The metal cation requirements of pyruvate, phosphate dikinase from Bacteroides symbiosus have been examined with respect to both the overall reaction and the three partial reactions. The overall reaction and all three partial reactions require a divalent cation (Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$). Although Mn$^{2+}$ and Co$^{2+}$ are very effective activators at low concentrations in two of the three partial reactions, they each inhibit one partial reaction at higher concentrations. Mg$^{2+}$ is not inhibitory to any of the partial reactions and thus is the most effective divalent ion activator for the complete overall reaction. The monovalent cation requirement of the dikinase (Tl$^{+}$, NH$_4^+$, or K$^+$) was found to reside completely in the P-enolpyruvate, pyruvate partial reaction, which is consistent with the involvement of monovalent cations in catalysis reactions.

Fully purified dikinase possesses a maximum of 18 sulfhydryl groups/dimeric molecule of which two or three are essential for enzymatic activity. The number of sulfhydryl groups decreased during loss of enzyme activity with aging. The relative reactivity of the sulfhydryl groups of the native enzyme (E), phosphorylated enzyme (E$_p$), and pyrophosphorylated enzyme (E$_{pp}$) was compared. The sulfhydryl groups of E$_p$ react with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) at a higher rate than those of either E or E$_{pp}$ with a concomitant increased rate of loss of enzyme activity. However, E$_p$ in the form of the transition state analog complex (consisting of E$_p$, Me$^{2+}$, oxalate, and an activating monovalent cation) shows a decreased reactivity of the sulfhydryl groups and decreased inhibition of the enzyme by DTNB. Apparently, a conformation change occurs during formation of the complex which protects the essential sulfhydryl groups.

Dikinase is dimeric and exhibits some properties of half-site reactivity. The sulfhydryl groups of the di-phosphorylated enzyme ($E_p$) were found to be more reactive with DTNB than those of the monophosphorylated enzyme (E$_p$) but the sulfhydryl groups of the transition state analog complex of $E_p$ were less reactive and the enzymatic activity was less subject to inactivation by DTNB. Apparently, when only one subunit of the enzyme is phosphorylated, the nonphosphorylated subunit may still react with DTNB.

The pyruvate, phosphate dikinase reaction involves (1–3) a three-site Tri Uni Uni Ping-Pong mechanism via partial Reactions 1a, 1b, and 1c:

\[
E^3 + ATP \rightleftharpoons E_{pp} + AMP \quad (1a)
\]

\[
E_{pp} + P_i + E \rightleftharpoons E_p + PP_i \quad (1b)
\]

\[
E_p + pyruvate \rightleftharpoons E + P-enolpyruvate \quad (1c)
\]

ATP + P$_i$ + pyruvate $\rightleftharpoons$ AMP + PP$_i$ + P-enolpyruvate

This reaction sequence predicts the existence of three independent exchange reactions and three independent forms of the enzyme (E, E$_p$, and E$_{pp}$). Although the studies of the dikinase of plants have not supported the above mechanism (4), kinetic data, exchange reactions, and the isolation of the three forms of the enzyme have demonstrated the validity of this mechanism for the enzymes from Bacteroides symbiosus (5, 6) and Propionibacterium shermanii (3, 6).

The dikinase of B. symbiosus appears to fulfill the position in glycolysis normally occupied by pyruvate kinase (6–8). Like pyruvate kinase, the dikinase from most sources has an absolute requirement for both a monovalent and a divalent cation (6, 9). Since the dikinase reaction can be separated into partial reactions, it has permitted analysis of the cation activation of the dikinase in a way that has not been possible with pyruvate kinase.

The dikinases from various sources are generally unstable and this has been a major difficulty in their study (6, 9). It has been suggested that the dark inactivation and light inactivation of the dikinase of plants involves the oxidation and reduction, respectively, of essential sulfhydryl groups (10, 11). Sugiyama and Shirahashi (11) have shown that the tetrameric dikinase from maize has 34 sulfhydryl groups. The present communication is the first study of the reactivity of the sulfhydryl groups of the dikinase from a highly purified bacterial enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

Pyruvate, phosphate dikinase was prepared from B. symbiosus by the method of Milner et al. (12). Imidazole, Tris base, tricyclohexyl-

**ammonium P-enolpyruvate, trisodium P-enolpyruvate, sodium salts of AMP and ATP, and sodium pyruvate were obtained from Sigma. All substrates were titrated to the appropriate pH before use except for pyruvate, which was kept at pH 3 before use so as to minimize polymer formation. Thallium nitrate was from K & K, and other metal salts were analytical grade obtained either from Fisher or

---

* This investigation was supported by Grant No. BMS 74-22692 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address, Department of Pediatrics, University of Cincinnati, Cincinnati, Ohio 45229.

