Boyer, P. D., & Bryan, D. M. (1967) *Methods Enzymol. 10*, 60-71.
Cassidy, P. J., & Kahan, F. M. (1973) *Biochemistry 12*, 1364-1374.
Deleo, A. B., & Sprinson, D. B. (1968) *Biochem. Biophys. Res. Commun. 32*, 873-877.
Dinovo, E. C., & Boyer, P. D. (1971) *J. Biol. Chem. 246*, 4586-4593.
Furniss, B. S., Ed. (1978) *Vogel's Textbook of Practical Organic Chemistry*, Longman Group Ltd., London.
Gollub, E., Zalkin, H., & Sprinson, D. B. (1967) *J. Biol. Chem. 242*, 5323-5328.
Grimshaw, C. E., Sogo, S. G., & Knowles, J. R. (1982) *J. Biol. Chem. 257*, 596-598.
Gunetileke, K. G., & Anwar, R. A. (1968) *J. Biol. Chem. 243*, 5770-5778.
Ife, R. J., Ball, L. F., Lowe, P., & Haslam, E. (1976) *J. Chem. Soc., Perkin Trans. 1*, 1776-1783.
Knowles, P. F., & Sprinson, D. B. (1970) *Methods Enzymol. 17A*, 351-352.
Levin, J. G., & Sprinson, D. B. (1964) *J. Biol. Chem. 239*, 1142-1150.
McConnell, R. L., & Coover, H. W., Jr. (1956) *J. Am. Chem. Soc. 78*, 4453-4455.
Middlefort, C. F., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5881-5887.
Millican, R. C. (1970) *Methods Enzymol. 17A*, 352-354.
Morgan, P. N., Gibson, M. I., & Gibson, F. (1963) *Biochem. J. 89*, 229-239.
O'Leary, M. H., DeGooyer, W. J., Dougherty, T. M., & Anderson, V. (1981) *Biochem. Biophys. Res. Commun. 100*, 1320-1325.
O'Neal, C. C., Jr., Bild, G. S., & Smith, L. T. (1983) *Biochemistry 22*, 611-617.
Sandifer, R. M., Thompson, M. D., Gaughan, R. G., & Poulter, C. D. (1982) *J. Am. Chem. Soc. 104*, 7376-6366.
Steinrucken, H. C., & Amrhein, N. (1980) *Biochem. Biophys. Res. Commun. 94*, 1207-1212.
Stubbe, J. A., & Kenyon, G. L. (1971) *Biochemistry 10*, 2669-2677.
Stubbe, J. A., & Kenyon, G. L. (1972) *Biochemistry 11*, 338-345.
Stubbe, J. A., & Ables, R. H., (1980) *Biochemistry 19*, 5505-5512.
Tener, G. M., Wright, R. S., & Khorana, H. G. (1957) *J. Am. Chem. Soc. 79*, 441-443.
Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384-421.
Wauchope, D. (1976) *J. Agric. Food Chem. 24*, 717-721.
Zemell, R. I., & Anwar, R. A. (1975) *J. Biol. Chem. 250*, 4959-4964.

Rabbit Muscle Phosphofructokinase. 1. Activation by Affinity Labeling Approximately Two Adenosine Cyclic 3′,5′-Monophosphate Binding Sites per Tetramer†

James W. Ogilvie

ABSTRACT: The smallest enzymatically active form of rabbit muscle phosphofructokinase consists of four identical or nearly identical subunits, and each subunit contains one binding site specific for the activating adenine nucleotides cAMP, AMP, and ADP. These activator binding sites on the enzyme have been covalently labeled to various degrees, ranging from an average value of less than one label/tetramer to four labels/tetramer, with the affinity label 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine, and the kinetic and regulatory properties of these modified phosphofructokinase preparations have been investigated. The kinetic and regulatory properties of the affinity-labeled phosphofructokinase are essentially identical with those of native enzyme activated by cAMP, and a near maximum activation of the affinity-labeled enzyme is observed in those preparations modified to the extent of two or more groups/tetramer, suggesting that the covalent attachment of approximately two affinity labels/tetramer is necessary and sufficient for full activation of the enzyme. This requirement for approximately two groups/tetramer for full activation of the enzyme has been substantiated by dissociating a solution containing both native enzyme and affinity-labeled enzyme modified to the extent of approximately four groups/tetramer and then allowing the resulting solution of labeled and unlabeled monomers to reassemble into active tetramers and observing that the extent of activation in the solution of reassembled enzyme is greater than that initially observed in the enzyme solution before dissociation.

Rabbit skeletal muscle phosphofructokinase (EC 2.7.1.11) is comprised of identical or nearly identical subunits of M_r 80 000, and the smallest enzymatically active form of the enzyme is a tetramer (Paetkau & Lardy, 1967; Pavelich & Hammes, 1973; Aaronson & Frieden, 1972; Lad et al., 1973). Below pH 7.5, phosphofructokinase is activated and inhibited by a number of metabolites, enabling the enzyme to play a key role in the regulation of the glycolytic pathway. Among the metabolites that influence the kinetic properties of phosphofructokinase are the adenine nucleotides ATP, ADP, AMP, and cAMP.[1] Not only is ATP a substrate for the enzyme but

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received February 24, 1983.* This work was supported by a grant from the National Institutes of Health (GM 27840).

