH 8, containing 1 mM EDTA. This treatment inactivated the enzyme 90%.

Carboxymethylation of Phosphofructokinase. The reaction procedure was similar to that for succinylation except that 2 mM iodoacetic acid in water was added and the reaction was performed at room temperature. The reaction was terminated with excess dithiothreitol. This procedure inactivates the enzyme 50–60%. When assayed at pH 7, the residual activity was still inhibited by increasing the concentration of ATP above 0.5 mM in the presence of 50 μM fructose 6-phosphate.

Results

Characterization of Fluorescence of the Phosphofructokinase–Sulfonate Complex. In 0.1 M potassium phosphate, pH 7, excitation of the sulfonate (100 μM) at 406 nm results in a low fluorescent emission with a maximum at 530 nm. Addition of phosphofructokinase (0.5 μM) caused a marked increase in fluorescence which was accompanied by a blue shift in the emission spectrum to a maximum of 480 nm. Under similar conditions, the bovine serum albumin–sulfonate complex had an emission maximum of 477 nm. The sulfonate in ethanol gave an emission maximum of 475 nm.

The absolute quantum yield of the fluorescence of the sulfonate bound to phosphofructokinase has not been determined directly. However, it was shown that at pH 7 phosphofructokinase caused a 30-fold increase in the relative quantum yield compared to the sulfonate in free solution. At the same molar protein concentration, sulfonate concentration, and at pH 7, the bovine serum albumin–sulfonate complex had a relative quantum yield which was 5.3-fold greater than that of the phosphofructokinase–sulfonate complex.

Figure 1 shows the influence of pH on the fluorescence of the sulfonate. Over the pH range studied, the fluorescence of the sulfonate in aqueous solution is unaffected; however, the fluorescence of the phosphofructokinase–sulfonate complex increases markedly at pH values below 6. In the pH range 6–10, where the enzyme is active, the fluorescence varies only slightly with a minimum at pH 7.5, which is close to the pH for maximum enzyme activity. The increase in fluorescence at acid pH could be related to changes in phosphofructokinase structure since the enzyme is known to dissociate at acid pH (Paetkau and Lardy, 1967). Examination of the binding (Klotz, 1947) of the sulfonate to phosphofructokinase showed

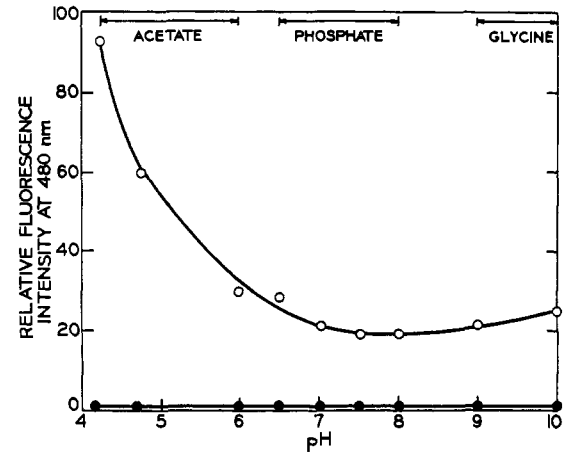

FIGURE 1: The effect of pH on the fluorescence of the phosphofructokinase–sulfonate complex. Fluorescence was recorded in 0.1 M buffer at the appropriate pH: (●) free dye (100 μM); (○) sulfonate (100 μM) complexed with phosphofructokinase (0.5 μM).

that decreasing the pH did not increase the number of binding sites for the sulfonate. Possibly the enhancement of fluorescence is related to changes in the ionization of groups at the sulfonate binding sites which results in an increased quantum yield.

