BBA 66476

# STUDIES ON HEART PHOSPHOFRUCTOKINASE

# THE EFFECT OF PYRIDOXAL 5'-PHOSPHATE ON ENZYME ACTIVITY AND DISSOCIATION

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(Received July 20th, 1971)

## SUMMARY

Reaction of pyridoxal 5'-phosphate with ε-amino groups of lysine residues in sheep heart phosphofructokinase causes a loss in the catalytic activity of the enzyme. Inactivation is slowed by the presence of Fru-6-*P*. Maximum inactivation occurs when 4 moles of pyridoxal 5'-phosphate are bound per 100 000 g of enzyme. Reaction with pyridoxal 5'-phosphate in the absence of Fru-6-*P* causes the enzyme to dissociate at 0°. Warming the dissociated enzyme to room temperature causes it to reassociate. Enzyme with a small amount of bound pyridoxal 5'-phosphate (< 1 mole per 100 000 g) is more sensitive to allosteric inhibition by ATP than native enzyme. This is associated with a shift in the acidic portion of the pH-activity curve of the enzyme to higher pH's.

## INTRODUCTION

Chemical modification of phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase) (2.7.1.11) has demonstrated the importance of sulfhydryl and histidine groups in the allosteric control and catalytic activity of the enzyme[1-3]. The purpose of this study was to investigate the effects of modification of lysine residues by pyridoxal 5'-phosphate on enzyme activity and structure. UYEDA[4] recently reported on the effects of lysine reagents on phosphofructokinase from skeletal muscle. The results reported here for the sheep heart enzyme emphasize the importance of lysine residues for the quaternary structure and activity of phosphofructokinase.

## METHODS

Phosphofructokinase from sheep heart was purified and crystallized as described before[5,6]. The solubilized crystals were freed of nucleotides by treatment with acid-washed charcoal[6]. The enzyme solution was incubated at 0° in the dark with the desired concentration of pyridoxal 5'-phosphate in a solution containing 10 mM

imidazole (pH 7.8), 1 mM EDTA, 1 mM dithioerythritol and, where indicated, a concentration of Fru-6-*P* equimolar to that of the pyridoxal 5'-phosphate. At the desired time (usually 20–90 min) freshly prepared $NaBH_4$ was added to the incubation mixture in 2-fold excess over the pyridoxal 5'-phosphate. The reduction was allowed to proceed for 10 min at 0° in the dark. The reduced phosphopyridoxal enzyme solution was then dialyzed at 4° against a 500-fold excess of a solution containing 50 mM potassium phosphate buffer (pH 8.0), 1 mM mercaptoethanol, 1 mM EDTA and 0.01 mM Fru-1,6-$P_2$. Fru-1,6-$P_2$ was present as an enzyme stabilizer. The dialysate was changed 2–3 times over a period of 15–18 h. A control enzyme sample was treated in an identical fashion except in the absence of pyridoxal 5'-phosphate. The amount of phosphopyridoxal lysine on the enzyme was determined from the difference in the absorbance at 322 nm between the phosphopyridoxal and control enzyme samples using a molar extinction coefficient of 9000 (ref. 7). Protein concentrations were determined by the method of LOWRY *et al.*[8].

The procedure of MARTIN AND AMES[9] for determining the sedimentation coefficient of proteins by sucrose gradient centrifugation was used. Further details for its use with phosphofructokinase were reported previously[10]. Crystalline aldolase (EC 4.1.2.6) from rabbit skeletal muscle which has a sedimentation coefficient of 7.35 S was used as a marker.

Enzyme activity at pH 8.2 or at pH 6.9 was determined spectrophotometrically as described before[5].

Sources of materials used in this investigation and not reported previously[5,6] were: pyridoxal 5'-phosphate, Calbiochem.; and $NaBH_3$, Sigma.

