Determination of the Free-Energy Coupling between ATP and an Affinity Label Attached to Rabbit Muscle Phosphofructokinase†

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Received June 22, 1984

ABSTRACT: The smallest enzymatically active form of rabbit muscle phosphofructokinase is a tetramer of four identical or nearly identical monomers. The enzyme is inhibited by ATP, and this inhibition by ATP is relieved by the activating adenine nucleotides adenosine cyclic 3′,5′-phosphate, AMP, and ADP. Each monomer contains one binding site specific for the inhibitor ATP and another site specific for the activating adenine nucleotides. The enzyme can also be activated by covalently labeling the activating adenine nucleotide binding sites with the affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine. These activator binding sites on the enzyme have been covalently labeled to various degrees, ranging from an average value of less than one label per tetramer to four labels per tetramer, and the free-energy coupling, ΔG_{xy}, between the covalently bound affinity label and ATP binding at the inhibitory site was determined. For enzyme preparations containing four labels per tetramer, ΔG_{xy} is approximately 1 kcal/mol at pH 6.95 and 25 °C. A very significant free-energy coupling is observed in those preparations containing an average of one label per tetramer and less, and the change in ΔG_{xy} in going from native tetramers to ones containing an average of two labels per tetramer is twice as great as the change in ΔG_{xy} observed in going from tetramers containing an average of two labels per tetramer to ones containing four labels per tetramer, suggesting that modification of the final two monomers in the tetramer contributes much less to the antagonistic effect on ATP binding than does modification of the first two monomers in the tetramer.

The smallest enzymatically active species of rabbit muscle phosphofructokinase (EC 2.7.1.11) is a tetramer comprised of identical or nearly identical monomers of M_r 80 000 (Paetkau & Lardy, 1967; Pavelich & Hammes, 1973; Aaronson & Frieden, 1972; Lad et al., 1973). The enzyme is subject to allosteric regulation by a number of metabolites including the adenine nucleotides. ATP, a substrate of the enzyme, is also an allosteric inhibitor of the enzyme (Lardy & Parks, 1956), and the inhibition produced by ATP is relieved by the activating adenine nucleotides ADP, AMP, and adenosine cyclic 3′,5′-phosphate (cAMP).[1] Each monomer appears to contain an inhibitory allosteric binding site for ATP (Lardy & Parks, 1956; Hofer & Pette, 1968; Kemp, 1969; Wolfman et al., 1978; Pettigrew & Frieden, 1979) and an allosteric binding site specific for the activating adenine nucleotides (Passonneau & Lowry, 1962; Kemp & Krebs, 1967). The activating adenine nucleotide binding site can be specifically labeled by 5′-FSO_2BzAdo, and the covalent attachment of the affinity label to this site results in a relief of inhibition by ATP similar to that produced by the noncovalent binding of cAMP to the site (Pettigrew & Frieden, 1978; Ogilvie, 1983). The activating adenine nucleotide binding site of sheep heart PFK also undergoes specific labeling by 5′-FSO_2BzAdo (Mansour & Colman, 1978), and the site of modification is a lysine residue (Weng et al., 1980).

In a previous study (Ogilvie, 1983), 5′-FSO_2BzAdo was employed to specifically label the activating adenine nucleotide binding sites of PFK to various degrees, ranging from an average value of less than one label per tetramer to four labels per tetramer, and the effect of the extent of modification of the enzyme on the inhibition of the enzyme by ATP was investigated. In that study, the percent inhibition produced by ATP and the percent inhibition relieved by affinity labeling the enzyme were calculated by comparing the specific enzymatic activity at 0.15 mM ATP, where native PFK activity is near maximum, with the enzymatic activity at 1.2 mM ATP, where native PFK is essentially totally inhibited. From these results and others presented therein, it was concluded that activation of the enzyme as so defined, i.e., total relief of the inhibition produced by 1.2 mM ATP, required that approximately two of the four activating adenine nucleotide binding sites on each tetramer be affinity labeled by 5′-FSO_2BzAdo. An investigation of the effects of ATP concentrations in excess of 1.2 mM was not conducted at that time; hence, a thermodynamic characterization of the interaction between the affinity label covalently bound at the activator binding site and ATP binding at the inhibitory binding site was not possible. To rectify this shortcoming in the previous study, the effect of higher concentrations of ATP on affinity-labeled PFK preparations has been investigated, and the results obtained are reported herein.