Using the formula developed by Klotz (1947), which assumes that all the binding sites are equivalent and noninteracting, phosphofructokinase was shown to have 40 binding sites per 380,000 daltons with a statistical binding constant, K_D, of 10 μM (an example is shown in the control for Figure 3). The number of binding sites for the sulfonate is well in excess of the numbers of binding sites calculated for the known effectors of phosphofructokinase (Kemp and Krebs, 1967), so it seems unlikely that the sulfonate is binding specifically to either the catalytic or regulatory sites on the enzyme. In agreement with this suggestion it was shown that when phosphofructokinase (2.8 nM) was assayed at pH 7 in the presence of 100 μM fructose 6-phosphate and variable ATP (4–100 μM), then the sulfonate (100 μM; sufficient to saturate 90% of available binding sites) had no influence on enzyme activity. This is important to later arguments since it suggests that the sulfonate does not interfere with conformational changes in the enzyme associated with catalytic activity.

Influence of Substrates and Effectors of Phosphofructokinase on the Enhancement of Fluorescence. Figure 2 shows the effect of substrates (ATP and fructose 6-phosphate), products (ADP and fructose 1,6-diphosphate), and effectors (AMP and citrate) of the enzyme reaction on the fluorescence of the phosphofructokinase–sulfonate complex. Several of these compounds decreased fluorescence. The most potent in this action were AMP and ADP followed by ATP. The concentrations required to produce a half-maximal decrease in fluorescence were 5, 5, and 15 μM, respectively.

Fructose 6-phosphate and fructose 1,6-diphosphate caused a slight decrease in fluorescence whereas citrate was virtually without effect. The concentration of ATP to produce a half-maximal decrease in fluorescence is of the same order as the K_M for ATP (20 μM) reported for muscle phosphofructokinase (Hanson, 1970; Hanson *et al.*, 1973). AMP is much more potent in modifying phosphofructokinase–sulfonate fluorescence than it is in reversing ATP inhibition which often re-

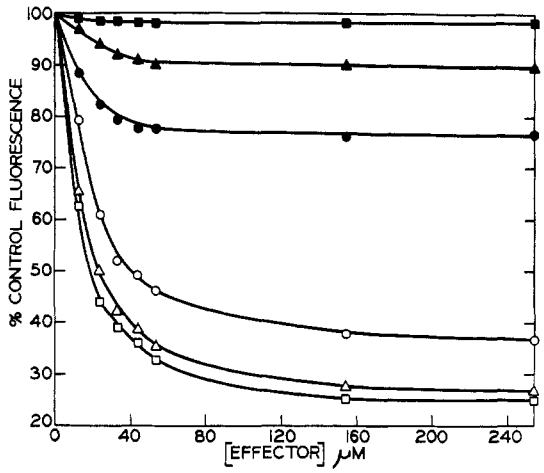

FIGURE 2: Influence of substrates and effectors on the fluorescence of the phosphofructokinase–sulfonate complex. Fluorescence of the sulfonate (100 μM) was recorded in 0.1 M potassium phosphate buffer, pH 7, in the presence of phosphofructokinase (0.5 μM), $MgSO_4$ (100 μM), and appropriate concentrations of effector: (□) AMP; (△) ADP; (○) ATP; (●) fructose 6-phosphate; (▲) fructose 1,6-diphosphate; (■) citrate.

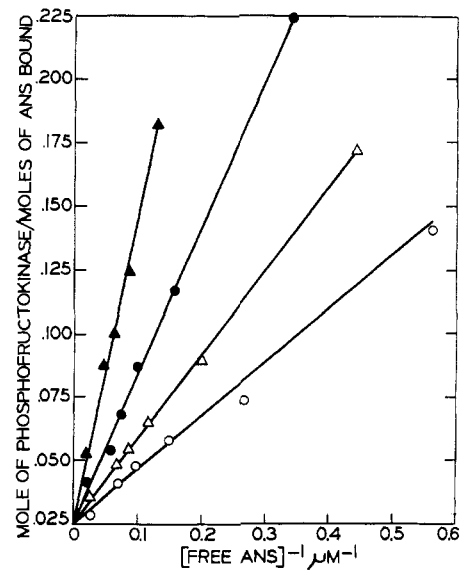

FIGURE 3: Influence of ATP on the binding constants of the sulfonate to phosphofructokinase. Phosphofructokinase (0.5 μM) in 0.1 M potassium phosphate buffer, pH 7, containing $MgSO_4$ (100 μM) was titrated with 5–50 μM sulfonate in the presence of the following concentrations of ATP: (○) none; (△) 10 μM; (●) 30 μM; (▲) 50 μM.

quires a concentration greater than 50 μM (Passoneau and Lowry, 1962). Kemp and Krebs (1967) have shown that the binding of AMP to phosphofructokinase has a dissociation constant of 1.8 μM which is of the same order as the concentration of AMP effective in decreasing fluorescence.

