Interaction of Phosphorylase Kinase with the 2′,3′-Dialdehyde Derivative of Adenosine Triphosphate. 1. Kinetics of Inactivation†

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ABSTRACT: The 2′,3′-dialdehyde derivative of ATP (oATP) was found to be a valid affinity label for rabbit skeletal muscle phosphorylase kinase. Inactivation by oATP at pH 6.8 followed pseudo-first-order and saturation kinetics. An apparent K_i of approximately 6.7 μM was obtained in the presence of 0.6 mM Ca^{2+} plus 10 mM Mg^{2+}. Protection against the rate of inactivation was provided by the natural substrate ATP. In addition, at pH 8.2, oATP could be used as a substrate to phosphorylate phosphorylase *b*, thus providing evidence that oATP can bind to the active site of phosphorylase kinase. Inactivation of phosphorylase kinase by oATP was sensitive to various effectors of the enzyme such as Ca^{2+}, Mg^{2+}, and pH. Ca^{2+} plus Mg^{2+} synergistically enhanced the rate of inactivation severalfold; each metal ion by itself had little effect on the rate of inactivation. This synergism was seen both at pH 6.8 and at pH 8.2; however, the rates of inactivation were much greater at pH 6.8. The enhancement of inactivation by Ca^{2+} plus Mg^{2+} was also more pronounced with activated than with nonactivated kinase.

Phosphorylase kinase (ATP:phosphorylase-*b* phosphotransferase, EC 2.7.1.38) is a key enzyme in the cascade of reactions associated with glycogenolysis. The enzyme is reportedly a hexadecamer of four different subunits, $\alpha_4\beta_4\gamma_4\delta_4$ (Cohen et al., 1978). The roles of these subunits are known to some extent. The δ subunit has been found to be similar to, or identical with, calmodulin (Shenolikar et al., 1979) and is probably responsible for the Ca^{2+} requirement of the enzyme. The α and β subunits are phosphorylated by cAMP-dependent protein kinase and by phosphorylase kinase itself (Walsh et al., 1971; Hayakawa et al., 1973; Cohen, 1973). Phosphorylation of the β subunit more closely correlated with the increase in enzymatic activity (Hayakawa et al., 1973; Cohen, 1973) whereas the α subunit has been reported to have regulatory properties (Cohen & Antoniew, 1973). Several groups of investigators have reported either β or γ to be a catalytic subunit of phosphorylase kinase [for a review, see Carlson et al. (1979)]. Gulyaeva et al. (1977) have, in fact, reported labeling of both the β and γ subunits by ATP analogues containing alkylating groups in place of the triphosphate moiety. The loss of enzyme activity, measured exclusively by phosphorylase conversion, was found to follow incorporation of label into the β subunit. However, more than 1 mol of analogue was also incorporated into the γ subunit, but with no loss of activity.

For further investigation of the catalytic function of phosphorylase kinase through affinity labeling, an ATP analogue is required that can be used in low concentrations that would favor specificity of labeling. To further ensure specificity, the analogue should be proven to be an active site directed inhibitor. Furthermore, if the inactivation of phosphorylase kinase by the analogue could be influenced by effectors of the enzyme, then differentiation of ATP binding sites through variation in conditions might be possible, and nonspecific reactions could thus be largely ruled out. In this report, we characterize the interaction of phosphorylase kinase with the 2′,3′-dialdehyde derivative of ATP and show that this analogue is a valid affinity label that fulfills all of these requirements.

Experimental Procedures

Nonactivated phosphorylase kinase was prepared from rabbit skeletal muscle by the method of Brostrom et al. (1971) and in all cases further purified by DEAE-cellulose[1] chromatography (Cohen, 1973; Jennissen & Heilmeyer, 1975).

Phosphorylase *b* was isolated from rabbit skeletal muscle as described by Fischer & Krebs (1958). Residual AMP was removed by treatment with coconut charcoal.

Concentrations of phosphorylase *b* and phosphorylase kinase were routinely determined spectrophotometrically by using absorbance indexes of 13.0 (Kastenschmidt et al., 1968) and 12.4 (Cohen, 1973), respectively, for 1% protein solutions at 280 nm. [γ-^{32}P]ATP and [U-^{14}C]ATP were obtained from New England Nuclear.