[1] Abbreviations: 5′-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine; SO_2BzAdo, covalently bound 5′-(*p*-sulfonylbenzoyl)adenosine group; cAMP, adenosine cyclic 3′,5′-monophosphate; PFK, phosphofructokinase; F-6-P, fructose 6-phosphate; F-1,6-P_2, fructose 1,6-diphosphate; Gdn·HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-5908$01.50/0

also at higher concentrations it is an inhibitor (Lardy & Parks, 1956). The inhibition of the enzyme by ATP is relieved by cAMP, AMP, and ADP; hence, these three adenine nucleotides are activators of the enzyme, and each M_r 80000 subunit of the enzyme appears to contain one binding site that is specific for these activating adenine nucleotides (Passonneau & Lowry, 1962; Lowry & Passonneau, 1966; Kemp & Krebs, 1967). Three adenine nucleotide analogues, 2′-*O*-(ethyl-2-diazomalonyl)adenosine cyclic 3′,5′-monophosphate (Cooperman & Brunswick, 1973), 5′-[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine (Craig & Hammes, 1980), and 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (Pettigrew & Frieden, 1978; Weng et al., 1980) have been reported to function as affinity labels specific for the cAMP, AMP, or ADP binding sites of phosphofructokinase. Pettigrew and Frieden have observed that the regulatory kinetic properties of rabbit muscle phosphofructokinase modified by 5′-FSO_2BzAdo to the extent of 3.6 groups/tetramer are similar to those of native phosphofructokinase in the presence of a saturating concentration of cAMP. In the present study, 5′-FSO_2BzAdo has been employed to covalently modify the cAMP or activating adenine nucleotide binding site of phosphofructokinase to various degrees, ranging from an average value of less than one SO_2BzAdo/tetramer to approximately four groups/tetramer. The kinetic and regulatory properties of these modified phosphofructokinase preparations have been investigated in order to ascertain (a) how these properties of phosphofructokinase vary as a function of the extent of modification of the enzyme, (b) the minimum number of SO_2BzAdo groups/tetramer required for full activation of the enzyme, and (c) the probable distribution pattern of labeled subunits among the tetramers in partially modified preparations.

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. Hexamethylphosphoric triamide and *p*-(fluorosulfonyl)benzoyl chloride were obtained from Aldrich Chemical Co. Sephadex G-25 fine was a product of Pharmacia. 5′-FSO_2BzAdo containing 1 mol of hexamethylphosphoric triamide of crystallization was synthesized by the procedure of Wyatt & Colman (1977).

Rabbit muscle phosphofructokinase (type III, lot 30F–9720) purchased from Sigma was employed throughout this study. This preparation of 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). 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–1 mM dithiothreitol, pH 7.0), and chromatographed at 4 °C on a 1 cm × 30 cm Sephadex G-25 fine column equilibrated with buffer A. Phosphofructokinase concentrations were determined spectrophotometrically with $E_{279} = 1.02$ mg^{-1} mL (Parmeggiani et al., 1966). When preparing phosphofructokinase to be used in affinity-labeling experiments, the concentration of dithiothreitol in buffer A was reduced to 0.1 mM.

Enzyme Assay Procedure. Phosphofructokinase activity was determined at pH 6.95 by monitoring the oxidation of NADH at 340 nm with a Gilford spectrophotometer thermostated at 25 °C and by employing a modification of the coupled assay procedure described by Lascu et al. (1979). The mixed crystalline auxiliary enzymes, isolated from ammonium sulfate suspensions by centrifugation, were dissolved in 25 mM glycylglycine–25 mM sodium β-glycerophosphate–1 mM EDTA buffer, pH 7.0, and dialyzed against the same buffer at 4 °C for 24 h before use. The standard assay mixture 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 phosphofructokinase. Since there was a slight lag during the first 30 s of the reaction, all initial velocity measurements were made between 30 s and 3 min when the reaction rate was constant. These initial velocities were then used to calculate specific enzymatic activities [μmol of F-1,6-P_2 formed min^{-1} (mg of protein)$^{-1}$].

Chemical Modification of Phosphofructokinase. 5′-FSO_2BzAdo containing 1 mol of dimethylformamide of crystallization was dissolved in absolute ethanol to yield stock solutions of approximately 5 mM 5′-FSO_2BzAdo. The concentration of 5′-FSO_2BzAdo was determined spectrophotometrically by employing an extinction coefficient at 259 nm of 1.58×10^4 cm^{-1} M^{-1} (Pal et al., 1975). These stock solutions were stored at −20 °C.

The chemical modification of phosphofructokinase with 5′-FSO_2BzAdo was carried out at 22 °C by slowly adding with stirring 0.15 mL of an ethanolic solution of 5′-FSO_2BzAdo to 0.85 mL of 0.1 M potassium phosphate–1.0 mM EDTA–0.1 mM dithiothreitol, pH 7.0, containing 0.9–1.1 mg of phosphofructokinase/mL. The final concentration of 5′-FSO_2BzAdo was 16–106 μM. Although designated as modifications at pH 7.0, the measured pH of the reaction mixture was 7.3. Attempts to carry out the reaction in the total absence of dithiothreitol were not successful as the reaction mixture became slightly turbid during the incubation even in the absence of 5′-FSO_2BzAdo. In general, low concentrations of 5′-FSO_2BzAdo (16–53 μM) and short reaction times (10–25 min) were used to modify the enzyme to the extent of two SO_2BzAdo/tetramer and less; 106 μM 5′-FSO_2BzAdo and reaction times of 15–125 min were employed to modify the enzyme to an extent greater than two SO_2BzAdo/tetramer. The reaction was stopped by chromatographing the 1 mL of 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. Termination of the reaction was taken as that time when the reaction mixture had completely entered the gel column. The column was then eluted at 4 °C with the 0.1 M potassium phosphate–1 mM EDTA–0.1 mM dithiothreitol buffer, pH 7.0, and the eluate monitored at 280 nm with a Bio-Rad Laboratories Model 1300 monitor. Fractions of 1-mL volume were collected, and an aliquot of each fraction was assayed for phosphofructokinase activity. The three fractions with the highest enzymatic activity were combined, and the absorbance of the combined fraction at 259 and 279 nm and the regulatory and kinetic properties of the combined fractions were determined. Several modifications of the above procedure were also investigated. Some samples of phosphofructokinase were chemically modified by 5′-FSO_2BzAdo at pH 7.0 and 8.0 in reaction mixtures containing 10% (v/v) dimethylformamide–2% (v/v) ethanol rather than 15% (v/v) ethanol (see Figure 2). Other samples of phosphofructokinase were modified by adding the 5′-FSO_2BzAdo in several portions during the 2-h incubation period.

