Modification of the ATP Inhibitory Site of the *Ascaris suum* Phosphofructokinase Results in the Stabilization of an Inactive T State†

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ABSTRACT: Treatment of the *Ascaris suum* phosphofructokinase (PFK) with 2′,3′-dialdehyde ATP (oATP) results in an enzyme form that is inactive. The conformational integrity of the active site, however, is preserved, suggesting that oATP modification locks the PFK into an inactive T state that cannot be activated. A rapid, irreversible first-order inactivation of the PFK is observed in the presence of oATP. The rate of inactivation is saturable and gives a K_{oATP} of 1.07 ± 0.27 mM. Complete protection against inactivation is afforded by high concentrations of ATP, and the dependence of the inactivation rate on the concentration of ATP gives a K_i of 326 ± 26 μM for ATP which is 22-fold higher than the K_m for ATP at the catalytic site but close to the binding constant for ATP to the inhibitory site. Fructose 6-phosphate, fructose 2,6-bisphosphate, and AMP provide only partial protection against modification. The pH dependence of the inactivation rate gives a pK_a of 8.4 ± 0.1. Approximately 2 mol of [^{3}H]oATP is incorporated into a subunit of PFK concomitant with 90% loss of activity, and ATP prevents the derivatization of 1 mol/subunit. The oATP-modified enzyme is not activated by AMP or fructose 2,6-bisphosphate. oATP has no effect on the activity of a desensitized form of PFK in which the ATP inhibitory site is modified with diethyl pyrocarbonate but with the active site intact [Rao, G. S. J., Wariso, B. A., Cook, P. F., Hofer, H. W., & Harris, B. G. (1987) *J. Biol. Chem. 262*, 14068–14073]. This desensitized enzyme incorporates only 0.2–0.3 mol of [^{3}H]oATP/subunit, suggesting that in the native enzyme inactivation perhaps results from the modification of the ATP inhibitory site rather than the catalytic site. Modification of an active-site thiol by 4,4′-dithiodipyridine is prevented by ATP before and after oATP treatment. Finally, gel filtration HPLC studies show that the oATP-modified enzyme retains its tetrameric state and neither the tryptophan fluorescence nor the circular dichroic spectra of the modified enzyme are affected by fructose 2,6-bisphosphate, suggesting that the enzyme is locked into a tetrameric inactive T state.

A form of the *Ascaris suum* phosphofructokinase (PFK)[1] has been prepared by treatment with diethyl pyrocarbonate that is selectively desensitized to hysteresis in the time course, positive homotropic cooperativity by F6P, and ATP inhibition (Rao et al., 1987a). This form of PFK is fully active, is not inhibited by ATP even up to concentrations of 5 mM, and is activated by AMP and F2,6P_2 (Payne et al., 1991). The desensitized PFK is amenable to studies of the catalytic site, independent of the interactions at the ATP inhibitory site. As such, it is a positive point of reference for studies with the native enzyme, serving as an active (relaxed) conformer. The kinetic (Rao et al., 1987b; Payne et al., 1991) and spectral (Rao et al., 1991) properties of the desensitized enzyme have been determined in detail. A stabilized form of an inactive (taut) conformer would be equally useful to serve as a negative point of reference.

Periodate-oxidized nucleotide analogues such as oATP and others have served as useful tools in labeling nucleotide binding sites in proteins (Colman, 1983; Bazaes, 1987; Schraw & Post, 1989; Dallachio et al., 1976; Mas & Colman, 1983). They are known to specifically modify lysine residues by forming a Schiff's base (Dallachio et al., 1976; Easterbrook-Smith et al., 1976) or dihydroxymorpholino adduct (Colman, 1983; Lowe & Beechey, 1982; Gregory & Kaiser, 1979). The reaction of oATP with rabbit muscle PFK, reported by Gregory and Kaiser in 1979, is one of the earlier examples of studies using this affinity analogue. These studies, carried out with the native enzyme, have shown that the reagent inactivates the enzyme by a mechanism other than the dissociation of the tetramers to dimers, and the authors have concluded that the lysine residues modified are important to catalytic activity or binding of ATP.

In this paper, we report studies of oATP inactivation of *A. suum* PFK. Data indicate that the native enzyme is inactivated as a result of irreversible binding of oATP at the ATP inhibitory site. The end result is the stabilization of PFK in an inactive conformation with an intact active site.

Materials and Methods

Chemicals. Imidazole, Tris, 2-mercaptoethanol, DTT, ATP, AMP, F6P, F2,6P_2, ethanolamine, and oATP were from Sigma. [2,8-^{3}H]ATP (30 Ci/mmol) was purchased from ICN Radiochemicals. [^{3}H]oATP was prepared from [^{3}H]ATP according to the method of Lowe and Beechy (1982). NADH was from P-L Biochemicals, while 4-PDS was obtained from Aldrich.

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[1] Abbreviations: PFK, phosphofructokinase; oATP, 2′,3′-dialdehyde ATP; DTT, dithiothreitol; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; F2,6P_2, fructose 2,6-bisphosphate; 4-PDS, 4,4′-dithiodipyridine; AMPPCP, 5′-adenylyl methylenediphosphonate; Mes, 2-(*N*-morpholino)ethanesulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Taps, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonate; Caps, 3-(cyclohexylamino)propanesulfonate.

