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The ATP/AMP Binding Site of Pyruvate,Phosphate Dikinase: Selective Modification with Fluorescein Isothiocyanate†

Nelson F. B. Phillips
Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106
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ABSTRACT: Pyruvate,phosphate dikinase from *Propionibacterium shermanii* is strongly inhibited by fluorescein 5′-isothiocyanate (FITC). The time course of inactivation is biphasic, but the dependence of the pseudo-first-order rate constants on the inhibitor concentration indicates the formation of a reversible complex with the enzyme prior to covalent modification. The substrate/product nucleotide pairs MgATP and MgAMP protected against inactivation, while in the absence of Mg^{2+}, both the nucleotides were ineffective. Previously, an essential lysine at the ATP/AMP subsite of the enzyme from *Bacteroides symbiosus* had been implicated by use of the 2′,3′-dialdehyde of AMP (oAMP) [Evans, C. T., Goss, N. H., & Wood, H. G. (1980) *Biochemistry 19*, 5809]. The inhibition by FITC was competitive with MgAMP, and a multiple inhibition analysis plot indicated that binding of oAMP and FITC was mutually exclusive. These observations suggest that FITC and oAMP bind at the nucleotide binding site and probably to the same reactive lysine that is modified by oAMP. With peptide mapping by high-performance liquid chromatography, FITC was found to be a suitable probe for isolating the peptide from the ATP/AMP subsite.

The overall reaction catalyzed by pyruvate,phosphate dikinase (EC 2.7.9.1, pyruvate,orthophosphate dikinase) from *Propionibacterium shermanii* and *Bacteroides symbiosus* (now classified as *Clostridium symbiosus*) involves three partial reactions, each of which is catalyzed at a distinct subsite:

$$\text{ENZ-His} + \text{ATP} \rightleftharpoons \text{ENZ-His}\sim{}^{\beta}\text{P}^{\gamma}\text{P} + \text{AMP} \quad (1a)$$

$$\text{ENZ-His}\sim{}^{\beta}\text{P}^{\gamma}\text{P} + \text{P}_i \rightleftharpoons \text{ENZ-His}\sim{}^{\beta}\text{P} + {}^{\gamma}\text{PP}_i \quad (1b)$$

$$\text{ENZ-His}\sim{}^{\beta}\text{P} + \text{pyruvate} \rightleftharpoons \text{ENZ-His} + {}^{\beta}\text{P-enolpyruvate} \quad (1c)$$

The phosphoryl moiety of the phosphoryl-enzyme intermediate (ENZ-His∼P)[1] was shown to be bound to the enzyme through a phosphoramidate linkage to the 3′-nitrogen of a histidine residue (Spronk et al., 1976). The pyrophosphoryl group of the ENZ-His∼PP intermediate has also been shown to be linked to a histidine residue after stabilization of the ENZ-His∼PP by diazomethylation (Phillips & Wood, 1986). The amino acid sequence surrounding the phosphorylated histidine residue has been sequenced (Goss et al., 1980). This essential histidyl residue is considered to be centrally located

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[1] Abbreviations: PPDK (dikinase), pyruvate,phosphate dikinase; ENZ-His∼P, phosphoryl pyruvate,phosphate dikinase; ENZ-His∼PP, pyrophosphoryl derivative of the dikinase; P-enolpyruvate (PEP), phosphoenolpyruvate; oAMP, 2′,3′-dialdehyde of AMP; FITC, fluorescein 5′-isothiocyanate; FTC, fluorescein thiocarbamyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NEM, *N*-ethylmorpholine; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; NADH, reduced nicotinamide adenine dinucleotide; TEA, triethylamine; AUFS, absorbance units full scale.

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in the enzyme active site and to mediate the transfer of the phosphoryl and pyrophosphoryl moieties between the three partial reaction subsites [for a review see Goss and Wood (1982)]. Recently, the sequence surrounding the pivotal histidine of pyruvate,phosphate dikinase from maize leaf has been sequenced (Burnell, 1984). There is complete homology with that of *B. symbiosus*, except that the bacterial peptide has one additional valine.[2]

Affinity labeling studies have been performed to investigate substrate interaction at the three subsites of PPDK from *B. symbiosus*. By modification with the 2',3'-dialdehyde of AMP (oAMP) an essential lysine at the ATP/AMP subsite (site for reaction 1a) has been implicated (Evans et al., 1980). Another critical lysine residue was identified by use of pyridoxal 5'-phosphate (a modifier of the phosphate binding site) (Phillips et al., 1983). A cysteine residue has been identified as a critical functional group at the pyruvate/P-enolpyruvate subsite (1c) by modification with bromopyruvate (Yoshida & Wood, 1978).

