Biochemistry

 Volume 17, Number 10 May 16, 1978

Study of the Interaction of Adenylyl Imidodiphosphate with Rabbit Muscle Phosphofructokinase†

Neil M. Wolfman,‡ W. Reid Thompson,§ and Gordon G. Hammes*

ABSTRACT: Equilibrium binding studies have been used to characterize the interaction of adenylyl imidodiphosphate, an adenosine 5′-triphosphate analogue, with rabbit muscle phosphofructokinase. Binding isotherms were obtained at pH 7.0, 4 °C, in 50 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, plus 5 mM fructose 6-phosphate, 5 mM fructose 1,6-bisphosphate, 10 mM potassium phosphate, or 5 mM fructose 1,6-bisphosphate–5 mM citrate. In these cases, the binding could be characterized by one site of high affinity (with a dissociation constant of ~1 μM), and one site of low affinity (with a dissociation constant of ~100 μM) per polypeptide chain, with varying degrees of negative cooperativity within each type of site. With 10 mM phosphate–50 μM cAMP present, the binding at the weaker site was abolished. Since cAMP is a known activator of the enzyme, this suggests that the "tight" site may be identified with the catalytic site, while the "loose" site is probably the regulatory site. The enzyme is predominantly tetrameric under the conditions employed, except with 5 mM citrate present where the enzyme is a mixture of dimers and tetramers. The binding isotherm was unaffected by doubling the enzyme concentration in the presence of 5 mM fructose 6-phosphate. However, the affinity of the enzyme for adenylyl imidodiphosphate was greatly weakened after modification of a single sulfhydryl group of the enzyme with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. When the citrate binding site of the enzyme was modified with pyridoxamine 5′-phosphate, the binding at the "tight" site in 10 mM phosphate was enhanced; the pyridoxamine 5′-phosphate modified enzyme was primarily dimeric under the experimental conditions employed. The binding of adenylyl imidodiphosphate to the enzyme causes a quenching of the protein fluorescence. Although the fluorescence changes can be interpreted in terms of a binding isotherm in 10 mM phosphate, the isotherm obtained is greatly different from that derived by direct measurement: the fluorescent changes apparently are caused by binding at a relatively low affinity site. This illustrates the danger in determining binding isotherms with indirect methods.

Phosphofructokinase plays a key role in the regulation of glycolysis. The activity of the enzyme from rabbit skeletal muscle has been shown to be dependent on several factors including the presence or absence of various effectors (Passoneau & Lowry, 1962, 1963). The enzyme exhibits association–dissociation equilibria which may be physiologically important in the control of the enzymatic activity (Lad et al., 1973; Hofer & Krystek, 1975). The multisubunit protein is composed of identical polypeptide chains, and the active tetramer has a molecular weight of about 320 000 (Coffee et al., 1973; Pavelich & Hammes, 1973). The monomer and dimer are essentially inactive (Lad et al., 1973).

In the work presented here, the interaction of adenylyl imidodiphosphate (AMP-PNP)[1] with rabbit muscle phosphofructokinase has been studied. This analogue of ATP was chosen for study since it has been shown to be very similar structurally to ATP (Yount et al., 1971a,b) but is not a substrate of the enzyme and is not hydrolyzed by the minor ATPase activity of the enzyme (Uyeda, 1970; Colombo et al., 1975). At a concentration where the enzyme is primarily tetrameric (0.6–0.7 mg/mL) and at pH 7.0 where the enzyme displays allosteric kinetic behavior (Hofer & Pette, 1968), one "tight" site and one "weak" site for AMP-PNP per 80 000 molecular weight of enzyme are observed in the presence of 5 mM fructose 6-phosphate, 5 mM fructose 1,6-bisphosphate, 10 mM potassium phosphate, or 5 mM fructose 1,6-bisphosphate–5 mM citrate at 4 °C. Some of the binding isotherms are complex and suggest cooperative effects, but the stoichiometry is the same in all cases. The presence of 10 mM phosphate–50 μM cAMP (an allosteric activator) eliminates the binding at the "loose" site, suggesting the "tight" site is the

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received December 7, 1977.* This work was supported by grants from the National Institutes of Health (GM 13292).