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Enzymes. Aldolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase from rabbit muscle were from Sigma. These three coupling enzymes, initially purchased as ammonium sulfate suspensions, were dialyzed against 50 mM imidazole hydrochloride, pH 6.8, containing 5% glycerol and 10 mM 2-mercaptoethanol and stored frozen at −20 °C. *Ascaris suum* PFK was purified according to Starling et al. (1982) and stored at −20 °C in 50 mM KH_2PO_4, pH 7, containing 2% glycerol and 10 mM 2-mercaptoethanol. The desensitized PFK was prepared from the native enzyme according to the method of Rao et al. (1987a). The enzyme (1 mg/mL) was first dialyzed against 50 mM KH_2PO_4, pH 7, containing 2% glycerol and was then incubated with 1 mM diethyl pyrocarbonate for 10 min at 30 °C in the presence of 100 mM F6P, 0.2 mM AMPPCP, and 1 mM $MgCl_2$. The reaction was terminated by the addition of 5 mM DTT, and the modified enzyme was dialyzed against the same buffer to remove excess reagent. The desensitized enzyme was stored at −20 °C.

Enzyme Assays. The activity of phosphofructokinase was routinely measured spectrophotometrically in the direction of phosphorylation of F6P coupling the production of FBP to the aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase reactions (Racker, 1947). A typical assay contained the following in 1 mL: 50 mM Tris-HCl, pH 8, 40 mM $(NH_4)_2SO_4$, 8 mM $MgCl_2$, 0.2 mM NADH, 4 units of aldolase, 5 units of α-glycerophosphate dehydrogenase, 13 units of triosephosphate isomerase, 0.2 mM ATP, and 20 mM F6P. The reaction was initiated by the addition of the enzyme and was monitored by the decrease in absorbance of NADH at 340 nm using a Gilford 250 recording spectrophotometer, equipped with a circulating water bath to maintain the temperature of the thermospacers of the cell compartment at 30 °C. For studies of the effect of AMP and $F2,6P_2$ on enzyme activity, assays were performed in imidazole hydrochloride, pH 6.8.

Chemical Modification with oATP. Stock solutions of oATP were freshly prepared in distilled water. Phosphofructokinase was dialyzed into 50 mM KH_2PO_4, pH 8, containing 2% glycerol and 2 mM DTT. The dialyzed enzyme was then incubated at 30 °C with oATP as described in the figure legends, and aliquots of the reaction mixture were assayed at pH 8.0 for residual enzyme activity. Protection against oATP modification was carried out by incubating the enzyme with the protective ligand for 2 min prior to the initiation of the reaction with oATP.

The pH dependence of the inactivation rate was determined using 1 mM oATP with the following buffers at a final concentration of 50 mM: Mes, pH 6.5–7.5; Hepes, 7.5–8.5; Taps, pH 8.5–9.5; Caps, pH 9.5–10.

Stoichiometry of oATP Modification. The number of moles of oATP incorporated into native PFK was measured by using [^{3}H]oATP in the inactivation reaction. A 0.5-mg aliquot of enzyme was modified as above for 20 min. The reaction was terminated by the addition of 50 μL of 0.5 M ethanolamine, pH 8. The free [^{3}H]oATP in the sample was removed by chromatography on a Sephadex G-50 column (1.3 × 120 cm) equilibrated with 50 mM KH_2PO_4, pH 7, at a flow rate of 12 mL/h. An aliquot of this labeled PFK was then counted, and another aliquot was used for estimation of protein. The stoichiometry was calculated from these values and the specific activity of [^{3}H]oATP in the original reaction mixture.

Reaction of PFK with 4-PDS. A stock solution of 4-PDS (10 mM) was prepared in 50% ethanol. The effect of the reagent on the enzyme was studied by incubating the enzyme with the reagent at different concentrations in 50 mM KH_2PO_4, pH 7, at 30 °C. The time course of inactivation was monitored by assaying aliquots of the inactivation mixture with time using the coupled assay. The stoichiometry of thiol modification was obtained by measuring the increase in absorbance at 324 nm due to the formation of 4-thiopyridine (ϵ_{324} = 19 800 M^{-1} cm^{-1}; Grassetti & Murray, 1967).

Fluorescence Measurements. All fluorescence spectra were recorded using an Aminco-Bowman SPF-500 spectrofluorometer in the normal mode. The final enzyme concentration was 18 μg/mL in 20 mM KH_2PO_4, pH 6.8. Temperature was maintained using a water-jacketed cuvette holder maintained at 30 °C with a circulating water bath. The excitation monochromator was set at 290 nm (4-nm band-pass), and the emssion spectrum was scanned from 300 to 400 nm with an 8-nm band-pass.

CD Spectral Measurements. CD spectra were recorded on an Aviv Model 62 HDS spectropolarimeter, equipped with an RC6 Lauda refrigerated circulating bath to maintain constant compartment temperature. The enzyme (0.2 mg/mL) in 20 mM KH_2PO_4, pH 6.6, was placed in 0.2-cm quartz cuvettes, and spectra were recorded from 250 to 190 nm, at intervals of 1 nm and a dwell time of 3 s. Each spectrum was the average of two repetitions. Appropriate buffer blanks were determined for each spectrum and subtracted to obtain corrected spectra.

