observed at all wavelengths. The pH dependence of the second reaction implicates the involvement of two ionizable groups. When the hydrogen bonds associated with these groups are broken, a conformational change could occur with a simultaneous binding of the substrate carboxyl group to the anion site.

The formation of the *gem*-diamine, structure **3,** proposed as an intermediate in the interaction, may produce the observed absorption at 343 nm (O'Leary, 1971). A rapid proton transfer, structure **3** to structure **3'**, would allow the formation of the substrate Schiff base, species 4. This species is expected to have a spectrum similar to that of the free enzyme. If equilibration with the bulk solution is prevented, another species, structure **4'**, might be formed which might also absorb at 343 nm. The spectrum of L-leucine with 5-deoxypyridoxal in basic solution contains a peak at 343 nm which has been attributed to this type of structure (Johnson and Metzler, 1970).

The final step in the glycine reaction is shown as the formation of a carbanion, species 5, which represents the long-wavelength-absorbing species observed 495 nm. Jordan and Akthar (1970) have shown that tritium is lost specifically from [2S-^{3}H]glycine to the solvent. The nature of the remaining intermediate formed in the serine reaction with the enzyme is not known. Without tetrahydrolic acid, the enzyme apparently is unable to break the carbon–carbon bond with serine. The last step with serine could be the interaction of a basic group with the serine hydroxy group which could then help in the breaking of the carbon–carbon bond in the presence of tetrahydrofolic acid.

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Modification of Fructose-1,6-diphosphatase with Pyridoxal 5′-Phosphate. Evidence for the Participation of Lysyl Residues at the Active Site†

Giovanna Colombo and Frank Marcus*

ABSTRACT: Treatment of purified pig kidney fructose-1,6-diphosphatase with pyridoxal 5′-phosphate (in the presence of substrate) followed by reduction with sodium borohydride leads to the selective alteration of the regulatory properties of fructose-1,6-diphosphatase due to the modification of up to four lysyl residues/mole of enzyme (G. Colombo *et al.* (1972), *Biochemistry 11,* 1798). Now, we have studied the inactivation of fructose-1,6-diphosphatase which occurs when modification with pyridoxal phosphate is carried out in the presence of the allosteric inhibitor AMP. Under these conditions up to four lysyl residues/mole of enzyme were modified, and the activity loss was linearly related with the formation of N^{ϵ}-(P-pyridoxyl)lysine. Enzyme inactivation was protected by the substrate fructose 1,6-diphosphate or the inhibitor fructose 6-phosphate, but not by the analog substrate fructose 1-phosphate. These results, as well as those obtained in kinetic studies of partially inactivated enzyme with the substrates fructose 1,6-diphosphate and fructose 1-phosphate, strongly suggest that the site of reaction of pyridoxal phosphate with fructose-1,6-diphosphatase (in the presence of AMP) is a lysyl residue at (or near) the 6-phosphate substrate binding site. These experiments provide another example of the usefulness of pyridoxal phosphate as a means of modifying lysyl residues in or near phosphate binding sites of enzymes, a subject which is herein shortly discussed.

Fructose-1,6-diphosphatase (EC 3.1.3.11, D-fructose-1,6-diphosphate 1-phosphohydrolase) is a regulatory enzyme which plays a key role in the control of gluconeogenesis (for a review, see Pontremoli and Horecker, 1971). The enzyme isolated from mammalian liver and kidney in its native form is composed of four presumably identical subunits with molecular weights of approximately 35,000 (Mendicino *et al.,* 1972; Tashima *et al.,* 1972; Traniello *et al.,* 1972). The existence of four substrate binding sites and four allosteric sites for the inhibitor AMP per enzyme molecule has been demonstrated (Pontremoli *et al.,* 1968a,b; Sarngadharan *et al.,* 1969). Amino acid analysis reveals a relatively large content of lysine (Byrne

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et al., 1971; Tashima *et al.*, 1972; Traniello *et al.*, 1972), which in the case of the pig kidney fructose-1,6-diphosphatase used in the present studies amounts to 96 lysyl residues per mole (Mendicino *et al.*, 1972).