§ Present address, Department of Biological Chemistry, Hebrew University, 20 MaMila St., Jerusalem, Israel.

The abbreviations used are: E, nonphosphorylated enzyme; E$_p$, phosphorylated enzyme; E$_{pp}$, pyrophosphorylated enzyme; E$_{pp}$, diphasphorylated enzyme (presumably one phosphate on each of the subunits); DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); P-HMB, p-hydroxymercuribenzoate.
for rapid ATP, AMP exchange. It is interesting that Mn\(^{2+}\) at Co\(^{2+}\) in the overall reaction can be explained by the inhibition is shown in Fig. 2. The relative ineffectiveness of Mn\(^{2+}\) and other divalent cations did not activate the enzyme, including Ba\(^{2+}\), Sr\(^{2+}\), Ca\(^{2+}\), Fe\(^{2+}\), Cu\(^{2+}\), and Zr\(^{2+}\).

Mn\(^{2+}\) appears to be the lowest of the three. Other divalent cations did not activate the enzyme, including Ba\(^{2+}\), Sr\(^{2+}\), Ca\(^{2+}\), Fe\(^{2+}\), Cu\(^{2+}\), and Zr\(^{2+}\).

p-HMB (not shown), N-ethylmaleimide, and DTNB, but it is not reactivated by dithiothreitol. However, a role for low concentrations gives somewhat the same activation profile for the P\(_{i}\)-PP, exchange as it does in the overall reaction in the direction of pyruvate formation.

**Monovalent Cations**—Monovalent cations are required for activity in the overall reaction in both directions, as shown in Fig. 3. The apparent K\(_{m}\) values appear to be lower in the direction of pyruvate formation, which is similar to the results which Sugiyama obtained using the dikinase from corn leaves (15). The requirement for monovalent cation in the exchange reactions of the dikinase is shown in Fig. 4. It is clear that only the P-enolpyruvate, pyruvate exchange reaction requires the presence of monovalent cations and the requirement is absolute. The K\(_{m}\) values for the activating monovalent cations are of the same relative magnitudes as for the overall reaction.

**Sulfhydryl Groups and the Activity of Dikinase**

**Inactivation of the Dikinase**—The dikinase is inhibited by various agents known to react with sulfhydryl groups, including p-HMB (not shown), N-ethylmaleimide, and DTNB, but not by iodoacetate or iodoacetamide (Table I).

Ultracentrifugation of the enzyme with various concentrations of p-HMB demonstrated that there was formation of the monomeric 5 S species from the active dimeric 8 S form of the enzyme (Fig. 5) and that the amount increased as the percentage inhibition of the dikinase increased. When the enzyme is reactivated by incubation at room temperature in 50 mM dithiothreitol for 30 min, the sedimentation coefficient again becomes 8 S.

The dikinase slowly inactivates when kept at 4°C in buffered solution, particularly when the protein concentration is less than 0.5 mg/ml. This inactive enzyme, like the p-HMB-inactivated enzyme, has a sedimentation coefficient of 5 S but it is not reactivated by dithiothreitol. However, a role for
sulfhydryl groups in age inactivation is suggested by the results presented in Table II. Enzyme of original specific activity 24 IU/mg was incubated for 14 days at 4°C in the presence of the indicated concentrations of p-HMB. At the end of this time, the activity remaining was determined by assaying all the samples in the presence of dithiothreitol. The enzyme originally most inhibited by the p-HMB retained the most activity, suggesting that essential sulfhydryl groups of the enzyme may have been protected from oxidation by the presence of the attached p-HMB. If so, the oxidation must be intrasubunit since the inactivation is accompanied by dissociation which would not occur if an intersubunit disulfide were formed.

Number of Sulfhydryl Groups of the Dikinase—Determination of the number of sulfhydryl groups of the purified active enzyme has given variable results, ranging from 12 to 18/dimeric molecule. Results, however, are quite reproducible for a given enzyme preparation at a given time.  