Data Processing. Apparent rate constants for inactivation (k_{inact}) were obtained by fitting the data for inactivation to eq 1 containing the expression for a first-order process and a constant. Data for the oATP dependence of the inactivation rate were fitted using eq 2. Data for the pH dependence of k_{inact} were fitted using eq 3:

$$A = Be^{-kt} + C \quad (1)$$

$$1/k = (K_i/k_{max})(1/I) + 1/k_{max} \quad (2)$$

$$\log y = \log [C/(1 + H/K_1)] \quad (3)$$

In eq 1, A represents the percent remaining activity at intervals of time, t, B and C are constants, and k is the rate of inactivation (k_{inact}). In eq 2, k is the inactivation rate observed at any oATP concentration, k_{max} is the inactivation rate at saturating oATP, K_i is the K_m for oATP, and I represents the concentration of oATP. In eq 3, y is the observed value of k_{inact} at any pH, C is the pH-independent value of k_{inact}, K_1 is the dissociation constant for the group being modified, and H represents the hydrogen ion concentration.

Results

Inactivation of Native Enzyme by oATP. When *Ascaris suum* phosphofructokinase is incubated with oATP in 50 mM Taps, pH 8, at 30 °C, a time-dependent inactivation of the enzyme is observed (Figure 1). Under identical conditions, the control without the reagent shows less than 10% inactivation. The inactivation rate increases with increasing concentrations of oATP. The semilog plots of percent remaining activity vs time (Figure 1) are nonlinear at higher time points, suggesting incomplete inactivation of the enzyme. Hence, instead of using a simple first-order expression to evaluate the rate constants for inactivation, an expression of the form shown in eq 1 containing a first-order expression plus a constant term was used. The constant term serves to define the portion of activity unaffected by the reagent. The values of k_{inact} thus obtained were 0.074 ± 0.020 min^{-1} at 0.25 mM, 0.120 ± 0.016 min^{-1} at 0.5 mM, 0.235 ± 0.026 min^{-1} at 1.25 mM, and 0.310 ± 0.029 min^{-1} at 2.5 mM oATP. A double-reciprocal plot of

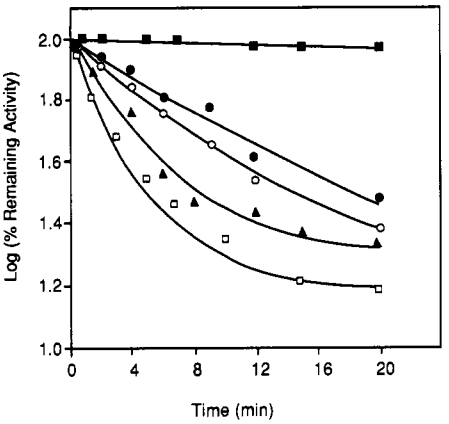

FIGURE 1: Reaction of *Ascaris suum* PFK with oATP. Dependence of the rate of inactivation on the concentration of oATP. Enzyme (30 μg/mL) was incubated with oATP at the concentrations indicated in 50 mM Taps, pH 8, with 1 mM DTT at 30 °C. Aliquots were withdrawn at the time intervals shown and assayed for PFK activity as described under Materials and Methods. The concentrations in millimolar oATP used are 0 (■), 0.25 (●), 0.5 (○), 1.25 (▲), and 2.5 (□). The points are experimental values while the solid curves are theoretical values from a fit of the data using eq 1 and the fitted parameters.

the inactivation rate vs oATP was linear (figure not shown), and a fit of these data using eq 2 gave a K_{oATP} of 1.07 ± 0.27 mM, a k_{max} of 0.36 ± 0.03 min^{-1}, and a k_{max}/K_i of 337 ± 62 M^{-1} min^{-1}.

As stated above, complete inactivation of the enzyme could not be achieved even at the highest concentration of oATP used. The inactivation obtained is irreversible, and the modified enzyme does not regain activity even after long periods of incubation at room temperature or after extensive dialysis to remove excess reagent. Addition of 30 mM borate to the modification reaction also did not result in significant enhancement of the rate of inactivation, and the modified adduct was stable without reduction with $NaBH_4$.

Effect of Ligands on the Rate of Inactivation of Native PFK by oATP. In order to demonstrate the specificity of the site modified by the reaction of oATP with the enzyme, the effect of various ligands on the inactivation rate was studied using 1 mM oATP at pH 8.0. Since the *Ascaris suum* PFK is known to exhibit lags and bursts in its reaction time course when preincubated with MgATP or F6P (Cook et al., 1987), the enzyme was preincubated with the ligand under study for at least 2 min prior to addition of oATP. First-order plots of log percent remaining activity vs time were used to determine values of k_{inact}. The results of these protection experiments are shown in Table I. The rate of inactivation is lowered by the presence of ATP at concentrations above 100 μM, indicating protection by this ligand. The extent of protection increases with increasing concentrations of ATP and reaches a maximum of 90% at 20 mM. The observed dependence of k_{inact} on the concentration of ATP enabled the determination of the apparent K_i for ATP from a Dixon plot of $1/k_{inact}$ vs ATP concentration. The appK_i value thus obtained from the data shown in Table I is 0.63 ± 0.05 mM. Among the other ligands tested, F6P, AMP, and $F2,6P_2$ provided significant but partial protection (Table I). The concentrations of these ligands used were at least 10 times their $S_{0.5}$ values determined in separate studies (Rao et al., 1991).

