Interaction of Macroions and Dioxane with the Allosteric Phosphoenolpyruvate Carboxylase*

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SUMMARY

Phosphoenolpyruvate carboxylase from Salmonella typhimurium is activated by basic compounds such as polylysine, protamine, histone, and spermidine. Ionic strength and pH of the assay medium are contributory factors for the activation of the enzyme by polylysine. Gradient centrifugation experiments show that polylysine binds with the enzyme. The polylysine-enzyme aggregate remains susceptible to the modulation of its activity by the allosteric effectors, aspartate (inhibitor) and fructose 1,6-diphosphate (activator).

Various nonpolar solvents such as dioxane, 2-propanol, ethanol, dimethyl sulfoxide, and propylene glycol also activate the enzyme reversibly. In the presence of 10% dioxane but not the other solvents the enzyme gets desensitized to the effect of aspartate and fructose 1,6-di-P. This desensitization is a reversible process and does not seem to be due to a dissociation of the enzyme polymer into subunits.

It is concluded that the enzyme possibly has accessible hydrophobic regions coupled with exposed areas with a preponderance of negatively charged groups. Long range electrostatic interactions or weakening of the hydrophobic bonds of the enzyme in its native conformation both lead to the activation of the enzyme.

In the earlier publications from this laboratory it has been demonstrated that phosphoenolpyruvate carboxylase (orthophosphate:oxalacetate carboxy-lyase (phosphorylating) EC 4.1.1.31) from Salmonella typhimurium is an important regulatory enzyme the activity of which is controlled by feedback inhibition by aspartate (1), precursor activation by fructose 1,6-diphosphate (2), compensatory activation by nucleoside di- and triphosphate "pools" (3), and a few other compounds. The fact that so many chemically diverse compounds are capable of interacting with the enzymic protein raises some important questions. It may be asked, for instance, whether each of the modifiers of the enzyme activity has a specific site on the enzyme surface, and whether the enzyme has some unusual structural properties which confer on it the function of regulation. These questions are, of course, basic to an understanding of all allosteric phenomena (4, 5). It seemed possible to approach these questions by observing alterations in the catalytic properties of the enzyme by treatment with agents which change the bond alignments of protein polymers. Such an approach has previously been made with a number of other allosteric enzymes (6-11).

We report in the following communication the characteristics of activation of partially purified P-enolpyruvate carboxylase from S. typhimurium by certain high molecular weight polymers and by dioxane and some other organic solvents.

EXPERIMENTAL PROCEDURE

Reagents—Poly-L-lysine (mol wt 195,000) and copoly-L-phenylalanyl-L-lysine (1:1; mol wt, 180,000) were obtained from Yeda Research and Development Company, Israel. Polyglucose sulfate as sodium salt (number average mol wt, 55,750) was kindly provided by Dr. P. T. Mora of Bethesda. Solutions of these polymers in glass distilled water were extensively dialyzed before use. Spermidine and protamine sulfate were purchased from Calbiochem. Calf thymus histone was a product of Mann Research. Pig heart L-malate dehydrogenase (specific activity, 720 i.u. per mg) was supplied by Boehringer. All other reagents used in this work were obtained from Sigma. Reagent grade dioxane was purified by reflux with KOH followed by distillation.

Enzyme Preparation and Assay—A 200- to 250-fold purified enzyme was prepared from glucose-grown S. typhimurium cells essentially in the same way as described earlier (3). A coupled spectrophotometric assay with malate dehydrogenase was used throughout (1, 2). Enzyme velocity was expressed as a change of absorbance of 0.001 at 340 nm per min. Unless indicated otherwise the assay mixture contained in a final volume of 3 ml, 20 mM NaHCO₃, 10 mM MgCl₂, 1.66 mM sodium P-enolpyruvate, 0.15 mM DPNH, 6 μg of malate dehydrogenase, and 0.1 M Tris-HCl. The final pH of the reaction mixture was 9.0. To ensure that P-enolpyruvate carboxylase remained rate limiting in assays in the presence of potent activators such as dioxane, the concentration of the carboxylase, whenever desired, was adjusted so that the initial velocities never exceeded 0.2 absorbance unit per min. This assay was proportional to the activity of malate dehydrogenase (the second en-
TABLE I
Effect of various compounds on activity of P-enolpyruvate carboxylase

The assay mixture (3 ml) consisted of 0.1 M Tris-HCl, pH 9.0, 20 mM NaHCO₃, 10 mM MgCl₂, 1.66 mM sodium P-enolpyruvate, 0.15 mM DPNH, 6.2 µg of malate dehydrogenase, various additives when required, and 36 units (1 unit corresponding to an absorbance change of 0.001 per min) of the enzyme. In this system the oxidation of DPNH was measured in cuvettes of 1-cm light path with a Gilford 2000 recording spectrophotometer at 24 and 25°. The substrate and activators were added simultaneously.

