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Pyruvate Phosphate Dikinase: Affinity Labeling of the Adenosine 5′-Triphosphate–Adenosine 5′-Monophosphate Site†

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ABSTRACT: The 2′,3′-dialdehyde of adenosine 5′-phosphate (oAMP) is shown to specifically modify the adenosine 5′-triphosphate (ATP)–AMP site of pyruvate phosphate dikinase from *Bacteroides symbiosus*, and in the presence of sodium cyanoborohydride the enzyme is irreversibly inactivated. The overall dikinase reaction involves three partial reactions, each of which is catalyzed by a functionally independent site; one site catalyzes the ATP–AMP exchange, the second the P_i–PP_i exchange, and the third the pyruvate–phosphoenolpyruvate exchange. The specificity of oAMP for the ATP–AMP site was demonstrated by examining the effect of inactivation on the three exchange reactions. When enzyme was incubated in the presence of oAMP and reducing agent, the ATP–AMP exchange was completely inhibited, the P_i–PP_i exchange was inhibited 15%, and the pyruvate–phosphoenolpyruvate exchange was not affected, showing that oAMP specifically inhibited the ATP–AMP site. The weak inhibition of the P_i–PP_i exchange may be because of its close proximity to the ATP–AMP site. In addition, oAMP fit the criteria of an affinity label. The compound is a competitive inhibitor with respect to AMP. The rate of inactivation of the enzyme followed pseudo-first-order kinetics, and a plot of the rate of inactivation vs. oAMP concentration gave a typical saturation curve. These results are consistent with a two-step reaction in which oAMP is rapidly and specifically bound to the ATP–AMP site and then a Schiff's base is formed inactivating the enzyme. AMP completely protected against the inactivation at concentrations >1.0 mM. Inorganic phosphate did not protect, while 1.0 mM pyruvate protected somewhat, against inactivation of the dikinase. Binding studies with [U-^{14}C]oAMP showed that there was 50% inactivation when 1 mol of the analogue was bound per mol of dimeric enzyme. At higher concentrations of oAMP, the inactivation became nonlinear as though modification of the first nucleotide site decreased the reactivity on the second site for oAMP but left it enzymatically active. These results are in contrast to previous experiments with bromopyruvate which reacted at the pyruvate–phosphoenolpyruvate site and gave complete inactivation of the enzyme when one site was modified, so suggesting half-site reactivity.

Pyruvate phosphate dikinase (EC 2.7.9.1) catalyzes the reversible phosphorylation of pyruvate and inorganic phosphate by utilizing the β and γ phosphates of ATP.[1]

$$\text{pyruvate} + \text{ATP} + P_i \rightleftharpoons \text{phosphoenolpyruvate} + \text{AMP} + PP_i \quad (1)$$

Several lines of evidence support the conclusion that the overall reaction catalyzed by enzyme preparations from *Bacteroides symbiosus* and *Propionibacterium shermanii* involves three partial reactions.

$$E + \text{ATP} \rightleftharpoons E\text{–PP} + \text{AMP} \quad (1a)$$

$$E\text{–PP} + P_i \rightleftharpoons E\text{–P} + PP_i \quad (1b)$$

$$E\text{–P} + \text{pyruvate} \rightleftharpoons E + \text{phosphoenolpyruvate} \quad (1c)$$

Equilibrium exchange studies (Evans & Wood, 1968; Milner & Wood, 1972; Milner & Wood, 1976), initial velocity steady-state kinetics (Milner & Wood, 1972, 1976), and product inhibition patterns (Milner & Wood, 1972) are consistent with a three-site tri-uni-uni ping-pong mechanism. The presence of phosphoryl- and pyrophosphoryl-enzyme forms has been demonstrated (Milner & Wood, 1976; Spronk et al., 1976). According to these observations, three functionally distinct sites catalyze, first, the ATP–AMP exchange, second, the P_i–PP_i exchange, and, third, the pyruvate–phosphoenolpyruvate exchange. Additionally, a histidyl residue is located so that it serves as a phosphoryl and pyrophosphoryl group carrier between these sites (Milner et al., 1978; Yoshida & Wood, 1978).

The present work was undertaken to further investigate the structural relationships among the three substrate sites by covalently modifying the ATP–AMP site with a chemically reactive substrate analogue of AMP. Inactivation experiments have shown that the 2′,3′-dialdehyde derivative of AMP (oAMP)[1] specifically modifies the ATP–AMP site of the dikinase. Several investigators have used dialdehyde derivatives of ATP, ADP, and UDP as site-specific covalent mod-

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[1] Abbreviations used: AMP, adenosine 5′-phosphate; oAMP, 2′,3′-dialdehyde AMP; ATTP, adenosine 5′-tetraphosphate; Cl_3AcOH, trichloroacetic acid; $NaCNBH_4$, sodium cyanoborohydride; ATP, adenosine 5′-triphosphate; E-P, phophoryl-enzyme intermediate; E-PP, pyrophosphoryl-enzyme intermediate; NADH, reduced nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; GMP, guanosine 5′-phosphate; IMP, inosine 5′-phosphate.