Preparation of Autophosphorylated Phosphorylase Kinase. Autophosphorylated phosphorylase kinase was prepared from nonphosphorylated kinase (1.02 mg/mL) by autophosphorylation in Hepes buffer (68.7 mM, pH 8.0) in the presence of sucrose (6.3%), EDTA (0.13 mM), $CaCl_2$ (0.2 mM), and MgATP [10 mM $Mg(CH_3CO_2)_2$; 1.0 mM [γ-^{32}P]ATP at 9.5 Ci/mol]. The reaction was carried out at 30 °C for 12 min and resulted in the incorporation of 8.6 mol of phosphate per tetramer ($\alpha\beta\gamma\delta$) of phosphorylase kinase. The phosphorylated kinase was separated from the reaction mixture by filtration at 4 °C over Sephadex G-25. The enzyme was eluted with Hepes buffer (50 mM, pH 6.8) containing sucrose (10%) and EDTA (0.2 mM).

Enzyme Assays. Phosphorylase kinase activity was determined following the incorporation of ^{32}P into protein from [γ-^{32}P]ATP utilizing the filter paper assay described by Reimann et al. (1971), except that 1% sodium pyrophosphate was included in the rinses with 5% trichloroacetic acid.

Synthesis of oATP. The 2′,3′-dialdehyde derivative of ATP (oATP) was prepared by periodate oxidation of ATP (Easterbrook-Smith et al., 1976). [γ-^{32}P]oATP and [U-^{14}C]oATP

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[1] Abbreviations used: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; AMPPNP, adenylyl β,γ-imidodiphosphate; NaDodSO$_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; oATP, 2′,3′-dialdehyde of ATP; oAMPPNP, 2′,3′-dialdehyde of adenylyl β,γ-imidodiphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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were prepared from [γ-^{32}P]ATP (19.7 Ci/mol) and [U-^{14}C]ATP (5.7 Ci/mol), respectively. The purity of oATP was checked by thin-layer chromatography on poly(ethylenimine) sheets (0.8 M NH_4HCO_3 as the solvent) as described by Easterbrook-Smith et al. (1976). The spots were located by UV light and showed no unreacted ATP. When radioactive starting material was used, the areas corresponding to ATP and oATP were also cut out and counted in Liquiflour toluene scintillation fluid to determine the purity of the oATP. The concentration of oATP was determined spectrophotometrically at 258 nm, using a value of 14900 cm^{-1} M^{-1} for the extinction coefficient (Hansske et al., 1974).

Modification of Phosphorylase Kinase. Phosphorylase kinase was incubated at 30 °C for 3 min in Hepes buffer (30–90 mM, pH 6.8). Chelators and metal ions were included where indicated in the figure legends. Inactivation was started by addition of a small aliquot of oATP at a final concentration of 0.01 to 0.1 mM. The final concentrations in the modification mixtures are given in the figure legends. The activity of samples incubated in the absence of oATP was set as 100% activity.

Treatment of Modified Enzyme with $NaBH_4$ and $NaBH_3CN$. Phosphorylase kinase was incubated with oATP as described above. The reaction mixture was cooled to 0 °C, and an aliquot of $NaBH_4$ or $NaBH_3CN$ in 10 mM NaOH was added. The final concentration of reductant was 5–100 mM. The reduction was carried out on ice for 30 min to 1 h before reactivation of the enzyme was attempted, as described in the text. When the samples were prepared for $NaDodSO_4$ gel electrophoresis, the reduction was carried out for 2 h. Stopping the inactivation with mercaptoethanol before addition of reductant had no effect on the results.

$NaDodSO_4$ Gel Electrophoresis. Phosphorylase kinase was inactivated with [^{14}C]oATP and treated with $NaBH_4$ as described earlier. The subunits of the modified enzyme were separated on 7.5% polyacrylamide gels in the presence of $NaDodSO_4$ according to the procedure of Weber & Osborn (1969). The gels were stained with Coomassie brilliant blue and destained electrically in 7% acetic acid for 15 min. The protein bands were cut from the gels and decolorized and solubilized by heating with 0.2 mL of 30% H_2O_2 for 2 h at 80 °C. ^{14}C was counted after addition of 10 mL of Aquasol scintillation fluid. Only background radioactivity was found associated with the protein bands. Visualizing the protein bands with 1-anilino-8-naphthalenesulfonate (Hartman & Udenfriend, 1969) under UV light was milder than the above staining and destaining procedure but did not prevent the loss of [^{14}C]oATP from the protein bands.