<table>
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<th>Additions</th>
<th>Concentration</th>
<th>Velocity</th>
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<tbody>
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<td>0.036</td>
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<tr>
<td>Histone</td>
<td>13 µg/ml</td>
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<td>Polyglucose SO₄</td>
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<td></td>
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</tr>
<tr>
<td>NaCl</td>
<td>0.03 M</td>
<td>0.036</td>
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</table>

zymic component in our assay mixtures), but none was found to effect it.

Zone Centrifugations in Sucrose Density Gradients—The method described by Martin and Ames (12) was used. The 4% and 20% sucrose solutions used in making gradients contained 0.05 M Tris-HCl, pH 8.0, and 1 mM MgSO₄. Whenever required, the sucrose solutions also contained various activators and other compounds. The enzyme solutions were dialyzed before use against 0.05 M Tris buffer (pH 8.0). Pig heart malate dehydrogenase was used as a marker in all runs. Centrifugations were performed with a swinging cup rotor in a Spinco model L ultracentrifuge for 10 hours at an average speed of 38,500 rpm and at a temperature of 0–2°. Each centrifuge tube yielded 78 to 80 drops.

RESULTS

Effect of Some Polynons on Activity of P-enolpyruvate Carboxylase
—It was during the purification of P-enolpyruvate carboxylase with protamine sulfate (3) that it was noticed that suspensions of protamine enzyme aggregates invariably exhibited higher activities compared with the soluble enzyme. In order to find whether other polynons were also capable of activating the enzyme, we tested a number of compounds listed in Table I on the activity of P-enolpyruvate carboxylase. It will be noted that the basic proteins and spermidine activate the enzyme, the most potent activator being polylysine. One of the polyanions, polyglucose sulfate, also activated the enzyme when used in high concentrations. Although inorganic sulfate also activates the enzyme (Table I), it is not certain whether polyglucose sulfate activation is connected with its polymeric structure or is just due to its sulfate content. This point has not been pursued further. In contrast to sulfate, monomeric L-lysine is incapable of activating the enzyme (Table I).

Effect and Nature of Binding between Polylysine and Enzyme
When the enzyme activity is measured in the presence of increasing concentrations of polylysine, the saturation curve (Fig. 1) reaches a plateau at a polymer concentration of about 0.65 µg per ml. Concentration higher than this leads to slight decreases in activation. By contrast, copolyphenylalanyllysine activates the enzyme only at concentration ranges many fold higher than those of polylysine. Thus, to bring about a 5.7-fold increase in activity the required concentrations of polylysine and the copolymer are 0.5 µg per ml and 36 µg per ml, respectively (Fig. 1). Introduction of nonpolar side chains in the polyelectrolyte molecule thus leads to a decrease in its properties as an activator.

Polylysine has a marked effect on the affinity of the substrate (P-enolpyruvate) for its binding site. The $K_m$ value of about 12 mM for P-enolpyruvate in the absence of polylysine changes to about 2 mM in the presence of 1.3 µg per ml (saturating) polylysine (Fig. 2). It is also noteworthy that the $V_{max}$ of the enzyme is not substantially altered in the presence of the macromolecular activator (Fig. 2).

Two obvious questions now arise. First, it may be inquired whether the activation of P-enolpyruvate carboxylase is due to a specific binding with polylysine, and second, whether other
kinetic parameters of the enzyme change as a result of this binding. To answer the first question, zone centrifugations in sucrose density gradients were performed with pig heart malate dehydrogenase as a marker both in the presence and absence of polylysine. When required, P-enolpyruvate carboxylase was mixed with polylysine and layered on top of the gradient. Under our experimental conditions during which separation of malate dehydrogenase and P-enolpyruvate carboxylase was achieved (Fig. 3), addition of polylysine always resulted in the recovery of P-enolpyruvate carboxylase as a pellet at the bottom of the centrifuge tube, with malate dehydrogenase remaining at the expected position in the gradient. It may be mentioned that

![Figure 2](image2.png)

**Fig. 2.** Effect of polylysine (1.3 µg per ml) on the activity of P-enolpyruvate (PEP) with P-enolpyruvate as the variable substrate. Tris-HCl, 0.1 M, at pH 9.0 was used as the buffer. Each cuvette contained 18 units of the enzyme and 20 mM bicarbonate (saturating).

