Specific Modification of an Effector Binding Site of Phosphofructokinase by Pyridoxal Phosphate†

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ABSTRACT: Conditions are described for the covalent modification of rabbit skeletal muscle phosphofructokinase by pyridoxal phosphate plus sodium borohydride to produce an enzyme that appears by a number of criteria to be modified at the citrate binding site. Evidence that modification occurs at this site is as follows. (1) Protection against activity loss due to modification is provided by the combination of MgATP and citrate, whereas neither low concentrations of citrate nor MgATP alone is effective. This is consistent with the increased affinity for citrate that is observed in the presence of MgATP. (2) The extensive changes in activity and equilibrium binding result from the incorporation of only 1 mol of pyridoxal phosphate per mol of protomer. (3) Modification greatly increases sensitivity to MgATP inhibition, an effect consistent with the known synergism between MgATP and citrate. (4) The affinity of the enzyme for both MgATP and MgITP at the catalytic site is increased by the modification. (5) The sensitivity of the enzyme to citrate inhibition is greatly diminished following covalent modification. (6) Modification abolishes the equilibrium binding of citrate. (7) Enhanced binding of MgATP is observed following modification, a result consistent with the enhancement of MgATP binding by citrate. Phosphofructokinase protected by citrate plus MgATP can also be modified by the incorporation of 1 or more mol of pyridoxal phosphate, but the enzyme so produced is capable of interacting with citrate and shows none of the properties herein described for the enzyme modified in the absence of citrate.

In two earlier studies (Uyeda, 1969; Setlow and Mansour, 1972), it was shown that modification of lysine residues of phosphofructokinase with pyridoxal phosphate in the presence of sodium borohydride led to an extensive loss of enzyme activity. Various effectors gave partial protection against this loss of activity. Setlow and Mansour (1972) noted that, with very limited incorporation of pyridoxal phosphate (less than 1 mol per 100 000 g), an enzyme with increased sensitivity to inhibition by ATP was produced. We have noted that the sensitivity to ATP inhibition is greatly increased in the presence of citrate, P-enolpyruvate, and 3-P-glycerate, all of which are competitive for the same site on the enzyme (Colombo et al., 1975). This suggests the presence of a binding site consisting of several positively charged groups. Benesch et al. (1972) found that pyridoxal phosphate binds rather specifically at the 2,3-P-glycerate site of hemoglobin. This suggested to us that, if the P-glycerate (citrate) site of phosphofructokinase contained at least one amino group among the charged groups, then pyridoxal phosphate may, under appropriate conditions, modify this site and thereby produce an enzyme locked in an ATP-sensitive conformation. The present communication describes the covalent modification of phosphofructokinase that produces an enzyme whose properties are consistent by a number of criteria with the presence of a P-pyridoxyl residue at the citrate interaction site.

Experimental Procedure

Enzymes. Aldolase, glycerol-3-P dehydrogenase, and triosephosphate isomerase were purchased from Sigma Chemical Company. Preceding their use in the coupled assay for phosphofructokinase, these enzymes were dialyzed for 24 h against 25 mM sodium glycero-P, 25 mM glycylglycine, 1 mM EDTA, all at pH 7.0, to remove ammonium sulfate. Phosphofructokinase was prepared from frozen rabbit skeletal muscle (Pel Freez, Rogers, Arkansas) by the procedure of Kemp (1975) and was crystallized three times. Crystals of phosphofructokinase for an experiment were collected by centrifugation and dissolved in a buffer (pH 7.0) consisting of 25 mM sodium glycero-P, 25 mM glycylglycine, 1 mM EDTA, 0.1 mM ATP, and 0.1 mM dithiothreitol. Material that did not dissolve within 30 min was removed by centrifugation. The supernatant was dialyzed overnight at 4 °C against a medium at pH 7.0 consisting of 50 mM Tes[1], 0.1 mM EDTA, 0.1 mM ATP, and 1 mM dithiothreitol. When it was necessary to remove ATP, the dialyzed enzyme solution was passed through a 20 × 4 mm column containing a mixture of one part of acid-washed charcoal and one part of coarse powdered cellulose. A 280:260 nm ratio of above 1.6 indicated that ATP had been removed from the enzyme (Parmeggiani et al., 1966).

Kinetic Analysis. Kinetic analyses were performed on a Gilford spectrophotometer at 30 °C and at the indicated pH in 3 ml of a medium containing 50 mM Tes, 0.1 mM dithiothreitol, 0.15 M KCl, 1 mM EDTA, 0.6 unit of aldolase, 0.3 unit of glycero-P dehydrogenase, 0.3 unit of triosephosphate isomerase, and 0.2 mM NADH. The concentrations of ATP or ITP, fructose-6-P, and other effectors were as indicated in the Results. A concentration of $MgCl_2$ was employed that was 5 mM in excess of the concentration of nucleoside triphosphate. Phosphofructokinase was diluted to the desired concentration in 25 mM sodium glycero-P, 25 mM glycylglycine, 1 mM EDTA, 0.1 dithiothreitol, 0.1 mM ATP, all at pH 7.0, and was added to the incubation in a volume of 50 μl. Reactions were started by the addition of fructose-6-P.