‡ National Institutes of Health Predoctoral Trainee (GM 00834; GM07273).

§ National Science Foundation Predoctoral Fellow.

[1] Abbreviations used: AMP-PNP, adenylyl imidodiphosphate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PMP, pyridoxamine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; cAMP, cyclic adenosine 3′,5′-monophosphate.

0006-2960/78/0417-1813$01.00/0

catalytic site and the "loose" site is the regulatory site. When a single sulfhydryl group of the enzyme is modified with NBD-Cl, the binding of AMP-PNP to the enzyme is greatly reduced; on the other hand, modification of the citrate binding site with pyridoxal 5′-phosphate and subsequent borohydride reduction led to a dimeric enzyme in which the affinity of AMP-PNP for the "tight" site was enhanced.

Experimental Section

Materials. The [^{3}H]AMP-PNP (30.6 Ci/mmol) and AMP-PNP were obtained from ICN and purified according to Yount et al. (1971a,b). The AMP-PNP containing fraction was lyophilized and stored as a powder or in 0.1 mM EDTA (pH 9.0) at −23 °C. The pyridoxal 5′-phosphate, ATP, cAMP, fructose 6-phosphate, fructose 1,6-bisphosphate, dithiothreitol, aldolase, glucose oxidase, α-glycerophosphate dehydrogenase, pyruvate kinase, triosephosphate isomerase, and serum albumin (bovine) were purchased from Sigma Chemical Co. The NBD-Cl was obtained from Pierce Chemical Co., and all other chemicals were the best available commercial grades. All solutions were prepared with deionized distilled water.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was purified by the method of Ling et al. (1966). The final ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate, 1.0 mM EDTA, 1 mM dithiothreitol (pH 8.0) and dialyzed against the same buffer to give a stock solution of 10–16 mg/mL. The protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 1.02 mL/(mg cm) (Parmeggiani et al., 1966).

Enzymatic activity was determined using coupled enzyme reactions (Ling et al., 1966; Lad et al., 1973). Assays were carried out in the following mixture: pH 8.0, 33 mM Tris-Cl, 2 mM ATP, 5 mM $MgCl_2$, 2 mM fructose 6-phosphate, 0.1 mM NADH, 2 mM dithiothreitol, 0.20 units/mL of aldolase, 35 units/mL of triosephosphate isomerase, 3.2 units/mL of α-glycerophosphate dehydrogenase, and 0.1–0.2 μg/mL of phosphofructokinase in a total volume of 3.0 mL. Assays were initiated by the addition of phosphofructokinase, and the velocity of the enzymatic reaction was recorded spectrophotometrically at 340 nm on a Cary 118 spectrophotometer thermostated at 23 °C. The specific activity of the native enzyme in 0.1 M potassium phosphate, 1.0 mM EDTA (pH 8.0) was 100–120 units/mg, where a unit of enzyme activity is defined as the production of 1 μmol of product per min.

Phosphofructokinase Derivatives. Phosphofructokinase was modified with pyridoxal 5′-phosphate as previously described (Wolfman & Hammes, 1977) except that 1.0 mM fructose 1,6-bisphosphate was substituted for 1.0 mM ATP in the dialysis buffer and reaction medium. The NBD modified enzyme was prepared as described elsewhere (Lad et al., 1977) in 25 mM potassium phosphate, 25 mM diglycine, 5 mM fructose 6-phosphate, 5 mM $MgCl_2$, 1 mM EDTA (pH 7.0).

Gel Chromatography. The average molecular weight of the native enzyme was determined using column chromatography as previously described (Wolfman & Hammes, 1977). Aldolase (mol wt 150 000), glucose oxidase (mol wt 186 000), pyruvate kinase (mol wt 240 000), and native phosphofructokinase (0.19 mg/mL, mol wt 320 000) in 0.1 M potassium phosphate (pH 8.0) were used as molecular weight standards.