## RESULTS

### *Inhibition of enzyme activity by pyridoxal 5'-phosphate and protection by various ligands*

When phosphofructokinase was incubated with 1 mM pyridoxal 5-phosphate there was a progressive loss of catalytic activity. Spectrophotometric analysis of the modified enzyme showed an absorption maximum at 280, 340 and 435 nm and a minimum at 377 nm confirming the formation of Shiff base by the interaction of the

TABLE I

EFFECT OF VARIOUS LIGANDS ON PYRIDOXAL 5'-PHOSPHATE INACTIVATION OF PHOSPHOFRUCTOKINASE

Enzyme (1 mg/ml) was incubated at 0° with the indicated compounds under the conditions described under METHODS. After 100 min each sample was assayed for catalytic activity at pH 8.2. Initial activity was determined before enzyme incubation and was approximately 130 units/mg. None of the ligands significantly affected enzyme activity when incubated alone with the enzyme.

| *Additions* | *% Initial activity* |
|---|---|
| None | 97 |
| 1 mM pyridoxal 5'-phosphate | 11 |
| 1 mM pyridoxal 5'-phosphate *plus* 0.01 mM Fru-1,6 $P_2$ | 65 |
| 1 mM pyridoxal 5'-phosphate *plus* 1 mM Fru-6-*P* | 88 |
| 1 mM pyridoxal 5'-phosphate *plus* 0.1 mM ATP | 54 |
| 1 mM pyridoxal 5'-phosphate *plus* 1 mM AMP | 13 |
| 1 mM pyridoxal 5'-phosphate *plus* 1 mM citrate | 20 |
| 1 mM pyridoxal 5'-phosphate *plus* 50 mM potassium phosphate | 73 |

$\varepsilon$-amino groups of lysine residues in the protein and pyridoxal 5'-phosphate[11]. Inhibition of enzyme activity after such treatment was found to be reversible. For example, incubation of the enzyme with pyridoxal 5'-phosphate for 90 min resulted in the loss of 86% of enzyme activity. This was completely regained following exhoustive dialysis. When the phosphopyridoxal enzyme was reduced with $NaBH_4$ it gave an absorption maximum at 322 nm which is characteristic for pyridoxal phosphate covalently linked with lysine. Under these conditions loss of activity is not reversible.

Table I summarizes the effects of various ligands on the inactivation of phosphofructokinase by pyridoxal 5'-phosphate. The substrate Fru-6-*P* (1 mM) affords the best protection against activity loss. Fru-1,6-$P_2$ at a concentration of 0.01 mM protects the enzyme to a significant degree. Other experiments not summarized here, show that the hexose diphosphate is as effective as Fru-6-*P* at a concentration of 1 mM. The substrate ATP at a concentration of 0.1 mM also offers some protection against inactivation by pyridoxal 5'-phosphate. This protection was not increased when the ATP concentration was raised to 1 mM. The activator AMP and the inhibitor citrate do not protect against activity loss. At high concentrations, potassium phosphate is an effective protector.

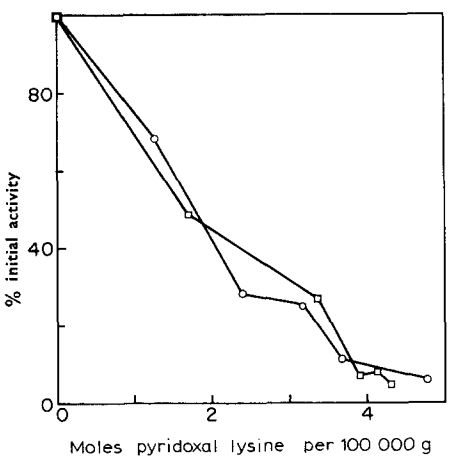


Fig. 1. Inactivation of phosphofructokinase by pyridoxal 5'-phosphate. Enzyme crystals were solubilized and incubated in the absence (○) and in the presence (□) of 1 mM Fru-6-*P* with 1 mM pyridoxal 5'-phosphate as described under METHODS. Aliquots were withdrawn from the sample incubated in the absence of Fru-6-*P* at 0, 5, 10, 15, 30 and 60 min and at 0, 15, 30, 60, 120 and 180 min from the sample incubated in the presence of Fru-6-*P* and were reduced with $NaBH_4$ and dialyzed. The number of phosphopyridoxal groups on the enzyme was determined as described under METHODS.