Although oAMP fulfilled the criteria as an affinity label on the basis of inhibition kinetics, all attempts to isolate the peptide containing the modified lysine have been unsuccessful, apparently due to instability of the adduct during proteolytic digestion and peptide isolation procedures. We have, therefore, explored the suitability of another nucleotide analogue, fluorescein 5'-isothiocyanate (FITC), as an affinity probe for the ATP/AMP subsite of the dikinase. FITC has been shown to specifically modify nucleotide binding sites of several ATPases, including sarcoplasmic reticulum Ca^{2+}-ATPase (Mitchinson et al., 1982), (Na,K)-ATPases (Karlish et al., 1979; Farley et al., 1984; Kirley et al., 1984), and phosphorylase kinase (Sotiroudis & Nikolavopoulos, 1984). This paper demonstrates the inactivation of the dikinase by the binding of FITC to the ATP/AMP subsite, apparently to the same lysine that is modified by oAMP. The suitability of FITC as a probe in isolation of the peptide from the nucleotide subsite is also demonstrated.

Experimental Procedures

Materials

Fluorescein 5'-isothiocyanate (isomer 1) was obtained from Molecular Probes; NADH, phosphoenolpyruvate, ATP, AMP, sodium pyrophosphate, lactate dehydrogenase, 2-mercaptoethanol, and ammonium acetate were from Sigma Chemical Co.; HPLC-grade acetonitrile was from EM Science; trifluoroacetic acid (sequanal grade) was from Pierce; L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin was obtained from Worthington Biochemical Corp.; *N*-ethylmorpholine from Sigma was redistilled and stored under nitrogen at −15 °C. All other chemicals were of analytical grade.

Methods

Purification and Assay of Pyruvate,Phosphate Dikinase. Pyruvate,phosphate dikinase was prepared from *P. shermanii* by a method similar to that described by Goss et al. (1980). The dikinase was assayed spectrophotometrically by measuring the rate of pyruvate formation from P-enolpyruvate by coupling with the lactate dehydrogenase reaction.

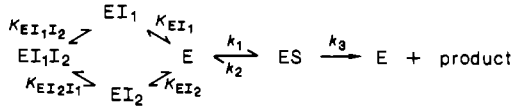
Synthesis of oAMP. The dialdehyde of AMP (oAMP) was synthesized and purified as described by Easterbrook-Smith et al. (1976).

Inactivation of Pyruvate,Phosphate Dikinase by Fluorescein 5'-Isothiocyanate. Inactivation was carried out by incubating 45 μg of enzyme (specific activity 11 units/mg) in 100 mM Tris-HCl (pH 6.8) buffer containing 20 mM NH_4Cl and various concentrations of FITC in a final volume of 250 μL. Incubation was at 30 °C in the dark. Residual enzyme activity was determined at different times as indicated in the figures.

Determination of Stoichiometry of Binding of FITC. Pyruvate,phosphate dikinase (5.6 μg, 0.6 nmol) having a specific activity of 11 units/mg was incubated at 30 °C with different concentrations of FITC in the buffer described above. After 40 min, the reaction solutions were desalted by the rapid microcentrifuge desalting technique as described by Helmerhost and Stokes (1980). A 5-mL syringe containing Sephadex G-25 (SF) preequilibrated with 100 mM NEM-acetate (pH 7.0) containing 2.5 mM EDTA was used for this purpose. Aliquots (10 μL) of the desalted solutions were withdrawn for determination of residual enzyme activity. Aliquots of 100 μL were diluted with 200 μL of 0.1 M NaOH containing 1% (w/v) SDS and absorbances at 500 nm determined. The amount of FITC bound to the protein was calculated by assuming a value of 80 000 cm^{-1} M^{-1} for fluorescein bound to protein (Pick & Karlish, 1980) and a molecular weight of 180 000 for the dikinase (subunit molecular weight was 90 000 by SDS–PAGE).

Peptide Mapping by HPLC. The dikinase (0.32 mg, 3.5 nmol) was reacted with 0.5 mM FITC under the conditions described above with the specific additions as indicated in the figure legends. After 80% of the dikinase was inactivated (60 min), the reaction mixture was desalted as described above. (When MgAMP was present in the reaction solution, no significant loss of dikinase activity was observed.) The desalted modified enzyme in 100 mM NEM-acetate (pH 8.5) was trypsinized by addition of TPCK-trypsin (2% w/w, final concentration) and incubated at 37 °C for 2 h. The digest was directly fractionated on a reverse-phase column by HPLC, as described in the legends of Figure 6.

Kinetic Analysis of Multiple Inhibition Data. The method of Yonetani and Theorell (1964) was used for the present analysis. The interactions of the two substrate-competitive inhibitors oAMP and FITC (I_1 and I_2) and the substrate AMP (S) with the enzyme (E) can be described by



where k_1, k_2, and k_3 are rate constants and K_{EI_1}, K_{EI_2}, $K_{EI_1I_2}$, and $K_{EI_2I_1}$ are the dissociation constants of the respective enzyme–inhibitor complexes shown above.

