The binding of nucleoside triphosphates to rabbit muscle phosphofructokinase has been determined in 0.05 M phosphate buffers by changes in intrinsic protein fluorescence and by direct binding measurements. These experiments have been performed over a wide range of pH, temperature, and effector concentration. Quenching of protein fluorescence is shown to measure binding of nucleotides to a site which is not the active site but rather a site responsible for inhibition of the kinetic activity. This site is relatively specific for either ATP or MgATP with free ATP binding about 10-fold more tightly than MgATP. A model to describe binding to this site as a function of pH and temperature is proposed. This model assumes that the apparent affinity for ATP is determined by protonation of two ionizable groups (per subunit) and that ATP binds exclusively to protonated enzyme forms. Several ligands which affect the apparent affinity for nucleotide binding at the inhibitory site act by shifting the apparent pK of the ionizable groups. NH₄⁺ and citrate do not influence nucleotide binding to the inhibitory site. At pH 6.9 in 0.05 M phosphate, low concentrations of MgATP or MgGTP enhance the protein fluorescence due to binding at the active site. The fluorescence studies and direct binding studies show that there is one active site and one inhibitory site per subunit. As described elsewhere (Pettigrew, D. W., and Frieden, C. (1978) J. Biol. Chem. 253, 3623-3627), there is a third nucleotide binding site on each subunit which is specific for cAMP, AMP, and ADP.

Phosphofructokinase (EC 2.7.1.11) is a key enzyme in the control of glycolysis (1-3). In an effort to understand its regulatory behavior, a number of studies of the ligand binding properties of the rabbit muscle enzyme have been performed (4-8). However, each binding studies may be complicated by several factors. For example, under some conditions, the enzyme may undergo a reversible dissociation from an active enzyme of four subunits to an inactive one of two subunits (9-14) and indeed this dissociation may play an important role in the regulation of the total enzyme activity (15). However, different molecular weight forms of the enzyme, or forms which lead to the dissociation, have been shown to bind some ligands differently (6, 9, 16, 17). Thus, equilibrium binding studies may reflect some type of average of the binding properties of different forms rather than those of a single form. Furthermore, there are several types of nucleotide binding sites on the enzyme (4, 5, 8, 17-24) and these may overlap in specificity, thus confusing any interpretation. Finally, the ability of some of these sites to bind nucleotides may depend markedly on the pH, temperature, and buffer composition (17, 25, 26) and these factors have not always been taken into account.

In the present paper, we examine binding of nucleotides and their magnesium complexes by the rabbit muscle enzyme in phosphate buffers under conditions where the oligomeric structure of the enzyme is known. In agreement with other studies (4, 5, 8, 17-24), we show that there are three types of nucleotide binding sites which overlap somewhat with respect to specificity. In terms of kinetic behavior, these three sites are the active site, an inhibitory site, and an activating site. A model is presented which describes nucleotide binding, and it is specifically shown that the pH, temperature, and effector dependence of binding to the inhibitory site of tetrameric enzyme forms can be described in terms of protonation of two identical groups per subunit. The results described here are entirely consistent with a model previously proposed by Frieden et al. (15) for the pH-dependent properties of the enzyme.

In the following paper (27), results of these binding studies are used to develop a model which quantitatively describes the regulatory kinetic behavior of the enzyme.

**MATERIALS AND METHODS**

All nucleotides, substrates, and enzymes were purchased from Sigma Chemical Co., unless otherwise indicated. AMP-PNP¹ was purchased from Boehringer Mannheim and purified according to procedures described by Yount (28). High pressure liquid chromatography showed the AMP-PNP to be 96% pure following these procedures. 1-Deoxyfructose was a generous gift from Dr. William L. Meyer of the University of Vermont College of Medicine. 1-Deoxy-Fru-6-P was prepared and purified according to procedures described by Bar-Tana and Cleland (29).

Throughout these binding studies, equine muscle ATP (former Sigma grade) was used routinely. It has been reported that this ATP preparation is contaminated with trace amounts of vanadate (30), and, recently, that sheep heart phosphofructokinase is inhibited by vanadate (31). The ATP binding properties of rabbit skeletal muscle phosphofructokinase were examined using vanadate-free ATP (Sigma Chemical Co.), and found to be identical with those obtained with the former Sigma grade ATP. Thus, under the conditions of these experiments, trace amounts of vanadate do not detectably affect the binding properties of rabbit skeletal muscle phosphofructokinase.

Phosphofructokinase from fresh rabbit skeletal muscle was prepare⁴

¹ The abbreviations used are: Fru-6-P, fructose 6-phosphate; Fru-1,6-dP, fructose 1,6-bisphosphate; F-5'-FOS-BenzA, 5'-p-fluorosulfonymidylenbenzoyl adenosine; eATP, 1,N₆-ethenoadenosine triphosphate; AMP-PNP, adenylyl imidodiphosphate.
pared as previously described (22). For the binding experiments, aliquots of the second crystals were collected by centrifugation and charcoal-treated as previously described (22). Following the charcoal treatment and extensive dialysis to remove ammonium sulfate, the ratio of absorbance of the protein at 280 nm to that at 260 nm ranged from 1.65 to 1.75, indicating removal of bound ATP (32). The concentration of phosphofructokinase was determined from the absorbance at 280 nm using $E_{280}^{nm}$ of 10 cm$^{-1}$. Molar concentrations of phosphofructokinase were calculated based on a subunit molecular weight of 80,000 (33). Dithioerythritol was then added routinely to a final concentration of 5 mM, except as indicated below, and the solution was stored at 4°C. Enzyme prepared in this manner had a specific activity of 105 units/mg at 25°C in the standard pH 8 ADP-coupled assay (22), and the behavior of the enzyme with respect to fluorescence titrations and regulatory kinetic properties was stable for at least 1 week.