In a previous paper of this laboratory (Colombo *et al.*, 1972) we have demonstrated that the modification of pig kidney fructose-1,6-diphosphatase with pyridoxal-P can lead to the selective alteration of the regulatory properties of the enzyme, with no alteration of the catalytic properties. These changes in properties appeared to be the result of the modification of up to four unique lysyl residues per mole of enzyme, which are necessary structural elements for the cooperative interaction between AMP binding sites of fructose-1,6-diphosphatase. To obtain this selective modification, the presence of the substrate fructose 1,6-diphosphate was required during the reaction with pyridoxal-P, since in its absence the modification led also to a partial loss of enzyme activity. This result suggests that perhaps another set of lysyl residues is involved in the active site region, in accordance with previous findings of modification of two different fructose-1,6-diphosphatases with pyridoxal-P (Marcus and Hubert, 1968; Krulwich *et al.*, 1969) which showed either loss of inhibition by excess substrate and/or partial loss of enzyme activity. Evidence suggesting the participation of lysyl residues in the binding of substrate or for maintenance of the required conformation of the active site has been also presented for *Candida utilis* fructose-1,6-diphosphatase (Rosen and Rosen, 1966).

The present work constitutes a further exploration of the problem by analyzing the results of modification of pig kidney fructose-1,6-diphosphatase with pyridoxal-P, under those conditions (presence of AMP) which prevent the alteration of the regulatory properties of the enzyme. Thus, we provide evidence that ϵ-aminolysyl groups are essential for catalytic activity of fructose-1,6-diphosphatase and that these residues are probably involved in the binding of substrate by the enzyme.

Experimental Section

Chemicals. The materials used in this study were obtained from the following sources: fructose 1,6-diphosphate (stock 750-1), fructose 1-phosphate, fructose 6-phosphate, β-glycerophosphate (grade I), AMP, NADP, glucose-6-phosphate dehydrogenase (type VII), phosphoglucose isomerase (grade III), Tris, EDTA, pyridoxal-5′-P, and $NaBH_4$, Sigma Chemical Co.; ammonium sulfate (Art. 1211) for biochemical use, Merck AG, Darmstadt; P-cellulose (Cellex-P), Bio-Rad Laboratories; Sephadex G-50 (fine) and Sephadex G-200, Pharmacia; N^{ϵ}-(pyridoxyl)lysine was a gift from Dr. E. H. Fischer; all others were proanalysis Merck AG, Darmstadt, Chemicals.

Enzyme Preparation. Fructose-1,6-diphosphatase with optimal activity at neutral pH was purified from pig kidney cortex, as previously described (Colombo and Marcus, 1973).

Assays. Fructose-1,6-diphosphatase concentration was determined by its absorbancy at 280 nm using a value of 0.755 for absorbancy mg^{-1} ml^{-1} (Marcus and Hubert, 1968). Fructose-1,6-diphosphatase activity was measured by the rate of formation of inorganic phosphate from fructose 1,6-diphosphate, as previously described (Marcus, 1967). Unless otherwise indicated, enzyme assays were carried out at pH 7.5 and 30°. The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 75 mM K_2SO_4, 0.1 mM EDTA, 0.10 ml of a solution containing fructose-1,6-diphosphatase, and the substrate fructose 1,6-diphosphate, as indicated in the text. The same method indicated above was used for enzyme activity measurements with fructose 1-phosphate as the substrate, except that K_2SO_4 was not added to the assay system (Colombo and Marcus, 1974). Dilutions of fructose-1,6-diphosphatase solutions were always made in 20 mM Tris-HCl (pH 7.5) containing 2 mM $MgSO_4$ and 0.1 mM EDTA.

For fructose-1,6-diphosphatase assays determined spectrophotometrically by following the rate of formation of NADPH at 340 nm in the presence of excess phosphoglucose isomerase and glucose-6-P dehydrogenase, the assay system of 1 ml contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 75 mM K_2SO_4, 0.1 mM EDTA, 0.15 mM NADP, fructose 1,6-diphosphate as indicated in the figures, excess phosphoglucose isomerase (4 units/ml) and glucose-6-P dehydrogenase (0.8 unit/ml), and fructose-1,6-diphosphatase. Assays were carried out at 30°. The initial rate of formation of NADPH was measured at 340 nm on a Gilford 2400 spectrophotometer with the absorbance recording system set at maximum sensitivity so that full scale deflection of the 10-in. recording chart corresponded to an absorbance of 0.1.

A unit of fructose-1,6-diphosphatase activity is defined as that amount of enzyme which catalyzes the formation of 1 μmol either of inorganic phosphate or fructose 6-phosphate/min under the conditions described. Specific activity is expressed in terms of units per milligram of protein.