The steady-state treatment of the reaction, originally worked out by Slater and Bonner (1952), yields

$$\frac{1}{v_i} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S]}\left(1 + \frac{[I_2]}{K_{EI_2}}\right) + \frac{K_m}{V_{max}[S]K_{EI_1}}\left(1 + \frac{[I_2]}{\alpha K_{EI_2}}\right)[I_1] \quad (2)$$

where [S] = [AMP], $[I_1]$ = [oAMP], and $[I_2]$ = [FITC]. α is an interaction constant between I_1 and I_2 in the EI_1I_2 complex and has the following correlations: $\alpha = K_{EI_1I_2}/K_{EI_2} = K_{EI_2I_1}/K_{EI_1}$. If I_1 and I_2 interact with the same site of E, they prevent each other from binding to E; i.e., EI_1I_2 is not formed and thus $\alpha = \infty$. If I_1 and I_2 interact with different sites of E, then $\infty > \alpha > 0$ (Yonetani, 1982).

[2] Added in proof: The maize leaf sequence was recently shown to be identical with that of the bacterial peptide (Roeske et al., 1988).

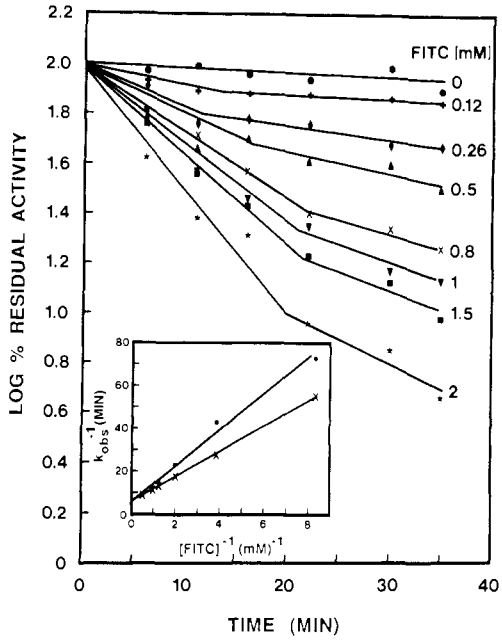


FIGURE 1: Inactivation of pyruvate,phosphate dikinase by FITC. The reaction mixture contained 100 mM Tris-HCl (pH 6.8), 20 mM NH_4Cl, 25 μg of the dikinase, and [FITC] as indicated, in a final volume of 250 μL. Incubation was at 30 °C in the dark. At the times shown, aliquots of the reaction mixture were assayed for residual activity by the spectrophotometric assay. (Inset) The observed first-order rate constant k_{obsd} as a function of FITC concentration: k_{obsd} was calculated from $t_{1/2}$ as described in the text. The data points were fit to a straight line by the method of least squares: (●) plot when k_{obsd} was calculated by using the $t_{1/2}$ obtained from both phases of inactivation; (×) plot when k_{obsd} was calculated by using the $t_{1/2}$ obtained from the rapid inactivation phase.

Equation 2 indicates that when $1/v_1$ is plotted against $[I_1]$ at fixed $[I_2]$, a straight line will be obtained with a slope of $[1 + [I_2]/(\alpha K_{EI_2})]K_m/V_{max}[S]K_{EI_1}$. When $\alpha = \infty$, the slope will be constant, i.e., $K_m/V_{max}[S]K_{EI_1}$, and thus plots of $1/v$ vs $[I_1]$ at different $[I_2]$ will become parallel straight lines. When $\infty > \alpha \geq 0$, both slope and intercept of the plot will increase as linear functions of $[I_2]$.

RESULTS

Inactivation of Pyruvate,Phosphate Dikinase by FITC. Incubation of PPDK (45 μg) with FITC in the absence of Mg^{2+} results in progressive inactivation of the dikinase. The inactivation follows pseudo-first-order rates over a concentration range of 0.12–2.0 mM FITC (Figure 1). The kinetic behavior deviates from a simple first-order reaction. There is a slight nonlinearity in the rate of inactivation, consisting of a fast phase and a slow phase. The reason for the decrease in the rate of inactivation is not clear, and this biphasic nature has been observed during the inactivation of Ca^{2+}-ATPases from sarcoplasmic reticulum (Pick, 1981) and phosphorylase kinase (Sotiroudis & Nikolavopoulos, 1984) by FITC. It is possible that FITC modifies two essential residues with differing sensitivities, which would give rise to the observed biphasic inactivation curve. However, the results from the peptide mapping data described below suggest that although two residues are modified by FITC, the modification of only one residue leads to complete inactivation of the dikinase. The second reactive residue is modified without the loss of enzyme activity, i.e., in the presence of MgAMP (see Figure 6b). Biphasic kinetics was also observed when inactivation is carried out in the presence of Mg^{2+}.

The observed first-order rate constant (k_{obsd}) was determined from the expression $k_{obsd} = 0.693/t_{1/2}$ by using the data points from both phases of inactivation to determine the $t_{1/2}$. A plot of $1/k_{obsd}$ vs $1/[FITC]$, using eq 3, is a straight line with a

$$1/k_{obsd} = K_i/k_3[FITC] + 1/k_3 \quad (3)$$

finite vertical intercept [Figure 1, inset (●)]. The K_i and k_3 values were calculated to be 1.28 mM and 0.16 min^{-1}, respectively, by using eq 3 (Kitz & Wilson, 1962). When the inactivation was carried out in the presence of Mg^{2+}, the K_i of FITC was increased to 2.04 mM (data not shown).