The effect of ATP, ADP, and AMP on the binding characteristics of the phosphofructokinase–sulfonate complex was determined by titrating the fluorescence of the sulfonate with phosphofructokinase at several fixed nucleotide concentrations. Figure 3 shows an example of the results which were obtained with ATP. In each case, the nucleotide had no significant effect on the number of binding sites, but rather the nucleotides decreased the affinity of the dye for the enzyme.

The nucleotides decreased the relative quantum yield of the phosphofructokinase–sulfonate complex on the basis of the total concentration of the sulfonate in solution. However, this decrease in relative quantum yield was matched by the decrease in the fraction of dye bound to the enzyme, so presumably the quantum yield of the phosphofructokinase–sulfonate complex was unaffected. Since the major change associated with quenching of fluorescence is to decrease the affinity of the sulfonate for phosphofructokinase, it would be expected that as the dye concentration is increased the quenching properties of the nucleotides should decrease. In agreement with this proposal, reciprocal plots of F_{obsd} *vs.* sulfonate concentration showed that the nucleotides did not decrease the maximum fluorescent intensity at infinite concentrations of the sulfonate.

To assess the significance of the decrease of fluorescence in the presence of adenine nucleotides, the fluorescence of the phosphofructokinase–sulfonate complex was investigated under conditions when the enzyme was denatured. In 6 M urea, dissolved in 50 mM potassium phosphate, pH 7, the fluorescence of the sulfonate (100 μM) mixed with phosphofructokinase (0.5 μM) developed slowly reaching a maximum after 20 min. In the absence of urea, maximal fluorescence developed within the time required for mixing. The relative fluorescence intensity was the same in either the presence or absence of urea; however, in the presence of urea the addition of ATP (500 μM), ADP (500 μM), or AMP (500 μM) had no effect on fluorescence showing that the decrease of fluorescence caused by nucleotides is dependent on the structural integrity of phosphofructokinase.

The decrease of fluorescence caused by the various effectors of phosphofructokinase could represent different structural transformations of the enzyme. This was evaluated by studying the ability of these agents to produce additive effects. In the presence of AMP (200 μM), the addition of a saturating concentration of ADP, ATP, or fructose 6-phosphate produced no further decrease of phosphofructokinase–sulfonate fluorescence. In fact, ATP (200 μM) in the presence of saturating AMP produced a small but reproducible increase in the fluorescence of the phosphofructokinase–sulfonate complex. In contrast to this result, the decrease of fluorescence caused by ATP and fructose 6-phosphate was additive and their added effect was similar to that of AMP alone.

If the ability of ATP to decrease the fluorescence of the phosphofructokinase–sulfonate complex is related to the binding of the nucleotide to the active site, then it would be expected that this effect should be related to the Mg^{2+} concentration since MgATP is the effective substrate. Figure 4 shows that in the absence of Mg^{2+}, ATP did decrease fluorescence, showing that it has some affinity for the enzyme; however, the addition of Mg^{2+} markedly increased its ability to decrease the fluorescence of the phosphofructokinase–sulfonate complex.

Nucleotide Specificity in the Regulation of Phosphofructokinase Activity. In order to study the nature of the nucleotide binding sites involved in decreasing phosphofructokinase–sulfonate fluorescence, it was necessary to find a number of nucleotides with varying regulatory properties. Uyeda and Racker (1965) have already shown that inosine triphosphate (ITP) participates in the catalytic reaction but does not inhibit phosphofructokinase, showing that the nucleotide specificity at the active site is different from the regulatory