Preparation of Pyridoxyl-P-fructose-1,6-diphosphatases. Fructose-1,6-diphosphatase (3 ml) as obtained in fraction IV of the purification procedure (Colombo and Marcus, 1973) was dialyzed at 4° against 1000 ml of 0.5 M KCl containing 0.1 mM EDTA. The enzyme (1.7 mg/ml) in 100 mM sodium borate buffer (pH 8.0), and other additions as indicated in the text, was incubated at 4° for 10 min before adding pyridoxal-P. Then pyridoxal-P was added and incubation was continued for 18 min at 4°.[1] After this period, 2 drops of octyl alcohol was added and the reaction was stopped by the addition of a few milligrams of solid $NaBH_4$ (just enough to produce decoloration of the reaction system). The solution was dialyzed at 4° for 16 hr against 1000 ml of 20 mM Tris-HCl (pH 7.5), containing 2 mM $MgSO_4$ and 0.1 mM EDTA. The enzyme was then desalted on a column (1.6 × 22 cm) of Sephadex G-50 fine. Fractions of 1.5 ml were collected, and absorbancy was measured at 280 and 325 nm. Only those fractions having an absorbance higher than 0.500 at 280 nm and a constant ratio of absorbancies (absorbance at 325 nm/absorbance at 280 nm) were stored frozen until used. The number of pyridoxyl-P amino groups present in pyridoxyl-P-fructose-1,6-diphosphatases was estimated from the absorbance at 325 nm by using the molar extinction coefficient of 10,150 for N^{ϵ}-(P-pyridoxyl)-lysine (Fischer *et al.*, 1963), and by assuming that pyridoxyl-P-fructose-1,6-diphosphatases have a comparable absorption. The molecular weight of pig kidney fructose-1,6-diphosphatase was taken as 140,000. This value was based on a subunit molecular weight of 35,000 (Mendicino *et al.*, 1972; F. González, A. M. González, and F. Marcus, unpublished results).

Identification of N^{ϵ}-(Pyridoxyl)lysine in Pyridoxyl-P-fructose-1,6-diphosphatase. An enzyme derivative (8.8 mg) containing 2.2 mol of pyridoxyl-P/mol of fructose-1,6-diphosphatase was dialyzed exhaustively in the dark against several changes of distilled water and then hydrolyzed in sealed tubes in the presence of 6 N distilled HCl for 20 hr at 110°. The hydrolysate was concentrated under reduced pressure, dissolved in water, and lyophilized. This procedure was repeated three times to eliminate residual HCl. Aliquots of the hydrolysate were applied to sheets of Whatman 3 MM paper and subjected

[1] It should be noted that since the amount of enzyme added to the reaction system of 3 ml is 0.5 ml, the concentration of KCl in the modification system amounts to 83 mM.

TABLE I: Effect of Substrate and AMP on Modification of Fructose-1,6-diphosphatase by Pyridoxal-P.[a]

Enzyme	Specific Activity[b] (units/mg)	Inhibition by Excess Substrate[c] (%)	Inhibition by AMP[d] (%)
1. Native	32.4	45	95
2. Treated with $NaBH_4$[e]	29.3	44	96
3. Modified	13.5	10	9
4. Modified in the presence of 10 mM fructose 1,6-diphosphate	28.0	43	12
5. Modified in the presence of 2.5 mM AMP	8.2	0	90
6. Modified in the presence of 10 mM fructose 1,6-diphosphate plus 2.5 mM AMP	28.9	48	92

[a] Modification of fructose-1,6-diphosphatase with pyridoxal-P was carried out at 30° as previously described (Colombo *et al.*, 1972) using 5 mM pyridoxal-P and other additions as indicated in this table. [b] Specific activity was measured by the rate of formation of inorganic phosphate from 0.5 mM fructose-1,6-diphosphate, as described under Assays. [c] For substrate inhibition, activity was measured at 10 mM fructose-1,6-diphosphate and a relative value of 100% was given to the activity measured at 0.5 mM substrate. [d] AMP inhibition was measured at 0.5 mM fructose 1,6-diphosphate plus 0.1 mM AMP. [e] Control enzyme treated with $NaBH_4$ in the absence of pyridoxal-P.

to: (a) high-voltage electrophoresis for 120 min at 1500 V in pyridine–acetic acid–water (1:10:89, v/v), pH 3.5 (Schnackerz and Noltmann, 1971); (b) chromatography in 2-propanol–pyridine–acetic acid–water (30:20:6:24) (Forrey *et al.*, 1971). In both experiments, after drying of the paper sheet, a fluorescent spot with a mobility identical with that of synthetic N^{ϵ}-(pyridoxyl)lysine was visible under uv light.