When only the early time points (0–16 min) from the rapid inactivation phase are used to plot $1/k_{obsd}$ vs $1/[FITC]$, a straight line with a finite vertical intercept is also obtained [Figure 1, inset (×)]. The K_i in this plot is lowered to 0.96 mM, while the intercept (k_3), however, is not altered. These results show that, regardless of whether the time points from the rapid inactivation phase or those from both phases of inactivation are used to replot the Figure 1 inset, the same limiting rate constant of inactivation (k_3) is obtained at saturating [FITC]. This result is indicative of saturation kinetics and is consistent with reversible binding of FITC prior to covalent modification, according to eq 4. This evidence fulfills one of the criteria normally used for affinity labeling.

$$ENZ + FITC \overset{K_i}{\rightleftharpoons} [ENZ{\cdot}FITC] \xrightarrow{k_3} [ENZ\text{-}FITC]_{inact} \quad (4)$$

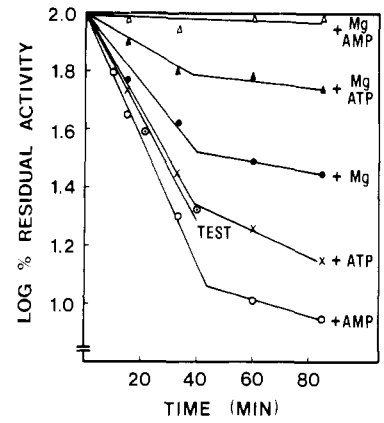


FIGURE 2: Effect of ATP/AMP subsite ligands on the inactivation of the dikinase by FITC. The dikinase (25 μg) was incubated with 0.5 mM FITC as described in the legend of Figure 1 except for the inclusion of 20 mM $MgCl_2$ and 10 mM AMP (△), 20 mM $MgCl_2$ and 10 mM ATP (▲), 20 mM $MgCl_2$ (●), 10 mM ATP (×), and 10 mM AMP (○). The control (⊙) or "test" lacked any additions. At the indicated times, aliquots were assayed for residual activity.

Specificity of FITC for the ATP/AMP Subsite. Three parameters were examined to assess the specificity of FITC for the ATP/AMP subsite.

(i) The effect of various substrates and products of the dikinase on the inactivation by FITC was tested and is shown in Figure 2. The substrate/product pair of reaction 1a, MgATP and MgAMP, affords almost complete protection, while AMP or ATP did not protect against inactivation. Mg^{2+} at 20 mM confers partial protection, and the protection observed with MgATP or MgAMP, but not the free nucleotides, is not due to protection by Mg^{2+} per se (Figure 2). The small loss of activity seen with MgATP as the protectant is presumably due to the formation of the ENZ-His~PP form of the enzyme (reaction 1a), which is known to be unstable (Phillips & Wood, 1986). The substrate/product pairs of the other partial reactions (1b and 1c), in contrast, did not protect against inactivation (Table I). The above results suggests that the inactivation by FITC may occur in the region of the ATP/AMP binding site.

(ii) When the inhibition was carried out under steady-state conditions as described in Figure 3, FITC acted as a compe-

Table I: Effect of Ligands of the P_i/PP_i and Pyruvate/PEP Subsites on the Inactivation of the Dikinase by FITC[a]

ligands	residual activity (%) (after 80 min)	$t_{1/2}$ (min)
none	19	27
P_i (5 mM)	19	27
PP_i (1 mM)	13	20
PEP (1 mM)	16	23
MgAMP (5 mM)	84	243

[a] The dikinase was reacted with FITC in the presence of the added ligands as indicated. The reaction conditions are as described for Figure 2.

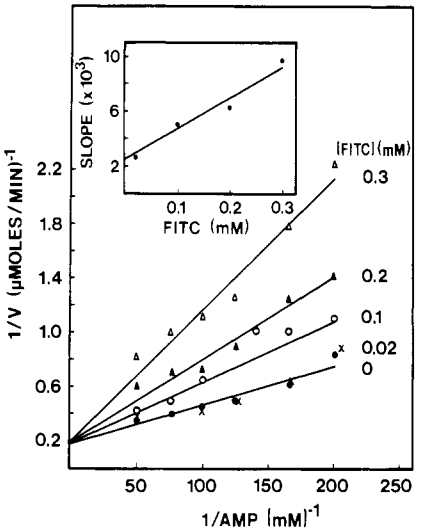


FIGURE 3: Competitive inhibition of the dikinase by FITC with respect to AMP. Initial rates of the dikinase activity were measured by the spectrophotometric assay with different fixed concentrations of FITC and varying [AMP]. The reaction mixture contained 50 mM imidazole hydrochloride (pH 6.8), 20 mM $MgCl_2$, 20 mM NH_4Cl, 1.0 mM PEP, 0.8 mM pyrophosphate, 0.1 mM NADH, 1 unit of lactate dehydrogenase, and various concentrations of AMP and FITC. The reaction at 30 °C was initiated with 0.7 unit (65 μg) of the dikinase, and the change in absorbance at 340 nm was monitored for 1–2 min. Initial velocity (V) was expressed as micromoles of NADH oxidized per minute. Lines were fitted by the least-squares method.