Results

Kinetics of the Inactivation of Phosphorylase Kinase by oATP. Incubation of phosphorylase kinase at 30 °C and pH 6.8 with micromolar concentrations of the 2′,3′-dialdehyde derivative of ATP (oATP) resulted in the loss of more than 90% of the kinase activity in less than 20 min. Figure 1 shows a time course of the inactivation in the presence and absence of 10 μM oATP. The semilog plot is linear to an inactivation of 70%, indicating that the inactivation follows first-order kinetics. The inactivation half-time ($t_{0.5}$) obtained in Figure 1 is related to the observed first-order rate constant (k_{obsd}) by eq 1.

$$t_{0.5} = \frac{\ln 2}{k_{obsd}} \quad (1)$$

Figure 2 shows the dependence of k_{obsd} on the concentration of oATP. Saturation kinetics were observed, thus providing

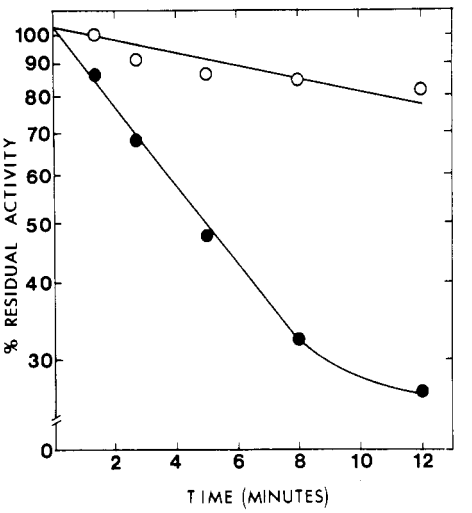

FIGURE 1: Time course of inactivation by oATP. Nonactivated phosphorylase kinase (0.18 mg/mL) was inactivated in Hepes buffer (34 mM) at pH 6.8 by oATP (10 μM). The reaction included EGTA (0.2 mM), EDTA (0.1 mM), sucrose (5%), $Mg(CH_3CO_2)_2$ (10 mM), and $CaCl_2$ (0.4 mM). At the indicated times, aliquots were initially diluted 100-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8) and then diluted 6-fold into an assay mixture containing, in final concentrations, kinase (0.3 μg/mL), phosphorylase *b* (4 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 15.9 Ci/mol], $CaCl_2$ (0.25 mM), EDTA (0.15 mM), mercaptoethanol (5.2 mM), and buffer (90 mM Hepes, pH 8.0). After 5 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. (○) Incubation without oATP; (●) incubation with oATP.

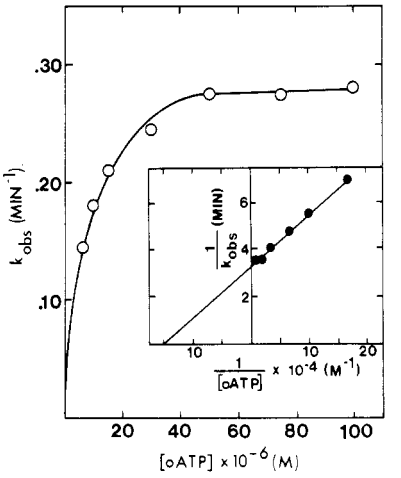

FIGURE 2: Concentration dependence of the inactivation by oATP. Nonactivated phosphorylase kinase (0.19 mg/mL) was inactivated with oATP (5–100 μM) in Hepes buffer (83 mM, pH 6.8) for 2–6 min. The modification included EDTA (0.3 mM), $Mg(CH_3CO_2)_2$ (10 mM), and $CaCl_2$ (0.6 mM). Aliquots of this reaction were initially diluted 34-fold with cold buffer (40 mM β-glycerophosphate and 30 mM mercaptoethanol, pH 6.8) and then diluted 6-fold into an assay mixture containing, in final concentrations, kinase (0.93 μg/mL), phosphorylase *b* (6.9 mg/mL), MgATP (10 mM $Mg(CH_3CO_2)_2$; 2.5 mM [^{32}P]ATP at 4.7 Ci/mol), mercaptoethanol (15 mM), $CaCl_2$ (0.3 mM), and buffer (42 mM Tris and 62 mM β-glycerophosphate, pH 8.2). After 2 min 40 s at 30 °C, an aliquot was removed for the determination of ^{32}P incorporation into protein. Kinase incubated without oATP was set as 100% activity. The inset shows a replot of the same data.

evidence that phosphorylase kinase and oATP form a reversible complex prior to inactivation according to (Meloche, 1967)

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_{inact} \xrightarrow{k_2} E_{inact} \quad (2)$$

where E represents free enzyme, I, oATP, C_{inact}, the en-

zyme-oATP complex, and E_{inact}, inactivated enzyme. Michaelis–Menten type derivations (Meloche, 1967) lead to

$$k_{obsd} = \frac{k_2}{(K_i/[I]) + 1} \quad (3)$$

where $K_i = (k_{-1} + k_2)/k_1$ and represents that concentration of oATP giving the half-maximum inactivation rate. A plot of the reciprocal of k_{obsd} vs. the reciprocal of the concentration of oATP allowed determination of an apparent K_i of 6.7 μM for oATP (Figure 2, inset). According to Figure 2 (inset), the maximum rate of inactivation (k_2) at saturating concentrations of oATP was 0.3 min^{-1}.