Results

Modification of Fructose-1,6-diphosphatase with Pyridoxal-P. The effects of pyridoxal-P modification on the activity of kidney fructose-1,6-diphosphatase and on its inhibition by either excess substrate or by AMP are shown in Table I. This introductory experiment was essential to corroborate the results previously obtained under similar experimental conditions (Colombo *et al.*, 1972), but this time using an enzyme preparation of neutral pH optimum (Colombo and Marcus, 1973). It has been clearly demonstrated during the last few years that the native form of liver and kidney fructose-1,6-diphosphatase exhibits maximal activity at neutral pH (Traniello *et al.*, 1971, 1972; Tashima *et al.*, 1972). However, proteolytic modification leads to the formation of an enzyme with optimal activity at alkaline pH (Nakashima *et al.*, 1971). Thus, with the exception of some of the last reported methods of purification (Traniello *et al.*, 1971; Byrne *et al.*, 1971; Tashima *et al.*, 1972; Colombo and Marcus, 1973), earlier purification methods yielded either alkaline fructose-1,6-diphosphatase or enzymes which had properties somewhere in between the neutral and alkaline forms. In general, the results shown in Table I corroborate our previous data (*cf.* Table II, Colombo *et al.*, 1972), demonstrating that unless the substrate fructose 1,6-diphosphate is present during modification, there is a loss of enzyme activity as well as of inhibition by excess substrate. However, there is a significant difference in the results obtained when modification of fructose-1,6-diphosphatase by pyridoxal-P is carried out in the presence of 2.5 mM AMP, since now a complete protection against the loss of AMP inhibition was obtained. This result opened the possibility for the selective alteration of the catalytic properties of fructose-1,6-diphosphatase by modification of the enzyme with pyridoxal-P. Figure 1A shows the number of moles of pyridoxyl-P incorporated per mole of enzyme when increasing amounts of pyridoxal-P are used for modification of fructose-1,6-diphosphatase at 4° in the presence of AMP. The result shows an apparent saturation curve, from which a maximum value of 4 mol of pyridoxyl-P incorporated/mol of enzyme is obtained from the vertical intercept of a double reciprocal plot (not shown). However, analysis of the data by means of a Scatchard plot reveals that incorporation of reagent follows a pattern typical of negative cooperativity (Koshland, 1970) which extrapolates to 4 mol of pyridoxyl-P incorporated/mol of enzyme (Figure 1B). The Hill plot of the same data of

TABLE II: Protection of Fructose-1,6-diphosphatase against Inactivation by Pyridoxal-P.[a]

Compd Added	Specific Activity[b] (units/mg)	Pyridoxyl-P Incorporated (mol/mol)
Control[c]	29.5	
None	9.7	2.7
Fructose-1,6-P_2 (0.1 mM)	12.6	2.5
Fructose-1,6-P_2 (1 mM)	23.0	1.4
Fructose-1,6-P_2 (10 mM)	27.5	1.0
Fructose-1-P (10 mM)	9.5	2.3
β-Glycero-P (30 mM)	12.4	2.7
Phosphate (10 mM)	8.9	2.5
Fructose-6-P (0.1 mM)	13.6	2.2
Fructose-6-P (1 mM)	17.2	1.6
Fructose-6-P (10 mM)	29.4	1.1

[a] Modification of fructose-1,6-diphosphatase with 5 mM pyridoxal-P was carried out as described in the Experimental Section, in the presence of 2.5 mM AMP and other compounds as indicated. [b] Specific activity was measured by inorganic phosphate formation from 0.5 mM fructose-1,6-diphosphate, as described under Assays. [c] Control enzyme treated with $NaBH_4$ in the absence of pyridoxal-P.

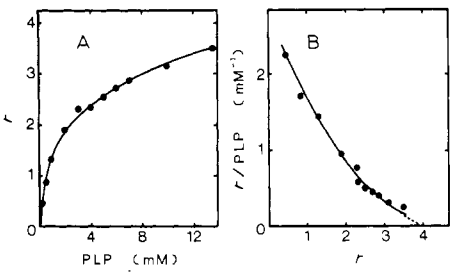

FIGURE 1: Plots of experimental data of incorporation of pyridoxyl-P into fructose-1,6-diphosphatase in the presence of AMP. Enzyme derivatives were prepared as described in the Experimental Section, but in the presence of 2.5 mM AMP and pyridoxal-P as indicated. (A) Plot of *r* (mol of pyridoxyl-P incorporated/mol of enzyme) as a function of pyridoxal-P (PLP) concentration. (B) Scatchard plot of *r*/[pyridoxal-P] *vs. r*.