Equilibrium Binding Studies. Binding studies were performed by the technique of Hummel and Dreyer (1962) using the conditions described by Kemp and Krebs (1967). Briefly, a 1.5-cm column was packed to the height of 20 cm with Sephadex G-50 Fine (Pharmacia) and was equilibrated with a buffered solution at pH 6.95 consisting of 25 mM glyclyglycine, 25 mM sodium glycero-P, 1 mM EDTA, 0.1 mM di-

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[1] Abbreviations used are: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; cAMP, cyclic adenosine 3′,5′-monophosphate.

thiothreitol, and [1,5-^{14}C]citric acid or [γ-^{32}P]ATP (New England Nuclear) at the desired concentration and other additions as indicated. Native or modified phosphofructokinase in 1.0 ml of the solution used to equilibrate the column was applied to the top of the column and was allowed to enter the gel. Elution was carried out with the same solution and fractions of 0.8–1.0 ml were collected. Flow rates of 15–20 ml/h were used and the columns were run at ambient room temperature (22–24 °C). One-tenth milliliter of each fraction was counted in a scintillation spectrometer. The validity of each determination was checked by demonstrating complete recovery of protein in the void volume, by the attainment of equilibrium as indicated by the return of the baseline concentration of the citrate to its initial value after the emergence of the protein, and by the agreement between the total areas of the peak and trough in the elution profile. In several experiments, assays of activity in the eluted protein fractions indicated no activity losses during the chromatography.

Incorporation of Pyridoxal Phosphate. Prior to the addition of pyridoxal phosphate, the enzyme, 2.5–3 mg in 1 ml, was incubated in an ice bath for 10 min in a pH 7.0 medium containing 50 mM Tes, 0.1 mM EDTA, 1 mM dithiothreitol, and the other additions indicated in the Results. Pyridoxal phosphate was added in a volume of 50 μl at a concentration necessary to achieve the final concentration indicated in the Results and the reaction was allowed to proceed for 5 min in the ice bath. At that time, 50 μl of octanol was added, followed immediately with 50 μl of freshly prepared aqueous solution of sodium borohydride (10 mg/ml). The reaction mixture was transferred to dialysis tubing and the solution was dialyzed overnight at 4 °C against a medium at pH 7.0 consisting of 50 mM Tes, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM ATP. The enzyme was then passed through a column of Sephadex G-50 Fine (1.6 × 16 cm) previously equilibrated at room temperature with the dialysis medium. Fractions of 0.5–0.7 ml were collected and the absorbancy was measured at 280 and 325 nm. On the basis of the earlier studies of pyridoxylation of phosphofructokinase (Uyeda, 1969; Setlow and Mansour, 1972) and of the spectra of the modified enzyme, it was assumed that incorporation occurred at ϵ-amino groups of lysine residues. The amino termini of muscle phosphofructokinase are known to be blocked by acetyl residues (Younathan et al., 1973). The number of phosphopyridoxylamino groups were estimated from the absorbance at 325 nm using the millimolar extinction coefficient of 10.15 for N^{ϵ}-(phosphopyridoxyl)lysine (Fischer et al., 1963). All data are expressed relative to a protomer molecular weight of 90 000. This value is based on the binding unit for fructose-6-P, cyclic 3′,5′-AMP, and citrate (Kemp and Krebs, 1967; Colombo et al., 1975) and of the minimal molecular weight from thiol reactivity studies (Kemp and Forest, 1968).

Results

Protection of Phosphofructokinase by Various Ligands. Setlow and Mansour noted that fructose-6-P, fructose-1,6-P_2, ATP, and inorganic phosphate all provide partial protection against the inactivation of the enzyme by pyridoxal phosphate at pH 7.8. Because we were interested in exploiting potential effector interactions that are only seen near neutral pH (Kemp, 1969a), the effect of various metabolites on the modification by pyridoxal phosphate plus borohydride was evaluated at pH 7.0. In a number of experiments, partial protection against loss of activity measured at pH 8.2 was provided by citrate (10 mM), fructose-1,6-P_2, fructose-6-P, and high concentrations of inorganic phosphate. Very little protection was provided by 1 mM AMP, 0.1 mM ATP, low concentrations of citrate (1 mM), or by low concentrations of MgATP. Some of these experiments are presented in Table I. The most interesting observation is that, whereas little protection was provided by low concentrations of either citrate or MgATP alone, complete protection from loss of activity was achieved by the two ligands together. We have shown in equilibrium binding studies (Colombo et al., 1975) that phosphofructokinase binds citrate very poorly in the absence of MgATP, but in the presence of the metal–nucleotide complex the affinity for citrate is high (K_d = 20 μM). If indeed pyridoxal phosphate is binding at the citrate site, then one would predict that the interaction would be more efficient in the presence of MgATP. For this reason in all subsequent studies, $MgCl_2$ at 1 mM and ATP at 20 μM were present during the modification with pyridoxal phosphate and sodium borohydride.