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ifying agents (Easterbrook-Smith et al., 1976; Powell & Brew, 1976; Fayat et al., 1978; Kumar et al., 1979). Easterbrook-Smith et al. (1976) presented evidence that the dialdehyde forms a Schiff's base with a lysine residue in the active site of the enzyme which is reduced in the presence of sodium borohydride.

Experimental Procedures

Materials

[U-^{14}C]AMP, sodium [^{32}P]pyrophosphate, and [2-^{14}C]pyruvate were from New England Nuclear, nucleotides, phosphoenolpyruvate, and NADH were from P-L Biochemicals, Inc., pyruvate was from Sigma Chemical Co., sodium pyrophosphate was from Fisher Scientific, and lactate dehydrogenase from Calbiochem-Behring Corp. All other chemicals used in this work were analytical grade.

Methods

Purification of Pyruvate Phosphate Dikinase. Pyruvate phosphate dikinase was purified from 110 g (wet weight) of cells of *B. symbiosus* (ATCC 14940). Following lysis with lysozyme and precipitation with streptomycin sulfate, the supernatant fraction was precipitated between 50 and 62% saturated ammonium sulfate. Additional purification of pyruvate phosphate dikinase was obtained after successive chroatography through DEAE-Bio-Gel, hydroxylapatite, and A-0.5m Bio-Gel columns. This procedure represented a 120-fold purification and a 50% recovery of the dikinase. The enzyme was judged pure on the basis of its mobility in Na-DodSO_4 and nondenaturing gels. The specific activity of the isolated enzyme was 18 units/mg which slowly diminished during storage as a precipitate in 80% saturated ammonium sulfate at 4 °C. A complete description of this purification procedure has been given elsewhere (Goss et al., 1980).

Assay of Enzyme. Pyruvate phosphate dikinase was assayed by measuring the rate of pyruvate formation from phosphoenolpyruvate by coupling with lactate dehydrogenase (Milner et al., 1975). Assay mixes were prepared daily. Reactions were initiated by the addition of enzyme and were incubated at 30 °C. Controls lacked either AMP or PP_i, and the value was subtracted from that of the complete reaction.

Protein Determination. Protein was determined by measuring the absorbance at 280 nm using a factor of 1.0 optical density unit = 1.3 mg/mL protein (Milner et al., 1975), by the modified biuret procedure (Mukrasch & McGilvery, 1956) using bovine serum albumin as a standard, and by the Coomassie procedure (Bradford, 1976) using known concentrations of pyruvate phosphate dikinase for standard curves.

Assay of the ATP–AMP Exchange. The incubation mixture contained in a total volume of 20 μL of 100 mM Tris–acetate buffer (pH 6.8) the following concentrations of substrates: AMP, 3.0 mM; ATP, 2.5 mM; $MgCl_2$, 20 mM; 100 μg of bovine serum albumin neutralized to pH 6.8. An appropriate amount of enzyme was added to the incubation mixture and permitted to preequilibrate for 5 min at 30 °C. A trace amount (80000 cpm) of [U-^{14}C]AMP (sp act. = 479 mCi/mmol) then was added, and the exchange reaction was monitored in time intervals of 10 min for up to 120 min. Substrate pairs were separated by spotting 2-μL aliquots on Eastman 13255 cellulose plates which had been previously spotted with 1.0 μmol of EDTA to terminate the reaction. Thin-layer chromatography was run in a solvent system consisting of *tert*-amyl alcohol, formic acid, and water (3:2:1 v/v) (R_f of AMP = 0.54 and R_f of ATP = 0.16). Spots were visualized by ultraviolet light and shown to migrate coincident with nonradioactive standards. Radioactive chromatograms were cut into 1-cm^2 fractions and counted in a liquid scintillation counter. Radioactive peaks corresponding to AMP and ATP were quantitated and the percent exchange was determined.