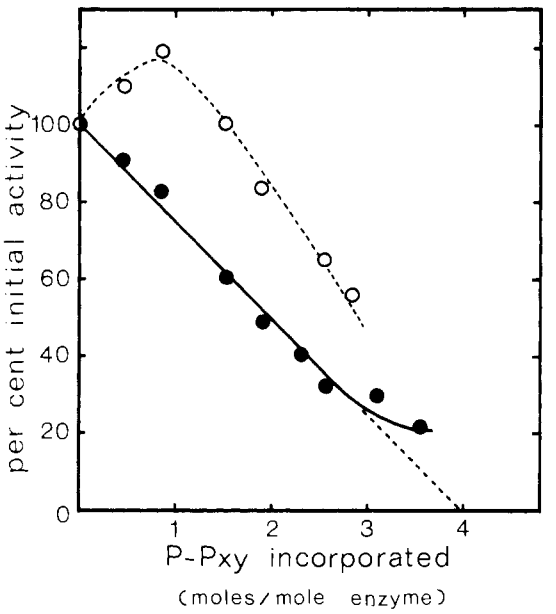

FIGURE 2: Stoichiometry of inactivation of fructose-1,6-diphosphatase by modification with pyridoxal-P. Enzyme derivatives were prepared as described in the Experimental Section, in the presence of 2.5 mM AMP and various amounts of pyridoxal-P ranging from 0.2 to 13.6 mM. Enzyme activity was determined by inorganic phosphate formation using 0.5 mM (●) or 10 mM (○) fructose 1,6-diphosphate in the assay. A value of a 100% initial activity was given to a control enzyme which was treated with $NaBH_4$ in the absence of pyridoxal-P.

Figure 1A (not shown) gives a Hill coefficient of 0.82, also indicative of negative cooperativity.[2]

To demonstrate that the reaction had occurred at ε-aminolysyl residues, fructose 1,6-diphosphate containing 2.2 mol of pyridoxyl-P/mol of enzyme was dialyzed exhaustively against water, hydrolyzed with HCl, and N^{ϵ}-(pyridoxyl)lysine was then identified in the acid hydrolysate as described under the Experimental Section. The analysis revealed the presence of a single fluorescent spot with a mobility identical with that of synthetic N^{ϵ}-(pyridoxyl)lysine. The fluorescent spot could be stained with ninhydrin, confirming the presence of a free amino group, and was also found to be quenched by NH_3.

Stoichiometry of Inactivation of Fructose-1,6-diphosphatase by Pyridoxal-P. The results of studies relating the extent of inactivation of the enzyme by modification with pyridoxal-P to the number of modified lysyl residues are given in Figure 2. When assayed at 0.5 mM (solid line) or lower fructose 1,6-diphosphate concentrations, a linear relationship is obtained in which extrapolation to a 100% inactivation indicates that the modification of four lysyl residues/mole of enzyme would be expected to result in complete inactivation of fructose-1,6-diphosphatase. Since the enzyme contains four apparently identical subunits (Mendicino *et al.*, 1972), these findings strongly suggest that one essential lysyl residue in each subunit may have been modified by the reagent. Figure 2 also shows the activity *vs.* extent of modification when activity is assayed at high (inhibitory) substrate concentrations (broken line). Under these conditions there is an initial rise followed by loss of enzymatic activity as modification proceeds, but extrapolation of the linear portion also gives the value of four lysyl residues

[2] This result could be interpreted as if subunit conformational changes occur upon pyridoxal-P binding, in which the binding of each molecule of pyridoxal-P makes it more difficult for the next molecule to bind. However, fructose 1,6-phosphate binding experiments at 4° with pig kidney fructose-1,6-diphosphatase also show some indication of negative cooperativity (n_H = 0.91, n_m = 4; G. Colombo and F. Marcus, unpublished observations).