Materials and Methods

Materials. All enzymes, substrates, nucleotides, and 5′-FSO_2BzAdo containing 1 mol of dimethylformamide of crystallization were purchased from Sigma Chemical Co. Sephadex G-25 fine was a product of Pharmacia.

Rabbit muscle phosphofructokinase (type III, lot 30F-9720) purchased from Sigma was employed throughout this study. The enzyme, obtained as a crystalline suspension in ammonium sulfate solution, was isolated by centrifugation, dissolved in buffer A (0.1 M potassium phosphate–1 mM EDTA–0.1 mM dithiothreitol, pH 7.0), and chromatographed at 4 °C on a 1 cm × 30 cm Sephadex G-25 fine column equilibrated with

† This work was supported by a grant from the National Institutes of Health (GM 27840).

[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; 5′-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; SO_2BzAdo, covalently bound 5′-(*p*-sulfonylbenzoyl)adenosine group; PFK, phosphofructokinase; EDTA, ethylenediaminetetraacetic acid.

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buffer A. The enzyme appeared to be essentially homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 9% separating gel and a 3% stacking gel as described by Laemmli (1970). PFK concentrations were determined spectrophotometrically with E_{279} = 1.02 mL mg^{-1} cm^{-1} (Parmeggiani et al., 1966).

Enzyme Assay Procedure. PFK activity was assayed in a 3.0-mL assay mixture, pH 6.95, by monitoring the oxidation of NADH at 340 nm with a Gilford spectrophotometer thermostated at 25 °C as previously described (Ogilvie, 1983). The standard assay solution contained 40 mM imidazole hydrochloride, pH 6.95, 50 mM KCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.053 mM NADH, 0.133 mM fructose 6-phosphate, 0.15 mM ATP, 0.9 unit/mL α-glycerophosphate dehydrogenase, 2.9 units/mL triosephosphate isomerase, and 1.3 units/mL aldolase. The reaction was initiated by the addition of PFK. Since there was a slight lag period, initial velocity measurements were made between 1 and 4 min when the reaction rate was constant. Specific enzymatic activities (micromoles of fructose 1,6-bisphosphate formed per minute per milligram of PFK) were calculated from the initial velocity measurements.

Affinity Labeling of PFK by 5′-FSO$_2$BzAdo. Stock solutions of approximately 4 mM 5′-FSO$_2$BzAdo in ethanol were prepared and stored at −20 °C. The concentration of 5′-FSO$_2$BzAdo was determined spectrophotometrically by employing an extinction coefficient at 259 nm of 1.58 × 10^4 cm^{-1} M^{-1} (Pal et al., 1975).

The labeling of PFK by 5′-FSO$_2$BzAdo was carried out at 25.0 °C by slowly adding with stirring 0.15 mL of an ethanolic solution of 5′-FSO$_2$BzAdo to 0.85 mL of 0.1 M potassium phosphate–1.0 mM EDTA–0.1 mM dithiothreitol, pH 7.0, containing 0.8–1 mg of PFK/mL. Final concentrations of 17–22 μM 5′-FSO$_2$BzAdo and reaction times of 3–35 min at 25 °C were employed to label the enzyme to the extent of two SO$_2$BzAdo per tetramer and less. Final concentrations of 17–110 μM 5′-FSO$_2$BzAdo and reaction times of 35 min–24 h were employed to label the PFK to an extent greater than two SO$_2$BzAdo per tetramer. The reaction was stopped by chromatographing the reaction mixture on a 1 cm × 30 cm Sephadex G-25 fine column equilibrated with 0.1 M potassium phosphate–1 mM EDTA–0.1 mM dithiothreitol, pH 7.0, at 4 °C. Fractions of 1-mL volume were collected, the three fractions with the highest PFK activity were combined, and the absorbance at 259 and 279 nm and the regulatory and kinetic properties of the combined fraction were determined. The concentration of PFK and the extent of labeling of the enzyme by 5′-FSO$_2$BzAdo were calculated from the millimolar extinction coefficients of the native PFK monomer at 259 and 279 nm, the millimolar extinction coefficients of 5′-FSO$_2$BzAdo at 259 and 279 nm, and the observed absorbances of the modified PFK at 259 and 279 nm by solving simultaneous equations. From E_{279} = 1.02 mL mg^{-1} cm^{-1} (Parmeggiani et al., 1966), an E_{279} = 81.6 mM^{-1} cm^{-1} was calculated for the M_r 80000 PFK monomer. An E_{259} = 15.8 mM^{-1} cm^{-1} was employed for 5′-FSO$_2$BzAdo (Pal et al., 1975), and the remaining two extinction coefficients were calculated from the observed A_{279}/A_{259} ratios for PFK and 5′-FSO$_2$BzAdo.