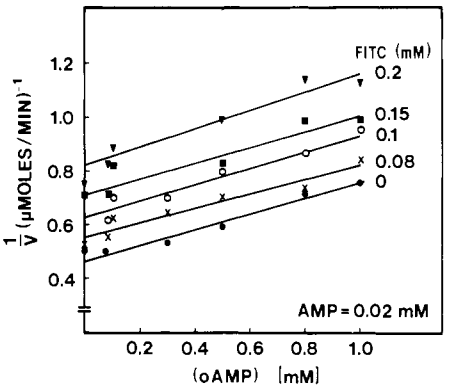


FIGURE 4: Yonetani–Theorell plot depicting multiple inhibition of the dikinase by the two substrate competitive inhibitors FITC and oAMP. Initial rates of the dikinase in the presence of FITC and oAMP were measured by the spectrophotometric assay as described in the Figure 3 legend. The concentrations of FITC and oAMP were varied as indicated, while the concentration of AMP was held constant at a subsaturating level of 0.02 mM. The initial rates were plotted according to eq 2 described under Methods. Lines were fitted by the least-squares method.

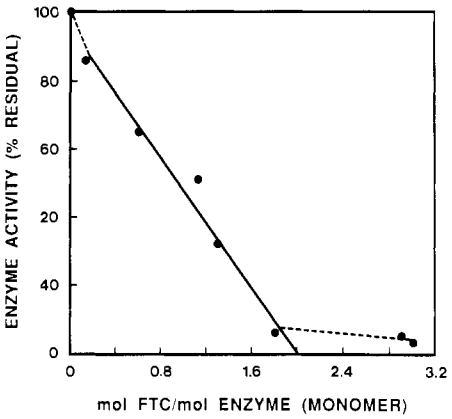


FIGURE 5: Relationship between inactivation and incorporation of FITC. The stoichiometry of the incorporation of FITC was determined as described under Methods. A value of 100% initial velocity was assigned to an enzyme control in the absence of FITC.

titive inhibitor of the dikinase with respect to AMP as the variable substrate. The K_i of FITC, determined from a replot of slopes vs [FITC] (Figure 3, inset), was found to be 0.11 mM, which is 10× lower than that determined from the inactivation kinetics (Figure 1). It is possible that FITC binds in a less favorable way when it undergoes the chemical reaction than when it binds as a reversible inhibitor (Plapp, 1982). This could account for the difference in the value of K_i determined under the two different conditions.

(iii) The multiple inhibition of the dikinase by the two competitive inhibitors FITC and oAMP was analyzed by the Yonetani–Theorell plot (Yonetani & Theorell, 1964) as described under Methods. As seen in Figure 4, a plot of $1/V_1$ vs [oAMP] at different [FITC] shows that the slope is independent of [FITC], thus indicating that oAMP and FITC bind to the same site and are mutually exclusive. The inhibition experiments described in Figures 3 and 4 were carried out under steady-state conditions, where, in the 1–2-min assay, only reversible inhibition occurs. Irreversible inhibition does not occur in this time period as determined by measuring enzyme activity of the FITC-treated enzyme (as described in the Figure 1 legend) after diluting the treated enzyme into a large excess of the spectrophotometric assay solution.

Stoichiometry of Inhibition by FITC. The stoichiometry of the reaction was determined by measuring the amount of FITC bound to the dikinase at different concentrations of the inhibitor, as described under Methods. By extrapolation to 100% loss of activity, about 2 equiv of FITC was determined as bound per monomer of the dimeric enzyme (Figure 5). The dimeric PPDK consists of identical subunits with one nucleotide binding site per monomer. It is shown below that FITC binds at two sites; apparently, only one of these is at the ATP/AMP subsite. At higher FITC concentrations (>0.8 mM) the nonspecific incorporation increases to more than 3 equiv.

Evaluation of Inactivation Kinetics by Peptide Mapping Using HPLC. Tryptic peptide mapping of the modified enzyme was performed to examine, in greater detail, the nonstoichiometric incorporation of FITC. The dikinase was reacted with FITC, desalted, and digested with trypsin as described under Methods. The digest was fractionated on a reverse-phase C_4 column by HPLC. The 496-nm absorption profile of the fractionated digest shows two major FTC-peptides (peaks 1 and 2 of Figure 6a), a finding that agrees with the results from stoichiometric incorporation. When MgAMP was included in the reaction, peak 1 is significantly reduced (Figure 6b), a result that is consistent with the data in Figures 2 and 3. This same peak is also absent when the dikinase is inactivated with oAMP prior to modification with FITC (Figure 6c), a finding that agrees with the multiple inhibition plot of Figure 4. The smaller 496-nm absorbing peaks probably represent nonspecific labeling of the dikinase.