pH Dependence of the Inactivation Rate. Figure 2 shows the effect of pH on the rate of inactivation of *A. suum* PFK by 1 mM oATP. The rate decreases from a pH-independent value of 1.01 min^{-1} above pH 10, giving a pK_a of 8.4 ± 0.1. In order to ascertain whether this pK_a represents that of the

Table I: Effect of Ligands on the Inactivation Rate of *A. suum* Phosphofructokinase by oATP[a]

ligand	k_{inact} (min^{-1})	% activity remaining[b]
none	0.29 ● 0.02	16
ATP (mM)[c]		
0.1	0.24 ± 0.06	18
0.2	0.21 ± 0.04	35
0.6	0.17 ± 0.04	43
1.0	0.10 ± 0.01	56
5.0	0.03 ± 0.005	79
20.0	very slow	90
F6P (100 mM)	0.12 ± 0.02	69
$F2,6P_2$ (25 μM)	0.23 ± 0.03	49
AMP (1 mM)	0.19 ± 0.03	38

[a] The enzyme (25 μg/mL) was incubated for 2 min with the ligand in 50 mM Taps, pH 8.0 at 30 °C, followed by oATP (1 mM). The values for k_{inact} were determined as stated under Materials and Methods and in the legend to Figure 1. [b] Values represent residual activity at 10 min after the addition of oATP. [c] In experiments where ATP was added, 1 mM $MgCl_2$ was also included up to 1 mM ATP, and then $MgCl_2$ stoichiometric to ATP was added.

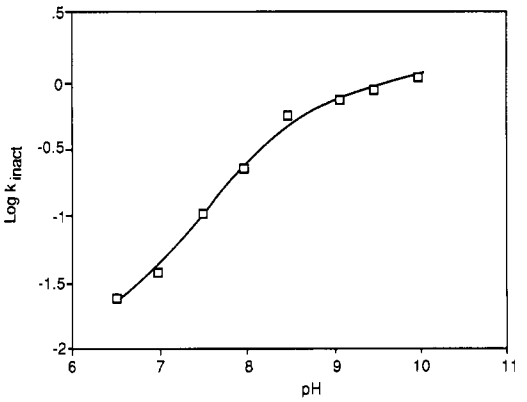

FIGURE 2: pH dependence of k_{inact}. The enzyme (30 μg/mL) was treated with 1 mM oATP at the pH values indicated, and the rate of inactivation was measured as described under Materials and Methods. The points are experimental values while the solid curve is the theoretical value from a fit of the data using eq 2 assuming a pK_a of 8.4 ● 0.1.

enzyme group modified, K_{oATP} was determined from the dependence of k_{inact} on the concentration of oATP at pH 7.1 and 9.3. The values obtained, 0.63 ± 0.1 and 0.78 ± 0.24 mM, respectively, are not significantly different from the value 1.07 ± 0.27 mM at pH 8. Thus, the pH dependence does not reflect the binding of oATP.

Stoichiometry of oATP Modification. The stoichiometry of the modification reaction was studied using [2,8-^{3}H]oATP as discussed under Materials and Methods. Stoichiometry was calculated from the measured protein and radioactivity of the labeled enzyme. The native enzyme incorporates 2.16 ± 0.04 mol of oATP/subunit concomitant with the loss of 84% of the original activity (data not shown). Prolonged incubation with excess reagent or the addition of $NaBH_4$ to the reaction mixture following the inactivation reaction does not result in any further inactivation or higher incorporation of the radiolabel. In addition, treatment of the labeled enzyme with activated charcoal does not result in any loss of radioactivity excluding the possibility of the presence of loosely bound oATP in the modified enzyme. When an aliquot of the labeled enzyme was treated with 5 M guanidine hydrochloride for 1 h at room temperature and then dialyzed to remove the denaturant, there was no loss of radioactivity, indicating that denaturation does not release the bound oATP. Thus, the results suggest that oATP covalently modifies two enzyme residues per subunit of the PFK. When the modification

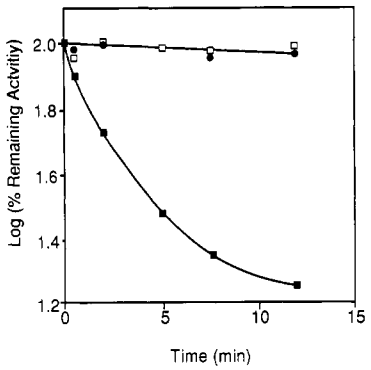

FIGURE 3: Effect of oATP on the desensitized form of *A. suum* PFK. A 40 μg/mL sample of dPFK (●) was incubated with 2 mM oATP in 50 mM Taps, pH 8, with 2 mM DTT. With time, 20-μL aliquots were withdrawn and assayed for PFK activity as described under Materials and Methods. A 30 μg/mL sample of the native enzyme, with (■) and without (□) oATP (2 mM), was treated in an identical manner for comparison.

reaction was carried out with [^{3}H]oATP in the presence of 20 mM ATP, the enzyme incorporated only 0.91 ± 0.05 oATP/subunit and retained about 78% of the original activity (data not shown). Thus, ATP protects only one of the two residues modified by oATP.