The protection by Fru-6-*P* against inactivation was found to be due to the fact that the hexose phosphate slowed the rate of reaction of pyridoxal 5'-phosphate with the enzyme. Fig. 1 shows that the amount of catalytic activity remaining is a function of the amount of pyridoxal 5'-phosphate linked to the enzyme and is independent of the presence of the protective agent, Fru-6-*P*. The figure also shows that 90 to 95% of the activity is lost when 4 moles of pyridoxal 5'-phosphate are covalently bound.

*Sedimentation properties of phosphopyridoxal phosphofructokinase*

Phosphofructokinase when incubated with pyridoxal 5'-phosphate at 0° and pH 8.0 had a sedimentation coefficient of 7.5 S, whereas that of the native enzyme was 11–13 S. However, enzyme treated with pyridoxal 5'-phosphate in the presence of Fru-6-*P* had a sedimentation coefficient of 11–13 S (Table II). Dissociation of the enzyme to 7.5 S was accompanied by a decrease in enzyme activity to 15% of initial activity. The presence of Fru-6-*P* protected the enzyme from such loss.

The sedimentation coefficients of the covalently modified samples when determined at 3° were identical to those prepared before reduction with $NaBH_4$ (Table II).

TABLE II

EFFECT OF PYRIDOXAL 5'-PHOSPHATE ON DISSOCIATION OF PHOSPHOFRUCTOKINASE

Enzyme was incubated at 0° with no additions in A; 1 mM pyridoxal 5'-phosphate in B and 1 mM pyridoxal 5'-phosphate *plus* 1 mM Fru-6-*P* in C. Aliquots of each incubation mixture were then layered on sucrose gradients with additions identical to those in the appropriate incubation mixtures. Sedimentation coefficients were determined at 0° in Expt. I. The remaining incubation mixtures were reduced with $NaBH_4$ and dialyzed as described under METHODS and the amount of pyridoxal phosphate on the enzyme was determined spectrophotometrically. Aliquots of these samples were layered on sucrose gradients and sedimentation coefficients were determined at 0° (Expt. II). The remaining reduced enzyme samples were incubated at room temperature overnight and were layered on sucrose gradients for determination of their sedimentation coefficient at 20° (Expt. III).

| *Experiment* | *Incubation mixtures* | *Moles pyridoxal 5'-phosphate per 100 000 g* | *Sedimentation coefficient (S)* |
|---|---|---|---|
| I | A | | 11–13 |
| | B | | 7.5 |
| | C | | 11–13 |
| II | A | 0 | 11–13 |
| | B | 3.5 | 7.5 |
| | C | 2.3 | 13 |
| III | A | 0 | 11–13 |
| | B | 3.5 | 11.5 |
| | C | 2.3 | 12.2 |

Enzyme prepared in the presence of Fru-6-*P* bound less pyridoxal 5'-phosphate (Table II) and lost less catalytic activity than enzyme prepared in the absence of Fru-6-*P*. Table II (Expt. III) shows the effects of incubating at room temperature the phosphopyridoxal enzyme samples which had been reduced with $NaBH_4$ and then centrifuged on sucrose gradients at 20°. Warming the enzyme prepared in the absence of Fru-6-*P* caused it to reassociate to a form which has a sedimentation coefficient similar to that of native enzyme. Reassociation of the enzyme by warming did not result in regaining of catalytic activity. Addition of ATP, Fru-1,6-$P_2$, cyclic 3',5'-AMP, mercaptoethanol or various combinations of these compounds to the enzyme during warming did not result in any significant increase in catalytic activity. These results illustrate that the loss of catalytic activity is due to binding of pyridoxal 5'-phosphate and not to the dissociation of the enzyme.