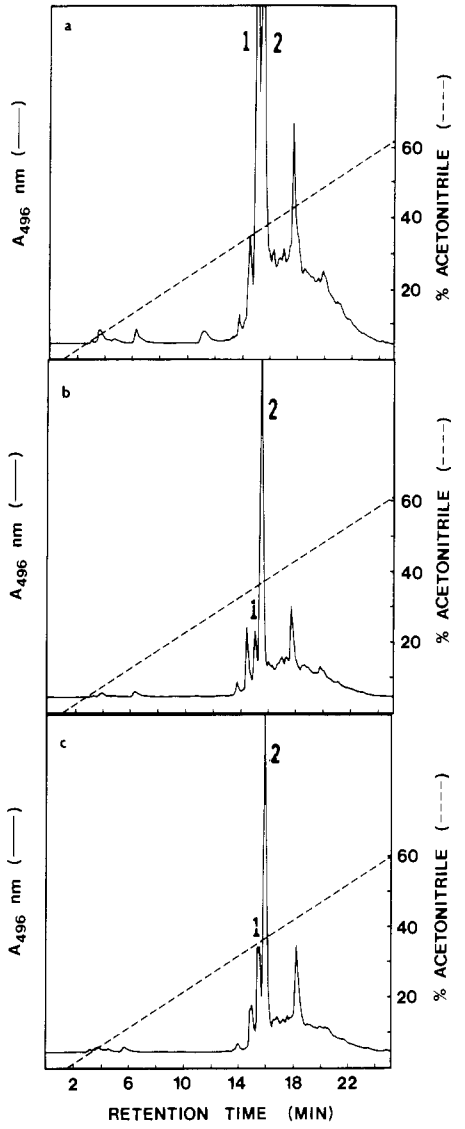


FIGURE 6: HPLC peptide maps of the tryptic digest of FITC-labeled pyruvate,phosphate dikinase. The dikinase was inactivated with FITC and digested with trypsin as described under Methods. The digest was fractionated on a Synchropak reverse-phase C_4 column (RP-4, 25 cm × 4.1 mm) with a Du Pont Model 850 liquid chromatograph. The column was eluted isocratically for 2.5 min with 0.1 M ammonium acetate (H 8.5, with triethylamine) at 0.9 mL/min. A linear gradient of 0–70% CH_3CN over 30 min was then applied by using 0.1 M ammonium acetate–TEA (pH 8.5) and CH_3CN solvent systems. FTC-labeled peptides were monitored at 496 nm (—) at a sensitivity setting of 0.32 AUFS for all three chromatograms. The dashed line (- - -) represents the CH_3CN gradient. (a) Tryptic peptide map of the dikinase inactivated with FITC in the absence of any protectant. (b) Peptide map of the dikinase inactivated with FITC in the presence of 20 mM $MgCl_2$ and 10 mM AMP. (c) Peptide map of the dikinase treated with FITC after inactivation (80%) of the enzyme by oAMP. The dikinase (3.5 nmol) was incubated with 0.5 mM oAMP in 100 mM Tris-HCl (pH 6.8) containing 20 mM NH_4Cl and 20 mM $MgCl_2$. After 10 min, sodium borohydride was added in a 5-fold molar excess over [oAMP] and the solution incubated for an additional 45 min. By this time, 80% of the dikinase is inactivated. The reaction mixture was then desalted by the microcentrifuge desalting method (Helmerhorst & Stokes, 1980) and treated with 0.5 mM FITC for 60 min. The subsequent treatment of the reaction solution was as described above. There was minimal loss or dilution of the enzyme by the desalting method described, as determined by subjecting the dikinase through the similar desalting conditions in the absence of any inhibitor.

This nonspecific labeling is also suggested by the kinetic data of Figure 5. However, the possibility that the smaller peaks were generated by rearrangement of a small portion of the fluorescein moiety of the major FTC-peptides cannot be ruled out. This is considered in more detail under Discussion.

Discussion

This study was undertaken to determine if FITC was a suitable affinity probe for the isolation of the peptide that comprises the ATP/AMP binding subsite. FITC was found to exhibit several characteristics of an affinity label for the nucleotide site, the more interesting features of which are discussed in more detail here.

Stoichiometry of Incorporation and Correlation with Peptide Mapping. The stoichiometry of incorporation of FITC extrapolated to 2 equiv bound per monomer. However, PPDK from *P. shermanii* and *B. symbiosus* is known to be a dimer with subunits of identical molecular weight (Goss et al., 1980). In addition, the enzyme from either source incorporates 2 mol of P_i and PP_i/mol of enzyme (Milner & Wood, 1972; Milner et al., 1978). These results are in accord with the view that each monomer contains one catalytic site. To resolve the above discrepancy, the nonstoichiometric incorporation of FITC was examined in greater detail by peptide mapping. The tryptic peptide map of the modified dikinase showed two FTC-peptides, but only one labeled peptide was seen when AMP was added during the modification. These results indicate that the second mole of FITC was incorporated by modification of a catalytically nonessential residue, since the presence of AMP protected PPDK against inactivation by FITC. This nonspecific binding may arise from the labeling of either the NH_2-terminal residue or another reactive lysine that is not essential for activity. Since there is a linear relationship between inactivation and incorporation, it is assumed that the two residues are equally sensitive to FITC but only one is essential for activity.