Assay of the P_i–PP_i Exchange. The procedure was as above except that P_i (3.5 mM), PP_i (4.0 mM), phosphoenolpyruvate (2.0 mM); NH_4Cl (200 mM), and [^{32}P]PP_i (90000 cpm) were added in place of AMP, ATP, and [U-^{14}C]AMP. The exchange reaction was monitored in 10-min intervals for up to 90 min. Thin-layer chromatography was run in a solvent system consisting of isopropyl alcohol, 10% trichloroacetic acid, and 25% NaOH (75:25:0.25 v/v) (R_f of P_i = 0.75 and R_f of PP_i = 0.28). Nonradioactive standards were visualized by spraying with Hanes–Isherwood solution (Mann et al., 1979). Radioactive fractions corresponded to P_i and PP_i standards.

Assay of Pyruvate–Phosphoenolpyruvate Exchange. The procedure was as in P_i–PP_i exchanges except that pyruvate, 3.8 mM, and phosphoenolpyruvate, 2.5 mM, were the substrate pair and [2-^{14}C]pyruvate (sp act. = 7.7 mCi/mmol, 60000 cpm) was the radioactive tracer. The exchange reaction was monitored in 5-min intervals for up to 60 min. Thin-layer chromatography was run in a solvent system consisting of 1-butanol, formic acid, and water (95:5:10 v/v) (R_f of pyruvate = 0.64 and R_f of phosphoenolpyruvate = 0.11). Nonradioactive pyruvate and phosphoenolpyruvate standards were visualized by spraying with a 0.05% solution of *o*-phenylenediamine in 10% Cl_3AcOH and the Hanes–Isherwood solution, respectively. Radioactive fractions correspond to authentic pyruvate and phosphoenolpyruvate.

Synthesis of oAMP and [U-^{14}C]oAMP. The synthesis of the dialdehyde derivative of AMP is based upon the method of Easterbrook-Smith (Easterbrook-Smith et al., 1976). Cleavage of the carbon–carbon bond between carbons 2 and 3 of the ribose moiety was achieved by periodate oxidation at neutral pH in distilled water. Reactions were performed at 4 °C in the dark. The synthesis of ^{14}C-labeled oAMP differed only in that a 200-fold excess of cold AMP (21 μmol) was added to radioactive samples prior to periodate oxidation. The analogue was purified from the reaction products by chromatography through a Sephadex G-10 (40 × 2.5 cm) column, equilibrated in 25 mM KCl. Peak fractions were monitored at 258 nm, pooled, lyophilized, and stored at −80 °C. This procedure gave a 50% recovery of radioactive material. Sample concentrations were calculated on the basis of the extinction coefficient of 1.49 cm mM^{-1} for oAMP at 258 nm (Easterbrook-Smith et al., 1976). Pooled samples showed little absorption at 225 nm, the λ_{max} for β-elimination products (Khym & Cohn, 1961). Thin-layer chromatography on cellulose plates run in *tert*-amyl alcohol, formic acid, and water in the proportions of 3:2:1 (v/v) (R_f of oAMP = 0.32) showed that the [U-^{14}C]oAMP was >90% pure.

Inactivation of Pyruvate Phosphate Dikinase by oAMP. Inactivation studies were performed by incubating 16 μg of enzyme having a specific activity of 8.0 units/mg of 25 °C in 100 μL of either 50 mM imidazole–HCl buffer, pH 6.8, or 50 mM Tris–acetate buffer, pH 6.8, to which various concentrations of oAMP were added. After 5 min, the reaction was quenched by adding $NaCNBH_4$ in a 2-fold molar excess over oAMP. The enzyme solutions were incubated an additional 35 min, diluted, and assayed. Divalent cations were required for inactivation, and therefore analogue solutions all contained 20 mM $MgCl_2$.

Determination of Enzyme-Bound [U-^{14}C]oAMP. Pyruvate phosphate dikinase (138 μg, 0.72 nmol) having a specific activity of 7.0 units/mg was incubated at 25 °C in 0.5 mL

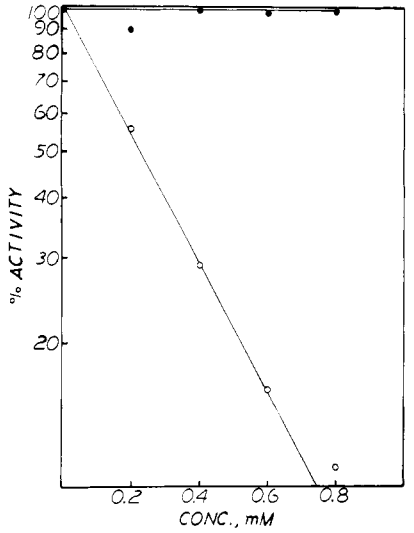

FIGURE 1: Inactivation of pyruvate phosphate dikinase by oAMP. 16 μg of enzyme was incubated at 25 °C in 100 μL of 50 mM imidazole–HCl buffer, pH 6.8, to which increasing concentrations of AMP (●) or oAMP (○) were added. A 2-fold excess of $NaCNBH_4$ was added after 5 min, and the enzyme was assayed 35 min later.