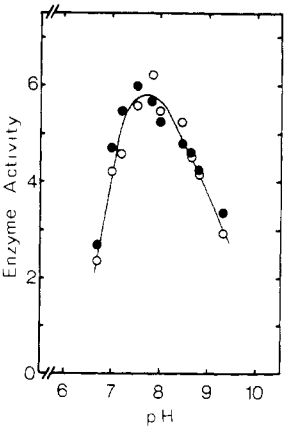

FIGURE 3: pH–activity profile of pyridoxyl-P-fructose-1,6-diphosphatase. Enzyme activity was determined by inorganic phosphate formation from 0.5 mM fructose 1,6-diphosphate at the indicated pH values; 50 mM Tris-HCl buffers, adjusted to pH with succinic acid, were used between pH 6.5 and 7.2. At pH 7.4 or above, 50 mM Tris-HCl buffers were used: (○) pyridoxyl-P-fructose-1,6-diphosphatase (1.9 mol of pyridoxyl-P/mol of enzyme); (●) control enzyme treated with $NaBH_4$ in the absence of pyridoxal-P. Enzyme activity is expressed in terms of specific activity per nonmodified enzyme active sites. Thus, specific activity/4 was used for the control enzyme, and specific activity/2.1 was used for the P-pyridoxyl derivative.

modified/mole of enzyme. These results, similar to those of Goldin and Frieden (1972) with glutamate dehydrogenase, account for the loss of high substrate inhibition previously noted for the modification of fructose-1,6-diphosphatase with pyridoxal-P (Marcus and Hubert, 1968; Colombo *et al.*, 1972).

Properties of Partially Inactivated Enzyme. Having demonstrated that modification of fructose-1,6-diphosphatase with pyridoxal-P results in the loss of enzyme activity, subsequent experiments were carried out to demonstrate that the result was not due to a shift of the pH optimum of the modified enzyme. Figure 3 shows that this is not the case, since the modified enzyme containing 1.9 mol of pyridoxyl-P/mol of enzyme shows the same pH profile of fructose-1,6-diphosphatase activity as the one shown by the control enzyme. The good fit obtained with a single pH curve expressed in terms of specific activity per nonmodified enzyme active sites can be interpreted as if modification of a subunit led to its complete inactivation without affecting the activity of the neighboring nonmodified subunit.

The inactivation of fructose-1,6-diphosphatase after modification with pyridoxal-P was neither due to dissociation nor association of the enzyme subunits, as studied by gel filtration. A single protein peak of V/V_0 of approximately 1.59 was obtained when a mixture of native enzyme (6.9 mg) and fructose-1,6-diphosphatase containing 2.2 mol of pyridoxyl-P/mol of enzyme (6.9 mg) was subjected to gel filtration at room temperature on a 1.8 × 54 cm column of Sephadex G-200.

Protection against Enzyme Inactivation. The effect of several compounds related to the reactions catalyzed by fructose-1,6-diphosphatase on the inactivation of the enzyme by pyridoxal-P in the presence of AMP is shown in Table II. As already demonstrated in Table I, the substrate fructose 1,6-diphosphate protected from inactivation by pyridoxal-P. The alternative substrates, fructose-1-P (Colombo and Marcus, 1974) and β-glycero-P (Cohen *et al.*, 1971), as well as the product (inorganic phosphate), did not give the protection one expects if the modification occurs at the active site,[3] unless the modification occurs at a portion of the active site which is not

[3] The term active site is used as defined by Koshland and Neet (1968).

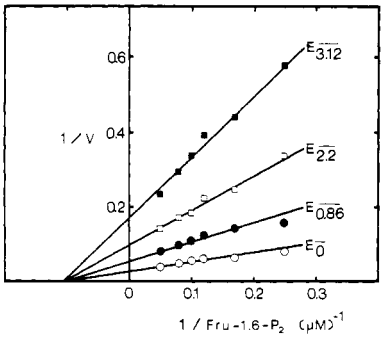

FIGURE 4: Double reciprocal plots for the hydrolysis of fructose 1,6-diphosphate by native and pyridoxyl-P-fructose-1,6-diphosphatases. The assays were performed spectrophotometrically as described in the Experimental Section: (○) native fructose-1,6-diphosphatase, (●, □, ■) pyridoxyl-P-fructose-1,6-diphosphatases, containing 0.86, 2.2, and 3.12 mol of pyridoxyl-P/mol of enzyme, respectively. V = μmoles of product formed min^{-1} mg of enzyme.$^{-1}$ In this figure, as well as in Figure 5, E_n denotes the state of modification of enzyme preparations by pyridoxal-P. The value of $\bar{n}$ indicates the number of moles of pyridoxyl-P incorporated/mole of enzyme.