The effect of ligands on the rate of modification of PFK by 17.5 μM 5′-FSO$_2$BzAdo at 25 °C was carried out as described above except that the final PFK concentration in the 15% ethanolic reaction solution was 40 μg/mL, the specified ligands were present in the concentrations indicated, and the extent of modification was followed as a function of time by removing aliquots of the reaction mixture at specified times and determining their enzymatic activity in the standard assay solution modified to contain 1.45 mM ATP.

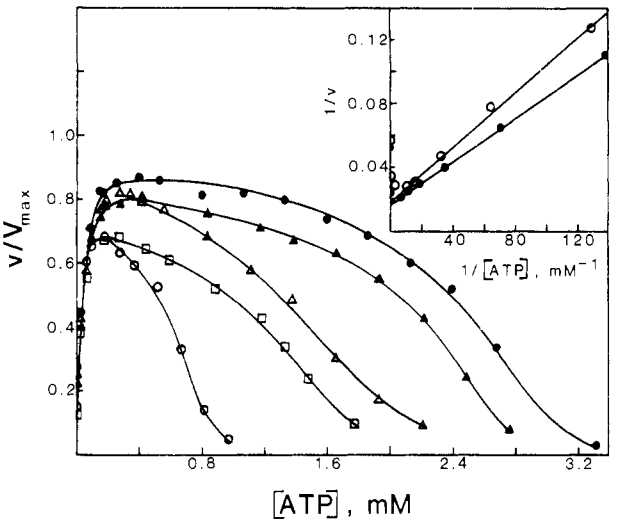

FIGURE 1: Normalized specific enzymatic activities of native and 5′-FSO$_2$BzAdo-modified PFK as a function of ATP concentration. Initial velocities were determined at 25 °C, pH 6.95, as described under Materials and Methods, with all other components of the assay system present at the concentrations given for the standard assay procedure. The observed enzymatic activities (micromoles of fructose 1,6-bisphosphate formed per minute per milligram of PFK) of each enzyme preparation were normalized to the maximum enzymatic activity of that preparation determined as described in the text. The enzyme preparations employed were native PFK (○) and PFK modified to the extent of 0.76 SO$_2$BzAdo group per tetramer (□), 1.65 SO$_2$BzAdo groups per tetramer (△), 3.35 SO$_2$BzAdo groups per tetramer (▲), and 3.97 SO$_2$BzAdo groups per tetramer (●). Inset: Double-reciprocal plots of the data for native PFK (○) and PFK modified to the extent 3.97 SO$_2$BzAdo groups per tetramer (●).

Results and Discussion

Some typical plots of the normalized specific enzymatic activity of native and affinity-labeled preparations of PFK vs. [ATP] are presented in Figure 1. These plots are similar to those previously presented (Ogilvie, 1983) except that the specific enzymatic activity, v, at each concentration of ATP has been normalized to V_{max}, the maximum specific enzymatic activity of that particular enzyme preparation, and the range of ATP concentrations investigated has been extended past 1.2 mM ATP. The value of V_{max} for each enzyme preparation was estimated by a regression analysis (Wilkinson, 1961) of the data obtained at [ATP] < 0.1 mM. As shown in the inset to Figure 1, at less than 0.1 mM ATP double-reciprocal plots of $1/v$ vs.$1/[ATP]$ appear to be linear for both native PFK and affinity-labeled PFK, indicating that inhibition by ATP is not significant at [ATP] < 0.1 mM and that the enzyme-catalyzed reaction follows Michaelis–Menten kinetics with respect to ATP concentration up to 0.1 mM ATP. At higher concentrations, ATP becomes inhibitory as illustrated by the deviation from linearity shown in the inset.