*Effects of reaction of enzyme with pyridoxal 5'-phosphate on enzyme kinetics*

The kinetics of phosphopyridoxal phosphofructokinase were investigated at pH 8.2 using enzyme which retained at least 50% of its catalytic activity. The modified enzyme at pH 8.2 showed a decrease in $v_{max}$ but there was no effect on the apparent $K_m$'s for either ATP or Fru-6-*P*. The sensitivity of phosphopyridoxal phosphofructokinase to ATP inhibition at pH 6.9 was tested. These experiments were carried out with enzyme which had 0.5–1 mole of pyridoxal 5'-phosphate covalently bound per 100 000 g. Fig. 2 shows that this form of the enzyme was more sensitive to ATP inhibition than the native enzyme. The apparent $K_i$ for ATP for the native enzyme (or for control enzyme incubated and reduced in the absence of pyridoxal 5'-phosphate) was about 0.6 mM, whereas the apparent $K_i$ for ATP for the phosphopyridoxal enzyme was about 0.12 mM. The addition of cyclic 3',5'-AMP to the ATP-

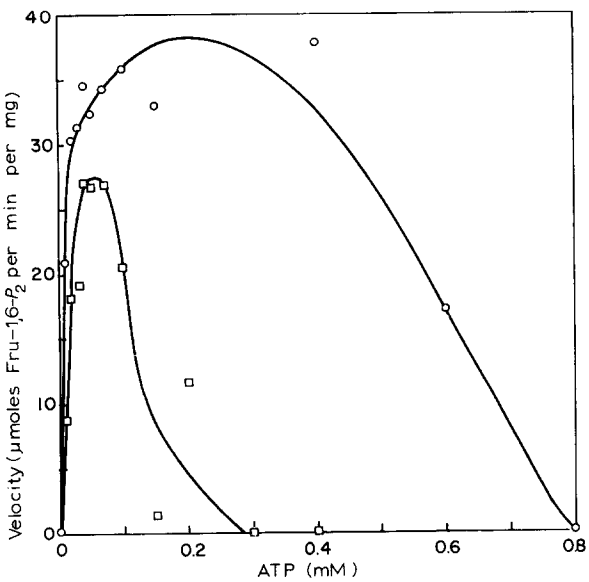


Fig. 2. Effect of pyridoxal 5'-phosphate treatment on the kinetics of ATP inhibition of phosphofructokinase. Enzyme containing 0.8 mole of phosphopyridoxal lysine per 100 000 g was prepared in the presence of Fru-6-*P* as described under METHODS. The catalytic activities of this enzyme (□) and of native enzyme (○) were compared at pH 6.9 at increasing ATP concentrations. The Fru-6-*P* concentration was 0.2 mM. The activity of the phosphopyridoxal enzyme at pH 8.2 was 15% lower than that of the native enzyme.

inhibited phosphopyridoxal enzyme resulted in activation of the enzyme similar to that observed with native enzyme. However, cyclic 3',5'-AMP did not activate the enzyme to a level above that obtained at lower ATP concentrations. In other words, cyclic 3',5'-AMP did not restore activity lost as a result of binding of pyridoxal 5'-phosphate to the enzyme. An investigation of the pH-activity relationships of native and phosphopyridoxal enzyme revealed that the enhanced sensitivity to ATP inhibition was correlated with a shift in the acidic portion of the pH activity curve to higher pH's (Fig. 3). This property of the phosphopyridoxal enzyme could account for its enhanced sensitivity to ATP inhibition when compared to native enzyme.