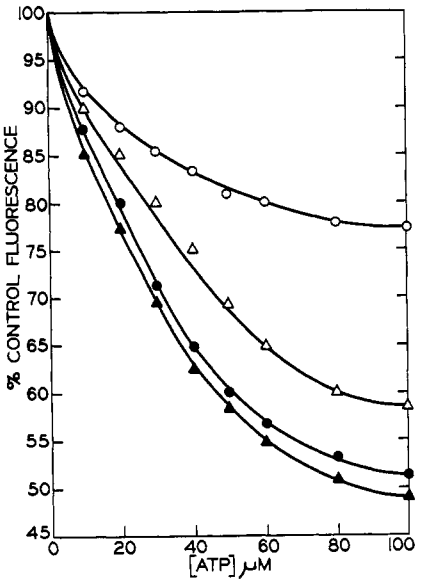

FIGURE 4: Enhancement of the ability of ATP to decrease fluorescence by Mg^{2+}. Fluorescence of the sulfonate (100 μM) in the presence of phosphofructokinase (0.5 μM) was decreased with ATP. The following concentrations of $MgSO_4$ were added: (○) none; (△) 10 μM; (●) 50 μM; (▲) 100 μM. $MgSO_4$ alone had no influence on the fluorescence of the phosphofructokinase–sulfonate complex.

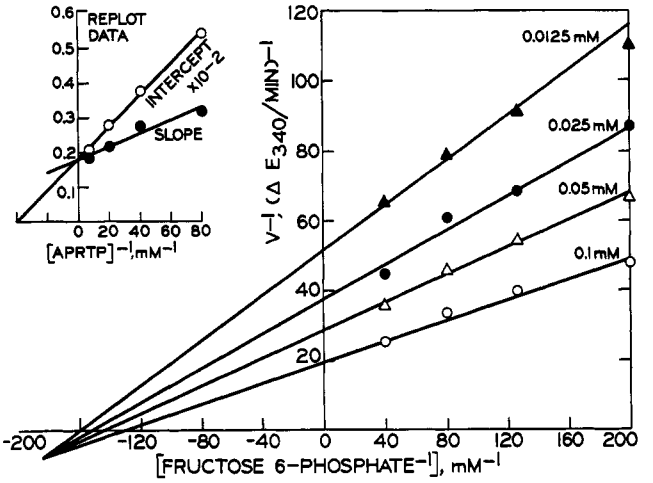

FIGURE 5: nRTP[1] as a phosphoryl donor for phosphofructokinase. The assay contained: Tris-HCl, pH 8, 50 mM; dithiothreitol, 1 mM; KCl, 40 mM; $MgSO_4$, 1 mM; aldolase, 30 μg; triosephosphate isomerase and α-glycerophosphate dehydrogenase, 1.5 μg each; phosphofructokinase, 0.3 μg; and appropriate concentrations of fructose 6-phosphate and nRTP. Assays were performed at 28° in a final volume of 1 ml. (nRTP = APRTP.)

site. In the present work, the 2-aminopurine nucleotide was tested to see if it had similar properties.

Increasing the concentration of nRTP at a fixed concentration of fructose 6-phosphate increased fructose 1,6-diphosphate production catayzed by phosphofructokinase. Reciprocal plots of initial velocity *vs.* fructose 6-phosphate concentration at several fixed concentrations of nRTP (Figure 5) gave rise to a series of intersecting lines from which the Michaelis constant and the dissociation constant for nRTP were calculated at 25 and 3 μM, respectively. These values are very similar to those reported for ATP (Hanson, 1970; Hanson *et al.*, 1973). It is obvious that the configuration of nRTP enables it to participate in the catalytic reaction.

When phosphofructokinase was assayed at pH 7, in the presence of 100 μM fructose 6-phosphate and increasing concentrations of ATP, the enzyme was progressively inhibited as the concentration of ATP exceeded 0.5 mM. In contrast, concentrations of nRTP up to 3 mM did not inhibit the enzyme under the same conditions. It is possible that nRTP binds to the inhibitory site, but that the altered position of the amino functional group prevents it from affecting the enzyme. In this case, it should antagonize the inhibitory effect of ATP. However, when phosphofructokinase was inhibited 80% by ATP (2 mM) the inclusion of nRTP had no influence on this inhibition showing that it cannot bind at the inhibitory site on the enzyme and that this site must be different from the catalytic site.