Protection of the Enzyme from Inactivation by oATP. One important criterion which determines whether an inhibitor might be active site directed is protection by the normal substrate against inactivation. ATP was found to protect phosphorylase kinase from inactivation by oATP. While the half-time of inactivation by 50 μM oATP was 4.9 min in the absence of ATP, it was increased to 6.9 min in the presence of 800 μM ATP, thus providing further evidence that oATP is a valid affinity label.

Synergistic Effect of Ca^{2+} and Mg^{2+} on the Inactivation of Phosphorylase Kinase by oATP. During characterization of the interaction of phosphorylase kinase with oATP, it was observed that Mg^{2+} was not required for inactivation to occur but that inactivation was enhanced in the presence of 10 mM Mg^{2+} and micromolar concentrations of Ca^{2+}. The effect of Ca^{2+} and Mg^{2+} was found to be synergistic; each metal ion by itself had no significant effect on the rate of inactivation (data not shown). When we repeated the experiment described in Figure 2 in the absence of Ca^{2+} plus Mg^{2+}, a more than 2-fold increase in the K_i for oATP and a decrease in the maximal rate of inactivation were observed. These results enabled us to lower the concentration of inhibitor in later experiments (King & Carlson, 1981b) to less than 100-fold above the concentration of enzyme, thereby decreasing the possibility of nonspecific interactions.

Comparison of the Effect of Ca^{2+} plus Mg^{2+} on the Inactivation of Phosphorylated and Nonphosphorylated Phosphorylase Kinase by oATP. The synergistic effect of Ca^{2+} plus Mg^{2+} was found to be influenced by the state of phosphorylation of the kinase. Figure 3 shows the inactivation of nonphosphorylated and phosphorylated phosphorylase kinase by oATP in the absence and presence of Ca^{2+} plus Mg^{2+}. In the absence of the two metals, the nonphosphorylated enzyme was inactivated by oATP at a significantly faster rate. In the presence of the metals, however, both forms of the kinase were inactivated more rapidly and at about the same rate. Consequently, the net effect of the two metals was more pronounced with the phosphorylated enzyme. The amount of inactivation achieved by incubating phosphorylated kinase with 10 μM oATP for 5 min was increased 40-fold by the presence of the two metal ions whereas nonphosphorylated kinase showed only an approximately 5-fold increase of the rate of inactivation under identical conditions (Figure 3).

Although complete inactivation of phosphorylase kinase could be achieved with higher concentrations of oATP, semilog plots of the time course of inactivation by 10 μM oATP in the presence of Ca^{2+} and Mg^{2+} were linear to approximately 60 or 70% inactivation and then leveled off (Figures 1 and 3). In separate experiments, we found that oATP was stable for at least 15 min under the conditions used in this work (data not shown); instability of the inhibitor is therefore not the explanation for the change in the rate of inactivation. A possible cause could be the involvement of two different

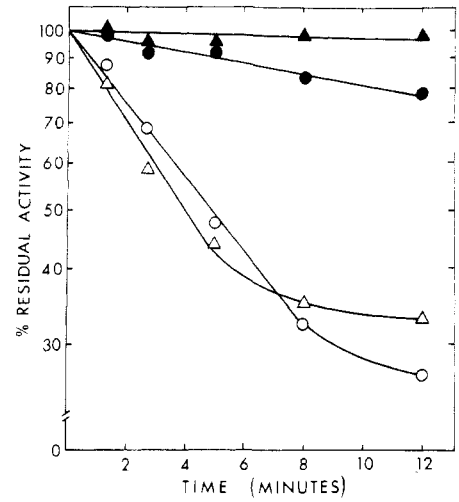

FIGURE 3: Synergistic effect of Ca^{2+} and Mg^{2+} on the inactivation of phosphorylated and nonphosphorylated phosphorylase kinase by oATP. Phosphorylated and nonphosphorylated phosphorylase kinase (0.18 mg/mL) were inactivated by oATP (10 μM) in Hepes buffer (30 mM, pH 6.8) in the presence of EGTA (0.2 mM) and Ca^{2+} plus Mg^{2+} (0.4 mM; 10 mM) where indicated. Aliquots were initially diluted 100-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8) and then diluted 6-fold into an assay mixture containing, in final concentrations, kinase (0.3 μg/mL), phosphorylase *b* (4 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 10.3 Ci/mol], $CaCl_2$ (0.25 mM), EDTA (0.15 mM), mercaptoethanol (5.2 mM), and buffer (90 mM Hepes, pH 8.0). After 5 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. (○) Nonphosphorylated kinase inactivated in the presence of Ca^{2+} plus Mg^{2+} and (●) in the absence of Ca^{2+} plus Mg^{2+}. (△) Phosphorylated kinase inactivated in the presence of Ca^{2+} plus Mg^{2+} and (▲) in the absence of Ca^{2+} plus Mg^{2+}.