Table I: Effect of Metabolites on the Modification of Phosphofructokinase by Pyridoxal Phosphate.[a]

Pyridoxal Phosphate (mM)	Addition (mM)	Mol Incorporated Per mol of protomer	Relative Activity
			100
0.5	Mg^{2+} (7)	0.76	56
0.5	Mg^{2+} (7), ATP (0.05)	0.76	50
0.5	Citrate (5)	0.86	81
0.5	Mg^{2+} (1), ATP (0.02), citrate (1)	0.65	103
0.5	Mg^{2+} (7), ATP (0.05), fructose-1,6-P_2 (0.1)	0.45	81
1.0		1.05	29
1.0	Mg^{2+} (1), ATP (0.02)	1.02	28
1.0	Citrate (1)	0.97	66
1.0	Mg^{2+} (1), ATP (0.02), citrate (1)	0.88	100
1.0	Fructose-6-P (1)	0.53	73

[a] Enzyme (2.5 mg/ml) was incubated at pH 7.0 in the presence of pyridoxal phosphate and the indicated additions for 5 min at 4 °C with other conditions described in the Experimental Procedure. Following $NaBH_4$ reduction, dialysis, and gel filtration, the enzyme was analyzed for P-pyridoxyllysine and for catalytic activity at pH 8.2 in the presence of 1 mM ATP and 1 mM fructose-6-P. In several instances samples were removed and assayed prior to the dialysis and gel filtration steps to determine whether losses occurred during the desalting. In each case, recovery was greater than 90%.

The dependence of inactivation upon pyridoxal phosphate concentration was examined and is described in Figure 1. Both the loss of activity and the modification of lysine residues occurred in a biphasic manner when the enzyme was modified in the absence of citrate as protective ligand. In the presence of 1 mM pyridoxal-P, 1 mol was incorporated per mol of protomer and approximately 70% of the activity at pH 8.2 was lost. Incorporation of a second mole led to only a slight further reduction in activity. The protection by citrate against loss of activity was almost complete and even with the formation of 2 mol of P-pyridoxyllysine only a slight decrease in activity at pH 8.2 was seen. Enzyme that was modified in the presence of MgATP will subsequently be referred to as "modified enzyme" whereas that produced in the presence of MgATP plus citrate will be called "modified–protected enzyme". Native phosphofructokinase will refer to enzyme that was incubated

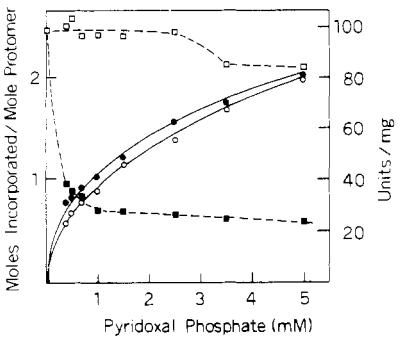

FIGURE 1: Pyridoxylation of phosphofructokinase. Phosphofructokinase (2.5 mg in 1 ml) was treated with the given concentrations of pyridoxal phosphate, reduced with sodium borohydride, dialyzed, and passed through Sephadex G-50, all as described in the Experimental Procedure. The data given by the closed symbols (●, ■) were obtained with enzyme treated with added $MgCl_2$ (1 mM) and ATP (20 μM). The data given by open symbols (○, □) were obtained in the presence of 1 mM $MgCl_2$, 20 μM ATP, and 1 mM sodium citrate. Incorporation of pyridoxal phosphate is given by the circle (○, ●). Enzymic activity (□, ■) was determined at pH 8.2 in the presence of 1 mM ATP and 1 mM fructose-6-P as described in the text.

in the absence of pyridoxal phosphate but was carried through the borohydride reduction, dialysis, and gel filtration steps.