Fluorescence Measurements. Fluorescence titrations of phosphofructokinase with AMP-PNP were performed with a Hitachi Perkin-Elmer MPF-3 fluorescence spectrophotometer thermostated at 4 °C. Dry nitrogen was circulated through the sample compartment to prevent condensation. The excitation wavelength was 280 nm, and the changes in the emission at 328 nm were recorded. The excitation slit was 4 nm, while the emission slit ranged from 2 to 4 nm. All measurements were made in square (1 × 1 cm) cuvettes with an initial solution volume of 2.5 mL. Fluorescence intensities were corrected for protein dilution due to the addition of ligand; these corrections were never more than 10%. A correction also was made for the increase in the absorbance of the solution at the exciting wavelength during the course of the titration. This was done by constructing a calibration curve from a fluorescence titration of tryptophan with ATP.

Equilibrium Binding Measurements. Binding measurements were carried out at 4 °C using the forced dialysis technique (Cantley & Hammes, 1973). Typically the enzyme, native or modified, was dialyzed at 4 °C into the appropriate buffer for 16 h, filtered through 0.45-μm Millipore filters, and diluted to the desired concentration. Varying amounts of the radioactive ligand were added to 0.5-mL samples of protein solution and allowed to equilibrate for 2 h. The total ligand concentration of each sample was determined by measuring the radioactivity of a 10-μL sample in Beckman Ready-Solv using a Beckman LS 255 liquid scintillation counter and using the known specific activity. The protein solutions were then placed in a Metalloglass ultrafiltration cell fitted with Diaflow XM-50 membranes, and a pressure of 25–40 psi of nitrogen was applied for several minutes until 20–30 μL of solution were forced through the membrane. Duplicate 10-μL samples were taken from the underside of the membrane, and the concentration of free ligand determined from the radioactivity as before. The amount of ligand bound to the enzyme was calculated by subtracting the free ligand concentration from the total ligand concentration. The XM-50 membranes do not bind the ligands, and the enzyme does not pass through the membrane (Hill & Hammes, 1975).

Data Analysis. The data were fit to the specified equations on a PDP-11 computer using a weighted nonlinear least-squares procedure. A few points greater than 1.9 standard deviations from the calculated curves were omitted in the final fitting iteration, although this made no appreciable change in the values of the fitting parameters.

Results

The isotherms for the binding of AMP-PNP to rabbit muscle phosphofructokinase at 4 °C in 50 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (pH 7.0) plus various effectors are shown in Figure 1 as plots of r, the moles of ligand bound per mole of polypeptide chain (mol wt 80 000) vs. the concentration of free AMP-PNP. The effectors used were 10 mM potassium phosphate–50 μM cAMP, 10 mM potassium phosphate, 5 mM fructose 6-phosphate, 5 mM fructose 1,6-bisphosphate, and 5 mM fructose 1,6-bisphosphate–5 mM citrate. The enzyme remained fully active throughout the binding experiment at the normal specific activity for each set of conditions, but the enzyme was quite unstable in the absence of any effectors.

The only simple binding isotherm obtained is that in the presence of cAMP (Figure 1A), and the data were fit to the equation

$$r = \frac{(L)}{K_1 + (L)} \tag{1}$$

where K_1 is the dissociation constant characterizing the interaction of the ligand L with the protein. A single binding site per polypeptide chain is found, and the dissociation constant obtained is given in Table I; the line in Figure 1A has been calculated according to eq 1 with the constant in Table I.

Under all other conditions, the binding isotherms are com-

TABLE I: Equilibrium Dissociation Constants.[a]

Effectors present	K_1 (μM)	K_2 (μM)	K_3 (μM)	K_4 (μM)
Phosphate (10 mM)-cAMP	0.909			
Fru 1,6-P (5 mM)	4.55	221		
Fru 1,6-P (5 mM)-citrate (5 mM)	0.730	67.1		
Phosphate (10 mM)	1.32	10.1	178	
Fru 6-P (5 mM)	0.917	19.6	270	502

[a] With 50 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0, plus effectors as listed at 4 °C.

plex, and the data were fit to a large variety of binding models. The only models which adequately describe the data require approximately two binding sites per polypeptide chain with either more than one class of binding sites or negative cooperativity. Since evidence already exists for both a catalytic site and a regulatory site, the data were fit assuming two classes of sites. In some cases, cooperativity within a given class of sites also was required to fit the data. The binding models presented here can be regarded as minimum, but not unique, representations of the data. In all cases the dissociation constants obtained from the data are summarized in Table I, and the lines in Figure 1 are calculated with the appropriate equations and the dissociation constants.