The enzyme modified with [2,8-^{3}H]oATP was subjected to digestion with endoproteinase Arg C, and the resultant major radiolabeled peptide was purified by reverse-phase HPLC on a C_{18} column at pH 4.0. The peptide was then subjected to automated Edman degradation on a solid-phase peptide sequencer, but no sequence was obtained. It thus appears that the modified residue is close to the N-terminus of the isolated peptide and that it is not processed by Edman degradation. Additional attempts using chymotrypsin and trypsin for digestion and subsequent peptide isolation procedures were also unsuccessful due to the instability of the oATP adduct with the enzyme at acid pH employed during peptide isolation procedures and also during sequencing.

Effect of oATP on Desensitized PFK. The high K_{oATP} value obtained from the dependence of the inactivation rate on oATP concentration and the requirement for millimolar concentrations of ATP for full protection of the enzyme against inactivation suggest that the enzyme is inactivated by modification of the ATP inhibitory site rather than the active site. This hypothesis was tested by studying the effects of oATP on desensitized enzyme. This enzyme form has practically lost its ability to be inhibited by ATP but is catalytically active. Figure 3 shows that under conditions that produce 90% inactivation of the native enzyme, i.e., 2 mM oATP in 50 mM Taps at pH 8, desensitized PFK is unaffected. This result is substantiated by the lack of incorporation of [^{3}H]oATP into this enzyme. A maximum of 0.3 ± 0.01 mol of oATP/mol of subunit was incorporated into desensitized PFK compared to the value 2.16 ± 0.14 obtained for the native enzyme (data not shown).

Reaction of PFK with 4-PDS. To further show that the catalytic site of the enzyme is not modified by oATP, the following thiol modification experiments were carried out with the native PFK and oATP-modified PFK using the thiol reagent 4-PDS. When the native enzyme is incubated with 4-PDS in phosphate buffer, pH 7 at 30 °C, a concentration-dependent first-order inactivation of the enzyme is observed. A double-reciprocal plot of k_{inact} against the concentration of 4-PDS, derived from the first-order plots, is linear and passes through the origin (data not shown), giving a second-order rate constant of 220 M^{-1} min^{-1}. Protection against 4-PDS inactivation by MgATP is observed even at very low concentrations (40–50 μM), implying that the reagent modifies a high-affinity ATP site on the enzyme. To further characterize this site, the rate of inactivation was measured as a function of ATP concentration at a fixed concentration of 10 μM 4-PDS. The Dixon plot (not shown) of $1/k_{inact}$ vs ATP concentration gave a K_i value of 15 μM for ATP at this site, comparable to the K_m for MgATP (20 μM; Rao et al., 1987b).

Table II: Stoichiometry of the Reaction of 4-PDS with Native and oATP-Modified Enzymes[a]

enzyme	additions	thiols/subunit	% activity remaining
native PFK	none	2.79 ± 0.06	8 ± 4
	ATP (0.2 mM), $MgCl_2$ (1 mM)	1.80 ± 0.02	78 ± 6
modified PFK	none	1.79 ± 0.2	
	ATP (0.2 mM), $MgCl_2$ (1 mM)	0.90 ± 0.05	

[a] An 80 μg/mL solution of the appropriate enzyme was treated with 10 μM 4-PDS in 20 mM KH_2PO_4, pH 7 at 30 °C, and the absorbance at 324 nm was continuously monitored. The number of thiols per subunit was calculated from the measured maximal absorbance change using ϵ_{324} = 19 300 M^{-1} cm^{-1}.

The number of thiols modified by 4-PDS was determined by measuring the absorbance of the product, 4-thiopyridine, at 324 nm. For native PFK, approximately three thiols per subunit are modified by the reagent, but only one of these is protected by MgATP (Table II). The protection results in the retention of about 80% of the initial enzyme activity. Under identical conditions, the reagent modifies two thiols per subunit of oATP-modified enzyme, and one of these is still protected by MgATP.

Characterization of oATP-Labeled PFK. When this form of the enzyme is subjected to gel filtration HPLC on an Ultrastyragel TSK-400 column along with molecular weight standards, the elution volume corresponds to the tetrameric form of the enzyme (data not shown). Thus, modification of PFK by oATP does not result in dissociation of the enzyme into subunits.