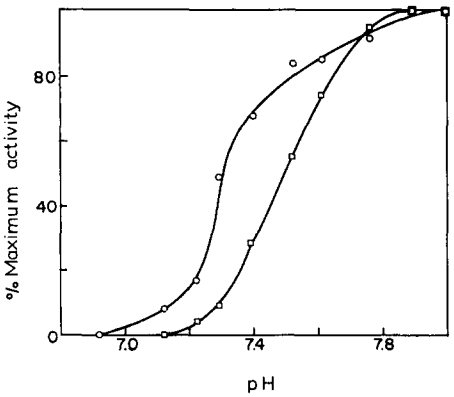


Fig. 3. Effect of pyridoxal 5′-phosphate treatment on the pH-activity relationship of phosphofructokinase. Enzyme containing 0.8 mole of phosphopyridoxal lysine per 100 000 g was prepared in the presence of Fru-6-*P* as described under METHODS. The catalytic activities of enzyme prepared in the absence of pyridoxal 5′-phosphate (○) were compared with those of the phosphopyridoxal enzyme (□) at several pH's between 6.9 and 8.0. Buffers for the assays were 50 mM maleate adjusted to the desired pH with Tris base. Substrate concentrations were: ATP, 0.5 mM; Fru-6-*P*, 1 mM.

## DISCUSSION

The data presented here provide evidence that lysine residues are important in the catalytic activity of the enzyme. This is supported by the inactivation of the enzyme upon binding of pyridoxal 5′-phosphate and by the protection by Fru-6-*P* against this inactivation. In agreement with a report by UYEDA[4] on the skeletal muscle enzyme, we found that virtually complete inactivation occurs when about 4 moles of pyridoxal 5′-phosphate per 100 000 g are covalently linked to the enzyme. The data showing that the degree of inactivation of the enzyme is dependent on the amount of pyridoxal 5′-phosphate bound and not upon the presence of Fru-6-*P* or upon the state of aggregation of the enzyme suggests that the lysine residues may be at the catalytic site, presumably at that portion involved in Fru-6-*P* binding.

The data on the sedimentation of phosphopyridoxal phosphofructokinase show that enzyme containing only 3.5 modified lysine residues can dissociate to a 7-S molecular form at 0° and reassociate at 20° to the size of the native enzyme. Dissociation of the skeletal muscle enzyme did not occur until about 8 moles of lysine per 100 000 g were modified[4]. However, these measurements were made at 20° which could explain the resistance of the skeletal enzyme to dissociation. Native phosphofructokinase is known to dissociate to a form with a sedimentation coefficient of 7.0 at pH 6.5 with a concomitant loss of activity[10]. Raising the pH to 8 causes reassociation and also restores much of the catalytic activity. This is different from the cold-induced dissociation of phosphopyridoxal phosphofructokinase which occurs at pH 8. In this case reassociation does not result in a significant restoration of catalytic activity.

The reason for the enhanced sensitivity of the enzyme to allosteric inhibition by ATP at pH 6.9 when about 1 mole of pryidoxal 5′-phosphate is bound is not clear. A possible explanation is that binding of 1 mole of pyridoxal 5′-phosphate may cause a conformational change in the molecule which enhances its sensitivity to ATP inhibi-

tion. Preliminary attempts to test this possibility were made by comparing the rates of reaction of native and phosphopyridoxal phosphofructokinase with two group-specific reagents. Ethoxyformic anhydride has been shown to react specifically with histidine residues in phosphofructokinase[3] and 5,5′-dithiobis(2-nitrobenzoic acid) reacts only with sulfhydryl groups[1]. However, there were no differences in the rates of reaction of either reagent with the two enzyme samples. The enhanced sensitivity of phosphopyridoxal phosphofructokinase to ATP inhibition seems to be correlated with a shift in the pH–activity curves of the enzyme to higher pH's.

ACKNOWLEDGEMENTS

This investigation was supported by Public Health Service Research Grant AI04214 from the National Institute of Allergy and Infectious Diseases and a grant-in-aid from the American Heart Association. B.S. was the recipient of a U.S. Public Health Service postdoctoral fellowship.

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