Under conditions when phosphofructokinase is inhibited by ATP, the addition of AMP enhances catalytic activity (Table I). In contrast, both nRMP and IMP are without effect on enzyme activity. Furthermore, the addition of these nucleotides does not decrease the activation by AMP. It follows that only nucleoside monophosphates containing an adenine ring are capable of activating the ATP-inhibited enzyme. The case for this suggestion is strengthened by the observation that CMP, GMP, UMP, and TMP are all incapable of activating ATP-inhibited phosphofructokinase (Passoneau and Lowry, 1962).

Nucleotide Specificity in Decreasing Phosphofructokinase–Sulfonate Fluorescence. Having established the regulatory properties of various purine nucleotides, the effect of these nucleotides in quenching fluorescence of the phosphofructokinase–sulfonate complex was studied (Table II). It is apparent that all of the nucleoside triphosphates substantially decreased fluorescence. In the nucleoside monophosphate series, AMP has far greater quenching properties than the other nucleotides which reflects the fact that it is the only nucleoside monophosphate that activates ATP-inhibited phosphofructokinase.

Influence of Chemical Modification of Phosphofructokinase on the Quenching Properties of Nucleotides. Succinylation of muscle phosphofructokinase results in a 90% loss of enzyme activity. The succinylated enzyme still enhances the fluorescence of the sulfonate and contains a similar number of

TABLE I: Comparison of Stimulatory Properties of Nucleoside Monophosphates.[a]

[Nucleoside Monophosphate] (mM)	ΔE_{340}/min		
	+AMP	+nRMP	+IMP
0	0.003	0.003	0.003
0.2	0.065	0.004	0.004
0.5	0.18	0.005	0.004
1.0	0.22	0.005	0.004

[a] The assay conditions were as described in Figure 7 except that ATP was fixed at 3 mM. Further additions of nucleoside monophosphates were as shown.

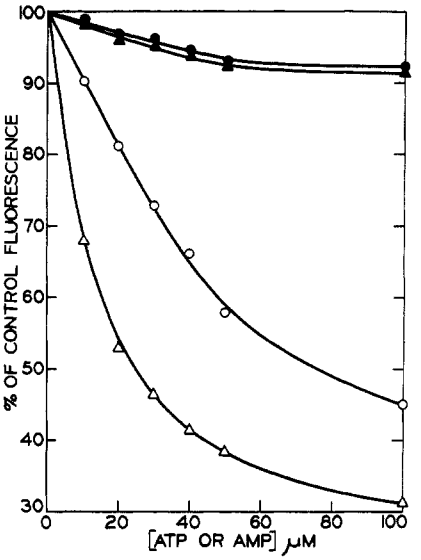

FIGURE 6: The effect of succinylation of phosphofructokinase on the ability of ATP and AMP to decrease fluorescence. Fluorescence was decreased with ATP or AMP using the conditions described in the legend to Figure 2. The following combinations of enzyme and nucleotide were used: (△) control phosphofructokinase (0.5 μM) + AMP; (○) control phosphofructokinase (0.5 μM) + ATP; (▲) succinyl phosphofructokinase (0.5 μM) + AMP; (●) succinyl phosphofructokinase (0.5 μM) + ATP.

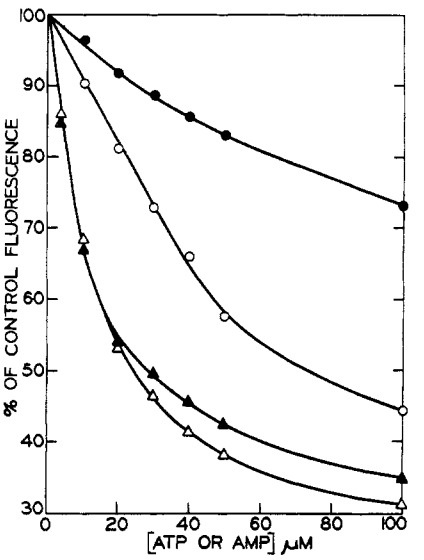

FIGURE 7: The effect of carboxymethylation of phosphofructokinase on the ability of ATP and AMP to decrease fluorescence. Fluorescence was decreased with ATP or AMP using the conditions described in the legend to Figure 2. The following combinations of enzyme and nucleotide were used: (△) control phosphofructokinase (0.5 μM) + AMP; (○) control phosphofructokinase (0.5 μM) + ATP; (▲) carboxymethyl phosphofructokinase (0.5 μM) + AMP; (●) carboxymethyl phosphofructokinase (0.5 μM) + ATP.