The smaller FTC-labeled peaks seen in Figure 6 could also have been generated by nonspecific binding, although to a lesser degree. It is quite possible that the smaller peaks were generated by a rearrangement of a small portion of the fluorescein group of the two major FTC-peptides (Figure 6). This rearrangement is thought to alter the hydrophobic properties of the FTC-peptide and hence its chromatographic retention times. Such observations have been reported for the fluorescein-labeled peptides of Ca^{2+}-ATPases (Mitchinson et al., 1982) and of (Na,K)-ATPases (Farley et al., 1984). In the latter case, the authors showed that when a single fluorescent peptide, purified from a C_{18} column, was injected onto a C_4 column, numerous fluorescent peptides were generated. Heterogeneity in the peptide structure was ruled out since amino acid compositions of each of the peptides were identical. The authors concluded that the heterogeneity in the peptides was probably due to rearrangement of the fluorescein group. In the present study, the possibility that one of the two peaks (of Figure 6a) may have been produced by this rearrangement is ruled out on the basis that peak 1 is absent when (a) MgAMP was included during the inactivation of the dikinase by FITC or when (b) the dikinase is inactivated with oAMP prior to modification with FITC. It is quite unlikely that MgAMP or oAMP can prevent the rearrangement of the fluorescein moiety. In addition, preliminary amino acid composition data of the two FTC-peptides (from peaks 1 and 2 of Figure 6a) indicate that they are different.

The protection by AMP against binding of FITC to one of the sites, in combination with the kinetic data discussed below, provides evidence that FITC acts as an affinity label for a single ATP/AMP site.

Kinetic Data Supporting the Validity of FITC as an Affinity Label for the ATP/AMP Site. (i) The inactivation of the dikinase by FITC shows saturation kinetics, indicating that

FITC forms a dissociable complex with the enzyme prior to covalent modification. The dissociation constant for FITC (K_i = 1.28 mM) is increased to 2.04 mM in the presence of Mg^{2+}. This effect could be brought about by the ability of Mg^{2+} to partially protect the dikinase against inactivation (Figure 2). Thus, Mg^{2+} would have the effect of rendering FITC a less effective inhibitor. This result is in contrast to the inactivation of Ca^{2+}-ATPase by FITC, where Mg^{2+} accelerated the rate of inactivation at pH 8.0 or lower (Pick, 1981).

(ii) The substrate/product pair MgATP and MgAMP protects the dikinase against inactivation by FITC, while ligands of the P_i/PP_i or pyruvate/PEP subsites are ineffective. Interestingly, neither AMP nor ATP afforded any protection in the absence of Mg^{2+}. This suggests that only the chelated form of the nucleotides can bind to the nucleotide binding region. Similar results were obtained with phosphorylase kinase (Sotiroudis & Nikolavopoulos, 1984); however, Mg^{2+} alone conferred almost complete protection against inactivation by FITC. In the case of the dikinase, only partial protection was seen with saturating Mg^{2+}.

(iii) FITC is a competitive inhibitor with respect to AMP.

(iv) Multiple inhibition analysis, performed to evaluate the relationship between the two competitive inhibitors of the ATP/AMP site, shows that oAMP and FITC are mutually exclusive; i.e., they prevent each other from binding to the same nucleotide site. This result suggests that the two inhibitors compete for the same residue that is involved in the binding of the nucleotide.

The evidence presented here suggests that FITC reacts at the nucleotide binding site, although the observed kinetic effects could also be brought about if FITC reacts at an "allosteric site", a possibility that cannot be ruled out on the basis of the present findings.

Comparison of oAMP and FITC as Affinity Probes in the Isolation of Peptides from Nucleotide Binding Sites. Previous attempts to isolate the tryptic peptide of the dikinase containing the [^{14}C]oAMP-modified lysine have been unsuccessful, apparently due to instability of the adduct during the isolation procedures (unpublished observation). Although oxidized nucleotides were first introduced as affinity labels for nucleotide binding enzymes more than 10 years ago (Easterbrook-Smith et al., 1976), there is only one published report (as far the author knows) of a successful isolation and sequencing of an active site peptide using the oxidized nucleotide as the probe (Bezares et al., 1987). There is, on the other hand, a relatively vast number of reports on the affinity labeling of enzymes using these compounds (Colman, 1983). It is generally accepted that the inhibition of enzymes by periodate-oxidized nucleotides involves formation of a Schiff base; however, there are many reports where the product is clearly not a Schiff base and the formation of dihydroxymorpholino derivatives has been observed (Lowe & Beechey, 1982; King & Colman, 1983). The bifunctional dialdehyde groups of the oxidized nucleotides can also lead to cross-linking of proteins, as has been observed with bovine serum albumin (Cysyk & Adamson, 1976). In the case of enzyme derivatives other than a Schiff base reducible by sodium borohydride, it has been shown that the product is unstable to acid hydrolysis (Gregory & Kaiser, 1979; King & Carlson, 1981) and possibly to prolonged proteolytic digestion and peptide isolation procedures [see the review by Colman (1983)].

The lack of published sequence information (with the one exception above) might suggest that, in most cases, affinity labeling using oxidized nucleotides leads to formation of derivatives other than Schiff bases. As suggested by Colman (1983), this instability may limit the value of these compounds as tools for isolating and identifying active site peptides containing the labeled residue.