All the binding studies to be described below were performed in 50 mM sodium phosphate, 0.2 mM dithioerythritol, buffers with 0.1 mM EDTA at pH 8.0 and 1 mM EDTA at pH 6.5 and pH 8.0, with other additions as indicated. The reported pH is corrected to the temperature of the particular experiment and it is the pH at that temperature ±0.03 pH unit.

Indirect ligand binding studies utilized titrations of intrinsic protein fluorescence to determine binding parameters (34). Fluorescence measurements were made using a fluorescence spectrophotometer. Excitation and emission wavelengths were 294 nm and 335 nm, respectively. The titrations were expressed as per cent of fluorescence change, given by

$$\% \text{Fluorescence change} = \left( \frac{F_i - F_0}{F_i} \right) \times 100$$

where $F_i$ is the corrected fluorescence (in arbitrary units) at ligand concentration $i$ and $F_0$ is the corrected fluorescence in the absence of added ligand. The fluorescence was corrected for dilution by multiplying the observed value by the ratio of the solution volume at ligand concentration $i$ to the volume in the absence of added ligand. Correction factors for inner filter effects (35) due to nucleotides were obtained from titrations of tryptophan fluorescence. This correction procedure assumed that there are no direct interactions between the nucleotides and the tryptophans.

The per cent fluorescence change was plotted as a function of total ligand concentration. Because the enzyme concentration (20 μg/ml, 0.25 μM, unless otherwise noted) was much smaller than the total dissociation constant in all cases, the difference between total and free ligand concentrations was negligible. For titrations with magnesium complexes of the nucleotide triphosphates, results were plotted as a function of the concentration of MgXTP$^-$, which was calculated as described in the miniprint supplement following this article.

For titrations between pH 8.0 and pH 6.9, charcoal-treated phosphofructokinase stored at pH 8.0 and 4°C was diluted directly into the fluorescence cuvette containing 3.0 ml of the appropriate 50 mM sodium phosphate buffer equilibrated to the temperature of the experiment. The solution was equilibrated for 8 min after addition of the protein before the titration was started. Titrations were performed by manual addition of small volumes of concentrated ligand solutions to the protein solution in the thermostated, continuously stirred cuvette, and they were normally completed within 30 min following addition of the protein. Kinetic measurements showed that the specific activity of the enzyme assayed at pH 8.0 did not change during the incubation or during the course of the titration, i.e. reversible pH-dependent inactivation of the enzyme, which involves dissociation to dimeric enzyme forms (9-14), does not occur under the conditions used for titrations of intrinsic protein fluorescence at pH 6.9. Therefore, the results represent titration of tetrameric enzyme forms.

For titrations at pH 6.0, phosphofructokinase was equilibrated to pH 6.0 by overnight dialysis at 4°C against 50 mM sodium phosphate, 1 mM EDTA, pH 6.0, 0.2 mM dithioerythritol. Determinations of the specific activity of this enzyme at pH 6.0 showed it to be 98% inactivated by this treatment. Because the enzyme is present as inactive dimers under these conditions (9-14), the results represent titrations of dimeric enzyme forms. At pH 6.0, however, titrations as described above were not possible because the enzyme was slowly adsorbed to the cuvette at the low protein concentration (3 μg/ml) required as a consequence of the high affinity for nucleotide binding. Each point then represents a single determination in which phosphofructokinase stored at pH 6.0 and 4°C was diluted into a cuvette containing a solution of the ligand of interest that was equilibrated to the temperature of the experiment. The fluorescence emission was then measured as described in the following sections of the text.

Direct ligand binding studies were performed by a sedimentation technique. One-milliliter solutions of phosphofructokinase and the ligand of interest in the appropriate 50 mM sodium phosphate buffer were prepared in 10-ml polycarbonate centrifuge tubes. Appropriate blanks without enzyme were prepared at the same time. The tubes containing the phosphofructokinase solutions were centrifuged for 21/2 h at 135,000 × g using a Beckman SW 27 swinging bucket rotor. Approximately 0.5 ml of the supernatant fluid was carefully removed. Free and total ligand concentrations were determined from measurements of the absorbance of the supernatant fluid and blanks, respectively. The concentration of bound ligand was given by the difference between the total and free ligand concentrations. Scatchard plots (36) of the data were used to obtain the apparent dissociation constants and number of ligand binding sites. Control experiments showed that the ligands were not adsorbed to the centrifuge tubes. Following the sedimentation, measurements of enzymatic activity showed negligible concentrations of enzyme in the supernatant fluid, indicating that human erythrocyte membranes did not reassociate the enzyme. Reassociation of the enzyme by trituration resulted in the recovery of 80% of the initial activity. Enzyme used in these direct binding studies was treated as described above (except that dithioerythritol was not added following dialysis) and was used for the direct binding studies within 1 day after the final dialysis.

Determinations of residual activity as a function of incubation pH were performed as described previously (16).

**Fluorescence emission curves were simulated using a Digital Equipment Corporation PDP-12/40 laboratory computer system (37).** FOCAL programs were written to calculate the per cent fluorescence change according to the relevant equation described below. Simulated curves and real data were simultaneously displayed on the computer oscilloscope. Parameters in the equations were varied to obtain the best fit as determined by visual comparison. Hard copies which were used in drawing the figures were obtained with an electrostatic printer/plotter manufactured by the Versatec Corp.

The concentration of ATP forms of different composition, net charge, and extent of protonation were calculated as described in the miniprint supplement following this article.