Comparison of the Rate of Reaction of DTNB with Different Forms of the Dikinase and the Effect of Oxalate on the

Reactions—As shown in Table III, there is a significant difference in the rates of reaction of the sulfhydryl groups of the three forms of the dikinase with DITNB. The phosphorylated form reacts the fastest and the nonphosphorylated form, the slowest. Furthermore, the reaction of the enzyme with DTNB is accompanied by inactivation of the enzyme; the phosphorylated dikinase is inactivated at the highest rate and the native form, the slowest (Fig. 6A). Experiments were also conducted (Fig. 6B) to determine the effect of oxalate on the inactivation of the enzyme with DTNB. It is seen by comparison of Fig. 6, A and B, that there was considerable stabilization of the $E_p$ form by oxalate and that the stabilization was
TABLE I

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Specific activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H2O)</td>
<td>7.8</td>
<td>0</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>7.2</td>
<td>8</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>1.0</td>
<td>87</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>8.0</td>
<td>0</td>
</tr>
<tr>
<td>DTNB</td>
<td>1.9</td>
<td>76</td>
</tr>
</tbody>
</table>

Fig. 5. Sedimentation velocity ultracentrifugation of pyruvate, phosphate dikinase in the presence of para-hydroxymercuribenzoate (p-HMB). Enzyme, 1.90 mg/ml, in 50 mM imidazole-HCl, pH 6.8, was incubated at 25°C for 15 min in the presence of the indicated concentrations of p-HMB. The samples were then placed on ice, assayed, and the ultracentrifugation begun within the hour. All samples were centrifuged at 60,000 rpm. A and B were at 1.25°C with the photographs being taken at 80 min after reaching speed. C and D were at 4.07°C and the photographs were taken 76 min after reaching speed. Sedimentation, right to left. A, No p-HMB; specific activity, 24.0 IU/mg. B, 10.0 pM p-HMB; specific activity, 22.7 IU/mg. C, 100 pM p-HMB; specific activity, 8.6 IU/mg. A and B 8000 = 8 S; C and D 8000 = 5 S and 8 S.

FIG. 6. Comparison of the inactivation of E, Epp, and Epp by DTNB in the presence and absence of oxalate. A, the incubation mixture contained, in a total volume of 0.50 ml: Tris/acetate (pH 7.2), 200 mM; DTNB, 0.5 mM; MgCl2, 4.0 mM; NH4Cl, 10 mM; and the dikinase (4.8 units). E form (O-----O). In addition, for the Epp form (E-----E), 1.0 mM P-enolpyruvate was added and for the Epp form (E-----E), 2.0 mM ATP was added. The experiments of B were similar except oxalate (1.0 mM) was added.

much less with the E or Epp forms of the enzyme.

Effect of Monovalent Cations on Sulphydryl Reaction and Inactivation of E, in the Presence and Absence of Oxalate—The rate of reaction of the sulphydryl groups with DTNB is approximately the same in the absence of oxalate with either NH4+ or Tl+. But, in the presence of oxalate, the rate is considerably slower with Tl+ present than it is with NH4+ present (Fig. 7A). The decreased reactivity of the sulphydryl group is also reflected in the decrease in the rate of inactivation of the enzyme (Fig. 7B). Thallium activates the dikinase with a K, about 1 order of magnitude less than that of NH4+ (Fig. 4). These results will be considered further under "Discussion."

Effect of the Degree of Phosphorylation of the Dikinase on the Reaction of the Sulphydryl Groups—Pyruvate, phosphate dikinase consists of two subunits and it has recently been found with a high ratio of P-enolpyruvate to enzyme, that two phosphates are incorporated into the dikinase (5, 16). It seemed possible that oxalate protection might be increased if both subunits were phosphorylated. A comparison of results obtained with the monophosphorylated (Ep) and diphosphorylated (Epp) enzymes is shown in Fig. 8. In this case, the respective phosphorylated enzymes were isolated by Sephadex filtration and no P-enolpyruvate was present in the reaction mixture. It is seen with both sites phosphorylated the sulphydryl groups are more susceptible to reaction with DTNB than when only one site is phosphorylated (Fig. 8, A compared to B). Also, the difference in the number of sulphydryl groups...
which react in the presence and absence of oxalate is greater with the diphosphorylated enzyme. Furthermore, the protection by oxalate against inactivation by DTNB is much greater with \( E_p \) than it is with \( E_p \) (Fig. 8, C compared to D).