In the presence of fructose 1,6-bisphosphate (Figure 1B) and fructose 1,6-bisphosphate-citrate (Figure 1C), the data are well described by assuming two classes of sites, that is

$$r = \frac{(L)}{K_1 + (L)} + \frac{(L)}{K_2 + (L)} \quad (2)$$

In the presence of 10 mM potassium phosphate (Figure 1A), the data at high ligand concentrations cannot be fit by assuming simple binding to the second site; therefore the additional assumption was made that binding to the weaker class of sites is cooperative with two dissociation constants characterizing the cooperativity. This gives rise to

$$r = \frac{(L)}{K_1 + (L)} + \frac{0.5(L)/K_2 + (L)^2/K_2K_3}{1 + (L)/K_2 + (L)^2/K_2K_3} \quad (3)$$

Finally, in the presence of fructose 6-phosphate (Figure 1D), the data require the assumption of cooperativity within each class of sites. The binding equation used to fit the data is

$$r = \frac{0.5(L)/K_1 + (L)^2/K_1K_2}{1 + (L)/K_1 + (L)^2/K_1K_2} + \frac{0.5(L)/K_3 + (L)^2/K_3K_4}{1 + (L)/K_3 + (L)^2/K_3K_4} \quad (4)$$

Although the enzyme concentration was kept constant in the binding experiments for which data are reported in order to maintain a constant enzyme aggregation state, in one set of experiments in the presence of 5 mM fructose 6-phosphate the enzyme concentration was doubled (from 0.7 to 1.4 mg/mL) with no discernible effect on the binding data (filled squares in Figure 1D).

The average molecular weight of the enzyme was determined by gel chromatography under the conditions of the binding experiments, 0.7 mg/mL enzyme, 50 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (pH 7.0), 4 °C. The average molecular weight was found to be 408 000 in the presence of 5 mM fructose 6-phosphate, 405 000 in the presence of 10 mM potassium phosphate, and 260 000 in the presence of 5 mM fructose 1,6-bisphosphate-5 mM citrate. Thus the enzyme is predominantly tetrameric under all conditions studied, except in the presence of citrate where a mixture of dimers and tetramers is probably present.

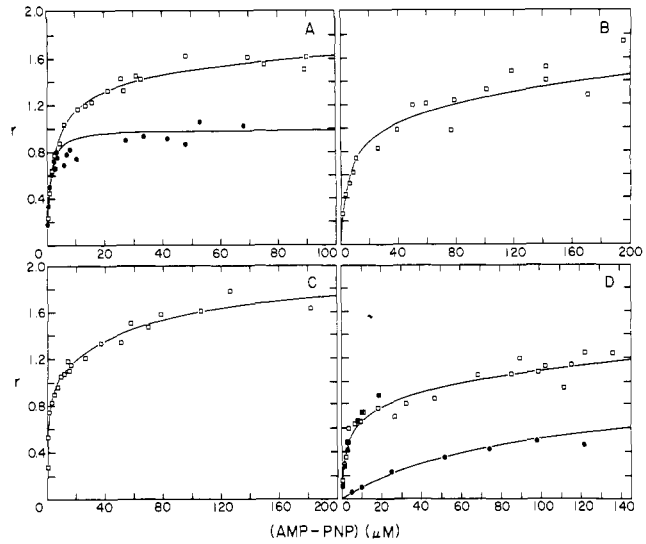

FIGURE 1: A plot of r, the number of moles of AMP-PNP bound per 80 000 molecular weight of phosphofructokinase (0.7 mg/mL except for the filled squares in D where the concentration was 1.4 mg/mL) vs. the free AMP-PNP concentration at 4 °C in 50 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0, and 10 mM potassium phosphate-50 μM cAMP (A, lower curve), 10 mM potassium phosphate (A, upper curve), 5 mM fructose 1,6-bisphosphate (B), 5 mM fructose 1,6-bisphosphate-5 mM citrate (C), or 5 mM fructose 6-phosphate (D, upper curve). The bottom curve in D is for AMP-PNP binding to NBD-modified enzyme (1.4 mg/mL) in the presence of 5 mM fructose 6-phosphate. The lines are calculated with the equations in the text and the constants in Table I for binding to the native enzyme; for binding to the NBD-modified enzyme, eq 1 and K_1 = 93 μM are used.