The concentration of chemically modified phosphofructokinase and the extent of labeling of the enzyme by 5′-FSO_2BzAdo were calculated from the millimolar extinction

coefficients of the native phosphofructokinase monomer at 259 and 279 nm, the millimolar extinction coefficients of 5′-FSO_2BzAdo at 259 and 279 nm, and the observed absorbances of the modified phosphofructokinase at 259 and 279 nm by solving simultaneous equations. An $E_{279} = 81.6\ mM^{-1}\ cm^{-1}$ was calculated for the M_r 80000 phosphofructokinase monomer with $E_{279} = 1.02\ mg^{-1}\ mL$ (Parmeggiani et al., 1966). An $E_{259} = 15.8\ mM^{-1}\ cm^{-1}$ was employed for 5′-FSO_2BzAdo (Pal et al., 1975). The remaining two extinction coefficients were calculated from the observed A_{279}/A_{259} ratios for phosphofructokinase and 5′-FSO_2BzAdo.

Dissociation and Reassembly of Native and Chemically Modified Phosphofructokinase. A modification of the procedure of Parr & Hammes (1976) was used to dissociate and reassemble solutions of native phosphofructokinase, phosphofructokinase modified by 5′-FSO_2BzAdo, and mixtures of native and modified phosphofructokinase. Dissociation to monomers was achieved by adding a concentrated solution of Gdn·HCl to solutions of phosphofructokinase in 0.1 M potassium phosphate–1 mM EDTA–1 mM dithiothreitol, pH 7.0, at 0 °C with stirring to yield solutions containing 0.8 M Gdn·HCl and 0.14–0.34 mg of phosphofructokinase/mL as final concentrations. After 1-min incubation at 0 °C, reassembly was initiated by diluting the solution 10-fold with 0.1 M potassium phosphate–1 mM EDTA–1 mM dithiothreitol, pH 7.0, at 25 °C and incubating the resulting solution at 25 °C. The extent of reactivation or reassembly as a function of time was followed by removing aliquots at specified times after the 10-fold dilution and assaying these aliquots by the standard assay procedure (0.15 mM ATP) and by the standard assay procedure modified to contain 1.2 mM ATP.

Results

Kinetic and Regulatory Properties of Native Phosphofructokinase and Phosphofructokinase Affinity Labeled with 5′-[p-(Fluorosulfonyl)benzoyl]adenosine. Plots of enzymatic activity at pH 6.95 vs. ATP concentration for native phosphofructokinase in the presence and absence of cAMP and for phosphofructokinase modified by 5′-FSO_2BzAdo to contain on average 1.8, 2.3, and 3.6 covalently bound SO_2BzAdo residues per phosphofructokinase tetramer are presented in Figure 1. The effect of increasing concentrations of ATP on the enzymatic activity of native phosphofructokinase at this pH (curve 1) is similar to that reported by other investigators and is characterized by an increase in enzymatic activity with increasing concentrations of ATP up to approximately 0.2 mM ATP, followed by a decrease in activity as the ATP concentration is increased above this level. This response of phosphofructokinase to ATP is generally believed to result from the presence of two types of ATP binding sites on the enzyme: (1) a high-affinity site at the active site of the enzyme where ATP serves as a substrate and (2) a lower affinity binding site or allosteric site for ATP, the saturation of which leads to inhibition of the enzyme (Hofer & Pette, 1968; Kemp, 1969; Lowry & Passonneau, 1966; Wolfman et al., 1978; Pettigrew & Frieden, 1979).

As shown in curve 2 of Figure 1, cAMP is an activator of phosphofructokinase by virtue of its ability to relieve the ATP inhibition of the enzyme. This type of activation has been observed for AMP, ADP, and cAMP by a number of investigators and has led to the hypothesis that the enzyme contains a third type of adenine nucleotide binding site that is specific for the activating allosteric modifiers AMP, ADP, and cAMP (Passonneau & Lowry, 1962; Lowry & Passonneau, 1966; Kemp & Krebs, 1967). Pettigrew & Frieden (1978) have demonstrated that 5′-FSO_2BzAdo is an affinity label for this