Regulatory Properties of the Modified Enzyme. The pH dependence of regulatory interactions of effector ligands with muscle phosphofructokinase is well known. Whereas positive or negative effector interactions are negligible above pH 7.5, below this pH inhibition by ATP increases as the pH decreases (Hofer and Pette, 1968; Kemp, 1969b). Maximal activity at pH 7.0 is achieved in the presence of 1 mM ATP and 1 mM fructose-6-P and is equal to about 50% of the activity at pH 8.2 (Kemp, 1971; Colombo et al., 1975). With ATP concentrations higher than about 1.5 mM at pH 7.0, native phosphofructokinase is inhibited (Colombo et al., 1975). Similarly, it has been previously shown that, because the actions of ATP and fructose-6-P oppose one another, the enzyme is inhibited at pH 7.0 and 1 mM ATP if fructose-6-P is present at concentrations less than 1 mM. In contrast to these known properties of native phosphofructokinase, the modified enzyme showed no activity at pH 7.0 and 1 mM ATP and 1 mM fructose-6-P. Only if the ATP concentration was considerably lowered did the modified enzyme have any significant activity. Even at ATP concentrations as low as 0.1 mM the modified enzyme had only 15% of the activity of the native enzyme in the presence of 1 mM fructose-6-P. Decreasing the ATP concentration further will decrease the activity of both the native and modified enzyme because the catalytic ATP site of the native enzyme is half-saturated in the region of 0.05 mM. Table II describes the results obtained at 0.1 mM ATP along with the effect of other variations in the assay conditions. Other experiments not shown employing native enzyme at this concentration of ATP showed that a velocity vs. fructose-6-P plot was sigmoid only at fructose-6-P concentration below 0.1 mM. The activity of native enzyme was maximal above 0.5 mM under these conditions and no increase in activity could be brought about by an increase in the concentration of fructose-6-P from 1 to 6 mM or by the addition of activators such as cAMP (Table II). Modified-protected enzyme (Table II) showed nearly the same activity as the native enzyme and only a very modest increase in activity was accomplished by increasing the concentration of fructose-6-P. On the other hand, enzyme modified by pyridoxal phosphate in the absence of citrate had considerably lower activity compared with protected enzyme. At 0.4 mM fructose-6-P, the modified enzyme had no activity and, as

Table II: Effect of Activators on Native and Modified Phosphofructokinase.[a]

Enzyme	Fructose-6-P (mM)	Additions	% Activity
Native	1		100
Native	6		100
Native	1	cAMP	100
Modified	1		15
Modified	1	AMP	52
Modified	1	cAMP	56
Modified	6		64
Modified	6	cAMP	76
Modified-protected	1		84
Modified-protected	6		100

[a] The preparation of the enzyme is described in Figure 2. Modified enzyme refers to phosphofructokinase modified in the presence of 0.4 mM pyridoxal phosphate, which led to incorporation of 0.78 mol per mol of protomer. Modified-protected enzyme refers to enzyme modified in the presence of 1 mM citrate and 0.7 mM pyridoxal phosphate, which led to the incorporation of 0.77 mol per mol of protomer. The enzyme was assayed at pH 7.0 in the presence of 0.1 mM ATP, the indicated concentration of fructose-6-P, and other conditions indicated in the Experimental Procedure. cAMP when added was present at 33 μM and AMP at 200 μM.

shown in Table II, at 1 mM fructose-6-P the enzyme was not saturated and was capable of being activated by the addition of adenine nucleotides. cAMP increased the activity more than threefold, and in the presence of both cAMP and 6 mM fructose-6-P the activity of the modified enzyme was only 25% less than that of native enzyme. These results are consistent with the idea that the modified enzyme is exceedingly sensitive to ATP inhibition to such a degree that inhibition occurs before the enzyme can be fully saturated with ATP at the catalytic site. It would be of interest to know, if in the absence of interaction at the inhibitory site, whether or not the native and modified enzymes have the same V_{max}. This, one might think, could be estimated from extrapolations of the data obtained at low concentrations of ATP. Unfortunately the nonlinear behavior of the enzyme at low ATP concentrations (see Figure 3) makes such extrapolations impossible.

Even with ITP as substrate, the modified enzyme showed inhibition at high concentrations of this phosphoryl donor. Native phosphofructokinase is not inhibited by ITP concentrations as high as 15 mM because of relatively weak interactions at the inhibitory site (Uyeda and Racker, 1965; Mathias and Kemp, 1972; Colombo et al., 1975). Modified phosphofructokinase (1.3 mol/mol) assayed at pH 7.0 and the presence of 1 mM fructose-6-P displayed maximal activity near 1 mM ITP and was inhibited by 50% at about 4 mM (Figure 2). The activity at 1 mM ITP could be doubled by the addition of cAMP whereas no increases in the activity of native enzyme can be observed by the addition of cAMP under these conditions. The foregoing results indicate an extreme sensitivity to ATP inhibition following modification by pyridoxal phosphate. Such sensitivity suggests that the pyridoxal phosphate has increased the affinity of the inhibitory site for ATP, presumably by occupying the citrate binding site and "freezing" the enzyme in an inhibited conformation.