**DISCUSSION**

The fact that the reaction of the dikinase consists of three distinct partial reactions has permitted the present study of the role of metals in its mechanism. The finding that monovalent cations are required only in the P-enolpyruvate, pyruvate exchange reaction (Fig. 4) is in accord with the observations by Suelter (17) that many enzymes which require monovalent cations involve keto-enol transformations in their transition states. In accord with this hypothesis, Nowak and Mildvan (18) found evidence that the monovalent cation activator of pyruvate kinase may be involved in forming a bridge between the enzyme and the carboxyl group of P-enolpyruvate. The studies of James et al. (19), with the same system, showed that an activating monovalent cation (K') increased the affinity of pyruvate kinase for both P-enolpyruvate and pyruvate, whereas a nonactivating monovalent cation ((CH₃)₂N⁺) did not.

Direct evidence of the role of monovalent cations in tautomeration of pyruvate has recently been obtained by Moskovitz and Wood (20) using [3-Tl]pyruvate and the phosphorylated form of the dikinase (\( E_p \)). They found that the monovalent cation is required for enolization (measured by formation of tritiated water) and the divalent cation for the phosphoryl transfer. Thus, partial reaction 1c actually consists of two parts:

\[
E_p + \text{pyruvate} \xrightarrow{\text{Me}^+} E_p + \text{enolpyruvate (1c')}
\]

\[
E_p + \text{enolpyruvate} \xrightarrow{\text{Me}^+} E + \text{P-enolpyruvate (1c'')}
\]

Study of the partial reactions has also provided information concerning action of the divalent cations. Mg²⁺, at its optimum concentration, is the most effective divalent ion for the overall reaction (Fig. 1). The lower activity of Mn²⁺ and Co²⁺ compared to Mg²⁺ may be explained by their effects on the three partial reactions. Although Mn²⁺ and Co²⁺ are more effective than Mg²⁺ at low concentrations in the P-enolpyruvate, pyruvate, and PP₃, P₃ partial reactions, they are not effective at the same concentrations in the ATP, AMP partial reaction. At higher concentrations, where they are effective in the ATP, AMP partial reaction, they inhibit one or the other of the partial reactions. Mg²⁺ is not effective in any of the partial reactions at the low concentrations but it activates all three partial reactions at higher concentrations (Fig. 2) and thus is the most effective in the overall reaction.

The poor activation of the P₃, PP₃ exchange reaction by Mn²⁺ at higher concentrations may be due to inhibition by the soluble high molecular weight complex of Mn²⁺ and PP₃, described by Cooperman and Mark (21). A similar activation profile by Mn²⁺ was observed with thiamin pyrophosphatase (22).

Activation profiles by Co²⁺ similar to those with pyruvate, phosphate dikinase have been observed in the overall reaction of pyruvate kinase (23). Since Co⁺ is known to change from octahedral to tetrahedral symmetry more easily than either Mg²⁺ or Mn²⁺, it was considered by Mildvan and Cohn (24) that the poor activation by Co²⁺ of pyruvate kinase might be caused by this change. However, no evidence for a blue shift in the visible spectrum of enzyme-bound Co⁺ consistent with the tetrahedral metal was discerned (24). A similar study of the enzyme-bound spectrum of dikinase-Co²⁺ in the presence of various substrates was likewise unrevealing. Thus, it remains unclear why Co²⁺ is a poor activator of the dikinase.

In his original observations regarding activation of the dikinase from B. symbiosus by divalent cations, Reeves (25) did not find activation of the enzyme by either Mn²⁺ or Co²⁺ in the direction of P-enolpyruvate formation, although both of these cations were activators in the direction of pyruvate formation. The most likely explanation of the discrepancy is

---

1. G. Michaels, unpublished results.
that assays in the direction of P-enolpyruvate formation require an alkaline pH and there is rapid inactivation of the dikinase in this pH range, particularly at low protein concentration. This problem was minimized in the present studies by stabilizing the dikinase with the addition of bovine serum albumin (5).

Pyruvate, phosphate dikinase from a variety of sources has been found to be unstable. In the case of the dikinase of B. symbioseus, this instability is due, at least in part, to the reactivity of its sulfhydryl groups. Accompanying the reaction of these groups, there is dissociation of the active dimeric enzyme with a sedimentation coefficient of 8 S to an inactive monomeric form with a sedimentation coefficient of 5 S. Furthermore, when the sulfhydryl groups react with p-HMB, the inactivation can be reversed (Table II). The reaction of the enzyme with p-HMB apparently protects the sulfhydryl groups from oxidation and prevents the irreversible inactivation which occurs when the enzyme is stored in the absence of p-HMB (Table II).