**RESULTS**

Studies of phosphofructokinase regulatory kinetic behavior to be described in the following paper (27) were performed at pH 6.9 in 0.05 mM phosphate buffers. The reasons for using phosphate buffer in the kinetic experiments have been discussed in detail elsewhere (25) and are also outlined briefly in the following paper (27). Clearly, it is best to perform the binding experiments under the same condition as the kinetic experiments. Since the enzyme undergoes a reversible time-dependent inactivation, as well as a concentration-dependent association (9-14), results obtained using methods which require long equilibration times or high enzyme concentrations would not reflect the binding properties of enzyme forms present in the kinetic assay (17). Using titrations of intrinsic protein fluorescence with the ligand of interest to obtain binding data accomplished this goal. This method is quite sensitive, requiring low protein concentrations (as little as 3 μg/ml), and titrations can be performed quickly. Importantly, as described under "Materials and Methods," reversible pH-dependent inactivation does not occur under the conditions used for the titrations.

**Fluorescence Titrations at pH 6.9 and 25°C—Results of titrations of phosphofructokinase intrinsic protein fluorescence with "ATP" and MgATP at pH 6.9 and 25°C in**

"ATP," as calculated in the miniprint supplement, represents all the forms of ATP not formed in a complex with magnesium, i.e. ATP$^+$, HATP$^-$, NaATP$^-$, KATP$^-$, while "GTP" represents all the forms of GTP not complexed with magnesium. Indication of the formal change of various complex is omitted in these discussions.
phosphate buffers are shown in Fig. 1. Under these conditions, binding of “ATP” quenches the fluorescence. The solid line through these data points was calculated based on the assumption of a single class of “ATP” binding sites with an apparent dissociation constant of 14 nM and maximum quenching of 19%. Low concentrations of binding sites with an apparent dissociation constant of 100 μM, MgGTP; ApA, “ATP”; &---A, “GTP.” Solid lines through the data points for “ATP” and “GTP” were calculated as described in the text. Magnesium nucleotide concentrations calculated as 87% of total nucleotide concentration.

Fig. 1. Nucleoside triphosphate dependence of change in phosphofructokinase fluorescence. Phosphofructokinase (20 μg/ml) fluorescence in 50 mM sodium phosphate, 1 mM EDTA, 0.2 mM dithioerythritol, 25 mM potassium chloride with and without 10 mM magnesium chloride at pH 6.9 and 25°C was titrated with ATP or GTP as described under “Materials and Methods.” ○—○, MgATP; ○—•, MgGTP; |—•, “ATP”; ▲—▲, “GTP.” Solid lines through the data points for “ATP” and “GTP” were calculated as described in the text. Magnesium nucleotide concentrations calculated as 87% of total nucleotide concentration.

Table I

<table>
<thead>
<tr>
<th>Nucleotide*</th>
<th>pH</th>
<th>Temperature °C</th>
<th>Quenching Enhancement</th>
<th>$K_i$ from fluorescence titrations μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP</td>
<td>8.0</td>
<td>20-25</td>
<td>&gt;1000</td>
<td>N.O.</td>
</tr>
<tr>
<td>+1-Deoxy Fr1-P (0.5 mM)</td>
<td>6.9</td>
<td>25</td>
<td>150</td>
<td>10</td>
</tr>
<tr>
<td>+cAMP (0.15 mM)</td>
<td>6.9</td>
<td>25</td>
<td>800</td>
<td>N.O.</td>
</tr>
<tr>
<td>+Fru-1,6-dP (0.5 mM)</td>
<td>6.9</td>
<td>25</td>
<td>&gt;500</td>
<td>N.O.</td>
</tr>
<tr>
<td>+5’-FSO2BzAdo</td>
<td>6.9</td>
<td>25</td>
<td>800</td>
<td>N.O.</td>
</tr>
<tr>
<td>+5’-FSO2BzAdo</td>
<td>6.4</td>
<td>25</td>
<td>150</td>
<td>10</td>
</tr>
<tr>
<td>+5’-FSO2BzAdo</td>
<td>6.0</td>
<td>15</td>
<td>5</td>
<td>N.O.</td>
</tr>
<tr>
<td>“ATP”</td>
<td>8.0</td>
<td>25</td>
<td>2000</td>
<td>N.O.</td>
</tr>
<tr>
<td>+NH4 (9 mM)</td>
<td>6.9</td>
<td>25</td>
<td>14</td>
<td>N.O.</td>
</tr>
<tr>
<td>+Citrate (0.25 mM)</td>
<td>6.9</td>
<td>25</td>
<td>140</td>
<td>N.O.</td>
</tr>
<tr>
<td>+Fru-6-P (2 mM)</td>
<td>6.9</td>
<td>25</td>
<td>140</td>
<td>N.O.</td>
</tr>
<tr>
<td>+AMP (0.25 mM)</td>
<td>6.9</td>
<td>25</td>
<td>90</td>
<td>N.O.</td>
</tr>
<tr>
<td>+Creatine phosphate (20 mM)</td>
<td>6.9</td>
<td>25</td>
<td>8</td>
<td>N.O.</td>
</tr>
<tr>
<td>+5’-FSO2BzAdo</td>
<td>6.9</td>
<td>25</td>
<td>100</td>
<td>N.O.</td>
</tr>
<tr>
<td>+5’-FSO2BzAdo</td>
<td>6.4</td>
<td>25</td>
<td>20</td>
<td>N.O.</td>
</tr>
<tr>
<td>+5’-FSO2BzAdo</td>
<td>6.0</td>
<td>25</td>
<td>0.8</td>
<td>N.O.</td>
</tr>
</tbody>
</table>

| MgAMP-PNP   | 8.0 | 20         | ~1000                  | 0.5*                              |
| +Fru-6-P (1 mM) | 6.9 | 25         | 100                    | 0.6                               |
| AMP-PNP     | 6.0 | 20         | N.O.                   | N.O.                               |
| MgGTP       | 8.0 | 20         | >1000                  | 10                                 |

* Where magnesium was included, 1 mM magnesium acetate was added at pH 8.0 while 10 mM magnesium chloride was added at pH 6.9 and pH 6.0. At pH 8.0, 0.5 mM ammonium chloride was also added.