Kinetics of ATP Inhibition at pH 7.35. As mentioned above, ATP inhibition of native phosphofructokinase is decreased at higher pH, an effect due to the reduced affinity of the inhibitory site for MgATP (Mathias and Kemp, 1972). As shown

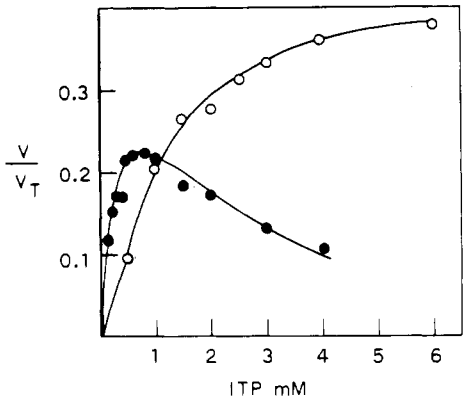

FIGURE 2: ITP inhibition of modified phosphofructokinase. Assays were performed at pH 7.0 with 1 mM fructose-6-P and the indicated concentrations of ITP with native enzyme (○) and with enzyme containing 1.35 mol of P-pyridoxyl/protomer. (●) V_T refers to the activity at pH 8.2 which was 150 units/mg for native enzyme and was 44 units/mg for modified enzyme.

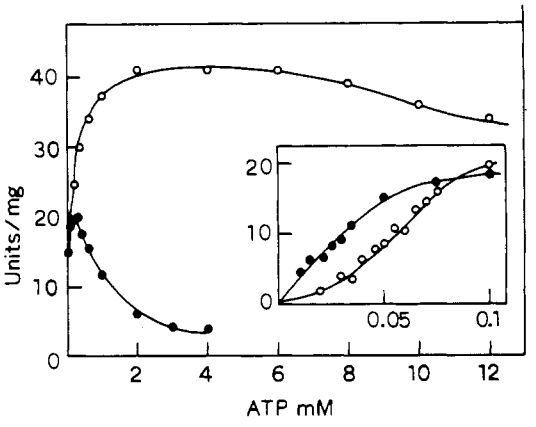

FIGURE 3: ATP inhibition of native and modified phosphofructokinase at pH 7.35. Assays were performed at 1 mM fructose-6-P and the indicated concentrations of ATP with native phosphofructokinase (○) and with enzyme containing 0.80 mol of P-pyridoxyl/protomer (●). The inset shows the data obtained with low-subsaturating concentrations of ATP.

in Figure 3, 12 mM ATP was only slightly inhibitory to the native enzyme in the presence of 1 mM fructose-6-P at pH 7.35. The modified enzyme was again much more sensitive to ATP, showing maximal activity at about 0.1 mM ATP and 50% inhibition at about 1.2 mM ATP. Of particular interest were the saturation properties of the catalytic site of the modified enzyme for ATP. At subsaturating levels of substrate nucleoside triphosphate, citrate has been shown to activate phosphofructokinase (Randle et al., 1968; Colombo et al., 1975). Similarly, as indicated by the inset of the figure, the P-pyridoxyl-enzyme had greater activity at low concentrations of ATP than did the native enzyme, suggesting that modification has increased the affinity of the catalytic site for ATP. For example, at 0.02 mM ATP, the activity of the modified enzyme was nearly fourfold higher than that of the native enzyme.

The increase in the affinity of the catalytic site for nucleoside triphosphate as a result of pyridoxylation was also seen with ITP as the phosphoryl donor (Figure 2). Analysis of the data for the native enzyme yielded a K_m for ITP of about 1.2 mM. Interpretation of saturation data for modified enzyme is of course complicated by the inhibition at higher concentrations of ITP, but half saturation appears to be in the region of 0.1–0.2 mM.

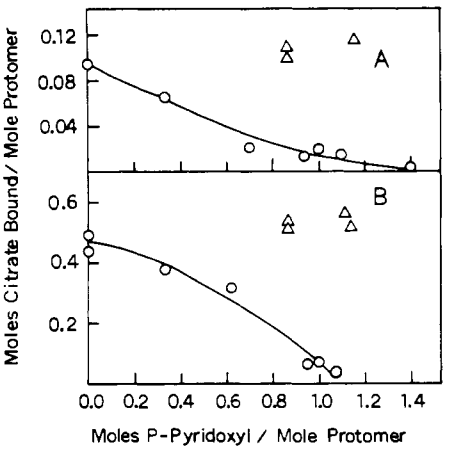

FIGURE 4: Loss of citrate binding accompanying incorporation of pyridoxal phosphate. Varying degrees of P-pyridoxylation were obtained by varying the concentration of pyridoxal phosphate as described in Figure 1. The enzyme was modified in the presence of MgATP (△) or in the presence of MgATP and citrate (○) as in Figure 2. Binding studies of each modified enzyme fraction were performed by the technique of Hummel and Dreyer (1962) as described in the Experimental Procedure employing 1.8–2.7 mg of protein per column run. Included in the equilibration buffer were 2 mM $MgCl_2$, 20 μM ATP, and either 20 μM [1,5-^{14}C]citrate (B) or 2 μM [1,5-^{14}C]citrate (A).