To study the binding of AMP-PNP to NBD modified phosphofructokinase, it was necessary to modify the enzyme in the absence of ATP. The reaction between NBD-Cl and enzyme in the absence of ATP was faster than with ATP present (Lad et al., 1977), but was still quite specific for a single sulfhydryl group. The NBD derivative used in binding experiments contained 1.2 mol of NBD per 80 000 molecular weight. The binding isotherm (1.4 mg/mL enzyme) in the presence of 5 mM fructose 6-phosphate is shown in Figure 1D, and the data were fit to a simple one site model (eq 1). The calculated dissociation constant is 93 μM.

The binding of AMP-PNP to PMP-modified phosphofructokinase also was studied; the PMP specifically blocks citrate binding (Colombo & Kemp, 1976). As with the NBD derivative, the modification was carried out in the absence of ATP: 1.0 mM fructose 1,6-bisphosphate was present to ensure enzyme stability. Both the specificity and the rate of reaction were not significantly altered (Wolfman & Hammes, 1977). Binding to the PMP-modified enzyme was carried out under conditions where the enzyme is predominantly dimeric (0.2 mg/mL, 50 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 mM potassium phosphate, pH 7.0, 4 °C). Although the data at low ligand concentrations could be fit to a model of a single binding site with a binding constant of 0.72 μM, the considerable scatter in the data associated with enzyme instability, particularly at high ligand concentrations, precluded a detailed analysis of the data.

The interaction of AMP-PNP with phosphofructokinase also was investigated by titrating a protein solution with

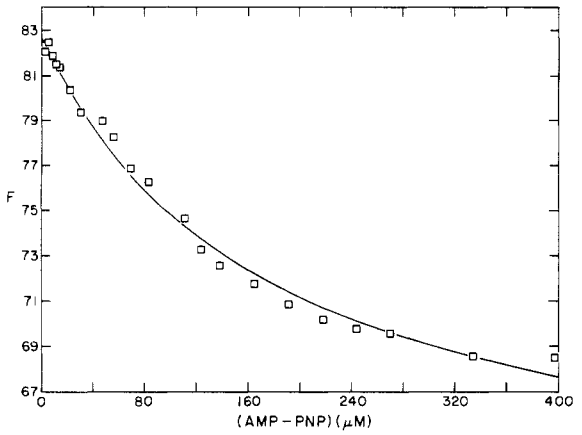

FIGURE 2: A plot of F, the fluorescence intensity, vs. the total AMP-PNP concentration present at 4 °C in 50 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0, and 10 mM potassium phosphate. The excitation wavelength was 280 nm, the emission wavelength was 328 nm, and the protein concentration was 0.7 mg/mL. The fitted line is calculated according to eq 5 with $F_0 = 82.7$, $F_\infty = 61$, and $K_1 = 177$ μM.

AMP-PNP and monitoring the protein fluorescence under conditions identical with those used in obtaining the binding data. With 10 mM potassium phosphate present, the quenching data presented in Figure 2 were obtained. These data can be fit to a single binding constant according to the equation

$$r = \frac{F - F_0}{F_\infty - F_0} = \frac{(L)}{K_1 + (L)} \quad (5)$$

where F is the measured fluorescence, F_0 is the fluorescence in the absence of ligand, and F_∞ is the fluorescence when the enzyme is saturated with the ligand. The best fit of the data was obtained with $K_1 = 177$ μM, $F_0 = 82.7$, and $F_\infty = 61$, where the units of fluorescence are arbitrary; the line in Figure 2 has been calculated with these parameters and eq 5. In the presence of fructose 6-phosphate, the extent of fluorescence quenching is extremely small (an average maximum of 7%), so that the data cannot be analyzed quantitatively in terms of a specific binding model.