From normalized plots of the type depicted in Figure 1, it would appear that even enzyme preparations containing four SO$_2$BzAdo groups per tetramer never achieve V_{max}, suggesting that significant inhibition by ATP occurs at concentrations less than that required to saturate the ATP binding sites at the active sites of the enzyme. To correct for this apparent onset of inhibition by ATP prior to the achievement of V_{max}, the observed specific enzymatic activity, v, at each concentration of ATP was normalized to v_{calcd}, the specific enzymatic activity calculated at that concentration of ATP employing K_m and V_{max}, the kinetic parameters for that particular enzyme

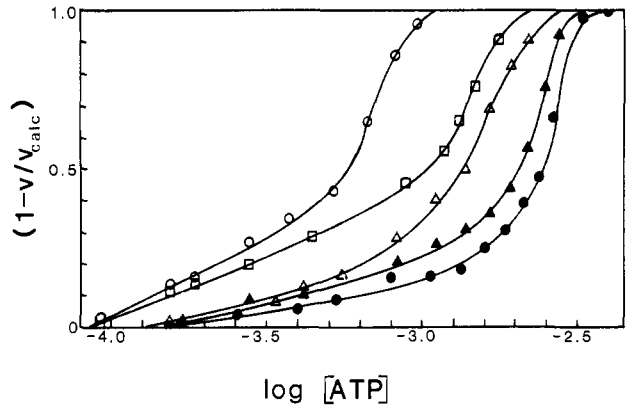

FIGURE 2: Fraction inhibition produced by ATP vs. log [ATP] for native and 5′-FSO_2BzAdo-modified PFK preparations. The PFK preparations and experimental data are the same as those in Figure 1; however, the observed enzymatic activities, v, for each enzyme preparation have been normalized to v_{calcd}, the enzymatic activity calculated from K_m, V_{max}, and the ATP concentration as described in the text. The PFK preparations were native PFK (○) and PFK modified to the extent of 0.76 (□), 1.65 (△), 3.35 (▲), and 3.97 (●) SO_2BzAdo groups per tetramer.

preparation obtained by a regression analysis (Wilkinson, 1961) of the data acquired at less than 0.1 mM ATP. The fraction inhibition produced by ATP ($1-v/v_{calcd}$) for each enzyme preparation at each concentration of ATP was then plotted vs. log [ATP] as shown in Figure 2, from which the free energy of interaction or free-energy coupling was estimated.

The free energy of interaction or free-energy coupling, ΔG_{xy}, as defined by Wyman (1964) and Weber (1975) in their classic papers on linked functions and the energetics of ligand binding to proteins, is the difference between the free-energy change for the binding of ligand X when ligand Y is bound to the protein and the free-energy change for the binding of X when Y is not bound to the protein. In the present case, ligand X would correspond to ATP binding at the four allosteric inhibitory binding sites of the phosphofructokinase tetramer, and ligand Y would correspond to the affinity label 5′-FSO_2BzAdo covalently attached to the activating adenine nucleotide binding sites of the tetramer. The free energy of interaction or free-energy coupling of the two ligands is

$$\Delta G_{xy} = (2.303RT)\ \Delta \log [ATP]_m$$

where $[ATP]_m$ is the median concentration of ATP (Wyman, 1964) and $\log [ATP]_m$ the centroid of the $1 - v/v_{calcd}$ vs. log [ATP] isotherm. The convention adopted with regard to the sign of ΔG_{xy} is that employed by Weber (1975); i.e., a positive ΔG_{xy} corresponds to a destabilizing or negative interaction between the two ligands. The values of ΔG_{xy} thus obtained for 40 different 5′-FSO_2BzAdo-modified phosphofructokinase preparations are plotted vs. the average extent of covalent labeling of the tetramer in Figure 3A. The isotherms in Figure 2 are clearly not symmetrical; hence, $[ATP]_m$ does not equal $[ATP]_{0.5}$, the concentration of ATP that results in 50% inhibition of the enzyme. However, the isotherms are similar enough in shape that calculation of ΔG_{xy} from $\Delta \log [X]_{0.5}$ yields very similar values, as shown in Figure 3B.