Binding of Citrate by Modified Phosphofructokinase. The crucial evidence for the interaction of pyridoxal phosphate at the citrate site was provided by studies of the equilibrium binding of citrate by modified enzyme. Figure 4 shows the reduction in the binding of citrate with increasing amounts of P-pyridoxyl bound to the enzyme. At both 2 and 20 μM citrate, the amount of ligand bound approached zero with slightly more than 1 mol of P-pyridoxyl per mol of protomer. If the enzyme was pyridoxylated in the presence of citrate (modified-protected enzyme), citrate binding was not blocked and, in fact, appeared to be slightly enhanced.

The findings shown in Figure 4 could also result from a modification that resulted in a great decrease in affinity rather than a complete blockade of the citrate site. In other words, did the residual binding of citrate that was observed when 1 mol of P-pyridoxyl was present result from a reduced number of sites or from reduced affinity at all of the sites? This was examined by studying the binding over a range of citrate concentrations. The results are shown in Figure 5 for citrate binding over the range of 1.5–20 μM citrate with native enzyme, modified protected enzyme with 0.88 mol of bound P-pyridoxyl, and modified enzyme with 0.95 mol of bound P-pyridoxyl. The results show that the reduction in citrate binding was due to a loss of binding sites. As plotted, the data give a K_d of 19 μM for modified enzyme, a value that is almost identical with that observed with native phosphofructokinase (20 μM). The intercept on the x axis indicates maximum binding of 0.15 mol of citrate compared with 1.0 for native enzyme. That 15% of the site was intact despite the incorporation of 0.95 mol is not surprising in view of the results in Figure 1 which show that the partial modification of at least one additional site occurs during the modification of the presumed citrate site (Figure 1). The binding data for the enzyme modified while protected with citrate show no loss in citrate binding capacity.

Binding of ATP by Native and Modified Phosphofructokinase. Earlier binding studies (Kemp and Krebs, 1967; Colombo et al., 1975) have shown that phosphofructokinase binds ATP and that the binding is increased in the presence of citrate,

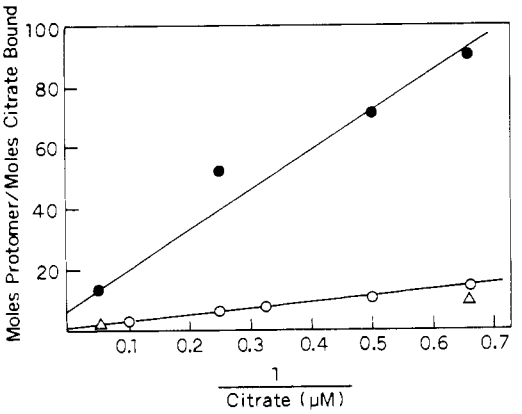

FIGURE 5: Citrate binding by native, modified, or modified-protected phosphofructokinase. Binding of the indicated concentrations of [1,5-^{14}C]citrate was measured by the technique of Hummel and Dreyer (1962) as described in the Experimental Procedure in the presence of 2 mM $MgCl_2$ and 20 μM ATP. Between 1.8 and 3.5 mg of enzyme was employed for each run. The phosphofructokinase employed was either unmodified (○), modified by P-pyridoxylation in the presence of MgATP (●), or modified in the presence of MgATP plus citrate (△).

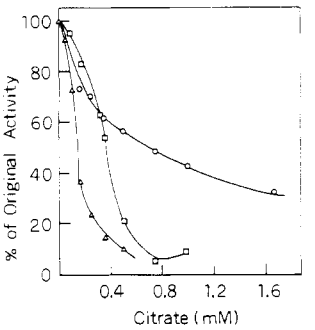

FIGURE 6: Effects of citrate on the activities of native and modified phosphofructokinase. Assays were performed at pH 7.35, 1 mM fructose-6-P, and other conditions described in the Experimental Procedure. (□) Native phosphofructokinase assayed in the presence of 3 mM ATP; (△) enzyme modified in the presence of MgATP and citrate, and assayed using 3 mM ATP; (○) enzyme modified in the presence of MgATP and assayed with 0.3 mM ATP.