sulfonate binding sites although the statistical binding constant for the sulfonate is increased to 50 μM. Figure 6 shows that neither ATP nor AMP is capable of decreasing the fluorescence of the succinylated phosphofructokinase–sulfonate complex. This result suggests that the structural changes produced by nucleotides which results in decreased fluorescence of the phosphofructokinase–sulfonate complex may be related to the ability of the enzyme to achieve a catalytically active conformation.

The catalytic activity of phosphofructokinase is reduced by alkylation of enzyme thiols (Younathan *et al.*, 1968; Froede *et al.*, 1968; Hofer, 1970). In the present work, carboxymethylation of phosphofructokinase resulted in a 50–60% inactivation of the enzyme; however, the residual enzyme activity was still inhibited by high concentrations of ATP (Chapman *et al.*, 1969). The fluorescence enhancement properties of carboxymethylated phosphofructokinase were virtually identical with the normal enzyme; however, Figure 7 shows that whereas the effect of AMP on fluorescence was unaffected, the ability of ATP to decrease fluorescence of the phosphofructokinase–sulfonate complex was significantly decreased by carboxymethylation (50%). The residual effect of ATP could be related to the fact that carboxymethylation does not produce complete inactivation of the enzyme. Thus, the modified enzyme must still have some affinity for ATP. The ability to distinguish between the properties of the binding sites for ATP and AMP by chemical modification of the enzyme is in accord with the results with nucleotide analogs showing that the structural specificity of the two sites was different.

TABLE II: Comparison of Effectiveness of Nucleotides in Decreasing Phosphofructokinase–Sulfonate Fluorescence.[a]

Purine Moiety	[Nucleotide] (μM)	% Decrease by Nucleoside Triphosphate Relative to ATP	% Decrease by Monophosphate Relative to AMP
Adenine	100	100	100
6-Mercaptopurine	100	72	22
Inosine	150	60	12
2-Aminopurine	100	79	14

[a] Fluorescence was measured in 0.1 M potassium phosphate, pH 7, containing 100 μM sulfonate, 100 μM Mg^{2+}, and 0.5 μM phosphofructokinase. Fluorescence was decreased with the concentration of nucleotide indicated. The concentration of inosine nucleotides was raised to compensate for the decreased affinity of thesl nucleotides for the enzyme.

Discussion

Phosphofructokinase causes a marked increase in the fluorescence intensity of the sulfonate which is accompanied by a blue shift in the emission maximum of fluorescence. Decreasing the polarity of the solvent produces a similar phenomenon and it has been presumed that the enhancement of sulfonate fluorescence results from binding of the dye to nonpolar regions in proteins (Stryer, 1965; Turner and Brand, 1968). Recent studies have revealed that binding to proteins probably results from an ionic interaction between the sulfonate group and a positively charged group on the protein (Ainsworth and Flanagan, 1969; Beyer *et al.*, 1972).

For several proteins the binding of the sulfonate occurs at very specific regions on the protein. For phosphofructokinase, this seems unlikely, due to the high number of binding

sites and because of the failure of several conditions which modify enzyme activity (6 M urea, succinylation, carboxymethylation) to significantly alter the fluorescence properties of the phosphofructokinase–sulfonate complex.