"Apparent dissociation constant determined from Scatchard plots when enhancement was not observed. When enhancement was observed, the following equation was used:

\[
% \text{Fluorescence change} = \frac{Q_1 \frac{[\text{MgATP}]}{K_1} + Q_2 \frac{[\text{MgATP}]}{K_2} + Q_3 \frac{[\text{MgATP}]}{K_3}}{1 + \frac{[\text{MgATP}]}{K_1} + \frac{[\text{MgATP}]}{K_2} + \frac{[\text{MgATP}]}{K_3}}
\]

where $Q_1$ is the maximum enhancement due to binding at the active site, $Q_2$ is the maximum quenching due to binding at the inhibitory site, and $Q_3$ is the quenching observed when both sites are occupied. $K_1$, $K_2$, and $K_3$ are the dissociation constants for binding at the two sites.

4 D. W. Pettigrew and C. Frieden, unpublished experiments.

affinity for binding of both MgATP (Fig. 2A) and “ATP” (Fig. 2B) to the inhibitory site as well as the apparent affinity to binding of MgATP to the active site. Fig. 3 shows effects of pH on binding of “ATP” to the inhibitory site. Decreasing the pH increases the apparent affinity.

The solid lines through the data points in Figs. 2B and 3...
the data points were calculated as described in the text. Chloride at pH 6.9 was titrated with ATP as described under "Materials and Methods." MgATP concentrations calculated as 87% of total ATP concentration. (A) M, 15°C; A---A, 25°C; A---A, 35°C.

Phosphofructokinase fluorescence. Phosphofructokinase (20 pg/ml) fluorescence in 50 mM sodium phosphate, 1 mM EDTA, 0.2 mM dithioerythritol, 25 mM potassium chloride, with (A) or without (B) 10 mM magnesium chloride at pH 6.9 was titrated with ATP as described under "Materials and Methods." MgATP concentrations calculated as 87% of total ATP concentration. (A) ○—O, 15°C; △—△, 25°C; △—△, 35°C. (B) ●—●, 18°C; ○—O, 25°C; △—△, 35°C; solid lines through the data points were calculated as described in the text.

FIG. 2. Temperature dependence of ATP effects on phosphofructokinase fluorescence. Phosphofructokinase (20 pg/ml) fluorescence in 50 mM sodium phosphate, 1 mM EDTA, 0.2 mM dithioerythritol, 25 mM potassium chloride, with (A) or without (B) 10 mM magnesium chloride at pH 6.9 was titrated with ATP as described under "Materials and Methods." MgATP concentrations calculated as 87% of total ATP concentration. (A) ○—O, 15°C; △—△, 25°C; △—△, 35°C. (B) ●—●, 18°C; ○—O, 25°C; △—△, 35°C; solid lines through the data points were calculated as described in the text.

were calculated as described in Table II, which summarizes the temperature and pH dependence of the apparent affinity for binding of "ATP" to the inhibitory site. The table shows that at pH 6.9, the apparent dissociation constant changes from 76 μM at 35°C to 2.5 μM at 15°C; and, at 25°C, it changes from about 2 mM at pH 8.0 to 0.8 μM at pH 6.0. At pH 6.9 and various temperatures, Scatchard plots of the data are linear. At higher pH values, such plots show a slight curvature indicative of negative cooperativity. The reasons for this are not clear, but do not affect the conclusions.

The results summarized in Table II also show the apparent affinity for "ATP" binding is markedly temperature-dependent at pH 6.0 and at pH 8.0, as well as at pH 6.9 as described above. The standard enthalpy change for the dissociation reaction calculated from the van't Hoff equation shows that in the range from pH 6.0 to pH 8.0, binding of "ATP" involves large enthalpy changes (25 to 30 kcal/mol).

The pH dependence for MgATP binding, as determined by fluorescence titrations, is analogous to that for "ATP" but the situation is somewhat more complex since both fluorescence quenching and enhancement can occur. Results summarized in Table I show that the affinity for binding at the inhibitory site decreases as the pH is raised. At pH 6.0 and 15°C, the apparent dissociation constant is 5 μM, while at pH 8.0 and 25°C, a value of over 1 mM is obtained. Fluorescence enhancement is not observed in titrations at pH 6.0 or pH 8.0.

The apparent low affinity for MgATP at pH 8.0 and 25°C is not consistent with the affinity for the active site determined from results of steady state kinetic studies performed under these conditions. Results of initial velocity studies at pH 8.0 and 25°C described in the following paper (27) are consistent with a random substrate addition rapid equilibrium mechanism, and an apparent dissociation constant for MgATP of 10 μM is obtained from double reciprocal plots (38). Direct binding studies were undertaken to determine whether there is high affinity binding of MgATP at pH 8 but were complicated by the intrinsic MgATPase activity of the enzyme (7, 39). This complication was avoided by using AMP-PNP, the imido analog of ATP, which is not cleaved by phosphofructokinase.

\[ \% \text{Fluorescence change} = \frac{Q \left[ \text{ATP} \right]}{\left[ \text{ATP} \right] + K_d} \]

where \( K_d \) is the apparent dissociation constant and \( Q \) is the maximum quenching observed at saturation of the "ATP" sites.

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature</th>
<th>( K_d )</th>
<th>%Q</th>
<th>( \Delta H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>25</td>
<td>2000</td>
<td>59</td>
<td>29</td>
</tr>
<tr>
<td>7.5</td>
<td>25</td>
<td>400</td>
<td>96</td>
<td>29</td>
</tr>
<tr>
<td>6.9</td>
<td>35</td>
<td>76</td>
<td>19</td>
<td>32</td>
</tr>
<tr>
<td>6.0</td>
<td>35</td>
<td>5</td>
<td>19</td>
<td>26</td>
</tr>
</tbody>
</table>

* Standard enthalpy change for dissociation calculated using the van't Hoff equation.