3-P-glycerate, P-enolpyruvate, and P-creatine. If indeed pyridoxal-P binds to the citrate site, then one might predict that modified enzyme will bind more ATP than native enzyme at subsaturating concentrations of the nucleoside triphosphate. The amount of ATP bound by native and modified (0.95 mol of P-pyridoxyllysine per protomer) enzyme at a concentration of 2.5 μM ATP was measured by the Hummel and Dreyer technique (1962) as described in the Experimental Procedure. Native enzyme bound 0.51 mol of ATP per mol of protomer in good agreement with previous data (Kemp and Krebs, 1967). Modified phosphofructokinase was not only fully capable of binding ATP, indicating no destruction of that site, but it bound more ATP (0.79 mol of ATP bound per mol) than native enzyme at the concentration employed. Whereas citrate at 0.5 mM enhanced the binding of ATP by native phosphofructokinase, no such enhancement was observed with the modified enzyme. The amount of ATP bound by the native enzyme in the presence of citrate was 0.72 mol per mol of protomer as compared with 0.78 mol per mol for the modified enzyme. These results are consistent with the proposal that the citrate site was already fully occupied by the P-pyridoxyl moiety and that the presence of the P-pyridoxyl, like citrate, resulted in increased affinity for ATP.

Citrate Inhibition of Modified Enzyme. One would assume that another consequence of occupancy of the citrate site on the enzyme would be that citrate should not affect the activity of the enzyme. A direct comparison of the effect of citrate on native and modified enzyme was complicated by the extreme ATP sensitivity of the modified enzyme and by the fact that the citrate effect is known to be synergistic with ATP. What is usually done in assessing citrate inhibition under various conditions is to determine the optimum concentration of ATP, that is, the peak of the ATP inhibition curve, and then measure the inhibition that results from citrate in the presence of the optimal level of ATP.

The effect of citrate on native and modified phosphofructokinase is described in Figure 6. Shown also for comparison is the citrate effect on modified-protected phosphofructokinase, that is, enzyme that was modified for pyridoxal phosphate in the presence of citrate to protect its binding site. The enzyme so treated contained 0.86 mol of modified lysine per mol of protomer. The modified-protected enzyme, assayed under the conditions as the native enzyme, was even more sensitive to citrate inhibition than was native phosphofructokinase. This is consistent with the binding data that showed that not only was the binding site intact when the enzyme was modified in the presence of citrate, but that the affinity of the enzyme for citrate was actually increased. The modified enzyme, treated in the absence of citrate, contained 0.76 mol of modified lysine per mol of protomer. Some inhibition by citrate was observed and it appeared biphasic (Figure 6). An initial phase of high sensitivity to citrate probably represents binding to unmodified enzyme. This initial inhibitory phase was followed by further inhibition that required considerably higher concentrations of citrate. To explain inhibition by these higher concentrations of citrate when it was previously shown that binding of citrate was blocked, one must invoke an interaction at a weak binding site not detected in the equilibrium binding studies, such as the creatine-P site, or at the ATP inhibitory site itself. Lorenson and Mansour (1969) noted two citrate binding sites in their study of heart enzyme as compared with our finding of one (Colombo et al., 1975) and they further suggested some competition between ATP and citrate. There was no competition between ATP and the high affinity citrate site described in our study (Colombo et al., 1975), but it is possible that citrate may be bound weakly by the ATP site.

Attempts to Detect Structural Changes following Modification. The elution profiles of native phosphofructokinase and modified enzyme (1.3 mol of P-pyridoxyl/mol) obtained from agarose gel chromatography (Bio-Gel 1.5m, Bio-Rad Laboratories) were identical, indicating no changes in the degree of polymerization due to pyridoxylation. These analyses, however, were performed with high concentrations of protein (7–8 mg/ml) relative to that used in kinetic assays. High protein concentrations favor the formation of higher aggregates of phosphofructokinase (Parmeggiani et al., 1966), even in the presence of citrate (Colombo et al., 1975). Further evidence that the degree of polymerization of the enzyme was not critical to the interpretation of the kinetic consequences of pyridoxylation was the observation that the rate curves obtained in kinetic assays of the modified enzyme were not significantly different from those obtained with native enzyme. Lags in the assay might be expected if the modified enzyme were depolymerized and that activity was dependent on the polymerized state. Furthermore, if such a situation existed, a nonlinear response of the enzyme to protein concentration might be expected. This was not observed.