of 50 mM Tris–acetate buffer, pH 6.8, containing increasing concentrations of [U-^{14}C]oAMP (sp act. = 2.87 mCi/mmol). The buffer acted as an effective scavenger by minimizing the nonspecific modification of the enzyme, and in its presence the enzyme was stable for over 60 min. A 2.5-fold excess of $NaCNBH_4$ was added after 5 min to enzyme–analogue solutions which were permitted to incubate an additional 35 min. Following incubation, a 10-μL aliquot was assayed and the remainder of the enzyme was precipitated with 10% Cl_3AcOH for 10 min at 4 °C. The precipitates were rapidly filtered, the tubes were rinsed twice with Cl_3AcOH, and the rinse was likewise filtered by using a Millipore vacuum filtration manifold fitted with HAWP 025 filters having a 0.45-μm pore size. The precipitates were washed twice with 10% Cl_3AcOH, dried under an infrared light, and counted for radioactivity. The radioactivity contributed by the nonspecific binding of the analogue to the filters was determined by precipitating samples without enzyme for each [U-^{14}C]oAMP concentration used and by subtracting these values from the corresponding enzyme precipitates. For all calculations, a molecular weight of ~180 000 was assumed for dimeric pyruvate phosphate dikinase (Goss et al., 1980).

Results

Inactivation of Pyruvate Phosphate Dikinase by oAMP. When pyruvate phosphate dikinase was incubated in the presence of concentrations of oAMP between 0.0 and 0.8 mM and cyanoborohydride, the enzyme rapidly lost activity (Figure 1). Under identical conditions, but substituting AMP for oAMP, the enzyme remained stable, and there was no loss of activity. Prolonged incubation with oAMP and reducing agent completely inactivated the enzyme. The inactivation was not reversed by gel filtration.

Specificity of the oAMP-Mediated Enzyme Inactivation. In order to establish that oAMP acts as an affinity label, it was necessary to demonstrate the specificity of the analogue for the active site of the enzyme. Three parameters were examined to assess the specificity of the oAMP for the ATP–AMP site of pyruvate phosphate dikinase.

In the absence of added reducing agent, oAMP acted as a competitive inhibitor of the enzyme with respect to AMP as shown in Figure 2. In addition, the inhibition characteristics

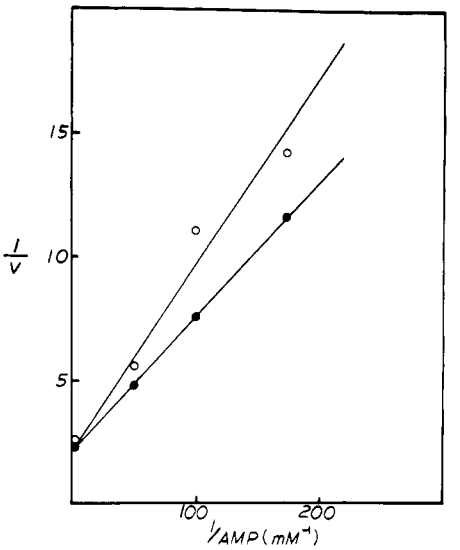

FIGURE 2: Inhibition of pyruvate phosphate dikinase by oAMP. The enzyme was assayed in the absence (●) and presence (○) of 0.13 mM oAMP. The reactions were carried out for 2 min at 30 °C in reaction mixtures containing in a final volume of 0.5 mL imidazole–HCl buffer (pH 6.8), 50 mM; NH_4Cl, 20 mM; $MgCl_2$, 20 mM; phosphoenolpyruvate, 1.0 mM; pyrophosphate 0.8 mM; NADH, 0.1 mM; lactate dehydrogenase, 1 unit; enzyme, 3.8 μg, (35 milliunits); and various concentrations of AMP as indicated. The change in absorbance at 340 nm was monitored, and velocity (V) was expressed in micromoles of NADH consumed per minute. Lines were fitted by the least-squares method.