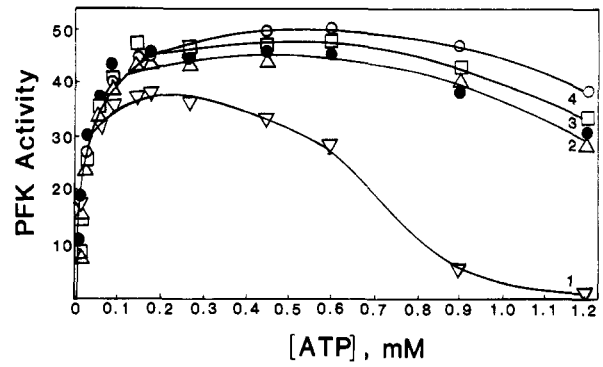

FIGURE 1: Enzymatic activity of native phosphofructokinase and 5′-FSO_2BzAdo-modified phosphofructokinase as a function of ATP concentration. Initial velocities were determined at 25 °C, pH 6.95, as described under Materials and Methods, with all other components of the assay system present at the concentrations given for the standard assay procedure. Enzymatic activities are expressed as μmol of F-1,6-P_2 formed min^{-1} (mg of PFK)$^{-1}$. (Curve 1) (▽) Native phosphofructokinase; (curve 2) (△) phosphofructokinase modified to the extent of 1.8 SO_2BzAdo groups/tetramer and (●) native phosphofructokinase + 133 μM cAMP; (curve 3) (□) phosphofructokinase modified to the extent of 2.3 SO_2BzAdo groups/tetramer; (curve 4) (○) phosphofructokinase modified to the extent of 3.6 SO_2BzAdo groups/tetramer.

site that is primarily specific for cAMP, AMP, or ADP. They also have reported that affinity labeling phosphofructokinase to the extent of 3.6 SO_2BzAdo residues/tetramer (0.9 residue/monomer) leads to a modified phosphofructokinase whose kinetic and molecular properties are similar to those of the native enzyme when saturated with an activator such as cAMP. As shown in curve 4 of Figure 1, similar results have been obtained in the present study. Moreover, results obtained in the present study (curves 2 and 3 of Figure 1) suggest that the regulatory kinetic properties of affinity-labeled enzyme preparations containing on average approximately two covalently bound SO_2BzAdo residues per phosphofructokinase tetramer are essentially identical with those of native phosphofructokinase in the presence of 133 μM cAMP, a near saturating concentration of cAMP. It is of interest to note that higher concentrations of ATP (3–5 mM) will overcome the activating effect of the affinity label, as suggested by the downward curvature of curves 2, 3, and 4, even though the label is attached covalently at one point.

The results of a more detailed investigation of the relationship between the extent of relief of ATP inhibition and the number of covalently bound SO_2BzAdo moieties per phosphofructokinase tetramer are presented in Figure 2. The extent of labeling was controlled by varying the initial concentrations of 5′-FSO_2BzAdo and the reaction time. The affinity-labeling reaction was stopped by chromatographing the reaction mixture on a Sephadex G-25 column to separate the enzyme from any unreacted 5′-FSO_2BzAdo as well as to remove any label not covalently bound to the phosphofructokinase. The extent of labeling and protein concentration of the covalently modified phosphofructokinase preparation thus isolated were determined spectrophotometrically as described under Materials and Methods. The effectiveness of the covalent modification in relieving the inhibition produced by 1.2 mM ATP was determined by measuring the enzymatic activity of both the native phosphofructokinase preparation and the affinity-labeled preparation at 0.15 and 1.2 mM ATP and calculating the percent inhibition produced by 1.2 mM ATP for the native and modified preparations. These percent inhibition values were then used to calculate the percent of ATP inhibition relieved as a result of the covalent modification. The data in Figure 2 suggest that (a) relief of the inhibition pro-

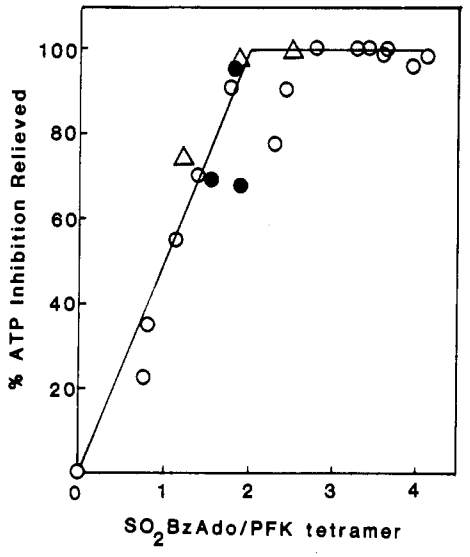

FIGURE 2: Percent ATP inhibition relieved as a function of extent of modification of phosphofructokinase by 5′-FSO_2BzAdo. The % ATP inhibition was calculated from initial velocities determined at ATP concentrations of 0.15 and 1.2 mM, pH 6.95, 25 °C, as described in the text. Modification of phosphofructokinase by 5′-FSO_2BzAdo was carried out at pH 7.0 in 15% ethanol (○), at pH 7.0 in 10% dimethylformamide–2% ethanol (●), and at pH 8.0 in 10% dimethylformamide–2% ethanol (△) as described under Materials and Methods. The line intersecting 100% ATP inhibition relieved at two SO_2BzAdo groups/tetramer was arbitrarily drawn to serve as a point of reference only.

duced by 1.2 mM ATP requires the covalent attachment of approximately two SO_2BzAdo residues/tetramer, (b) a further increase in the average number of SO_2BzAdo groups bound/PFK tetramer has little effect on the activity of the enzyme at 1.2 mM ATP, and (c) the distribution of labeled subunits among the tetrameric molecules in a preparation containing on average two labeled subunits/tetramer cannot be random.