catalytic sites with different affinities for oATP. The results in the following paper (King & Carlson, 1981b) are consistent with this explanation. Using oATP as a tool in differential inactivation experiments with various substrates of phosphorylase kinase, we obtained evidence that supports the hypothesis that two catalytic sites exist on the enzyme with different, but perhaps not absolute, specificities for different substrates.

pH Dependence of the Inactivation of Phosphorylase Kinase by oAMPPNP. Phosphorylase kinase has a significantly higher activity at pH 8.2 than at pH 6.8. The activity ratio at pH 6.8:pH 8.2 has traditionally been used to describe a particular kinase preparation and to monitor the extent of activation of the enzyme. The ratio is approximately 0.05 for nonactivated phosphorylase kinase and increases with increasing activation. Because effectors of the kinase reaction have usually been tested at one or both of these pH's, we chose these values to investigate the effect of pH on the inactivation by oATP and its synergistic enhancement by Ca^{2+} and Mg^{2+}. Fearing that phosphorylase kinase might be able to carry out autophosphorylation with oATP at high pH and in the presence of metals, we decided that oAMPPNP would be the inhibitor of choice for studies of the influence of pH on inactivation (Figure 4). Inactivation in the absence of Ca^{2+} and Mg^{2+} was found to be 3 times faster at pH 6.8 than at pH 8.2. In the presence of Ca^{2+} and Mg^{2+}, activation seemed to occur at pH 8.2 during the first 6 min of reaction time, as opposed to 60% inactivation at pH 6.8 during the same time period. Although not caused by autophosphorylation, the apparent activation at pH 8.2 did show a requirement for metal ions. When the enzyme was incubated with oAMPPNP at pH 8.2 in the absence of Ca^{2+} and Mg^{2+}, no activation was observed. It should be noted that the results of Figure 4 could be duplicated by oATP (results not shown).

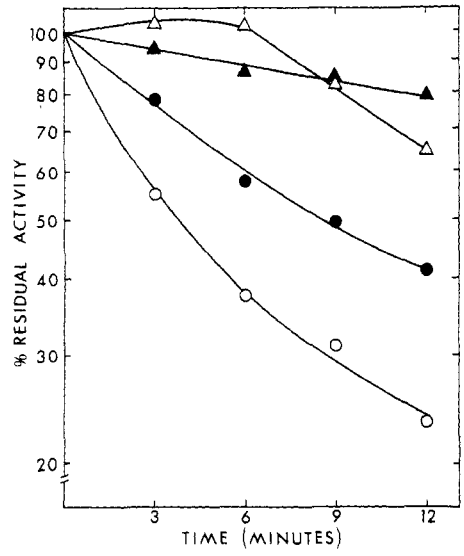

FIGURE 4: Time course of inactivation of phosphorylase kinase at pH 6.8 and 8.2 with oAMPPNP. Nonactivated phosphorylase kinase (0.18 mg/mL) was inactivated at 30 °C in Hepes buffer (70.2 mM) at pH 6.8 or 8.2 by oAMPPNP (10 μM). The reaction included EGTA (0.15 mM), EDTA (0.2 mM), β-glycerophosphate (5.3 mM), sucrose (1.1%), and $CaCl_2$ (0.4 mM) plus $Mg(CH_3CO_2)_2$ (10 mM) where indicated. At the indicated times, aliquots were initially diluted 60-fold with cold buffer (40 mM β-glycerophosphate and 30 mM mercaptoethanol, pH 6.8) and then diluted 6-fold into an assay mixture containing, in final concentrations, kinase (0.5 μg/mL), phosphorylase b (7.2 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 2.5 mM [^{32}P]ATP at 11.6 Ci/mol], $CaCl_2$ (0.2 mM), mercaptoethanol (11.8 mM), and buffer (49 mM β-glycerophosphate, 15 mM Hepes, and 42 mM Tris, pH 8.2). After 3 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. (●) Inactivation at pH 6.8; (○) inactivation at pH 6.8 in the presence of Ca^{2+} and Mg^{2+}; (▲) inactivation at pH 8.2; (△) inactivation at pH 8.2 in the presence of Ca^{2+} and Mg^{2+}.