1 The number of MgATP binding sites per subunit varied between 1.5 and 4 depending on the method used to determine ATP concentrations. This variable result is due to the intrinsic MgATPase activity under the conditions used for direct binding (pH 8.0, 20°C, 3 h, 10 μM enzyme). The ADP produced will bind to the enzyme, thus complicating experiments based on measurements of "free" ATP. Conditions used for fluorescence titrations (30 min, 0.25 mM enzyme) are such that any MgATPase activity is so low as not to be a complication.
Initial velocity studies at pH 8.0 and 25°C using the assay described in the following paper (27) showed MgAMP-PNP to be a competitive inhibitor with respect to MgATP with an apparent $K_i$ of 10 μM, which agrees well with other reports (6, 40). Results of direct binding studies summarized in Table III show that at pH 8.0 and 20°C, MgAMP-PNP binds to one high affinity site per subunit. Fluorescence titrations under the same conditions (except for protein concentration) showed the fluorescence is not affected by MgAMP-PNP up to a concentration of 800 μM (Table I), which is consistent with the lower affinity for MgAMP-PNP binding at the inhibitory site. Thus, binding of MgAMP-PNP or MgATP at the active site does not affect the fluorescence observed at pH 8.0. Identical results were obtained for MgGTP. Direct binding studies with MgGTP (Table III) show one high affinity site per subunit, while the data of Table I show that this binding does not affect the fluorescence.

Stoichiometry of “ATP” Binding—At pH 6.0 or at pH 6.9 and lower temperatures, the apparent dissociation constant for “ATP” (Table II) is small enough to allow direct determinations of binding stoichiometry from fluorescence titrations at high enzyme concentrations. Titrations with “ATP” were performed under the following conditions: pH 6.0, 15°C, 1 μM enzyme; pH 6.9, 15°C, 2.5 μM enzyme; pH 6.9, 4°C, 5 μM enzyme. Results of these titrations showed that the observed quenching of fluorescence is due to binding of 1 “ATP” per enzyme subunit. Binding of “ATP” to the active site is apparently too weak to be observed and thus does not complicate stoichiometry determinations.

Effect of Specific Chemical Modification on Fluorescence Titrations with Nucleotides—Results of titrations with ligands which relieve ATP inhibition (e.g. cAMP, Fru-1,6-diphosphate) showed that they quench the fluorescence to only a small extent. For example, it was previously reported (22) that cAMP binding to a high affinity site resulted in only a 3 to 4% quench. On the other hand, results summarized in Table I show that in the presence of saturating concentrations of these ligands, the apparent affinity for binding of “ATP” or MgATP at the inhibitory site is decreased. The fluorescence enhancement is not observed with MgATP in the presence of these ligands, indicating that they have the same effect on the fluorescence titration curves as do changes in pH or temperature. Titrations at pH 6.9 and 25°C in the presence of 9 mM NH₄Cl or 250 μM citrate show that the apparent affinity for “ATP” or MgATP binding to the inhibitory site is the same as in the absence of these ligands. Creatine phosphate, which potentiates ATP inhibition (41, 42), increases the affinity for binding of “ATP” to the inhibitory site (Table I).

Effect of Specific Chemical Modification on Fluorescence Titrations with Nucleotides—Results of titrations with ligands which relieve ATP inhibition (e.g. cAMP, Fru-1,6-diphosphate) showed that they quench the fluorescence to only a small extent. For example, it was previously reported (22) that cAMP binding to a high affinity site resulted in only a 3 to 4% quench. On the other hand, results summarized in Table I show that in the presence of saturating concentrations of these ligands, the apparent affinity for binding of “ATP” or MgATP at the inhibitory site is decreased. The fluorescence enhancement is not observed with MgATP in the presence of these ligands, indicating that they have the same effect on the fluorescence titration curves as do changes in pH or temperature. Titrations at pH 6.9 and 25°C in the presence of 9 mM NH₄Cl or 250 μM citrate show that the apparent affinity for “ATP” or MgATP binding to the inhibitory site is the same as in the absence of these ligands. Creatine phosphate, which potentiates ATP inhibition (41, 42), increases the affinity for binding of “ATP” to the inhibitory site (Table I).

Effect of Citrate on Fru-6-P Binding—Because citrate did not alter the affinity for binding of “ATP” or MgATP at pH 6.9 and 25°C (Table I), it was of interest to examine the effect of citrate on the affinity for Fru-6-P. Fluorescence titrations with Fru-6-P showed only a small extent of quenching, which is consistent with other reports (6), and reliable estimates of the apparent dissociation constant could not be obtained. However, the fluorescence enhancement observed in titrations with MgAMP-PNP is abolished by Fru-6-P (Table I). Using this change, it was found that at pH 6.9 and 25°C the apparent dissociation constant for Fru-6-P is increased about 2-fold in the presence of 250 μM citrate (35 μM versus 75 μM).

### DISCUSSION

Types and Specificity of Nucleotide Sites—Results of titrations of phosphofructokinase fluorescence at pH 6.9 are consistent with two types of MgATP binding sites. Binding of MgATP to one enhances the fluorescence while binding to the other quenches it. Since only enhancement is observed in titrations with MgGTP, which is a substrate but not an inhibitor, it can be concluded that the enhancement is associated with binding at the active site while quenching represents binding to the inhibitory site. The pH, temperature, and effector dependence of nucleotide binding measured by fluorescence quenching are entirely consistent with this conclusion. For example, direct binding studies at pH 8.0 show that the magnesium complexes of the nucleoside triphosphates bind to a single high affinity site per subunit, while quenching of the fluorescence indicates very low affinity for these magnesium complexes, consistent with the greatly decreased ex-

### TABLE III

Summary of determinations of nucleotide binding sites other than the inhibitory site

<table>
<thead>
<tr>
<th>Nucleotide*</th>
<th>pH</th>
<th>$K_i$ (μM)</th>
<th>Sites/subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP</td>
<td>8.0</td>
<td>See text</td>
<td></td>
</tr>
<tr>
<td>MgAMP-PNP</td>
<td>8.0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>cAMP</td>
<td>6.9</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>MgGTP</td>
<td>8.0</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

*Where magnesium was included, 1 mM magnesium acetate was added at pH 8.0 while 10 mM magnesium chloride was added at pH 6.9. 0.5 mM ammonium chloride was added at pH 6.0.