Subtle differences in conformation of the modified enzyme

were detectable by measuring reactivity of thiol groups with 5,5′-thiobis(2-nitrobenzoic acid). We have previously shown (Kemp, 1969a; Mathias and Kemp, 1972) that a single thiol group per protomer of phosphofructokinase is extremely reactive with several reagents. The reactivity of the thiol can be blocked by the presence of MgATP, and the relative protective effect of a given concentration of MgATP is enhanced in the presence of citrate (Mathias and Kemp, 1972). The reactivity with 5,5′-dithiobis(2-nitrobenzoic acid) of this thiol group of enzyme modified with pyridoxyl-P was found to be similar to that of native phosphofructokinase in the absence of added ligands. However, the thiol group of the modified enzyme was relatively more sensitive to the protective action of MgATP than was native enzyme. The protection afforded by 0.1 mM MgATP was similar to that provided by 0.1 mM MgATP plus 0.6 mM citrate for the unmodified phosphofructokinase. In other words, the modified enzyme behaved as though it were in the presence of citrate.

Discussion

Pyridoxal phosphate has been often used in the past 10 years for the modification of amino groups in proteins. Its structure directs it toward regions of positive charge by means of its phosphate group and the aldehyde is available for the formation of a Shiff base with an adjacent amino group. Subsequent reduction of the Shiff base to a secondary amine produces a stable covalent modification. Benesch et al. (1972) demonstrated the specific interaction of pyridoxal phosphate with the 2,3-P_2-glycerate site of hemoglobin, a site which consists of three positively charged groups including two amino groups. The specificity of an inhibitor binding site of phosphofructokinase suggests that it too may consist of several positively charged residues. This site binds citrate, 3-P-glycerate, and P-enolpyruvate, and, presumably, 2,3-P_2-glycerate (Colombo et al. 1975). Evidence is presented in the present communication that this site can be specifically modified with pyridoxal phosphate. The evidence that the modification does indeed occur at this specific site may be summarized as follows: (1) maximal protection against loss of activity following covalent modification is provided by the combination of MgATP and citrate. Separately these ligands are not effective. This is consistent with the enhanced affinity of the enzyme for citrate in the presence of MgATP. (2) The modification appears to be biphasic with 1 mol incorporated readily, whereas more extensive pyridoxylation proceeds more slowly. The most dramatic activity and binding changes occur simultaneous to the incorporation of the first mole. (3) Modification results in a profound increase in the sensitivity of phosphofructokinase to MgATP inhibition. This is consistent with the fact that MgATP and citrate act synergistically to inhibit phosphofructokinase. (4) Modification lowers the K_m for MgITP, an effect identical with the action of citrate. (5) Modification abolishes citrate binding, whereas modification in the presence of MgATP plus citrate protects against the loss in citrate binding capacity. (6) Modification results in an increase in the amount of MgATP bound by the enzyme in the presence of a fixed subsaturating concentration of the nucleotide. The ability of citrate to enhance the binding of ATP is abolished by the modification. (7) Although citrate inhibition is still observed with modified enzyme, the effectiveness of citrate as an inhibitor is greatly diminished.

In light of the conclusion that pyridoxal phosphate modifies an inhibitory site, the effect of metabolites on the modification of phosphofructokinase (Table I) may at first appear paradoxical. That the mixture of citrate and MgATP protects against modification of the inhibitory site is easily explained, but protection against activity loss by activators such as fructose-6-P and fructose-1,6-P_2 may not be immediately apparent. Protection of a site from modification, however, can be achieved by direct occupancy by a ligand, such as citrate, or by production of a conformation in which the site is unavailable for interaction with the modifying reagent. The latter alternative may explain the protective action of fructose-6-P and fructose-1,6-P_2. We have previously suggested on the basis of rates of reaction of thiol groups with two different reagents (Kemp, 1969a; Mathias and Kemp, 1972) that fructose-6-P and fructose-1,6-P_2 induce or stabilize one conformation and that MgATP and citrate induce or stabilize a second. Furthermore, the stabilization of one conformation by MgATP (and presumably citrate) can be reversed by fructose-6-P (Kemp, 1969a). The sugar phosphates should thus prevent interaction of pyridoxal phosphate at the citrate site.

Another aspect of the study that is not readily rationalized is the occurrence of considerable incorporation of pyridoxal phosphate even in the presence of protective ligands (Table I and Figure 1). This reaction may be the sum of a large number of partial interactions at various ϵ-amino groups or it could represent reaction with a particular group made more reactive by the presence of the binding ligand. Both fructose-6-P and fructose-1,6-P_2 inhibit overall incorporation by about 50%, but the mixture of citrate and MgATP only slightly inhibits uptake of pyridoxal phosphate despite the complete protection against activity loss. The enzyme produced in the presence of citrate and MgATP, called here the "modified-protected" enzyme, has altered properties as shown by the slightly increased affinity for citrate (Figures 4 and 6). Modification of different sets of lysine residues by pyridoxal phosphate was also observed in studies of fructose-1,6-diphosphatase by Colombo et al. (1972). In that study, modified enzymes with identical degrees of incorporation of pyridoxal phosphate but with vastly different regulatory properties were produced by modification in the presence of either substrate or the allosteric regulator, AMP.

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