Since the data presented so far suggest that inactivation of the *A. suum* PFK by oATP results in a nonactive site and irreversible inhibition of the enzyme, it was of interest to examine whether the modification caused a permanent loss of sensitivity to allosteric effectors, such as AMP and F2,6-P_2. Hence, the effect of 1 mM AMP and 10 μM F2,6P_2 on the activity of the modified enzyme was studied at pH 6.8, under conditions which produce >90% inhibition of the enzyme activity (1 mM MgATP and 1 mM F6P). It was observed that neither AMP nor F2,6P_2 had any activating effect on the modified PFK while the native enzyme was maximally activated (results not shown). Figure 4 shows the effect of F2,6P_2 on the intrinsic tryptophan fluorescence of the native and oATP-modified PFK. Addition of 10 μM F2,6P_2 to native enzyme at pH 6.8 causes a substantial quenching of fluorescence at 335 nm (Figure 4A). Under the same conditions, the effector causes very little change in the spectrum of the modified enzyme (Figure 4B).

Likewise, the CD spectrum of the native enzyme shows a large change when 10 μM F2,6P_2 is added (Figure 5A), but the spectrum of the modified enzyme does not change significantly when F2,6P_2 is added (Figure 5B). Comparison of the CD spectra of the two enzyme forms shows that the modified enzyme is largely in the same conformation as the unmodified enzyme, although there is some small deviation between the two (Figure 5C). The small changes observed in Figure 5C may suggest the presence of an equilibrium

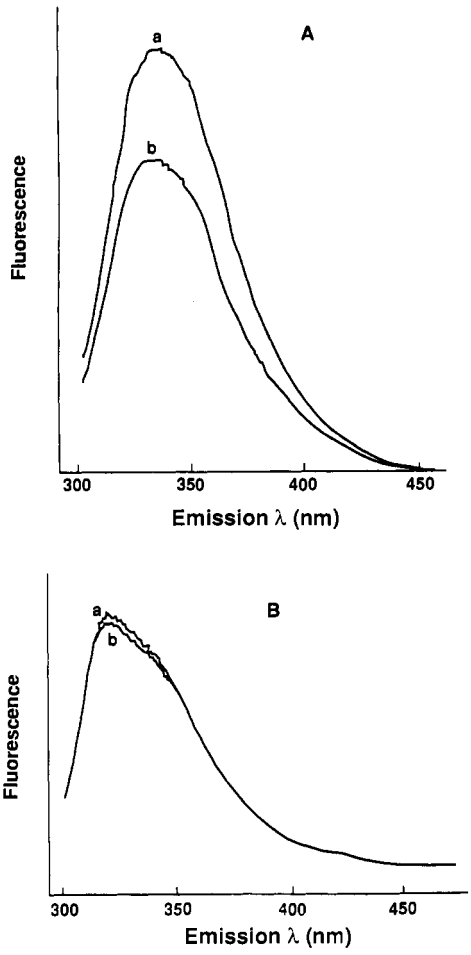

FIGURE 4: Comparison of the effect of F2,6P_2 on the intrinsic tryptophan fluorescence of native enzyme and oATP-modified enzymes. Spectra were recorded as described under Materials and Methods. Panels A and B represent spectra for native PFK and modified PFK, respectively, minus (a) and plus (b) F2,6P_2 (10 μM).

mixture of active and inactive states in the absence of effectors. Modification by oATP is responsible for shifting the equilibrium completely toward the inactive or less active T state.

DISCUSSION

The affinity analogue 2',3'-dialdehyde ATP rapidly inactivates the *Ascaris suum* PFK in a concentration-dependent manner. The overall process is saturable, implying that the reagent forms a reversible complex with the enzyme prior to modification. The inactivation conforms to first-order kinetics for some time and then levels off at each concentration of the reagent as is evident from the nonlinear semilog plots shown in Figure 1. This implies incomplete inactivation of the enzyme even with the highest concentrations of oATP used. This incomplete inactivation could be due to several causes such as (1) instability of the reagent. This possibility is eliminated by our observation that addition of excess reagent to the reaction mixture after the reaction levels off does not result in any further loss of activity. (2) The modification reaction could produce an enzyme form which is less active, but still retains a definite lower level of activity. This is a more likely possibility in the present case as the modification reaction carried out under several different conditions always resulted in an enzyme form which retained 10–15% of its original activity, even after extensive dialysis to remove the excess reagent.

Complete protection against inactivation is afforded only by high concentrations of ATP, suggesting that the site