**Apparent dissociation constant and number of binding sites were obtained from Scatchard plots (36).**

The substrate analog 1-deoxy-Fru-6-P was used to examine interactions between substrates as measured by fluorescence titrations. 1-Deoxy-Fru-6-P was shown to be a competitive inhibitor with respect to Fru-6-P with an apparent $K_i$ of 110 μM, which agrees well with other reports (29). Results summarized in Table I show that, at pH 6.9 and 25°C, 1-deoxy-Fru-6-P decreases the affinity for MgATP binding to the inhibitory site by about 6-fold. A similar decrease in affinity for “ATP” binding to the inhibitory site is observed in the presence of Fru-6-P (Table I).

Effect of Specific Chemical Modification on Fluorescence Titrations with Nucleotides—It was found previously that the apparent affinity for “ATP” at pH 6.9 is decreased by chemical modification of the enzyme with 5'-FSO.BzAdo (22). This affinity label modifies the enzyme at an activator site specific for AMP, cAMP, and ADP. The apparent $pK$ obtained from plots of the pH dependence of the residual activity (16) at 25°C is lowered from 6.9 to 6.4 by the modification. Results of titrations of modified enzyme at pH 6.9 summarized in Table I support the previous conclusion that the modification is equivalent to irreversible binding of cAMP, AMP, or ADP since the same behavior is obtained for modified enzyme as for native enzyme in the presence of these ligands, i.e. the apparent affinity for “ATP” and MgATP binding to the inhibitory site is decreased. However, at pH 6.4, the affinity of modified enzyme for “ATP” or MgATP is the same as that of native enzyme at pH 6.9 (Table I). The extent of quenching and enhancement is somewhat reduced for the modified enzyme at pH 6.4 which may reflect some pH dependence of the quenching processes, or some structural changes from the modification unrelated to the $pK$ shift. However, relative to the apparent $pK$ obtained from plots of the pH dependence of the residual activity, the apparent affinities of native and modified enzyme for binding “ATP” or MgATP are the same. This result strongly suggests that the apparent affinities for “ATP” and MgATP can be correlated with the $pK$ of specific ionizable groups.

### DISCUSSION

Types and Specificity of Nucleotide Sites—Results of titrations of phosphofructokinase fluorescence at pH 6.9 are consistent with two types of MgATP binding sites. Binding of MgATP to one enhances the fluorescence while binding to the other quenches it. Since only enhancement is observed in titrations with MgGTP, which is a substrate but not an inhibitor, it can be concluded that the enhancement is associated with binding at the active site while quenching represents binding to the inhibitory site. The pH, temperature, and effector dependence of nucleotide binding measured by fluorescence quenching are entirely consistent with this conclusion. For example, direct binding studies at pH 8.0 show that the magnesium complexes of the nucleoside triphosphates bind to a single high affinity site per subunit, while quenching of the fluorescence indicates very low affinity for these magnesium complexes, consistent with the greatly decreased ex-
tent of ATP inhibition observed at pH 8.0.

Previous results involving chemical modification by 5'-FSO2BzAdo have shown that in addition to the two types of sites for “ATP” and MgATP, there is a third site for the activators cAMP, AMP, and ADP, and that this site does not appear to bind “ATP” or MgATP (22). In terms of kinetic properties, the three types of sites can be identified as an active site, an inhibitory site, and an activator site.

It must be emphasized that either “ATP” or MgATP can be bound at the inhibitory site or the active site and are therefore competitive with respect to one another. Simulation of titration curves at pH 6.9 shows that the affinity for binding of “ATP” to the inhibitory site is about 10 times greater than that for binding MgATP (see below), while MgATP binds much greater affinity than “ATP” to the active site.4 However, the form of ATP responsible for inhibition depends on the pH, temperature, and total magnesium concentration used for kinetic studies. Thus, at the high magnesium concentrations typically used in kinetic studies near pH 7, the primary inhibitory form is MgATP (see miniprint), particularly under kinetic conditions where the apparent dissociation constant for the inhibitory form is MgATP (see miniprint), particularly under conditions where the apparent dissociation constant for "ATP" is greater than about 10 μM (e.g. pH 6.9, 25°C). But, under conditions where this apparent dissociation constant is small (e.g. lower temperature, Table II), inhibition by "ATP" could become more significant, as it also will at lower magnesium concentrations. Therefore, in the development of models for phosphofructokinase regulatory kinetic behavior, it is necessary to take binding of both "ATP" and MgATP to the inhibitory site into account.

Results described above showed that fluorescence quenching by "ATP" could be described by assuming the existence of a single type of binding site, the inhibitory site (Table II). For MgATP binding at pH 6.9 (Fig. 2A), in which there is both enhancement and quenching of the fluorescence, the situation is more complex. There are 10 tryptophans/subunit (43), and the observed fluorescence probably contains contributions from them all. The relationship between the emission from a given tryptophan residue and binding of MgATP at the two sites described here is unknown. Furthermore, binding of "ATP" will be quite significant at 15°C (Table II), and the relationship between the observed fluorescence and binding of both "ATP" and MgATP is unknown. Given these complications, quantitative description of titration curves for MgATP is uncertain. Nevertheless, curves simulated on the basis of the assumption that there are two binding sites per subunit can be fitted to the data and are consistent with about 10-fold lower affinity for MgATP binding to the inhibitory site than for "ATP" (Table I). While it is clear that the fluorescence enhancement observed in titrations with MgATP is associated with binding at the active site, the relationship between the true affinity for binding and the apparent affinity as indicated by the enhancement is not clear. Enhancement is not observed at pH 8.0, where direct binding studies show the presence of a high affinity MgATP binding site. Thus, the temperature dependence of the enhancement observed in Fig. 2A may not reflect temperature dependence of the binding of MgATP at the active site.