Covalent modification of phosphofructokinase with 5′-FSO_2BzAdo also alters the saturation curve for F-6-P, the other substrate for the enzyme, by decreasing the concentration of F-6-P required to achieve half-maximal velocity (data not shown). Again, it appears that a near maximum effect of affinity labeling is realized when the tetramer contains on average approximately two covalently bound SO_2BzAdo residues and that the regulatory effect produced by affinity labeling phosphofructokinase is similar to that produced by a near saturating concentration of cAMP.

Dissociation and Reassembly of Native Phosphofructokinase and Affinity-Labeled Phosphofructokinase. Parr & Hammes (1976) have demonstrated that phosphofructokinase rapidly dissociates to inactive monomers in 0.8 M guanidine hydrochloride at pH 8.0 and that these inactive monomers reassemble by a second-order kinetic process to yield fully active tetramers in good yield when diluted 1:10 under suitable conditions. They have reported also that the reassembly of the inactive monomers is consistent with the following mechanism:

$$4P \xrightarrow{k_1} 2P_2 \xrightarrow{k_2} P_4 \qquad (1)$$

where P represents the protomer or monomer of phosphofructokinase and k_1 and k_2 represent the second-order rate constants for dimerization and tetramer formation, respectively. If the tetramer, P_4, is the only enzymatically active species and if $k_2 \gg k_1$ or $k_1 \gg k_2$, they have shown that a plot of $(4/[P_0])(1 - A_t/A_\infty)^{-1}$ vs. time, where $[P_0]$ is the initial

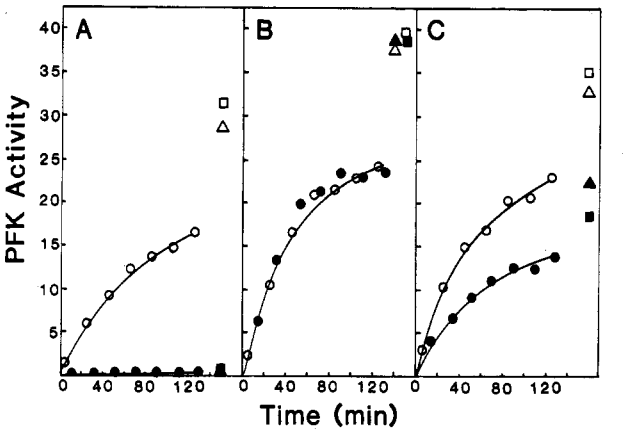

FIGURE 3: Plot of enzymatic activity vs. time after initiation of reactivation or reassembly of dissociated phosphofructokinase preparations. Dissociation to monomer was carried out at pH 7.0 in 0.8 M Gdn·HCl in an ice bath, and the reassembly was initiated by diluting 10-fold with pH 7.0 buffer at 25 °C as described under Materials and Methods. (A) Reactivation of dissociated native phosphofructokinase (33.6 μg/mL final protein concentration). (B) Reactivation of dissociated modified phosphofructokinase containing 3.6 SO_2BzAdo groups/tetramer (18.4 μg/mL final protein concentration). (C) Reactivation of a mixture of dissociated native phosphofructokinase (16.8 μg/mL final concentration) and dissociated modified phosphofructokinase containing 3.6 SO_2BzAdo groups/tetramer (14.7 μg/mL final concentration). The extent of reactivation in A–C was followed by determining the enzymatic activity [expressed as μmol of F-1,6-P_2 formed min^{-1} (mg of protein)$^{-1}$] at 0.15 (○) and at 1.2 mM ATP (●) with all other components of the assay system present at the concentrations given for the standard assay procedure under Materials and Methods. The enzymatic activities determined at 0.15 (□) and at 1.2 mM ATP (■) before dissociation to monomers and the enzymatic activities determined at 0.15 (△) and at 1.2 mM ATP (▲) 24 h after initiation of reactivation are shown near the right borders of A–C.

protomer concentration and A_t and A_∞ represent the percent reactivation at time t and at infinite time, respectively, should be linear with a slope directly related to the rate constant (slope = k_1 if $k_2 \gg k_1$; slope = $k_2/4$ if $k_1 \gg k_2$). Furthermore, from the effect of ATP on the rates of dissociation and reactivation, they have concluded that k_1, the formation of dimers, is probably the rate-limiting step.

In the present study, a modification of the procedure of Parr and Hammes was employed to investigate the dissociation and reassembly of native phosphofructokinase, affinity-labeled phosphofructokinase, and mixtures of native and affinity-labeled enzyme in an attempt to (a) obtain information as to the location of the third and fourth SO_2BzAdo group that can be covalently attached to a phosphofructokinase tetramer and (b) confirm that the covalent binding of approximately two SO_2BzAdo groups per tetramer leads to full relief of the inhibition of the enzyme by 1.2 mM ATP. The rationale behind these studies was as follows. If a mixture of native enzyme and affinity-labeled enzyme modified to contain approximately four SO_2BzAdo residues/tetramer were dissociated and then reassembled, the protection against inhibition by ATP observed in the reassembled preparation might be greater than that observed in the original mixture before dissociation provided that (a) all monomers in the original affinity-labeled enzyme contain a single SO_2BzAdo group covalently bound to the activator binding site and (b) full protection against inhibition by 1.2 mM ATP requires about two covalently bound SO_2BzAdo groups/tetramer.