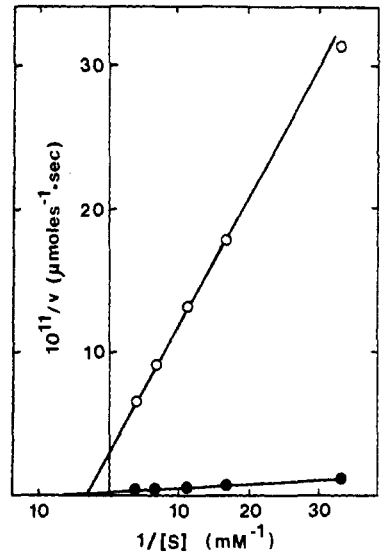

FIGURE 5: Phosphorylation of phosphorylase b by oATP and ATP. Phosphorylase b (6.7 mg/mL) was incubated at 30 °C with nonactivated phosphorylase kinase (33 μg/mL) in the presence of buffer (42 mM Tris and 62 mM β-glycerophosphate, pH 8.2), EDTA (0.06 mM), dithiothreitol (0.15 mM), $CaCl_2$ (0.2 mM), $Mg(CH_3CO_2)_2$ (10 mM), and [^{32}P]ATP (67.8 Ci/mol) (●) or [^{32}P]oATP (19.7 Ci/mol) (○). Aliquots were removed every 20 s up to an assay time of 1 min 40 s to determine the incorporation of ^{32}P into protein. The reciprocals of the initial rates were plotted against the reciprocals of the concentrations of ATP (●) and oATP (○).

Comparison of the Phosphorylation of Phosphorylase b by Phosphorylase Kinase with oATP and ATP. The results in Figure 4 show that no inactivation occurs with oAMPPNP at pH 8.2 in the presence of Ca^{2+} and Mg^{2+} during the first 6 min of inactivation time. This suggested that we might be able to phosphorylate phosphorylase b by using oATP as the substrate. Figure 5 shows a comparison of the phosphorylation by ATP and oATP. The V_{max} is 20-fold higher for ATP than for oATP, while the apparent K_m's for the two substrates seem similar. Comparison of the actual values was avoided because the effective concentration of oATP in the presence of phosphorylase b probably does not equal its total concentration. In addition to possible nonspecific interactions of oATP with the large excess of protein substrate, phosphorylase b has been reported to bind ATP (Graves & Wang, 1972; Johnson et al., 1979) and therefore has the potential of also specifically binding oATP. The potential reduction in effective concentration of oATP could be significant because the concentration of phosphorylase b in the assay is almost 70 μM and the highest concentration of oATP used was only 250 μM.

Care was taken to ensure complete absence of contamination by ATP in the oATP used in this experiment. Oxidation of [γ-^{32}P]ATP by periodate was repeated several times, even though thin-layer chromatography showed no unreacted ATP under UV light after the first oxidation. Furthermore, the developed chromatograms were cut into small strips after each successive oxidation and measured for ^{32}P. This analysis indicated that the synthesis of oATP was complete after the first oxidation. We are therefore confident that phosphorylation of phosphorylase b by oATP was not due to contamination of unreacted ATP. The ability of oATP to act as a substrate provides further evidence that the inhibitor can bind to the active site of phosphorylase kinase and therefore is a valid affinity label of the enzyme.

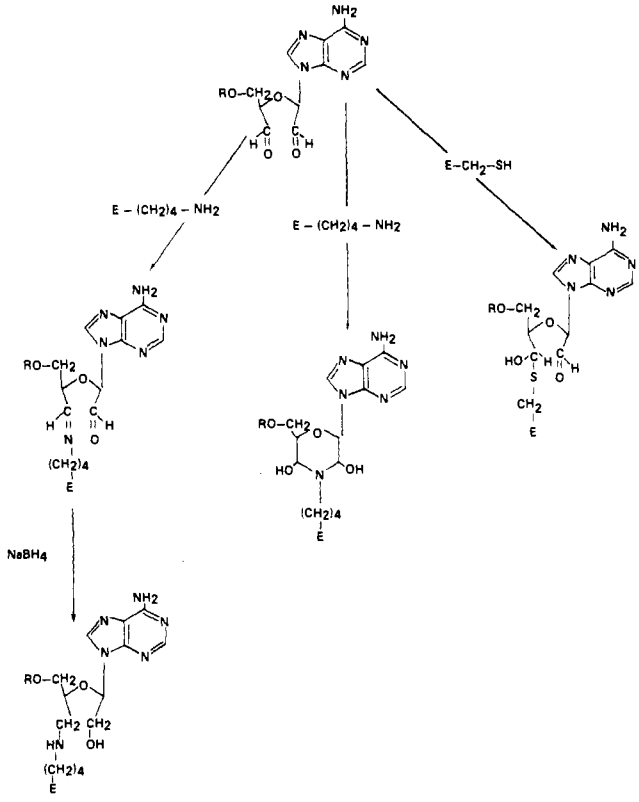

FIGURE 6: Possible reactions of oATP with enzymatic side chains.