Discussion

The use of AMP-PNP as an ATP analogue is well documented (Yount et al., 1971a,b; Maguire & Gilman, 1974; Melnick et al., 1975). In the case of rabbit muscle phosphofructokinase, steady-state kinetic results have shown that AMP-PNP is a strong competitive inhibitor of the substrate, MgATP (Bârzu et al., 1977; Lad et al., 1977). The binding isotherms reported indicate that two specific AMP-PNP binding sites are present per polypeptide chain: measured values of r do not exceed 2, and the data can be fit with reasonably simple binding models. These data cannot, of course, rule out the possibility of additional weaker binding sites. The results are most easily explained in terms of one "tight" binding site and one "loose" binding site per polypeptide chain. In view of the fact that ATP is both a substrate and an allosteric inhibitor, it is reasonable to assign one site as catalytic and the other as regulatory. This interpretation is supported by the binding isotherm obtained in the presence of 50 μM cAMP, an allosteric activator of the enzyme: only a single tight binding site per polypeptide chain is found. This suggests the "tight" site is the catalytic site and the "loose" site is the regulatory site. An alternative explanation is that the "tight" site is the regulatory site and that cAMP both blocks the regulatory site and greatly enhances binding to the "loose" site which is the catalytic site. While this interpretation cannot be excluded, it is less probable since the primary regulatory effect of cAMP appears to be relief of MgATP inhibition, rather than a tightening of the binding of MgATP at the catalytic site. Whether the blockage of the inhibitory site by cAMP is competitive or allosteric is not known.

A previous study of the binding of ATP to rabbit muscle phosphofructokinase has suggested that three ATP sites are present per polypeptide chain (Kemp & Krebs, 1967). When sulfhydryl group reactivity was used as a monitor of binding to the inhibitory site, the dissociation constant for MgATP binding to the inhibitory site was estimated to be less than 10 μM (Kemp, 1969). However, the results reported here represent the first direct detailed investigation of binding to ATP sites on the rabbit muscle enzyme. Studies of the binding of ATP to the sheep heart enzyme indicated 3.6 mol of ATP per 100 000 molecular weight, but the precise stoichiometry was dependent on the protein concentration (Lorenson & Mansour, 1969). We have found no dependence of the binding isotherm on the protein concentration under one set of conditions, although this aspect of the binding process was not studied in detail. The same binding stoichiometry was observed for the tetrameric enzyme and for the enzyme in a tetramer-dimer mixture (5 mM fructose 1,6-bisphosphate–5 mM citrate).

The binding models used to fit the data should not be taken too literally. This is especially true with regard to characterizing the nature of the cooperativity in binding to the "loose" site since reliable data could not be obtained at high r values and ligand concentrations while maintaining a constant protein concentration. However, the nature of the cooperativity observed clearly depends on the effectors present. The aggregation state of the enzyme does not seem to be a major factor in the nature of the cooperative binding. Negative cooperativity is observed within both classes of sites. A previous study of fructose 6-phosphate binding to rabbit muscle phosphofructokinase has indicated the occurrence of negative cooperativity, positive cooperativity, and no cooperativity (Hill & Hammes, 1975) depending on the specific conditions. Obviously a complex model will be needed to explain quantitatively the complex binding equilibria observed.

The dissociation constant characterizing binding to the "tight" site is about 1 μM: it is decreased in the presence of citrate and cAMP and is somewhat larger in the presence of sugar phosphates. Binding to the "loose" site follows a similar pattern. Citrate has been shown previously to enhance ATP binding (Colombo et al., 1975). The binding of AMP-PNP to the catalytic site is also enhanced in the dimeric PMP-modified enzyme, although this enzyme is essentially inactive. This finding further supports the idea that citrate inhibition and the lack of activity in the dimer are due to alterations in the intrinsic catalytic activity and are not due to major alterations in binding at the catalytic site (Lad & Hammes, 1974). The NBD-modified enzyme binds AMP-PNP very weakly. If the observed binding is attributed to the catalytic site, it is consistent with the large increase in the AMP-PNP steady state competitive inhibition constant (Lad et al., 1977).