A Model for Nucleotide Binding to the Inhibitory Site—Effects of temperature, pH, and activating ligands with respect to the apparent affinity for nucleotide binding to the inhibitory site can be explained within the context of the mechanism proposed (15) for the pH-dependent characteristics of phosphofructokinase. Thus, highly cooperative protonation of specific ionizable groups defines enzyme forms with different affinities for MgATP or "ATP" binding at the inhibitory site. Except at pH 6.0, where dissociation of the enzyme to dimers has occurred (9-14), the binding of nucleotides is considered to be to tetrameric enzyme forms. A quantitative correlation between the pK of these ionizable groups and the apparent affinity for "ATP" binding at the inhibitory site is based on the following assumptions: 1) "ATP" binds exclusively to the protonated enzyme form and the binding is noncooperative; 2) the protonation of two identical ionizable groups per subunit is infinitely cooperative; 3) the pK of the ionizable groups is given by the apparent pK observed in plots of the pH dependence of the residual activity (specific activity following incubation in a series of phosphate buffers at different pH), and varicos with temperature according to $\Delta H_{\text{act}} = -9 \text{ kcal/mol}$ (16).

Based on these assumptions, the following expression for the "ATP" dependence of the change in fluorescence can be derived by rapid equilibrium methods (44):

$$\text{Fluorescence change} = \frac{Q[ATP]}{[ATP] + K_a(1 + (K_a[H^+])^n)}$$

where $Q$ is the per cent of fluorescence change obtained at saturation of the "ATP" binding sites, $K_a$ is the ionization constant for the specific groups, and $K_d$ is the intrinsic dissociation constant for "ATP" binding. The solid lines through the data points in Figs. 2B and 3 were calculated with Equation 1 using the parameter values tabulated in Table IV. It should be noted that, in the range pH 6.9 to pH 8.0, the different values for the intrinsic dissociation constant, $K_a$, used to calculate the curves vary only about 2-fold. Given the enormous pH and temperature dependence of the apparent affinity (Table II), agreement within a factor of 2 is quite satisfactory. It should be clear that, with respect to binding of "ATP" at the inhibitory site, a change of pH is equivalent to a change of temperature. The large change in $K_a$ between 6.9 and 6.0 may reflect dependence of the apparent affinity on the association state of the enzyme (tetramer versus dimer).

The pH and temperature dependence of $K_d$ obviously depend on the number of ionizable groups assumed and the relation assumed between the pK of those groups and the pK observed in plots of residual activity versus pH. For example, the intrinsic dissociation constant, $K_d$, may be temperature-dependent. This is true for mammalian hemoglobins where the apparent heat of oxygenation contains two components: heat of ionization of specific groups, and heat of oxygenation proper (45). Assumption of two identical ionizable groups for "ATP" binding to phosphofructokinase is based on the pH dependence of the apparent dissociation constant (45, 46). This assumption accounts for about two-thirds of the observed enthalpy change (Table II) in terms of the heats of ionization of those groups and, thus, allows for heat of "ATP" binding.

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature °C</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
<th>$K_d$</th>
<th>pH</th>
<th>Temperature °C</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>25</td>
<td>6.90</td>
<td>15</td>
<td>6.9</td>
<td>30</td>
<td>6.67</td>
<td>18</td>
</tr>
<tr>
<td>7.5</td>
<td>25</td>
<td>6.90</td>
<td>23</td>
<td>6.9</td>
<td>30</td>
<td>6.67</td>
<td>18</td>
</tr>
<tr>
<td>6.9</td>
<td>25</td>
<td>6.90</td>
<td>7</td>
<td>6.0</td>
<td>30</td>
<td>6.90</td>
<td>0.7</td>
</tr>
</tbody>
</table>

$^a$ The value of the pK at 25°C is that determined from residual activity plots, pK values at other temperatures calculated assuming $\Delta H_{\text{act}} = -9 \text{ kcal/mol}$ (16).

$^b$ $K_d$ is the "intrinsic" dissociation constant.

Table IV
Parameters used to simulate "ATP" titration curves

Fluorescence titration curves in Figs. 2B and 3 were simulated using Equation 1 and parameter values tabulated below. Values of $Q$ were the same as listed in Table II.
proper. But, the actual partitioning of the observed enthalpy change between ionization of specific groups and “ATP” binding proper is unknown. For the model described above, it was assumed that the pK of the specific groups, and the temperature dependence of that pK, are those observed in plots of residual activity versus pH. However, because that apparent pK reflects equilibria between dimeric and tetrameric enzyme forms (14), neither it, nor its temperature dependence, may be the same as that for those forms controlling the equilibrium between tetrameric enzyme forms. Furthermore, lack of identity between the true pK and the apparent pK means that the apparent dissociation constant for “ATP” binding calculated on the basis of observed shifts of the apparent pK may differ from that obtained from fluorescence titrations.

The converse of the correlation between the apparent pK and affinity for “ATP” binding at the inhibitory site is that ligands which do not change the apparent affinity do not shift the apparent pK. Citrate and NH₄⁺ did not alter binding of “ATP” or MgATP (Table I), and NH₄⁺ does not shift the apparent pK, while citrate does (16, 17). This probably reflects the fact that citrate binds preferentially to dimeric enzyme forms (9). Thus, the apparent pK observed in the presence of ligands may reflect differential binding of the ligands to tetrameric and dimeric forms (17) and not the effect of the ligand with respect to direct displacements of the equilibrium between protonated and unprotonated tetrameric enzyme forms. Furthermore, the stabilization of dimeric enzyme forms by citrate is the most likely explanation for the difference between these results and other reports (4, 5, 7) with respect to the effect of citrate increasing the affinity for ATP or MgAMP-PNP. It was shown above that the affinity for binding of “ATP” or MgATP to the dimeric forms is very high (Table II). In other reports (4, 5, 7), results were obtained using methods requiring long equilibration times and high protein concentrations, and the enzyme used was typically exposed to a pH of about 7 in the cold for long periods of time. It has been pointed out (17) that studies performed under such conditions may be complicated by the concentration-dependent aggregation and the pH-dependent inactivation of the enzyme (9-14).