Chemical Mechanism of the Reaction of oATP with Phosphorylase Kinase. oATP can react with enzymatic side chains in at least three different ways. Reaction with the ϵ-amino group of lysine can lead to a Schiff base (Figure 6, left side) which subsequently can be reduced by $NaBH_4$, or $NaBH_3CN$, to form a stable covalent linkage (Easterbrook-Smith et al., 1976; Ranieri-Raggi & Raggi, 1976; Kumar et

al., 1979; Westcott et al., 1980). oATP can also react with the lysine to form a dihydroxymorpholino derivative (Figure 6, center), as has been suggested by Gregory & Kaiser (1979) for the inactivation of phosphofructokinase by oATP. A third possibility, not previously reported, is the reaction of oATP with the sulfhydryl group of cysteine, forming a hemithioacetal (Figure 6, right side).

Several experimental results suggest that the inactivation product of phosphorylase kinase and oATP is not a Schiff base. Following inhibition by oATP, we were not able to reactivate the enzyme by diluting 1500-fold with buffer (40 mM β-glycerophosphate and 30 mM mercaptoethanol, pH 6.8) or by dialyzing for 24 h at 4 °C against the same buffer. Mercaptoethanol functions as an aldehyde trap in these experiments and was used to stop inactivation as well as to reactivate the modified enzyme. We also attempted to reactivate the modified enzyme by incubation with buffer (40 mM β-glycerophosphate and 30 mM mercaptoethanol, pH 6.8) at 30 °C for 1 h but could not increase the residual activity that was observed before the incubation. Treatment of the enzyme–oATP complex with $NaBH_4$ or $NaBH_3CN$ prior to the attempts to reactivate the enzyme did not influence the extent of inactivation. We also attempted to reverse the inactivation by incubating the modified enzyme at 30 °C for 10 min with a 2700-fold excess of ATP over oATP, but had no success.

These results indicate that oATP is bound very tightly to the enzyme; however, when the kinase was inactivated with ^{14}C-labeled oATP followed by $NaDodSO_4$ gel electrophoresis, no radioactivity was found associated with any of the enzyme subunits. Treatment of the modified enzyme with $NaBH_4$ prior to $NaDodSO_4$ gel electrophoresis did not stabilize the oATP on the enzyme.[2]

The failure to reactivate the modified enzyme by established methods (Easterbrook-Smith et al., 1976; Ranieri-Raggi & Raggi, 1976; Kumar et al., 1979) and the loss of the radioactive label on $NaDodSO_4$ gels after treatment with $NaBH_4$ provide evidence that the inactivation product is not a Schiff base. It has not yet been established which alternative mechanism is responsible for the inactivation of phosphorylase kinase by oATP.

Discussion

Phosphorylase kinase was inactivated at pH 6.8 by micromolar concentrations of the 2′,3′-dialdehyde derivative of ATP (oATP). The reaction was pseudo first order and showed saturation kinetics, which provided evidence that oATP forms a dissociable complex with the enzyme prior to inactivation. The rate of inactivation was reduced by the presence of ATP, thus indicating protection of the enzyme. Inactivation by oATP was very slow at pH 8.2; at this pH, oATP could be used as a substrate to phosphorylate phosphorylase *b*, thus providing evidence that oATP can bind to the active site of phosphorylase kinase. We are therefore able to show that several generally accepted criteria could be applied to the interaction of phosphorylase kinase with oATP to provide evidence that oATP is an active site directed inhibitor of the enzyme (Meloche, 1967; Singer, 1967; Phillips, 1977; Wold, 1977).

A common criticism of the use of oATP as an affinity label is the inherently high reactivity of the dialdehyde group of the analogue which often results in decreased specificity of the reaction. However, the reaction of oATP with phosphorylase kinase seems to be an exception. The lower than normal pH employed in our system as well as the low concentrations of inhibitor required for the reaction minimizes the likelihood of nonspecific reactions.

Further evidence that oATP is a very specific inhibitor in our system was provided when the reaction of phosphorylase kinase with oATP showed dependence on pH, Ca^{2+} plus Mg^{2+}, and the phosphorylation state of the enzyme. An increase in the pH of the modification buffer resulted in decreased inactivation by oATP, which is opposite of what would normally be expected chemically. On the other hand, a very pronounced increase in the rate of inactivation was observed in the presence of Ca^{2+} plus Mg^{2+}. The two metal ions acted synergistically to cause an approximately 2-fold decrease in the apparent K_i for oATP as well as an increase in the rate of inactivation when present in concentrations of 0.6 and 10 mM, respectively.