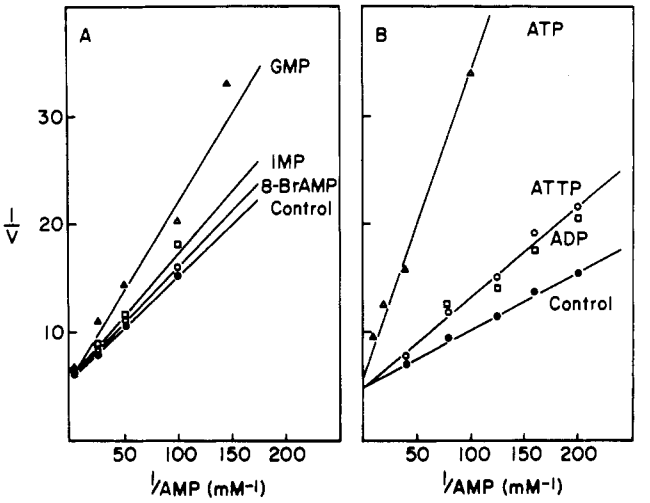

FIGURE 3: Inhibition of pyruvate phosphate dikinase by purine nucleotides. The enzyme was assayed in the presence and absence of various purine nucleotides. (A) The conditions were as described in Figure 2 except that 5.0 mM of either GMP (△), IMP (□), or 8-Br-AMP (○) replaced oAMP in assay solutions. (B) The conditions were as described in Figure 2 except that either ATP (△, 0.5 mM), ATTP (□, 1.0 mM), or ADP (○, 1.0 mM) replaced oAMP in assay solutions. Lines were fitted by the least-squares method.

of several purine nucleotides were examined and are depicted in Figure 3. Nucleotides in which the purine moiety differed from adenine were poor inhibitors, while the adenosine nucleotides with varying phosphate chains competed with AMP. By calculation of the inhibition constants from the slopes and intercepts found in Figures 2 and 3, the analogues could be ranked in decreasing order according to their effectiveness as competitive inhibitors: ATP > ATTP, ADP > oAMP > GMP > IMP > 8-Br-AMP. The K_i of oAMP was calculated as 0.8 mM.

By application of steady-state kinetics in which it is assumed that the dikinase and oAMP form a dissociable complex prior

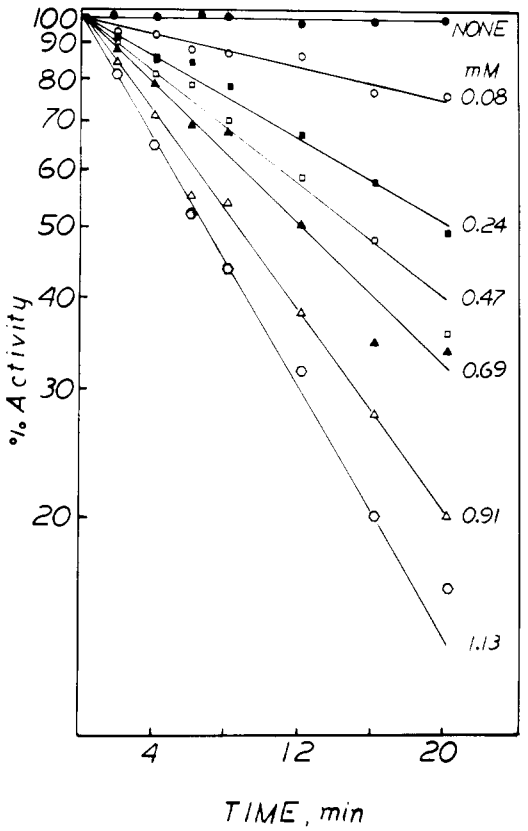

FIGURE 4: Time course of pyruvate phosphate dikinase inactivation by oAMP. The reaction mixture contained 50 mM imidazole–HCl buffer (pH 6.8), 20 mM $MgCl_2$, 16 μg of enzyme, and oAMP as indicated in a final volume of 0.1 mL. Incubation was carried out at 25 °C. At the time intervals shown, a 10-μL aliquot of enzyme was diluted and assayed for activity. The percent enzyme activity remaining was plotted vs. time.

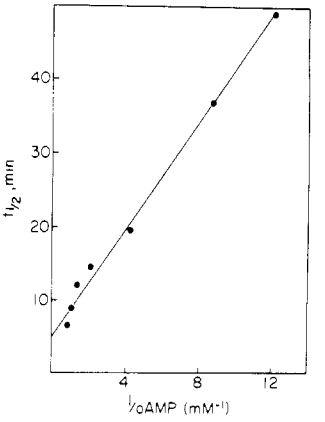

FIGURE 5: Double-reciprocal plot showing the effect of oAMP concentration on the rate of enzyme inactivation. Conditions of the reaction were the same as described in Figure 4. The inactivation half-times, $t_{1/2}$, were obtained from Figure 4 by detemining the time required for each oAMP concentration to cause a 50% reduction in enzyme activity.