Comparison to Results of Others—Results of the binding studies described in this paper may be compared with results reported by other investigators. Based on studies of MgAMP-PNP binding properties of phosphofructokinase at pH 7 and 5°C using a forced dialysis technique, Wolfman et al. (5) recently reported two binding sites per enzyme subunit. They concluded that a high affinity site is the active site while a low affinity site is the inhibitory site. This conclusion is in complete agreement with that reached in the binding studies described in this paper.

Based on studies of fluorescence quenching and polarization at pH 7.0 and 20°C, Liou and Anderson (8) reported three high affinity binding sites per enzyme subunit for both MgATP and Mg₃ATP. This finding is inconsistent with the report of Wolfman et al. (5) and with the findings described in this paper. In the studies reported by Liou and Anderson, enzyme concentrations of 10 to 19 μM were used. Results described earlier in this paper show that the intrinsic MgATPase (and presumably Mg₃ATPase, since it is a substrate (47)) activity of phosphofructokinase may be a complication under these conditions. Thus, one of the high affinity sites reported by Liou and Anderson may in fact represent binding of ADP, or eADP, to the adenine nucleotide activator site.

Kemp and Krebs (4) reported three high affinity “ATP” binding sites on the basis of extensive gel filtration binding studies at pH 6.95 and 23°C. The conclusion of a single high affinity “ATP” binding site per subunit described above is based on results of fluorescence quenching under conditions where the dissociation constant is quite small (0.3 to 2.5 μM) i.e. low temperature and pH (Table II). Binding sites with dissociation constants on the order of 10 μM may not be detected by this method. Results described in the following paper (27) show that only one “ATP” binding site, the inhibitory site, is required for the quantitative description of phosphofructokinase regulatory kinetic behavior. Thus, the role of other “ATP” binding sites, should they exist, with respect to kinetic behavior is unclear. Other “ATP” binding sites may reflect overlapping specificities of the adenine nucleotide binding sites which are observed only in binding studies and have, in fact, no kinetic role.

Jones et al. (48, 49) have reported results of studies of nucleotide binding to phosphofructokinase using resonance techniques. The dissociation constants for binding of “ATP,” MgATP, or MnATP obtained by these methods are quite high relative to constants derived from kinetic studies. Especially in the case of the spin-labeled enzyme, it is tempting to speculate that these techniques are monitoring binding to the inhibitory site, and the environment of the spin label is particularly sensitive to binding at the inhibitory site in a manner analogous to the sensitivity of the observed fluorescence to binding at the inhibitory site. However, prolonged exposure of the enzyme to lower pH in different buffers and the high protein concentrations required by these techniques precludes direct comparison with the results of the studies described here (17).

Acknowledgment—We thank Helen R. Gilbert for her excellent technical assistance.

REFERENCES
Phosphofructokinase: Ligand Binding Properties


Additional references are found on p. 1896.
Supplementary Material

"The Binding of Regulatory Ligands to Rabbit Muscle Phosphofructokinase" by
H. M. Perlmann and G. F. Fruton

In the presence of magnesium and near neutral pH (the range of interest with respect to phosphofructokinase), ATP is stoichiometrically converted to several forms of ATP in rat liver extract with little difference in composition, degree of protection, and rate of formation. The kinetic and stoichiometric properties of phosphofructokinase may differ with respect to different forms of ATP. However, the presence of ATP is a prerequisite for all reactions. For the enzyme from rat liver, ATP has been reported to be a substrate for the yeast enzyme (1), but it is essential to determine the concentrations of the different forms of ATP present in different experimental conditions in attempts to quantitatively describe the changes in the kinetic parameters. Table 1.2 shows the conditions used in these studies of phosphofructokinase kinetic and ATP binding behavior. It is described below.

The system of simultaneous equations used in the calculation of kinetic studies of ATP under the conditions of these studies is shown in Table 1.2. Table 1.2, which shows three types of concentrations for different ATPs, lists the conditions used in these studies of phosphofructokinase kinetic and ATP binding behavior. The system of equations is shown in Table 1.2, which lists the conditions used in these studies of phosphofructokinase kinetic and ATP binding behavior.

Table 1.2. Concentrations of Different Forms of ATP Present Under Different Conditions of pH and Temperature

<table>
<thead>
<tr>
<th>pH</th>
<th>1.5</th>
<th>6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ATP]</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>[ADP]</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>[AMP]</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

The concentrations of different forms of ATP were calculated using the method of Liou and Corbett-Bowers (1) and are expressed as percent of the total ATP concentration. Table 1.2 shows the conditions used in these studies of phosphofructokinase kinetic and ATP binding behavior. The system of equations is shown in Table 1.2, which lists the conditions used in these studies of phosphofructokinase kinetic and ATP binding behavior.

Table 1.2. Concentrations of Different Forms of ATP Present Under Different Conditions of pH and Temperature

<table>
<thead>
<tr>
<th>pH</th>
<th>1.5</th>
<th>6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ATP]</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>[ADP]</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>[AMP]</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

References
Binding of regulatory ligands to rabbit muscle phosphofructokinase. A model for nucleotide binding as a function of temperature and pH.  
D W Pettigrew and C Frieden  


Access the most updated version of this article at http://www.jbc.org/content/254/6/1887

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/6/1887.full.html#ref-list-1