![Figure 3](image3.png)

**Fig. 3.** The separation of malate dehydrogenase (MDH) and P-enolpyruvate carboxylase (PEP) in sucrose density gradients. The dotted lines represent activity of the enzymes in gradients with 6% dioxane.

![Figure 4](image4.png)

**Fig. 4.** The effect of pH on the activation of P-enolpyruvate carboxylase by polylysine and fructose-1,6-di-P (FDP). P-enolpyruvate and bicarbonate concentrations were 1.66 mM and 20 mM, respectively. Tris-HCl, 0.1 M, was used. Each cuvette contained 16 units of the enzyme. V₀ and Vₐ refer to the velocities in the presence and the absence of the activators, respectively.

in assay mixtures with polylysine as an activator, clear solutions (as inspected by eye) were always obtained and no turbidity changes could be detected by spectrophotometric methods. Assuming a molecular weight of 40,000 for the pig heart malate dehydrogenase (13), a molecular weight of 183,500 ± 8,000 can be assigned to P-enolpyruvate carboxylase. This value does not change in the presence of small molecular weight modifiers of enzyme activity (1-3), such as acetyl-CoA (1 mM), fructose-1,6-di-P (12 mM), and l-aspartate (2 mM), which means that "aggregation" of the enzyme is not necessarily a prerequisite for or a consequence of activation or inhibition.

The binding between polylysine and P-enolpyruvate carboxylase seems to be electrostatic in nature, as is suggested by experiments on the extent of activation of the enzyme as a function of pH and ionic strength (Figs. 4 and 5) of the assay medium. It will be noted from Fig. 4 that at an ionic strength of 0.084 (0.1 M Tris-HCl), about 7-fold activation is obtained at pH 9.0 (pH optimum of the enzyme), but at pH 7.0 this value changes to about 23-fold. As a contrast to macroionic activation, the extent of activation by fructose-1,6-di-P remains essentially unchanged at all pH values (Fig. 4). Similarly, maximum activation at pH 9.0 is produced by polylysine in assay medium of low ionic strength. The extent of activation decreases continually as the ionic strength is raised (Fig. 5). It may be pointed out that the activity of the enzyme itself does not change in the absence of polylysine between the ionic strength ranges of 0.1 and 0.5.

Does polylysine binding to the enzyme change other kinetic parameters of the enzyme? This question is important because large numbers of other allosteric enzymes are modified kinetically either reversibly or irreversibly by treatment with various re-
Activation of Phosphoenolpyruvate Carboxylase

I I I I I I

0.10 0.20 0.30 0.40 0.50
ionic strength

FIG. 5. The effect of the ionic strength of the assay medium on the extent of activation by polylysine. Tris-HCl of different ionic strengths, at pH 9.0, was used. P-enolpyruvate and bicarbonate concentrations were 1.66 mM and 20 mM, respectively. In each assay 14 units of enzyme were used. The concentration of polylysine was 0.65 µg per ml. V₀ and Vₐ refer to the velocities in the presence and the absence, respectively, of polylysine.

In the presence of polylysine the enzyme is protected from thermal denaturation at 48°C. Thus, in one experiment the enzyme lost 29% of its activity at 48°C in 3 hours, but in the presence of polylysine (2 µg per ml) no loss of activity occurred. The allosteric modifiers, aspartate (2.5 mM) and fructose-1,6-di-P (10 mM), failed to give any significant protection under similar conditions.

Activation of Enzyme by Ethanol and Dioxane—In order to probe further into the nature of the binding of polylysine with the enzyme, we decided to test the effect of a medium of low dielectric constant on the extent of activation of the enzyme by the polycation. In such experiments it was found that in the presence of ethanol or dioxane alone dramatic increases in the activity of the enzyme occur. This can be seen from Fig. 7. At a concentration of about 6% (v/v) ethanol or dioxane the enzyme activity increases approximately 35-fold with dioxane and about 28-fold with ethanol. The extent of activation by ethanol is consistently lower than that obtained with dioxane (Fig. 7). Also, the degree of activation is considerably decreased at concentrations of ethanol higher than 10%. That the activating effect of dioxane and ethanol is due to a lowering of the dielectric constant of the assay medium and is not due to their specific binding on the enzyme surface can be surmised from the observation that other unrelated compounds which effectively lower the dielectric constant of water also activate the enzyme. Thus, at a concentration of about 10% (v/v), 2-propanol, dimethyl sulfoxide, and propylene glycol activated the enzyme 21, 22, and 25-fold, respectively. It may be mentioned that all of these reagents are without effect on malate dehydrogenase, which is the other enzymic component of the assay system used here. In control experiments, no oxidation of DPNH is observed in the

agents and other allosteric ligands (4, 5, 14). At concentrations of polylysine sufficient to bind all of the free enzyme in solution (judged by a curve of the kind presented in Fig. 1), the allosteric effectors, fructose-1,6-di-P (2) and aspartate (1), are able to bring about activation and inhibition, respectively (Fig. 6).