involved in the binding of these compounds (*i.e.*, the binding site of phosphate-6 of fructose 1,6-diphosphate). The protective effect shown by the product fructose-6-P seems to support the above hypothesis. As expected, those compounds (fructose 1,6-diphosphate or fructose-6-P) which protect from enzyme inactivation by pyridoxal-P modification also reduce the number of amino groups modified by the reagent. However, since incorporation is not completely abolished, it appears that the incorporation observed in the presence of 2.5 mM AMP plus 10 mM fructose 1,6-diphosphate (or 10 mM fructose-6-P) is probably reflecting the exposure of another set of less reactive lysyl residues (neither involved in enzyme activity nor in AMP inhibition) that could be modified in the enzyme conformation induced by the presence of substrate plus the allosteric inhibitor.

Kinetic Studies with Pyridoxyl-P-fructose-1,6-diphosphatases. The data in Figure 4 show the effect of modification of fructose-1,6-diphosphatase with pyridoxal-P on the kinetic constants of the reaction of the enzyme with fructose 1,6-diphosphate as the substrate. The data show that V_{max} decreased after modification, while K_m of fructose 1,6-diphosphate (9.3 μM) did not change. These results can be interpreted as a situation in which the properties of a fraction of the enzyme molecules or subunits have not been changed (the unmodified ones), whereas the modified molecules or subunits are fully inactive. Although these results *per se* do not permit a distinction as to whether the loss of activity was due to the alteration of substrate binding or to the alteration of some step subsequent to binding (Zerner and Bender, 1964), the experiments reported above on protection of enzyme inactivation by either fructose 1,6-diphosphate or fructose 6-phosphate, but not by fructose 1-phosphate, suggested *a priori* that the lysyl residue modified by pyridoxal-P could be located at (or near) the 6-phosphate binding site of fructose-1,6-diphosphatase. If such be the case, kinetic studies with the analog substrate fructose 1-phosphate (Colombo and Marcus, 1974) might provide evidence to support the above hypothesis. As shown in Figure 5, K_m values for fructose 1-phosphate increased after modification, as if the modified molecules or subunits could still bind fructose 1-phosphate albeit poorly. Table III summarizes the K_m and V_{max} values for fructose 1,6-diphosphate and fructose 1-phosphate of fructose-1,6-diphosphatases modified to different extents with pyridoxal-P.

TABLE III: Kinetic Parameters of Pyridoxyl-P-fructose-1,6-diphosphatases.[a]

Substrate	Enzyme Derivative (mol of pyridoxyl-P/mol)	K_m (mM)	V_{max}
Fructose-1,6-P_2	0	0.0093	40
	0.86	0.0093	18
	2.20	0.0093	10
	3.12	0.0093	6
Fructose-1-P	0	2.2	1.1
	0.86	2.9	0.9
	1.29	3.5	0.7
	2.30	4.0	0.5

[a] Kinetic constants reported in this table were obtained from the data of Figures 4 and 5.

The results on protection by fructose 6-phosphate against enzyme inactivation led us also to an examination of the inhibition of fructose-1,6-diphosphatase by fructose 6-phosphate, a fact already pointed out by Mokrasch and McGilvery (1956) using fructose 1,6-diphosphate as the substrate of the rabbit liver enzyme. Now, by using fructose 1-phosphate as the substrate for the reaction it is demonstrated that the inhibition is linear competitive (Figure 6), with a K_i for fructose 6-phos-

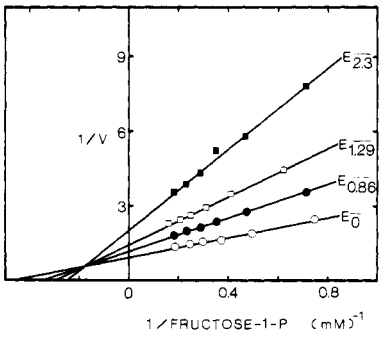

FIGURE 5: Double reciprocal plots for the hydrolysis of fructose 1-phosphate by native and pyridoxyl-P-fructose-1,6-diphosphatases. The assays were performed as described in the Experimental Section: (○) native fructose-1,6-diphosphatase, (●, □, ■) pyridoxyl-P-fructose-1,6-diphosphatases containing 0.86, 1.29, and 2.3 mol of pyridoxyl-P/mol of enzyme, respectively. V = μmoles of product formed min^{-1} mg of enzyme^{-1}.