The reactivity of the sulfhydryl groups of the pyrophosphorylated (E_phosphorylated (E_p), and nonphosphorylated forms of the enzyme (E) with DTNB have been found to differ. The E_p form being the most reactive (Table III). Apparently, phosphorylation causes conformational changes in the dikinase which increases the exposure of sulfhydryl groups. In the presence of oxalate, Mg^{2+}, and Tl^{+} or NH_4^{+}, the reactivity of the sulfhydryl groups of E_p with DTNB is decreased (Fig. 7A) and the inactivation is decreased (Fig. 7B). This protection of oxalate against inactivation apparently is specific for very reactive and essential sulfhydryl groups since, in 2 min in the absence of oxalate, the enzyme is 70 to 80% inactivated (Fig. 7D) when only two or three sulfhydryl groups had reacted (Fig. 7A). The slow inactivation in the presence of oxalate may be due to nonspecific oxidation of sulfhydryl groups which are not at the catalytic site.

Previous studies by paramagnetic resonance (26) have indicated that there is a specific strong interaction of oxalate with the phosphorylated form of the dikinase and that the E_p form of the enzyme is required for the oxalate binding. It appears by the criteria of Wollenden (27) that the E_p-Me^{2+}-oxalate complex represents a transition state analog complex. The EPR spectrum of the enzyme bound Mn^{2+} changes drastically in the presence of monovalent cations and the E_p-Mn^{2+}-oxalate complex resembles the spectrum of Mn^{2+} in the solid state, suggesting a highly immobilized Mn^{2+} which is protected from the buffeting action of solvent molecules. This interaction is believed to occur because of the strong structural resemblance of oxalate and enolpyruvate which is postulated to be the true transition state of the enzyme complex. It is suggested that the immobilized and very rigid structure of the E_p-Mn^{2+}-Tl^{+}-oxalate complex causes a conformation change in an area much larger than the divalent cation site thus causing numerous sulfhydryl groups to be protected from the action of DTNB. Reed and Morgan (28) have shown that oxalate is a potent inhibitor of pyruvate kinase; however, monovalent cations had no effect on the strong binding of oxalate to pyruvate kinase.

Pyruvate, phosphate dikinase exhibits some properties of half-site reactivity. The dikinase is dimeric but only one phosphoryl group is incorporated per mol of enzyme unless the ratio of P-enolpyruvate to enzyme is high (5, 16). In addition, homogeneous preparations exhibit a specific activity of either 12 or 24 units/mg, but the enzyme of specific activity of 24 usually reverts unpredictably and spontaneously to specific activity of 12 (5, 16). Only 1 mol of oxalate binds to the enzyme but 2 mol of Mn^{2+} are tightly bound (26). With diethylpyrocarbonate, it has been shown that two histidyl groups are directly involved in the catalysis but combination of only 1 mol of bromopyruvate with the enzyme causes complete inactivation of the enzyme (16). The inactivation by bromopyruvate apparently occurs by combination with a sulfhydryl group near the keto acid site (16). These results are in accord with the present results which show that the activity of the enzyme is dependent on the presence of certain free sulfhydryl groups.

In the EPR studies by Michaels et al. (26) only one oxalate was bound. In this paper the dikinase was probably monophosphorylated and the oxalate apparently only combines with the subunit which is phosphorylated. Presumably, it is the subunit which is phosphorylated which is protected by oxalate from the action of DTNB. If both subunits are phosphorylated (E_p), there should be a greater protection by oxalate against the action of DTNB than with the monophosphorylated (E_p) form. The results in Fig. 8 show that this is true. These results indicate that DTNB reacts most efficiently with nonphosphorylated (and therefore nonoxalated) sites. The use of oxalate offers the prospect of a relatively rapid assay of the percentage of phosphorylated sites on the enzyme without use of radioactive substrates. The actual importance of these findings in relation to the mechanism of the dikinase catalysis will require further experimentation.

REFERENCES
Pyruvate, phosphate dikinase. Metal cation requirements and inactivation of the enzyme by sulfhydryl agents.
G Michaels, Y Milner, B R Moskovitz and H G Wood


Access the most updated version of this article at http://www.jbc.org/content/253/21/7656.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/253/21/7656.citation.full.html#ref-list-1