Table I: Prevention against Inactivation with oAMP by Substrates[a]

incubation addition	act. (%)	protection (%)
control	100	
1.4 mM AMP	100	100
1.0 mM AMP	98	94
0.8 mM AMP	94	83
0.4 mM AMP	90	71
0.1 mM AMP	68	8
1.0 mM pyruvate	75	31
0.8 mM PP_i	64	0
control + oAMP	64	

[a] Inactivations were conducted at 26 °C with 14.4 μg of enzyme having a specific activity of 8.2 units/mg. The incubation mixture contained, in 100 μL of 50 mM imidazole-HCl buffer (pH 6.8), 0.13 mM oAMP, 20 mM $MgCl_2$, and the indicated concentrations of AMP, pyruvate, or PP_i. The control reaction mixture was as above except that substrates and oAMP was not included. After 5 min, 0.3 mM $NaCNBH_4$ was added to reaction mixtures which were incubated an additional 35 min, diluted, and assayed for enzyme activity. The percent protection was calculated by the following formula: (% act. − 64)/(100 − 64) × 100.

to inactivation, the specificity of oAMP for the ATP–AMP site of the enzyme was further demonstrated. Eq 2–4 summarize the steady-state condition (Meloche, 1967)

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

where E represents free enzyme; I, oAMP, E·I, the enzyme–analogue complex; and E_{inact}, the inactivated enzyme. The constants, k_1 and k_{-1} are the association and dissociation constants, respectively, while k_2 describes the rate of Schiff's base formation. The steady-state equation which describes this process is given in eq 3 and 4

$$t_{1/2} = T/[1 + K_{inact}(1 + [S]/K_s)/[I]] \qquad (3)$$

which may be rewritten as a linear relationship when [S] approaches zero.

$$t_{1/2} = (TK_{inact})1/[I] + T \qquad (4)$$

where $t_{1/2}$ = inactivation half-time, T = minimum inactivation time at infinite inactivator concentration, K_{inact} = rate of inactivation constant, K_s = substrate constant, [S] = substrate concentration, and [I] = inhibitor concentration.

Figure 4 shows that the inactivation of pyruvate phosphate dikinase is pseudo-first-order over a concentration range of 0.08–1.1 mM oAMP. The inactivation half-time, $t_{1/2}$, was calculated by determining the time required for oAMP to cause a 50% reduction in dikinase activity. As shown in Figure 5, a plot of $t_{1/2}$ vs. 1/[oAMP] is a straight line with intercepts of T = 5 min and K_{inact} = 0.8 mM oAMP. Thus, the inactivation follows saturation kinetics and provides evidence that oAMP forms a dissociable complex with the dikinase prior to Schiff's base formation.

For a chemical modifying reagent to be deemed specific, the active site of the enzyme must be protected against inactivation by its normal substrate. Since nonspecific modification of the enzyme occurs at high concentrations of oAMP, a concentration of oAMP was chosen for these studies which caused limited irreversible inactivation of the dikinase. It was found in the absence of AMP that 0.13 oAMP reduced the enzyme activity by ~40%. The inactivation was reduced by increasing the concentration of AMP in incubation mixtures, and with concentrations of AMP >1.0 mM, the protection was complete (Table I). PP_i at 0.8 mM failed to protect the dikinase from oAMP, but pyruvate, 1.0 mM, did prevent inactivation to some extent.

Stoichiometry of the oAMP Modification of Pyruvate Phosphate Dikinase. [^{14}C]oAMP was used in inactivation experiments to determine the relationship between the moles of oAMP bound and the loss of enzymatic activity. The relationship between the extent of inactivation and oAMP incorporated was linear to ~50% inactivation (Figure 6). When 47% of the catalytic activity remained, 1 mol of oAMP was bound per mol of enzyme. By the method of least squares,

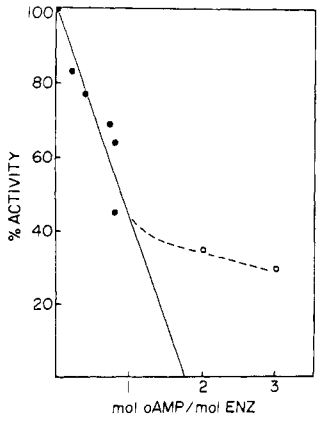

FIGURE 6: Relation of enzyme inactivation and oAMP bound. The enzyme (138 μg, 0.72 nmol) was incubated at 25 °C in 50 mM Tris–acetate buffer (pH 6.8), 20 mM $MgCl_2$, and increasing concentrations of [U-^{14}C]oAMP (sp act. = 2.87 mCi/mmol) in a final volume of 0.5 mL. After 5 min, a 2.5-fold excess of $NaCNBH_4$ was added to enzyme–analogue solutions which were permitted to incubate an additional 35 min. Following incubation, a 10-μL aliquot was diluted and assayed, while the balance of the enzyme was precipitated with 10% Cl_3AcOH, rapidly filtered, washed, dried, and counted for radioactivity. For each [U-^{14}C]oAMP concentration used, reaction mixes lacking enzyme were treated as above. The radioactivity contributing from these controls was subtracted from corresponding enzyme precipitates.