The results of a typical dissociation and reassembly experiment are depicted in Figure 3. The data for reassembly of dissociated native phosphofructokinase (Figure 3A) indicate that a 91% yield of reactivation or reassembly was achieved

Table I: Data Summary for Reactivation of Solutions Containing Monomers Derived from Phosphofructokinase and Modified Phosphofructokinase

expt	source of monomers	% reactivation	slope (M^{-1} s^{-1})	slope* (M^{-1} s^{-1})	% inhibition at 1.2 mM ATP predicted by[a] model I	model II	model III	model IV	% inhibition observed at 1.2 mM ATP
1A	0.377 nmol of native PFK tetramer (SA[b] = 30.1 at 0.15 mM ATP; 99% inhibited at 1.2 mM ATP)	85	1590		99	99	99	99	98
1B	0.195 nmol of modified PFK tetramer (3.4 SO_2BzAdo groups/tetramer; SA = 37.0 at 0.15 mM ATP; 0% inhibited at 1.2 mM ATP)	91	4700	4400	0	13	0	2	0
1C	0.188 nmol of PFK tetramer (identical with that in 1A) plus 0.156 nmol of modified PFK tetramer (identical with that in 1B)	94	3000	2130	47	55	21	32	32
2A	0.457 nmol of native PFK tetramer (SA = 31.5 at 0.15 mM ATP; 98% inhibited at 1.2 mM ATP)	91	1710		98	98	98	98	99
2B	0.250 nmol of modified PFK tetramer (3.6 SO_2BzAdo groups/tetramer; SA = 39.4 at 0.15 mM ATP; 1% inhibited at 1.2 mM ATP)	97	4900	4900	1	9	1	2	1
2C	0.229 nmol of PFK tetramer (identical with that in 2A) plus 0.200 nmol of modified PFK tetramer (identical with that in 2B)	93	3050	2310	46	50	14	28	32
3A	0.457 nmol of PFK tetramer (identical with that in 2A)	77	1950		98	98	98	98	98
3B	0.250 nmol of modified PFK tetramer (identical with that in 2B)	76	4740	4740	1	9	1	2	0
3C	0.152 nmol of PFK tetramer (identical with that in 2A) plus 0.217 nmol of modified PFK tetramer (identical with that in 2B)	80	3750	2880	36	42	1	19	21

[a] See Discussion for a description of each model and assumptions employed to arrive at % inhibition predicted. [b] SA, specific enzymatic activity [μmol of F-1,6-P_2 formed min^{-1} (mg of PFK)$^{-1}$].

in this experiment and that full sensitivity to inhibition by ATP was retained in the reassembled enzyme. The data for the reassembly of affinity-labeled phosphofructokinase containing 3.6 SO_2BzAdo groups/tetramer (Figure 3B) show a 97% yield for reassembly and full retention of the protection against inhibition by ATP in the reassembled tetramer (only 1% inhibition at 1.2 mM ATP). The data for dissociation and reassembly of a solution containing 0.229 nmol of native phosphofructokinase tetramers identical with that used in Figure 3A *plus* 0.200 nmol of affinity-labeled enzyme identical with that in Figure 3B are depicted in Figure 3C. The percent reassembly or reactivation in this experiment was 93%, and the inhibition produced by 1.2 mM ATP decreased from 47% before dissociation to 32% after dissociation and reassembly. This observed decrease in the sensitivity of the preparation to inhibition by 1.2 mM ATP suggests that (a) the distribution of labeled and unlabeled subunits among the tetramers after dissociation and reassembly differs from that present before dissociation and (b) considerably fewer than four SO_2BzAdo groups/tetramer are required to overcome the inhibition by ATP. In the reassembled enzyme in Figure 3C, only 42% of the subunits are labeled, yet 68% of the inhibition by 1.2 mM ATP is relieved. The results of this dissociation and reassembly experiment, as well as those of other experiments employing different concentrations and different preparations of native and affinity-labeled phosphofructokinase, are summarized in Table I. A more detailed consideration of the implications of these results will be presented under Discussion.

Second-order plots of the data presented in Figure 3, prepared as described by Parr & Hammes (1976), were linear. The slopes of these plots, which are directly related to either k_1 or k_2, the second-order rate constants in eq 1, are presented in Table I also.

Discussion

The results of Pettigrew & Frieden (1978), as well as those of the present study, indicate that 5′-FSO_2BzAdo is an affinity label specific for the adenine nucleotide binding site that binds the activators cAMP, AMP, and ADP. Furthermore, the results of both of these studies indicate that the alterations in the molecular and regulatory kinetic properties of the enzyme produced by covalent modification with 5′-FSO_2BzAdo appear to be identical with those produced by the binding of cAMP. The only confirmed difference between activation by cAMP and activation by 5′-FSO_2BzAdo is the involvement of covalent bonding of the activator to the enzyme in the latter case.

Although four SO_2BzAdo groups can be covalently attached to a phosphofructokinase tetramer (one group per 80000-dalton monomer), the data presented in Figures 1 and 2 indicate that a near maximum effect on the regulatory kinetic properties of the enzyme may be realized when the affinity-labeled enzyme contains on average only two SO_2BzAdo groups per tetramer. This observation suggests that the distribution of labeled subunits among the tetramers in a preparation containing on average two SO_2BzAdo groups/tetramer is not random, for this would generate some tetramers that contained only native unlabeled subunits. Instead, the most likely distribution of labeled subunits in such a preparation would appear to be one in which most of the tetramers contain two labeled and two unlabeled subunits. Such a distribution might indicate that native phosphofructokinase is comprised

of two subunits or protomers of one type and two of another type, with one type being much more reactive with 5′-FSO_2BzAdo than the other. This would seem to be an unlikely possibility since the protomers appear to be identical in all other respects. It appears more probable that this distribution of two labeled subunits per tetramer could be the result of negative cooperativity between labeled and unlabeled subunits in the tetramer, which leads to a decrease in the binding affinity of the enzyme for the second two affinity labels or to a decrease in the rate of covalent bond formation between the second two bound affinity labels and the enzyme.