Despite the lack of specificity of sulfonate binding to phosphofructokinase, the fluorescence of the complex can be used to assess the binding properties of effectors of phosphofructokinase and to indicate possible conformational changes of the protein. The fluorescence of the phosphofructokinase–sulfonate complex is decreased by numerous effectors, including ATP, ADP, AMP, and fructose 6-phosphate. Citrate, an inhibitor of phosphofructokinase (Garland *et al.*, 1963; Parmeggiani and Bowman, 1963), has a minimal effect which indicates that the ability to decrease fluorescence may be related to the ability of effectors to place the enzyme in a catalytically active conformation. This view is enhanced by the observation that when the enzyme is denatured in 6 M urea or inactivated by succinylation, the ability of the various effectors to decrease fluorescence is abolished. Furthermore, all four nucleotides with catalytic activity are able to decrease fluorescence. Since nRTP and ITP do not inhibit phosphofructokinase, it may be concluded that nucleoside triphosphates decrease fluorescence of the phosphofructokinase–sulfonate complex by binding to the catalytic site. The observations that ATP causes a decrease in fluorescence in a similar concentration range to the K_M of the enzyme for ATP and is enhanced by Mg^{2+} are in agreement with this suggestion. Magnetic resonance studies on manganese-activated phosphofructokinase indicate that the enzyme binds directly to ATP and a metal bridge between the nucleotide and enzyme is very unlikely (Jones *et al.*, 1972). This offers an explanation for the decrease of fluorescence caused by free ATP. Presumably, Mg^{2+} enhances the effect of ATP due to formation of a ternary complex with a high affinity for the enzyme.

If the decrease of phosphofructokinase–sulfonate fluorescence is an indicator of the ability of the enzyme to achieve a catalytically active transition state, the potent ability of AMP to mimic the action of ATP and fructose 6-phosphate may indicate that AMP is capable of maintaining the enzyme in an active transition state. Possibly the enzyme cannot be inhibited by high concentrations of ATP when it is in this conformation, which could explain the activating properties of this nucleotide. Unfortunately, high concentrations of ATP do not significantly reverse the decrease in fluorescence of the phosphofructokinase–sulfonate complex so that this concept cannot be evaluated using this technique.

Chemical modification of phosphofructokinase has shown that it is possible to distinguish between the catalytic and regulatory sites of the enzyme. Thus, photooxidizing heart phosphofructokinase results in a loss of regulatory properties without modifying catalytic activity (Ahlfors and Mansour, 1969; Lorenson and Mansour, 1969; Mansour, 1970). In the reverse situation, Chapman *et al.* (1969) have shown that modification of enzyme thiols decreases the catalytic activity while the enzyme still retains its regulatory properties.

Study of the ability of nucleotides to decrease the fluorescence of the phosphofructokinase–sulfonate complex clearly distinguishes two distinct sites for nucleotide binding. One site that binds nucleoside triphosphates and may represent the active site has a wide specificity for nucleoside triphosphates, whereas the other site is highly specific for AMP. Carboxymethylation of the enzyme modifies the interaction of ATP with the enzyme whereas it does not influence the interaction with AMP. Kemp and his coworkers (Kemp and Forest, 1968; Kemp, 1969a,b; Mathias and Kemp, 1972) have provided extensive evidence that the binding of ATP to phosphofructokinase involves a thiol group which is exceptionally reactive toward DTNB. The conditions of carboxymethylation used in the present work resulted in the loss of all thiol groups that could be titrated with DTNB in 0.1 M potassium phosphate, pH 8 (six–eight thiols per 90,000 daltons in 30 min). If the carboxymethylation reaction time was decreased to 5 min, the number of modified thiols was reduced; however, the decrease of phosphofructokinase–sulfonate fluorescence by ATP was still inhibited. Under this condition the control enzyme contained 2.2 thiols per 90,000 daltons that reacted virtually instantaneously with DTNB, whereas the modified enzyme had only 0.5 similar thiol. Obviously this evidence does not conclusively prove that a specific thiol is involved in ATP binding. However, it is in agreement with the suggestion that a thiol may be involved in ATP binding at the active site. Since modification of thiols does not completely abolish the quenching properties of ATP nor does it completely inhibit enzyme activity, it must be concluded that other factors are also involved in the binding of nucleotides at the active site.

A whole range of studies including the present now point toward the fact that the binding sites for ATP and AMP are spatially separated in the enzyme. In order to prove this point conclusively it will be necessary to develop nucleotide affinity labels and to determine which sites are modified.