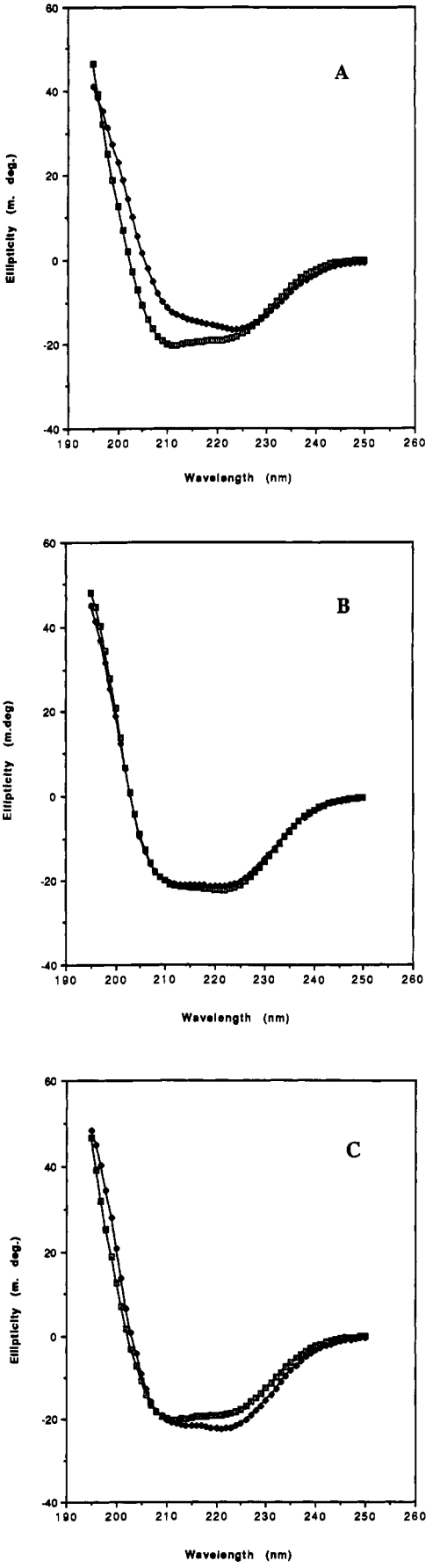

FIGURE 5: Effect of F2,6P_2 on the circular dichroic spectra of native and oATP-modified phosphofructokinase. (A) Native PFK in the absence (□) and in the presence (■) of 10 μM F2,6P_2. (B) oATP PFK in the absence (□) and in the presence (■) of 10 μM F2,6P_2. (C) Comparison between native (□) and oATP-modified enzyme (■).

modified is a low-affinity ATP site. Stoichiometry determinations indicate the modification of two enzyme residues by the affinity analogue, and only one of these is protected by ATP, suggesting that at least one of the two sites modified is in an ATP binding site. The reagent is shown to modify lysine residues in several enzymes (King & Colman, 1983; Easterbrook-Smith, 1976), including rabbit muscle PFK (Gregory & Kaiser, 1979), and if this is the case in *Ascaris suum* PFK, the modified group(s) has (have) an average pK of 8.4. Attempts at identification of the modified lysine(s) by isolating the labeled peptide were not successful due to the instability of the oATP adduct with the enzyme at acid pH and the inability to obtain a sequence when labeled peptide was isolated at higher pH. Such instability of the oATP adduct and problems associated with sequencing the labeled peptide are commonly reported in the literature (Colman, 1983). The absence of a need for $NaBH_4$ reduction for the stability of the modified adduct is consistent with the formation of a dihydroxymorpholino adduct as reported for rabbit muscle PFK (Gregory & Kaiser, 1979), adenosinetriphosphatase (Lowe & Beechy, 1982), and phosphorylase kinase (King & Carlson, 1981).

Modification is at the allosteric ATP inhibitory site as opposed to the active site based on a number of findings. (1) The concentration of oATP required to produce maximum inactivation is in the millimolar range (K_{oATP} = 1.07 mM), compared to micromolar concentrations of ATP required to saturate the catalytic ATP subsite (K_m = 15 μM; Rao et al., 1987b). Since oATP is a structural analogue of ATP, the large difference in the affinities is an indication that oATP may be interacting at a site different from the catalytic site. (2) There is an order of magnitude difference between protection against modification by oATP and protection against modification of a thiol in the ATP subsite of the catalytic site. The apparent K_i for ATP binding to the allosteric site is 630 ± 50 μM from the protection experiments of Table I. The oATP concentration in this experiment was 1 mM, and correcting for this [appK_i is equal to $K_i/(1 + [oATP]/K_{oATP})$] gives a K_i of 326 ± 26 μM, in agreement with the K_I of about 160 μM for substrate inhibition by ATP (Rao et al., 1987a). In contrast, binding of ATP at the active site, quantitated by protection against thiol modification by 4-PDS, gives a K_i of 15 μM (K_m for ATP = 15 μM) which is 22-fold lower than the K_i for ATP determined by protection against oATP modification. Therefore, it is obvious that the site modified by oATP is different from the catalytic site. (3) The pK_a for the residue modified by oATP is 8.4. The pH dependence of V/K_{MgATP} for the desensitized enzyme, determined in a separate study (unpublished observations of G. S. J. Rao in this laboratory), does not indicate the ionization of a group with this pK_a value in the catalytic site. (4) The diethyl pyrocarbonate modified form of PFK is desensitized to allosteric ATP inhibition (Rao et al., 1987a). The activity of the desensitized PFK is not affected by oATP, and only minimal incorporation of the reagent into this enzyme is observed. Since the ATP inhibitory site of this enzyme is blocked by reaction of histidines with diethyl pyrocarbonate, any interaction of oATP with this enzyme form should reflect the modification of the catalytic site. The absence of inactivation as well as incorporation is clear evidence that the reagent has little effect on the catalytic site. (5) The allosteric modulators AMP and F2,6P_2 attenuate the effect of ATP binding to its inhibitory site; that is, higher concentrations of ATP are required in the presence of these modulators to give an equivalent amount of inhibition (Srinivasan et al., 1990). There is no effect of these modulators at saturating concentrations on the K_i or K_m for ATP functioning as a substrate (Payne et al., 1991). Thus, partial protection against inactivation by oATP by F2,6P_2 and AMP[2] is consistent with modification at the allosteric ATP inhibitory site.