In 5′-FSO_2BzAdo-modified phosphofructokinase preparations containing four SO_2BzAdo groups per tetramer, each monomer in the tetramer contains one SO_2BzAdo group covalently attached to the activating adenine nucleotide binding site (Ogilvie, 1983). As shown in Figure 3, the free-energy coupling for such preparations at 25 °C is approximately 1 kcal/mol, a value very typical in magnitude to the values observed for ΔG_{xy} between ligands in other systems (Weber,

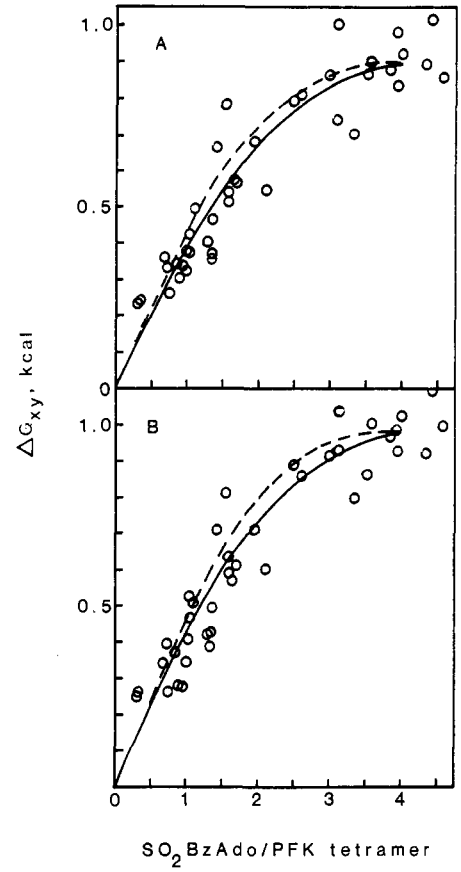

FIGURE 3: Free-energy coupling, ΔG_{xy}, between SO_2BzAdo covalently bound to the activating adenine nucleotide binding sites and ATP binding at the inhibitory binding sites plotted as a function of the extent of covalent modification of PFK. (A) ΔG_{xy} calculated from the median concentration of ATP. (B) ΔG_{xy} calculated from the concentration of ATP that produces 50% inhibition of PFK. The dashed and solid curves are theoretical curves calculated for two different models as described in the text.

1975). Since the SO_2BzAdo group at the activating site is antagonistic to the binding of ATP at the inhibitory site, ΔG_{xy} is positive.

In those preparations containing an average of less than four SO_2BzAdo groups per tetramer, the distribution of labeled monomers among the tetramers is unknown; however, the convex shapes of the curves in Figure 3 rule out the unlikely possibility that there are only two species of tetramers, a species containing four labeled monomers and a species containing four unlabeled monomers, present in these preparations. In those preparations containing an average of one SO_2BzAdo group per tetramer and less, a major fraction of the labeled tetramers would be expected to contain one label per tetramer. The existence of a very significant free-energy coupling in such preparations suggests that the presence of one SO_2BzAdo group per tetramer has a marked antagonistic effect on the binding of ATP at the inhibitory sites. In this respect, it is of some interest to note that in the oxygenation of hemoglobin, a major fraction of the change in the intersubunit interaction energy that results from oxygenation occurs at the first oxygenation step (Chu et al., 1984). Modification of the second monomer in the tetramer also appears to contribute significantly to ΔG_{xy}. The change in free-energy coupling in going from native tetramers to ones containing an average of two SO_2BzAdo groups per tetramer is twice as great as the change observed in going from tetramers containing an average of two SO_2BzAdo groups to fully modified tetramers, suggesting that modification of the final two monomers in the tetramer contributes much less to the antagonistic effect on ATP binding

than does modification of the first two monomers in the tetramer. In fact, as shown by the dashed curves in Figure 3, modification of the final two monomers in a tetramer may not contribute at all to ΔG_{xy}. These dashed curves are theoretical curves calculated on the basis of the following assumptions: (1) Partial covalent modification of PFK by 5′-FSO_2BzAdo leads to a binomial distribution of labeled monomers among the tetramers. (2) For any modified preparation of PFK, the observed free-energy coupling, ΔG_{xy}, is an average of the free-energy coupling values for all species of tetramers present, weighted for the relative number of molecules of each species, i.e.