extrapolation of the linear portion gave 1.8 mol of oAMP bound per mol of enzyme (Figure 6). At high [^{14}C]oAMP concentrations, the relationship deviated from linearity. When imidazole–HCl was substituted for Tris buffer, nonspecific binding of oAMP to the enzyme was more extensive, apparently because lysine residues other than those in the ATP–AMP site were modified.

Equilibrium Exchange Reactions of Modified Enzyme. The equilibrium exchange reactions of enzyme modified with oAMP were investigated to verify previous conclusions (Evans & Wood, 1968; Milner & Wood, 1972; Milner & Wood, 1976) that pyruvate phosphate dikinase catalyzes three functionally distinct partial reactions. Two micrograms of enzyme preincubated either in the presence or absence of 0.13 mM oAMP as described in Figure 1 was added to the incubation mixtures and permitted to preequilibrate for 5 min at 30 °C. A trace amount of [U-^{14}C]oAMP, [^{32}P]PP_i, or [2-^{14}C]pyruvate was then added. The exchange reactions were then monitored as described under Methods. For native enzyme, 1% total exchange was observed in 60 min for the ATP–AMP exchange, 5% in 45 min for P_i–PP_i exchanges, and 25% in 30 min for the pyruvate–phosphoenolpyruvate exchange. The fraction of these exchanges occurring in an equivalent amount of enzyme modified with oAMP was calculated and expressed as the percent exchange activity of the unmodified enzyme. When enzyme was incubated in the presence of oAMP and reducing agent, the ATP–AMP exchange was completely inhibited, the P_i–PP_i exchange was inhibited 15%, and the pyruvate–phosphoenolpyruvate exchange was not affected.

Discussion

We conclude that the dialdehyde derivative of AMP is a specific affinity label of the ATP–AMP site of pyruvate phosphate dikinase. The analogue competes with AMP for binding to the enzyme, and AMP protects the dikinase from inactivation by oAMP. The rate of inactivation is pseudo first order and exhibits saturation kinetics, implying that a rate-limiting step involving binding of the analogue to the enzyme occurs prior to the inactivation.

The inactivation of the enzyme by oAMP was proportional to the amount of oAMP bound and extrapolated to ~2 mol bound per mol of the dimeric enzyme. It has been demonstrated that pyruvate phosphate dikinase is a dimer containing subunits of identical molecular weight (Goss et al., 1980; Milner et al., 1975). It has been shown previously that 2 mol of Mn^{2+} bind tightly to the enzyme (Michales et al., 1975). In addition, the dikinase incorporates 2 mol of phosphate and pyrophosphate (Milner et al., 1978). These results are in accord with the view that each subunit of the enzyme participates in catalyzing the overall reaction. Other results have indicated that the enzyme may exhibit half-site reactivity. Pyruvate phosphate kinase binds 1 mol of oxalate (Michaels et al., 1975), and modification with 1 mol of bromopyruvate causes complete inactivation of the enzyme (Yoshida & Wood, 1978). In addition, phosphorylation and pyrophosphorylation protect the dikinase from inactivation by bromopyruvate (Yoshida & Wood, 1978) which suggests that structural changes occur in enzyme phosphorylated by ATP which hinder the ability of bromopyruvate to react at the pyruvate–phophoenolpyruvate site. With [^{14}C]oAMP, the loss of enzymatic activity was not linear after ~50% inhibition, and the inhibition was not complete even with high concentrations of oAMP (Figure 6). Thus, in contrast to expectations for half-site reactivity, the binding of oAMP at the AMP–ATP site of one subunit apparently left the second subunit catalytically active and relatively less reactive with oAMP. These results may reflect a change in the second nucleotide site making it inaccessible to oAMP after the first site is modified. Furthermore, pyruvate protected 30% of the enzyme activity from inactivation by oAMP which likewise may be explained by a decreased availability of the ATP–AMP site for oAMP. Thus, pyruvate phosphate dikinase does not present a single pattern of site reactivity.