A synergistic effect of Ca^{2+} and Mg^{2+} on phosphorylase kinase has previously been observed in our laboratory (King & Carlson, 1981a). Preincubation of the enzyme with Ca^{2+} plus Mg^{2+} caused the appearance of an EGTA-insensitive activity of phosphorylase kinase in subsequent assays. This activation was reversible and not due to proteolysis. Another synergistic effect of Ca^{2+} and Mg^{2+} was recently reported by Heilmeyer et al. (1980), who found induction of new Ca^{2+} binding sites on phosphorylase kinase by 20 mM Mg^{2+}. The binding of 1–2 mol of Ca^{2+} to these induced sites allowed expression of a phosphorylase kinase activity designated as a_4, which is one of three partial activities toward phosphorylase *b* that these authors were able to identify.

In a recent report by Crouch & Klee (1980), the four Ca^{2+} binding sites on calmodulin were investigated in the presence and absence of 3 mM Mg^{2+}. Mg^{2+} was found to increase the dissociation constants for Ca^{2+} 2–4-fold to a final value of 5 to 40 μM. Several additional reports further describe conformational changes and different conformational states of calmodulin caused by Ca^{2+} and Mg^{2+} (Men'shikov & Tkachuk, 1979; Forsen et al., 1980; Seamon, 1980). These results can be interpreted to suggest that the synergistic effect of Ca^{2+} and Mg^{2+} on the inactivation by oATP may be mediated through the δ subunit of phosphorylase kinase, which has been found to be similar to, or identical with, calmodulin (Shenolikar et al., 1979). If the binding of Ca^{2+} and Mg^{2+} to the enzyme causes conformational changes such that the affinity of the nucleotide binding site is increased, as is indicated by the decrease in the apparent K_i for oATP, then this should be observable in thermodynamic binding studies with ATP in the presence of Ca^{2+} and Mg^{2+}.

Our results with oATP also show that the synergistic effect of Ca^{2+} plus Mg^{2+} is even greater for the phosphorylated form of the enzyme but decreases with increasing pH. This suggests that the interaction of phosphorylase kinase with oATP can be used as a sensitive tool to detect conformational changes around active sites of the enzyme.

[2] Although we were unable to isolate an enzyme subunit labeled with oATP, preliminary experiments in which the 8-azido 2′,3′-dialdehyde of ATP was used as a photoaffinity label indicated preferential labeling of the β subunit.

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Interaction of Phosphorylase Kinase with the 2′,3′-Dialdehyde Derivative of Adenosine Triphosphate. 2. Differential Inactivation Measured with Various Protein Substrates†

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ABSTRACT: The 2′,3′-dialdehyde derivative of ATP was used as an affinity label to inactivate phosphorylase kinase in either the presence or absence of Ca^{2+} and Mg^{2+} ions. Following inactivation, the residual activity of phosphorylase kinase toward various protein substrates was measured and compared with that retained for conversion of phosphorylase *b*. Three different classes of substrates were distinguished by this analysis. For the first class (glycogen synthase), inactivation proceeded at the same rate as that measured with phosphorylase conversion, regardless of whether the inactivation was carried out in the presence or absence of the metal ions. For the second class of substrates (troponin I and troponin T), inactivation of the kinase in either the presence or absence of the metals was much more rapid with phosphorylase as substrate. Phosphorylation of the third class of substrates (phosphorylase kinase itself and a synthetic tetradecapeptide) was inactivated in parallel with phosphorylase *b* when modification was performed in the absence of metals; however, inclusion of Ca^{2+} and Mg^{2+} ions in the inactivation mixture caused activity toward phosphorylase *b* to be lost more rapidly than that toward the alternative substrates. Our results are consistent with a model in which glycogen synthase and phosphorylase *b* are preferentially phosphorylated at one type of catalytic site in phosphorylase kinase and troponin I and troponin T at another.

Phosphorylase *b* kinase (ATP:phosphorylase-*b* phosphotransferase, EC 2.7.1.38) of rabbit skeletal muscle is a hexadecamer composed of four different types of subunits, $\alpha_4\beta_4\gamma_4\delta_4$ (Cohen et al., 1978). The enzyme catalyzes the phosphorylation of phosphorylase *b* as well as the phosphorylation of itself (DeLange et al., 1968), troponin I (Stull et al., 1972), troponin T (Perry & Cole, 1974), and glycogen synthase (Roach et al., 1978).

There is not general agreement, however, as to which subunit(s) catalyzes (catalyze) the above reactions, or indeed, if phosphorylation of all the protein substrates is even carried out by a single type of catalytic site. Both the β and γ subunits have been reported to have catalytic capacity. Skuster et al. (1980) reported the isolation of catalytically active γ subunit after dissociation of the enzyme with LiBr. Gulyaeva et al.

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