The results of the investigation of the dissociation and reassembly of native and affinity-labeled phosphofructokinase preparations, undertaken to obtain information as to the locations of the third and fourth SO_2BzAdo groups that can be covalently attached to a phosphofructokinase tetramer, are summarized in Table I. In the experiments designated 1C–3C, the solutions that were dissociated and then allowed to reassemble contained both native phosphofructokinase and affinity-labeled phosphofructokinase. In all three experiments, the activation or relief of ATP inhibition observed after dissociation and reassembly was significantly greater than that observed before dissociation. This enhanced activation of the enzyme is not the result of a change in the sensitivity of the native phosphofructokinase to ATP due to the dissociation and reassembly process itself since no change in the sensitivity of native phosphofructokinase to inhibition by ATP was observed when the native enzyme alone was dissociated and allowed to reassemble (Table I, experiments 1A–3A; Figure 3A). The increased relief of ATP inhibition observed in experiments 1C–3C also is not the result of a preferential loss or low yield in the reassembly of the ATP-sensitive native phosphofructokinase since all losses have been taken into account in the calculated values presented in the column labeled model I in Table I, as will be explained later. On the other hand, a plausible explanation for the decrease in sensitivity of the preparation to inhibition by ATP after dissociation and reassembly is that the process results in a wider distribution of labeled subunits among the tetramers. This would decrease the inhibition observed at 1.2 mM ATP provided (a) two labeled subunits per tetramer (one labeled subunit in each of the two dimers that comprise the tetramer) leads to complete relief of ATP inhibition, as suggested by the data in Figure 2, and (b) all affinity-labeled preparations containing four covalently attached affinity labels per tetramer contain one SO_2BzAdo group attached to the allosteric activator binding site of each monomer.

Both dissociation of the phosphofructokinase tetramer to monomers and the reassembly of monomers to form tetramers appear to proceed through a dimer intermediate (Parr & Hammes, 1976). Four possible models for the reassembly of tetramers from a solution containing both labeled and native monomers have been analyzed in an attempt to arrive at a predicted value for the inhibition expected at 1.2 mM ATP were this model operative in the reassembly process. The four models are as follows. (Model I) The reassembly process leads to a distribution of labeled monomers among the reassembled tetramers that is identical with that present before dissociation of the solution containing both native tetramers and labeled tetramers, with the percent reactivation or reassembly of each being the same as that observed when the native and the affinity-labeled enzyme preparations were dissociated and reassembled separately. Although an unlikely model, model I should yield the percent inhibition observed at 1.2 mM ATP in the mixture before dissociation corrected for the fact that the percent yield for reactivation or reassembly into active tetramers is slightly different for native and modified phosphofructokinase preparations. (Model II) The reassembly process leads preferentially to the formation of dimers containing either all labeled subunits or all unlabeled subunits. These dimers then aggregate to form enzymatically active tetramers. (Model III) The reassembly process results in the preferential formation of dimer intermediates containing one labeled and one unlabeled monomer, which then aggregate to form active tetramers. (Model IV) The reassembly process is a random one leading to a binomial distribution of labeled subunits in the intermediate dimers which then aggregate to form active tetramers.

The following four assumptions were employed in the calculations of the expected inhibition at 1.2 mM ATP for all four models. (1) All affinity-labeled monomers are identical, and each contains one SO_2BzAdo group covalently bound to the activator binding site. (2) The tetramer is comprised of two dimers. (3) In a tetramer, any dimer containing either one or two labeled subunits will possess specific enzymatic activities (per milligram of protein) at 0.15 and 1.2 mM ATP identical with those of the original affinity-labeled phosphofructokinase that contained 3.4 or 3.6 labeled subunits/tetramer; i.e., 1.2 mM ATP will not be inhibitory. In addition, the percent yield in the reactivation or reassembly of dimers of this type will be the same as that observed for the reassembly of labeled enzyme containing 3.4 or 3.6 labeled subunits/tetramer. (4) In a tetramer, any dimer containing two unlabeled monomers will have specific enzymatic activities (per milligram of protein) at 0.15 and 1.2 mM ATP identical with those of the original native phosphofructokinase, i.e., 98–99% inhibition at 1.2 mM ATP. In addition, the percent yield in the reactivation or reassembly of dimers of this type will be the same as that observed for native phosphofructokinase.

If one knows the amounts of labeled and native tetramers present in the solution before dissociation, as well as the average number of labeled subunits/labeled tetramer, the number of labeled and unlabeled subunits in the solution after dissociation can be calculated. From these values, the number of intermediate dimers formed during reassembly that contain two unlabeled subunits, one labeled and one unlabeled subunit, and two labeled subunits can be calculated for each proposed model. By employment of the assumptions listed above as to the yield for reassembly of each kind of dimer to active tetramers and the specific activities at 0.15 and 1.2 mM ATP for each kind of dimer in a reassembled tetramer, the predicted percent inhibition produced by 1.2 mM ATP can be obtained for each model.