FIG. 6. Left, the effect of fructose-1,6-di-P (FDP) alone or in combination with polylysine on the activity of the enzyme. The buffer was Tris-HCl, pH 9.0. P-enolpyruvate and bicarbonate concentrations were 1.66 mM and 20 mM, respectively. In all assays 7 units of enzyme were used. Right, the inhibition of enzyme activity by l-aspartate in the presence of 1.3 µg per ml of polylysine.

FIG. 7. The activation of the enzyme by dioxane and ethanol. Substrate concentration and pH were the same as noted in the legend to Fig. 6. In all assays 22 units of enzyme were used. The activators and enzyme were added simultaneously to the assay medium.
assay mixtures in the presence of dioxane or the other reagents when malate dehydrogenase is omitted. The effect of dioxane on the enzyme is completely reversible. When P-enolpyruvate carboxylase is incubated at 4° with 10% dioxane or ethanol for 8 hours and subsequently tested after removal of the reagents by dilution, no modifications in enzyme activity are observed.

Dioxane has a pronounced effect on the $K_m$ of P-enolpyruvate. In the presence of 0% dioxane in the assay medium, the Michaelis constant (evaluated by the use of double reciprocal plots) is found to be 0.4 mM, a figure which is about 5 times the $K_m$ of P-enolpyruvate obtained in the absence of dioxane.

Since dioxane is a known “dissociating” agent for proteins, the question may be asked whether reversible activation of the enzyme is brought about by the dissociation of the enzyme subunits or some other conformational changes in its structure. In sucrose density gradients with 6% dioxane no significant changes in the sedimentation velocity of P-enolpyruvate carboxylase were detected (Fig. 3) other than those resulting from the density changes in the sucrose gradients caused by the addition of dioxane. It is, thus, likely that activation is not brought about by significant molecular weight changes of the enzyme. Since dioxane was found to activate the enzyme to the expected degree in the presence of 10% sucrose, it seems unlikely that sucrose may have altered the enzyme, masking an effect of dioxane.

However, some changes in the tertiary structure of the protein possibly occur in the presence of dioxane, as is demonstrated by the observation that the enzyme is completely “desensitized” to the effect of the feedback inhibitor aspartate (1) and the precursor activator fructose-1,6-di-P (2) in the presence of 10% dioxane. This can be seen for aspartate in Fig. 8. At a P-enolpyruvate concentration of 20 mM (2 times $K_m$) L-aspartate yields a $K_i$ value of 0.8 mM, but in the presence of dioxane, concentrations of aspartate 20 times $K_i$ fail to show any inhibition even though P-enolpyruvate concentration in this experiment is only 0.066 mM. Similarly, fructose-1,6-di-P tested at a concentration of 15 mM in the presence of dioxane fails to activate the enzyme. Under the same conditions, however, acetyl-CoA, another potent activator of P-enolpyruvate carboxylase (1), tested at a concentration of 0.20 mM, gives approximately a 2-fold increase in enzyme activity.

While nearly complete “desensitization” of the enzyme is brought about by dioxane, none of the other solvents described earlier show this effect. In all other solvents (ethanol, propylene glycol, dimethyl sulfoxide, each tested at a concentration of 10%) the enzyme is inhibited to the expected extent by aspartate.

**DISCUSSION**

On the basis of the admittedly qualitative results presented here, P-enolpyruvate carboxylase can be very simply pictured as a protein molecule which in its native tertiary configuration has accessible hydrophobic regions together with exposed areas with a preponderance of polar, negatively charged groups. As far as catalysis is concerned this native configuration, thermodynamically perhaps the most probable, does not seem to be the ideal one, because any change, whether it be brought about by Coulombic or hydrophobic interaction, or even possibly by a combination of both, leads to an activation of the enzyme as a consequence of a decrease in the Michaelis constant of the substrate. It is perhaps this peculiarity of its native configuration that makes P-enolpyruvate carboxylase an ideal enzyme for regulatory purposes and makes it susceptible to control by a variety of compounds, such as aspartate (1), fructose-1,6-di-P (2, 14), acetyl-CoA (1), and certain nucleoside polyphosphates (3).