Since the PFK is inactivated by oATP, the conformational integrity of the active site can be questioned. Thus, data for modification of the active site of the *Ascaris suum* PFK by the thiol reagent 4-PDS are also presented. Specifically, the thiol appears to be in the ATP portion of the active site as shown by protection using low concentrations of ATP and the K_i value of 15 μM for ATP derived from these protection experiments. Active-site thiol modification has also been shown for other PFKs (Kemp, 1969; Hofer, 1970; Schwartz et al., 1976; Latshaw et al., 1987). The stoichiometry determinations indicate that although three thiols are modified in the native enzyme and two thiols in the oATP-modified enzyme, only one thiol is protected by ATP in both cases under identical conditions. In agreement with the protected thiol being in the active site, protection against its inactivation results in retention of 80% activity in the native enzyme.

In recent years, our laboratory has been attempting to obtain *Ascaris suum* PFK in different forms and to characterize these forms by kinetic analysis as well as physical studies. Desensitized PFK is one such form which lacks hysteresis in its time courses, positive homotropic (F6P) and negative heterotropic (ATP) cooperativity (Rao et al., 1987a), but retains a high specific activity with respect to native enzyme as well as its heterotropic positive (e.g., AMP and F2,6P_2) cooperativity (Payne et al., 1991). In other words, desensitization yields an enzyme locked into an R state. In the present study, evidence is presented for the preparation of a form of the enzyme that apparently maintains an integral active site but is less active, i.e., a T state of the enzyme. Interestingly, both the desensitized and oATP-modified forms of the enzyme are thought to result from a modification of the ATP inhibitory site, although presumably at different residues. However, in the case of desensitized enzyme, the modification is carried out in the presence of a high concentration of F6P which favors the active state. Once modified, the enzyme loses its ability to be drawn to the inactive state. In the case of oATP-modified enzyme, the situation is somewhat different. In the presence of high concentrations of oATP, the enzyme is predominantly in an inactive state, and upon irreversible binding of oATP, the enzyme is locked into this inactive state and loses its ability to be converted to the active state. The absence of activation by the allosteric effectors AMP and F2,6P_2 is further evidence for this hypothesis. In addition, as discussed above, native PFK exhibits a substantial quenching of tryptophan fluorescence in the presence of F2,6P_2. The oATP-modified enzyme, however, shows no such quenching at the same F2,6P_2 concentrations. Similar results are observed with the circular dichroism spectra. The modified enzyme retains a tetrameric subunit structure as suggested by gel filtration experiments. Hence, the oATP-modified enzyme, although it retains its quaternary structure, loses its ability to respond to structural alterations induced by the allosteric ligands in the native enzyme. Therefore, oATP modification results in an enzyme which is irreversibly locked into a less active state which ap-

[2] The K_{act} for AMP is 25 μM for native PFK and increases to 100 μM for desensitized PFK (Rao et al., 1991; Payne et al., 1991). These data suggest a close proximity of the ATP inhibitory site where modification by diethyl pyrocarbonate takes place and the AMP activator site. Thus, diethyl pyrocarbonate likely modifies between the two sites.

proaches the T state of the R-T model of Monod et al. (1965).

Registry No. PFK, 9001-80-3; ATP, 56-65-5; oATP, 54970-91-1.

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Proton Release during Successive Oxidation Steps of the Photosynthetic Water Oxidation Process: Stoichiometries and pH Dependence†

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ABSTRACT: Flash-induced absorption changes of pH-indicating dyes were investigated in photosystem II enriched membrane fragments, in order to retrieve the individual contributions to proton release of the successive transitions of the Kok cycle. These stoichiometric coefficients were found to be, in general, noninteger and to vary as a function of pH. Proton release on the $S_0 \rightarrow S_1$ step decreases from 1.75 at pH 5.5 to 1 at pH 8, while, on $S_1 \rightarrow S_2$ the stoichiometry increases from 0 to 0.5 in the same pH range and remains close to 1 for $S_2 \rightarrow S_3$. These findings are analyzed in terms of pK shifts of neighboring amino acid residues caused by electrostatic interactions with the redox centers involved in the two first transitions. The electrochromic shift of a chlorophyll, associated with the S transitions, responding to local electrostatic effects was investigated under similar conditions. The pH dependence of this signal upon the successive transitions was found correlated with the titration of the proton release stoichiometries, expressing the electrostatic balance between the oxidation and deprotonation processes.

An important element for understanding the process of photosynthetic water oxidation is the mechanism of proton release. The overall reaction, $2H_2O \rightarrow O_2 + 4e^- + 4H^+$, involves the abstraction of four electrons which are taken up one at a time by the reaction center of photosystem II (PS II).[1] The oxygen-evolving complex (OEC) thus goes through four successive oxidation states before releasing molecular oxygen. The behavior of the system under illumination by short sat-

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[1] Abbreviations: PS II, photosystem II; OEC, oxygen-evolving complex; DNP-INT, dinitrophenyl ether of iodonitrothymol; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; BBY, PS II enriched membrane preparation according to Berthold et al. (1981).

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