A comparison of the final two columns of Table I indicates that the inhibition observed at 1.2 mM ATP is in remarkably good agreement with that predicted by model IV. This agreement between the predicted and observed values in all three experiments would appear to constitute support not only for model IV, the random reassembly process, but also for the four basic assumptions employed in the calculations of the predicted percent inhibitions. Hence, the complete activation at 1.2 mM ATP by approximately two covalently bound SO_2BzAdo groups per tetramer (one per dimer) suggested by the data in Figure 2 is further substantiated by the results of the reassembly experiments.

The rate constants for the reactivation or reassembly of the phosphofructokinase preparations, i.e., the slopes of the second-order plots prepared as described by Parr & Hammes (1976), are given in the columns of Table I labeled slope and slope*, with the former referring to the slope when the extent

of reactivation as a function of time was determined by measuring the enzymatic activity at 0.15 mM ATP and the latter to the slope when the extent of reactivation was determined by measuring the enzymatic activity at 1.2 mM ATP. These dissociation and reassembly experiments were carried out at pH 7.0, and the average value obtained for the slope in the reassembly of native phosphofructokinase (Table I, experiments 1A–3A) was 1750 M^{-1} s^{-1}, which is lower than the average value of 7900 M^{-1} s^{-1} observed by Parr & Hammes (1976) at pH 8.0. A decrease in the rate of assembly at lower pH values is compatible with the observations of others indicating that dissociation of the phosphofructokinase tetramer is promoted by low pH (Paetkau & Lardy, 1967; Aaronson & Frieden, 1972; Pavelich & Hammes, 1973; Hesterberg & Lee, 1982). The average value obtained for the slope in the reassembly of affinity-labeled phosphofructokinase preparations containing 3.4 and 3.6 SO_2BzAdo groups per tetramer was 4780 M^{-1} s^{-1}, which is 2.7-fold greater than that observed for native phosphofructokinase and essentially identical with the value of 4590 M^{-1} s^{-1} obtained for the reassembly of dissociated native enzyme in the presence of cAMP. The greater rate of reassembly observed with the affinity-labeled preparation or with native enzyme in the presence of cAMP is compatible with the observation that activators of phosphofructokinase stabilize the tetrameric form of the enzyme (Lad et al., 1973; Hesterberg & Lee, 1982). The rate constants or slope values obtained for the reassembly of dissociated mixtures of native and affinity-labeled phosphofructokinase were intermediate between those observed for the native enzyme and those observed for the affinity-labeled preparations. A value for slope* could not be obtained for the reassembly of dissociated native phosphofructokinase because the reassembled preparation, like native phosphofructokinase, is 98–99% inhibited at 1.2 mM ATP. Slope* values obtained for the reassembly of dissociated affinity-labeled enzyme preparations (experiments 1B–3B) were essentially identical with the slope values, indicating that when the affinity-labeled monomers reassemble to form active tetramers, they are fully protected against inhibition by 1.2 mM ATP. Interestingly, the slope* values observed for the reassembly of dissociated mixtures of native and affinity-labeled enzyme preparations (experiments 1C–3C), where a large fraction of the intermediate dimers formed could contain one labeled and one unlabeled subunit, are consistently about 25% less than the corresponding slope values, suggesting that the formation of enzymatically active tetramers is somewhat more rapid than the achievement of insensitivity to inhibition by 1.2 mM ATP.

The validity of the major conclusion drawn from this study, i.e., that the covalent attachment of only two SO_2BzAdo groups/phosphofructokinase tetramer (one group/dimer) produces a near maximum effect on the regulatory and kinetic properties of the enzyme, is dependent upon the accuracy of the spectrophotometric method employed to determine the number of SO_2BzAdo groups covalently incorporated/phosphofructokinase tetramer. The assumption implicit in the spectrophotometric method is that the extinction coefficients of 5′-FSO_2BzAdo at 259 and 279 nm are not altered upon covalent attachment to the protein. The method has been successfully employed with other proteins (Lee et al., 1981; Tobias & Strickler, 1981) and would appear to be valid for phosphofructokinase also since the maximum number of SO_2BzAdo groups incorporated, as determined by the spectrophotometric method, was one per subunit, which is equal to the number of cAMP binding sites per subunit and to the number reported by Pettigrew & Frieden (1978) for the incorporation of [8-^{14}C]-5′-FSO_2BzAdo.

Acknowledgments

I gratefully acknowledge the diligent and skillful technical assistance given by Siang Tsao.

Registry No. PFK, 9001-80-3; 5′-FSO_2BzAdo, 57454-44-1; cAMP, 60-92-4; ATP, 56-65-5.

References

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

Cooperman, B. S., & Brunswick, D. J. (1973) *Biochemistry 12*, 4079–4084.

Craig, D. W., & Hammes, G. G. (1980) *Biochemistry 19*, 330–334.

Hesterberg, L. K., & Lee, J. C. (1982) *Biochemistry 21*, 216–222.

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.

Lascu, I., Kezdi, M., Goia, I., Jebeleanu, G., Barzu, O., Pansini, A., Papa, S., & Mantsch, H. H. (1979) *Biochemistry 18*, 4818–4826.

Lee, Y., Esch, F. S., & DeLuca, M. A. (1981) *Biochemistry 20*, 1253–1256.

Lowry, O. H., & Passonneau, J. V. (1966) *J. Biol. Chem. 241*, 2268–2279.

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.

Parr, G. R., & Hammes, G. G. (1976) *Biochemistry 15*, 857–862.

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.

Tobias, B., & Strickler, R. C. (1981) *Biochemistry 20*, 5546–5549.

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

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

Wyatt, J. L., & Colman, R. F. (1977) *Biochemistry 16*, 1333–1342.