The following points justify the aforementioned picture of the enzyme.

**Electrostatic Interactions in Activation of Enzyme**—It is clear from the results that the activation of P-enolpyruvate carboxylase is very likely brought about by nonspecific electrostatic interactions between negatively charged groups on the enzyme surface and positively charged amino groups on polylysine or copolylysylphenylalanine. In support of this contention is the finding that the degree of activation is determined by the lysine content of the homo and heteropolymers, much as with the inhibition of lipoprotein lipase by basic polyamino acids described by Korn (15). Further, the extent of activation by polylysine increases as the pH is decreased from 9.8 to 7.0 (Fig. 4) which may, in part, be due to the protonation of an increasing number of e-amino groups of the lysine residues (16, 17). Finally, the activation of the enzyme by polylysine is decreased with an increase in the ionic strength of the assay medium (Fig. 5), which is very likely due to the reduction of electrostatic interactions as a result of the screening of the charged groups by the stabilizing counterions.

Although a considerable number of enzymes are known to be either inhibited or activated by polyamino acids (for a review, see Reference 18), the actual molecular mechanisms whereby modifications in activity are brought about are uncertain (18). However, as much as the activation of the enzyme by polylysine must, of necessity, be considered to be due to the binding of the polyamino acid at areas distinct from the substrate-binding groups, there is a possibility that the charge interactions cause an alteration in the geometry of the catalytic site, possibly as a
Hydrophobic Interactions in Activation and Desensitization of Enzyme—In the interpretation of the results with nonpolar solvents as activators, some problems connected with the mode of action of these solvents on proteins arise. In general, nonpolar solvents are expected to affect protein configuration not only by weakening the hydrophobic bonds (14), but also by altering the ionic interactions in the protein molecule by increasing the pK values of the ionizing groups (19, 20). However, the fact that the nonpolar solvents tested here have very different dielectric constants but are nearly equally effective in activating P-enolpyruvate carboxylase may mean that it is primarily the weakening of the hydrophobic interactions that leads to the activation of the enzyme. This is also supported by the observation that concentrations of dioxane and ethanol as low as 2% (when contribution to the dielectric of the assay medium may not be significant) lead to significant increases in the activity for the accessibility of the regions of hydrophobic interactions in the enzyme. Connected with this also may be the observation made earlier (1) that P-enolpyruvate carboxylase is indefinitely stable at 4° in the presence of 0.1 M ammonium sulfate, but loses its activity in its absence. As an electrolyte, ammonium sulfate is expected to strengthen hydrophobic bonding and thus preserve the native configuration of the enzyme.

It has been shown earlier that while all organic solvents used here share with dioxane the property of being able to activate P-enolpyruvate carboxylase, it is only in the presence of dioxane that the enzyme also becomes desensitized (4) to the action of the allosteric effectors, aspartate (Fig. 8) and fructose-1,6-di-P. Since dioxane has a very low dielectric constant compared with the other solvents, it is tempting to suggest that it is this extreme nonpolarity which is responsible for desensitization. This could conceivably happen either by the change in pK values of ionizable groups mentioned earlier, or, more likely (if the exposed negatively charged groups on the surface of the enzyme were identified with carboxylate ions), by the withdrawal of the polar, anionic groups away from the nonpolar environment with a concomitant change in the configuration of the enzyme. In this altered configuration the allosteric binding site (or sites) is possibly not accessible to the effectors. That a direct influence of dioxane on the pK values of the effectors is perhaps not responsible for desensitization is borne out by the simultaneous loss of effect for two compounds, the pK values of which are very different from one another. Further, that desensitization is not likely due to dissociation of the enzyme is shown by the results of ultracentrifugation presented earlier.

Certain other important conclusions may also be drawn from the experiments reported here. Our contention earlier (2) that acetyl-CoA and fructose-1,6-di-P occupy different sites on the enzyme seems to be strengthened by the demonstration here that under conditions in which fructose-1,6-di-P is ineffective in the modulation of enzyme activity, acetyl-CoA is still capable of activating the enzyme. The fact that the enzyme in the dioxane-induced configuration is simultaneously desensitized to aspartate and fructose-1,6-di-P may mean that both of these effectors share a common site.

REFERENCES

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