$$\Delta G_{xy} = \frac{n_0 \Delta G_{xy}^{0} + n_1 \Delta G_{xy}^{1} + n_2 \Delta G_{xy}^{2}}{n_0 + n_1 + n_2}$$

where ΔG_{xy}^{0}, ΔG_{xy}^{1}, and ΔG_{xy}^{2} are the free-energy coupling values for tetramers containing zero, one, and two or more labeled monomers, respectively, and n_0, n_1, and n_2 are the number of molecules of tetramers containing zero, one, and two or more labeled monomers, respectively. Values of 0, 0.45, and 0.90 kcal were employed for ΔG_{xy}^{0}, ΔG_{xy}^{1}, and ΔG_{xy}^{2}, respectively, to calculate the dashed curve in Figure 3A, while values of 0, 0.49, and 0.98 kcal, respectively, were employed in the calculation of the dashed curve in Figure 3B. As indicated by the solid curves in Figure 3, a somewhat better fit to the data is achieved by assuming, in addition to a binomial distribution of labeled monomers among the tetramers, that the tetramers consist of two dimers and that ΔG_{xy}^{0}, ΔG_{xy}^{1}, and ΔG_{xy}^{2} represent the free-energy coupling values for tetramers containing zero, one, and two labeled dimers, respectively, where a labeled dimer is any dimer containing at least one labeled monomer, and n_0, n_1, and n_2 represent the number of molecules of tetramers containing zero, one, and two labeled dimers, respectively. The values of ΔG_{xy}^{0}, ΔG_{xy}^{1}, and ΔG_{xy}^{2} employed in the calculation of the solid curves in Figure 3AB were the same as those employed in the calculation of the dashed curves.

In heterotropic interactions between two ligands, X and Y, reciprocity between X and Y must exist; i.e., energetically, the effect produced by the binding of X upon the binding of Y must be the same as the effect produced by the binding of Y upon the binding of X (Weber, 1975). When one of the ligands is covalently attached to the protein prior to the binding of the other ligand, as in the present case, reciprocity cannot be demonstrated experimentally. However, since 5′-FSO_2BzAdo is an affinity label *specific* for the activating adenine nucleotide binding site, it most likely binds reversibly at the activator binding site prior to covalently modifying this site. Due to reciprocity, the presence of ATP in the affinity labeling reaction should decrease the concentration of the affinity label–enzyme complex and hence decrease the rate of modification of PFK by 5′-FSO_2BzAdo. As shown in Figure 4, this is indeed the case when the modification reaction is carried out at pH 7.0, where PFK displays allosteric regulatory properties and is inhibited by 1 mM ATP. On the other hand, at pH 8.0, where PFK is not inhibited by 1 mM ATP, the presence of either 1 mM ATP or 1 mM MgATP does not alter the rate of modification of PFK by 5′-FSO_2BzAdo (Pettigrew & Frieden, 1978).

As to the nature of the inhibitory species of ATP, Pettigrew & Frieden (1979) concluded, on the basis of changes produced by ATP and MgATP in the intrinsic protein fluorescence of PFK, that both ATP and MgATP are capable of binding at the inhibitory sites of PFK at pH 6.9, with ATP binding 10-fold more tightly than MgATP. On the other hand, Kemp