The binding of AMP-PNP to the enzyme produces a marked quenching of fluorescence under some conditions. However, this fluorescence quenching cannot be reliably interpreted in terms of a binding isotherm. Although the data in Figure 2 can be quantitatively described by eq 5, the calculated binding isotherm bears little resemblance to that determined by a direct method under identical conditions (Figure 1A vs. Figure 2). In fact, the dissociation constant determined from the fluorescence data is close only to the constant K_3 used to charac-

terize the binding isotherm determined by forced dialysis. The agreement between K_3 and the dissociation constant derived from the fluorescence measurements is suggestive but most likely is fortuitous. This finding again emphasizes the danger in using an indirect method, such as fluorescence changes, to measure binding isotherms.

In summary, the binding of AMP-PNP to rabbit muscle phosphofructokinase can be interpreted in terms of a relatively tight binding catalytic site and a relatively loose binding inhibitory site per polypeptide chain.

References

Bârzu, O., Tilinca, R., Porutiu, D., Gorun, V., Jebeleanu, G., Ngoc, L. D., Kezdi, M., Goia, I., & Mantsch, H. H. (1977) *Arch. Biochem. Biophys. 182,* 42.

Cantley, L. C. Jr., & Hammes, G. G. (1973) *Biochemistry 12,* 4900.

Coffee, C. J., Aaronsen, R. P., & Frieden, C. (1973) *J. Biol. Chem. 248,* 1381.

Colombo, G., & Kemp, R. G. (1976) *Biochemistry 15,* 1774.

Colombo, G., Tate, P. W., Girotti, A. W., & Kemp, R. G. (1975) *J. Biol. Chem. 250,* 9404.

Hill, D. E., & Hammes, G. G. (1975) *Biochemistry 14,* 203.

Hofer, H. W., & Krystek, E. (1975) *FEBS Lett. 53,* 217.

Hofer, H. W., & Pette, D. (1968) *Hoppe-Seyler's Z. Physiol. Chem. 349,* 1378.

Kemp, R. G. (1969) *Biochemistry 8,* 3162.

Kemp, R. G., & Krebs, E. G. (1967) *Biochemistry 6,* 423.

Lad, P. M., & Hammes, G. G. (1974) *Biochemistry 13,* 4530.

Lad, P. M., Hill, D. E., & Hammes, G. G. (1973) *Biochemistry 12,* 4303.

Lad, P. M., Wolfman, N. M., & Hammes, G. G. (1977) *Biochemistry 16,* 4802.

Ling, K. H., Paetkau, V., Marcus, F., & Lardy, H. A. (1966) *Methods Enzymol. 9,* 425.

Lorenson, M. Y., & Mansour, T. E. (1969) *J. Biol. Chem. 244,* 6420.

Maguire, M. E., & Gilman, A. G. (1974) *Biochim. Biophys. Acta 358,* 154.

Melnick, R. L., Tavares De Sousa, J., Maguire, J., & Packer, L. (1975) *Arch. Biochem. Biophys. 166,* 139.

Parmeggiani, A., Luft, J. H., Love, D. S., & Krebs, E. G. (1966) *J. Biol. Chem. 241,* 4625.

Passoneau, J. V., & Lowry, O. H. (1962) *Biochem. Biophys. Res. Commun. 7,* 10.

Passoneau, J. V., & Lowry, O. H. (1963) *Biochem. Biophys. Res. Commun. 13,* 372.

Pavelich, M. J., & Hammes, G. G. (1973) *Biochemistry 12,* 1408.

Uyeda, K. (1970) *J. Biol. Chem. 245,* 2268.

Wolfman, N. M., & Hammes, G. G. (1977) *Biochemistry 16,* 4806.

Yount, R. G., Babcock, D., Ballantyne, W., & Ojala, D. (1971a) *Biochemistry 10,* 2484.

Yount, R. G., Ojala, D., & Babcock, D., (1971b) *Biochemistry 10,* 2490.