The present work demonstrates that FITC is a valid affinity label for the ATP/AMP subsite and that the labeled product is stable during the peptide isolation procedures. FITC has been successfully used to isolate and sequence peptides at the ATP binding sites of several ATPases [see Kirley et al. (1985) and references cited within] and shows good promise as a tool in the isolation and the sequencing of the peptide comprising the ATP/AMP subsite of the dikinase, a work that is currently under way.

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Isotope Exchange as a Probe of the Kinetic Mechanism of Pyrophosphate-Dependent Phosphofructokinase†

Yong-Kweon Cho,‖ Terry O. Matsunaga,‡ George L. Kenyon,‡ Byron L. Bertagnolli,§ and Paul F. Cook*,‖

Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143, Department of Biochemistry, University of Illinois, Champaign–Urbana, Urbana, Illinois 61801, and Department of Biochemistry, North Texas State University/Texas College of Osteopathic Medicine, Denton, Texas 76203

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ABSTRACT: Data obtained from isotope exchange at equilibrium, exchange of inorganic phosphate against forward reaction flux, and positional isotope exchange of ^{18}O from the bridge position of pyrophosphate to a nonbridge position all indicate that the pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii* has a rapid equilibrium random kinetic mechanism. The maximum rates of isotope exchange at equilibrium for the [^{14}C]fructose 1,6-bisphosphate $\rightleftharpoons$ fructose 6-phosphate, [^{32}P]P_i $\rightleftharpoons$ $MgPP_i$, and Mg[^{32}P]PP_i $\rightleftharpoons$ fructose 1,6-bisphosphate exchange reactions increasing all four possible substrate–product pairs in constant ratio are identical, consistent with a rapid equilibrium mechanism. All exchange reactions are strongly inhibited at high concentrations of the fructose 6-phosphate (F6P)/P_i and $MgPP_i$/P_i substrate–product pairs and weakly inhibited at high concentrations of the $MgPP_i$/fructose 1,6-bisphosphate (FBP) pair suggesting three dead-end complexes, E:F6P:P_i, E:$MgPP_i$:P_i, and E:FBP:$MgPP_i$, in agreement with initial velocity studies [Bertagnolli, B. L., & Cook, P. F. (1984) *Biochemistry 23*, 4101]. Neither back-exchange by [^{32}P]P_i nor positional isotope exchange of ^{18}O-bridge-labeled pyrophosphate was observed under any conditions, suggesting that either the chemical interconversion step or a step prior to it limits the overall rate of the reaction.

Pyrophosphate-dependent phosphofructokinase (PP_i-PFK)[1] catalyzes the reversible phosphorylation of D-fructose 6-phosphate at the C-1 hydroxyl to give D-fructose 1,6-bisphosphate with magnesium pyrophosphate as the phosphoryl donor. The enzyme thus catalyzes the same chemical reaction as does the mammalian ATP-dependent phosphofructokinase. The enzyme from *Propionibacterium* exhibits Michaelis–Menten kinetics in both reaction directions at pH 8 and 25 °C (Bertagnolli & Cook, 1984).

The kinetic mechanism of the enzyme from *Propionibacterium freudenreichii* is proposed to be rapid equilibrium random with E:F6P:P_i, E:$MgPP_i$:P_i, and E:FBP:$MgPP_i$ dead-end complexes (Bertagnolli & Cook, 1984). However, initial velocity studies are not a good probe of the steps occurring within the interconversion of central complexes and indicate only that one or more steps responsible for the conversion of E:$MgPP_i$:F6P to E:Mg:P_i:FBP limit(s) the overall reaction.

The present study makes use of three different isotope exchange techniques. Isotope exchange at equilibrium is used to determine whether the mechanism is truly rapid equilibrium. Isotope exchange of [^{32}P]P_i back into pyrophosphate with the reaction running in the direction of production of P_i and FBP (exchange against the flow) and positional isotope exchange of ^{18}O-bridge-labeled pyrophosphate into the nonbridge position are used to determine whether steps other than phosphoryl transfer limit the interconversion of central complexes. These studies confirm the rapid equilibrium random mechanism proposed from initial velocity data and further suggest that the chemical step(s) probably limit(s) the interconversion of central complexes.

Materials and Methods

Enzymes. Pyrophosphate-dependent phosphofructokinases from *Propionibacterium freudenreichii* and *Phaseolus aureus* were both obtained in crude form from Sigma and purified according to Bertagnolli and Cook (1984) and Bertagnolli et al. (1986). Fructose-1,6-bisphosphate aldolase, triosephosphate isomerase, phosphoglucose, isomerase, and α-glycerophosphate dehydrogenase from rabbit muscle, glucose-6-phosphate de-

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‡ University of California.
§ University of Illinois, Champaign–Urbana.
‖ North Texas State University/Texas College of Osteopathic Medicine.

[1] Abbreviations: PP_i-PFK, inorganic pyrophosphate dependent phosphofructokinase; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; Taps, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TEA, triethylamine.

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