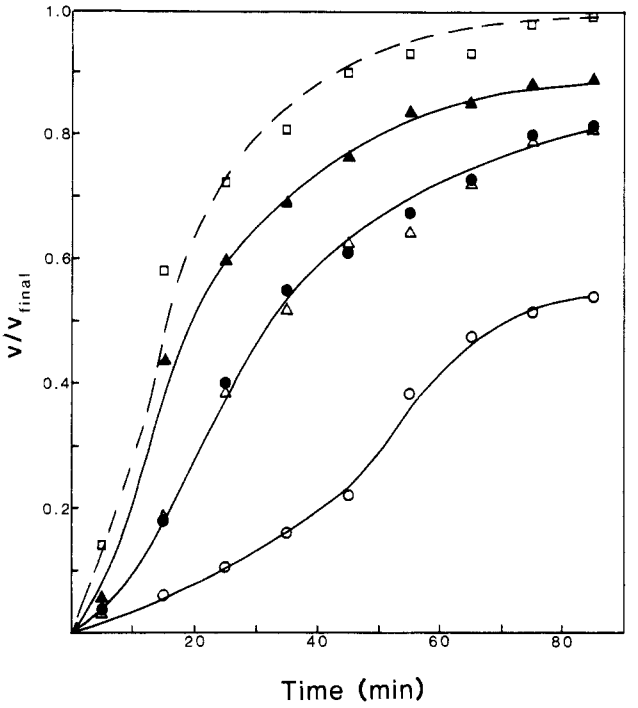

FIGURE 4: Plots of the normalized specific enzymatic activity of PFK when assayed at 1.45 mM ATP vs. time of preincubation of PFK with 17.5 μM 5′-FSO_2 BzAdo at 25 °C, pH 7.0. The modification of PFK (40 μg/mL) by 5′-FSO_2BzAdo was carried out as described under Materials and Methods, and 10-μL aliquots were removed at the times indicated and assayed in the standard assay solution modified to contain 1.45 mM ATP. The final specific enzymatic activity, v_{final}, was determined approximately 24 h after initiation of the modification by 5′-FSO_2BzAdo. Additional ligands present during the preincubation with 5′-FSO_2BzAdo were 3 mM ATP (○), 3 mM ATP + 6 mM $MgCl_2$ (●), 1.5 mM ATP (△), 1.5 mM ATP + 6 mM $MgCl_2$ (▲), and none (□). The dashed curve is a theoretical concentration–time curve for C formed by series first-order reactions of the type A $\xrightarrow{k_1}$ B $\xrightarrow{k_2}$ C where k_1 = 0.29 min^{-1} and k_2 = 0.06 min^{-1}.

(1969) concluded, on the basis of changes produced by ATP and MgATP in the reactivity of a protein sulfhydryl group, that the affinity of the inhibitory site of PFK is much greater for MgATP than for ATP. As shown in Figure 4, 6 mM $MgCl_2$ diminishes but does not abolish the antagonistic effect of 3 mM ATP on the rate of 5′-FSO_2BzAdo modification of PFK. In fact, 6 mM $MgCl_2$ plus 3 mM ATP produces the same effect on the rate of modification as 1.5 mM free ATP, suggesting that the inhibitory binding site has a greater affinity for ATP than for MgATP. The presence of 6 mM $MgCl_2$ had no effect on the rate of modification of PFK by 5′-FSO_2BzAdo in the absence of ATP. Since the data in Figures 1–3 were all obtained in the presence of 6 mM $MgCl_2$, the inhibition observed in these experiments may reflect predominantly the binding of MgATP to the inhibitory binding sites.

The apparent dissociation constant for the MgATP–PFK complex determined at pH 6.9 and 25 °C by fluorescence titrations (Pettigrew & Frieden, 1979) is approximately 10-fold greater than that determined for the ATP–PFK complex (150 μM vs. 14 μM), and the apparent dissociation constant for the MgATP-modified PFK complex containing four SO_2BzAdo groups per tetramer is 8-fold greater than that for the ATP-modified PFK complex (800 μM vs. 100 μM). For modified PFK preparations containing four SO_2BzAdo groups per tetramer, free-energy coupling values of 0.99 and 1.16 kcal/mol can be calculated for MgATP and ATP, respectively, from these apparent dissociation constants. These values are in good agreement with the 1 kcal calculated in the present study, indicating that there exists a good correlation between

the change in intrinsic protein fluorescence and the change in enzymatic activity produced by the binding of ATP or MgATP.

At pH 8.0, PFK does not display regulatory kinetic behavior in that it is not inhibited by 1 mM ATP and the labeling of the enzyme by 5′-FSO_2BzAdo at pH 8.0 is a first-order process with a half-time of 15 min (Pettigrew & Frieden, 1978). At pH 7.0, where the enzyme displays regulatory kinetic behavior, the labeling of the enzyme by 5′-FSO_2BzAdo is clearly not a simple first-order process since all of the labeling reactions show an induction period (Figure 4). One possible explanation for the existence of an induction period is that series first-order reactions of the type

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

are involved, where the integrated equation for [C] is (Frost & Pearson, 1961)

$$[C] = [A_0]\left[1 + \frac{1}{k_1 - k_2}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right]$$

Concentration–time curves for C in series first-order reactions characteristically show an induction period. That series first-order reactions could possibly account for the induction period is shown by the dashed curve in Figure 4, which is a concentration–time curve calculated for series first-order reactions by using rate constants of 0.29 and 0.06 min^{-1}, which would correspond to half-times of 2.4 and 11.5 min for the two reactions. If this is the mechanism responsible for the induction period, the conversion of B to C might represent the covalent labeling of the site by noncovalently bound 5′-FSO_2BzAdo and the A to B conversion could possibly represent a protein conformational change that is required before covalent modification can occur. If the A to B conversion were pH dependent and the enzyme existed predominantly in the B conformation at pH 8.0, then covalent modification at pH 8.0 would be a simple first-order reaction as has been reported (Pettigrew & Frieden, 1978).