Following inactivation of the dikinase by oAMP, the ATP–AMP exchange was completely inhibited and the pyruvate–phosphoenolpyruvate exchange remained unaffected, but the P_i–PP_i exchange was inhibited 15%. In earlier studies when enzyme preparations were modified with bromopyruvate, the pyruvate–phosphoenolpyruvate exchange was rapidly inhibited and the P_i–PP_i exchange was partially inhibited, while the ATP–AMP exchange activity was not affected (Yoshida & Wood, 1978). Thus, modification of pyruvate phosphate dikinase with either oAMP or bromopyruvate affects the function of the P_i–PP_i site, indicating that the P_i–PP_i site may be centrally located and alteration of either of the other subsites affects its function.

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Substrate-Mediated Inactivation of Urocanase from *Pseudomonas putida*. Evidence for an Essential Sulfhydryl Group†

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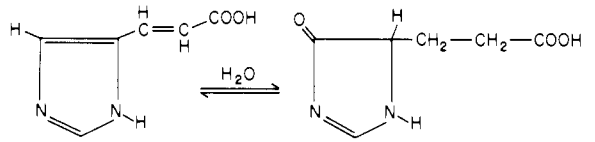
ABSTRACT: Incubation of urocanase from *Pseudomonas putida* with either its substrate, urocanic acid, or product, 4′(5′)-imidazolone-5′(4′)-propionic acid, resulted in an oxygen-dependent inhibition of enzyme activity. Coincident with the inactivation was the stoichiometric incorporation of radioactivity from [^{14}C]urocanate into the protein. NAD^+ which is required for activity of urocanase was not directly involved in the inactivation process. The inactivation of urocanase was irreversible, could be partially blocked by the competitive inhibitor imidazolepropionate, and involved the modification of a single active-site thiol. The inhibition resulted from oxidative decomposition of 4′(5′)-imidazolone-5′(4′)-propionate but was not due to the formation of the major degradative product, 4-ketoglutaramate, since this compound was not an irreversible inactivator of urocanase although it did produce some inhibition at high concentrations. A mechanism is presented in which a reactive imine intermediate in the decomposition scheme is subject to nucleophilic attack by an active-site thiol, thereby generating a covalent enzyme–thioaminal adduct. These results emphasize the importance of a catalytic center sulfhydryl group for urocanase activity.

Urocanase (EC 4.2.1.49) catalyzes the second step in the histidine catabolic pathway, namely, the conversion of urocanic acid to 4′(5′)-imidazolone-5′(4′)-propionic acid:



UROCANIC ACID — IMIDAZOLONE PROPIONIC ACID

The urocanase from *Pseudomonas putida* (Egan & Phillips, 1977; Keul et al., 1979) as well as the enzyme from beef liver (Keul et al., 1979) contains a tightly bound NAD^+ that is essential for catalysis. Other studies have suggested the importance of sulfhydryl residues in catalysis for urocanases from *P. putida* (Hug & Roth, 1973), *Pseudomonas testosteroni* (Hacking et al., 1978), and beef liver (Feinberg & Greenberg, 1959).

In the course of an investigation into the participation of NAD^+ in the mechanism of the urocanase reaction, we observed that the enzyme from *P. putida* became completely inactivated under conditions where large amounts of substrate were being converted to product. Kaminskas et al. (1970) had reported a similar inactivation of the enzyme from *Bacillus subtilis*; however, no mechanism was offered to account for this observation. Our interest in the chemistry of the active site led us to examine this substrate-derived inactivation in greater detail so as to understand its molecular basis and relationship to catalysis. In this report, we present evidence for the role of substrate in the inactivation of urocanase and additionally substantiate the importance of an active-site thiol for catalysis.

Materials and Methods

Chemicals. [^{14}C]Urocanic acid was prepared from L-[U-^{14}C]histidine (270 mCi/mmol) according to the procedure of Mehler et al. (1955). Imidazolonepropionic acid, both unlabeled and ^{14}C labeled, was prepared and purified as described by Brown & Kies (1959). 4-Ketoglutaramic acid was prepared by the method of Hassall & Greenberg (1963). *N*-Formylisoglutamine was synthesized as described by Borek & Waelsch (1953). All other chemicals were obtained from commercial sources.

Enzyme Preparation and Assay. Urocanase was isolated from *Pseudomonas putida*, ATCC 12633, essentially as described by George & Phillips (1970). The purified enzyme had a specific activity of 1.8–2.2 μmol min^{-1} mg^{-1} and was of 95% purity or greater as determined by polyacrylamide gel electrophoresis at pH 8.3 (Davis, 1965) and sodium dodecyl sulfate gel electrophoresis (Weber & Osborn, 1969).

The urocanase assay was the spectrophotometric measurement of urocanate disappearance at 277 nm, as described by George & Phillips (1970). Protein determinations were performed according to the method of Groves et al. (1968), with bovine serum albumin as the standard.

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