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Chemical Kinetic and Proton Magnetic Resonance Studies of 5′-Adenosine Monophosphate Binding to Ribonuclease A†

Peter H. Haffner* and Jui H. Wang‡

ABSTRACT: The effects of 5′-AMP on the kinetics of ribonuclease A catalyzed hydrolysis of cyclic CMP have been analyzed. The results suggest the existence of the ternary complex, enzyme–cyclic CMP–5′-AMP, which breaks down to product approximately three times faster than the enzyme–cyclic CMP complex at pH 5.57. The pH profile for the binding constant of 5′-AMP to ribonuclease A has been determined in the pH range 4–8.5. The shape of this profile is very similar to that found previously for the binding of orthophosphate and 3′-pyrimidine nucleotides to the enzyme. The titration curves of the individual histidine residues of ribonuclease A have been determined by measuring the pD dependence of the proton magnetic resonance chemical shifts of the imidazole C-2 proton resonance for each histidine. The shapes of the His-12 and His-119 curves were found to be abnormal in the pH region 3–5.2. These results call into question the assignments of the His-12 and His-119 resonance lines, but a linewidth analysis of these two resonances over the entire pD region has substantiated the originally proposed assignments. The abnormal shape of these two titration curves demonstrates a change in the environment of both His-12 and His-119 which accompanies the titration to low pD. The effects of 5′-AMP binding on the aromatic region of the ribonuclease A nuclear magnetic resonance (nmr) spectrum have been observed over the pD range 3–8.5. 5′-AMP binding perturbs the pK_a values of His-12 and -119 from their free enzyme values of 6.2 and 6.06 to 7.6 and 6.3, respectively. In addition, the His-119 resonance is shifted upfield some 20 Hz at low pD. The presence of 5′-AMP has no observable effect on the large Phe-120 peak associated with the pyrimidine nucleotide binding pocket at the enzyme surface. The results demonstrate that the phosphate moiety of 5′-AMP is bound at the same site on the enzyme surface at which the phosphate of 3′-pyrimidine nucleotide is bound. In contrast to the 3′-pyrimidine nucleotide case, there is only a very weak interaction between the phosphate of bound 5′-AMP and His-119. Like pyrimidine nucleotide, 5′-AMP phosphate has a strong acid stabilizing influence on His-12. The shielding effect on the fully protonated His-119 resonance at low pD is consistent with a base stacking interaction between the adenine ring of bound 5′-AMP and the imidazole ring of His-119.

Bovine pancreatic ribonuclease A catalyzes the hydrolysis of RNA and (3′,5′)-dinucleoside phosphate esters in two steps. The first step is an intramolecular transesterification to a stable (2′,3′) cyclic phosphate intermediate which is, in turn, hydrolyzed to a 3′-nucleotide (Brown and Todd, 1953; Brocklehurst *et al.*, 1967). Recently, X-ray crystallographic studies (Carlson, W. D., *et al.*, to be published) of ribonuclease S, a proteolytically modified derivative of ribonuclease A, have uncovered an adenosine nucleotide binding site at the enzyme surface. This site is directly adjacent to the site occupied by bound pyrimidine nucleotide and presumably by poly- and (2′,3′) cyclic pyrimidine nucleotide substrates as well. Previously, substrate specificity studies had revealed that in the ribonuclease A catalyzed transesterification of (3′,5′)-dinucleoside phosphate esters, the enzyme exhibits a marked preference for purine, particularly adenosine, as the 5′ alcohol (Witzel and Barnard, 1962; Gassen and Witzel,

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06510. *Received October 26, 1972.* This material was submitted by P. H. H. to the Graduate School of Yale University in partial fulfillment of the requirements for the Ph.D. degree. This work was supported by research grants from the National Institute of General Medical Sciences (GM 044 83 and GM 19990-01) and a Public Health Service Training grant (00748-09).

* Present address: Department of Molecular Biophysics and Biochemistry, Yale University.

‡ Present address: Bioenergetics Lab, State University of New York at Buffalo, Buffalo, New York 14214.