In the previous study of PFK and 5′-FSO_2BzAdo-modified PFK (Ogilvie, 1983), a number of tentative conclusions were proposed on the basis of the effect of the extent of labeling of PFK on the extent of inhibition of the enzyme by 1.2 mM ATP. The results of the present study of the energetics of the interaction between the adenine nucleotide analogue covalently bound at the activating binding site of PFK and ATP binding at the inhibitory binding site of the enzyme support most of the conclusions previously advanced. For example, the results of both studies clearly support the conclusion that the generation of a marked antagonistic effect on the inhibition of the enzyme by ATP does not require the labeling of all four subunits in the tetramer. In fact, the results of both studies are consistent with the hypothesis that the maximum antagonistic effect or maximum free-energy coupling may be achieved when only two monomers or one monomer in each of the two dimers that constitute the tetramer is labeled. On the other hand, the conclusions of the two studies are discrepant in one respect. The method employed in the previous study to calculate the percent inhibition produced by 1.2 mM ATP and the percent ATP inhibition relieved by covalent modification of the enzyme by 5′-FSO_2BzAdo led to the erroneous conclusion that partial modification of PFK could not result in a binomial distribution of labeled monomers among the PFK tetramers. The results of the present and more rigorous investigation of the energetics of the interaction between the two ligands are clearly compatible with the possibility that partial modification of PFK by 5′-FSO_2BzAdo may lead to a binomial distribution of labeled monomers among the PFK tetramers. In addition to supporting most of the conclusions of the previous study, the results of the present study suggest that the allosteric inhibitory adenine nucleotide binding sites on PFK may bind either ATP or MgATP and that the binding of either of these forms of the ligand produces similar effects upon the kinetic properties of the enzyme.

ACKNOWLEDGMENTS

I gratefully acknowledge the excellent technical assistance given by Siang Tsao and Larry Mesner.

Registry No. ATP, 56-65-5; 5′-FSO_2BzAdo, 57454-44-1; phosphofructokinase, 9001-80-3.

REFERENCES

Aaronson, R. P., & Frieden, C. (1972) *J. Biol. Chem. 247*, 7502–7509.

Chu, A. H., Turner, B. W., & Ackers, G. K. (1984) *Biochemistry 23*, 604–617.

Frost, A. A., & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd ed., pp 166–171, Wiley, New York.

Hofer, H., & Pette, D. (1968) *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1378–1392.

Kemp, R. G. (1969) *Biochemistry 8*, 3162–3168.

Kemp, R. G., & Krebs, E. G. (1967) *Biochemistry 6*, 423–434.

Lad, P. M., Hill, D. E., & Hammes, G. G. (1973) *Biochemistry 12*, 4303–4309.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lardy, H. A., & Parks, R. E., Jr. (1956) *Enzymes: Units of Biological Structure and Function*, pp 584–587, Academic Press, New York.

Mansour, T. E., & Colman, R. F. (1978) *Biochem. Biophys. Res. Commun. 81*, 1370–1376.

Ogilvie, J. W. (1983) *Biochemistry 22*, 5908–5914.

Paetkau, V., & Lardy, H. A. (1967) *J. Biol. Chem. 242*, 2035–2042.

Pal, P. K., Wechter, W. J., & Colman, R. F. (1975) *J. Biol. Chem. 250*, 8140–8147.

Parmeggiani, A., Luft, J. H., Love, D. S., & Krebs, E. G. (1966) *J. Biol. Chem. 241*, 4625–4637.

Passonneau, J. V., & Lowry, O. H. (1962) *Biochem. Biophys. Res. Commun. 7*, 10–15.

Pavelich, M. J., & Hammes, G. G. (1973) *Biochemistry 12*, 1408–1414.

Pettigrew, D. W., & Frieden, C. (1978) *J. Biol. Chem. 253*, 3623–3627.

Pettigrew, D. W., & Frieden, C. (1979) *J. Biol. Chem. 254*, 1887–1895.

Weber, G. (1975) *Adv. Protein Chem. 29*, 1–83.

Weng, L., Heinrikson, R. L., & Mansour, T. E. (1980) *J. Biol. Chem. 255*, 1492–1496.

Wilkinson, G. N. (1961) *Biochem. J. 80*, 324–332.

Wolfman, N., Thompson, W., & Hammes, G. G. (1978) *Biochemistry 17*, 1813–1817.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 223–286.