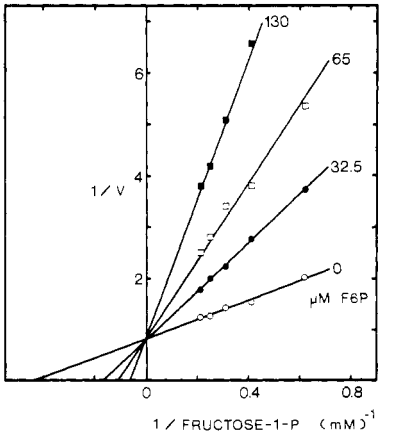

FIGURE 6: Double reciprocal plots for the fructose 6-phosphate inhibition of the activity of fructose-1,6-diphosphatase with fructose 1-phosphate as the substrate. The assays were performed as described in the Experimental Section, in the presence of the indicated concentrations of fructose 6-phosphate (F6P). V = μmoles of product formed min^{-1} mg of protein^{-1}.

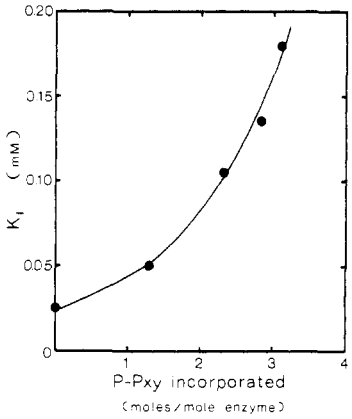

FIGURE 7: Effect of pyridoxal-P modification on the inhibition constant for fructose 6-phosphate. Inhibition studies with pyridoxyl-P-fructose-1,6-diphosphatases were carried out as shown in Figure 6 for the native enzyme. K_i values for fructose 6-phosphate were then obtained by replotting slope *vs.* *I* (Cleland, 1963).

phate of 25 μM obtained by replotting slope *vs.* *I* (Cleland, 1963). Linear competitive inhibition by fructose 6-phosphate was also obtained when the inhibition studies were carried out with pyridoxyl-P-fructose-1,6-diphosphatases. However, as expected if modification of fructose-1,6-diphosphatase with pyridoxal-P is altering a lysyl residue at (or near) the 6-phosphate binding site, fructose 6-phosphate inhibition of the enzyme reaction with fructose 1-phosphate is considerably decreased by modification of fructose 1,6-diphosphatase. This is clearly shown in Figure 7, a plot of the inhibition constant for fructose 6-phosphate as a function of pyridoxyl-P incorporation.

Discussion

Modification of Fructose-1,6-diphosphatase with Pyridoxal-P. The chemical modification of fructose-1,6-diphosphatase with pyridoxal-P (in the presence of substrate) has already proved useful for demonstrating the participation of lysyl residues in allosteric regulation of fructose-1,6-diphosphatase (Colombo *et al.*, 1972). Now, by performing the reaction in the presence of the allosteric inhibitor AMP, the modification by pyridoxal-P has been made selective for ϵ-aminolysyl residues essential for fructose-1,6-diphosphatase activity. The evidence presented herein indicates that, under these conditions, pyridoxal-P is acting as an active-site selective reagent (Means and Feeney, 1971a) for the modification of fructose-1,6-diphosphatase. This conclusion is based on the following experimental facts. (a) *Incorporation of reagent exhibits a saturation effect with increasing reagent concentration.* As demonstrated in Figure 1 the incorporation of reagent shows a saturation effect in which the extrapolated maximal value of four lysyl residues modified/mole of enzyme indicates that only a small group of the 96 lysines of fructose-1,6-diphosphatase (Mendicino *et al.*, 1972) are reactive with pyridoxal-P in the presence of AMP. (b) *Stoichiometric inactivation.* Kidney fructose-1,6-diphosphatase (Mendicino *et al.*, 1972; Tashima *et al.*, 1972), as well as liver and muscle fructose-1,6-diphosphatase (Traniello *et al.*, 1972; Black *et al.*, 1972), are composed of four presumably identical subunits, and contain four substrate binding sites/mol wt *ca.* 140,000 tetramer (Pontremoli *et al.*, 1968a; Sarngadharan *et al.*, 1969). Thus, the interpretation of the stoichiometry of inactivation, which indicates that complete loss of enzymatic activity extrapolates to 4 mol of modified ϵ-aminolysyl groups/mol of fructose-1,6-diphosphatase (one lysyl residue/subunit), is straightforward. Adequate controls demonstrated that loss of enzyme activity was neither due to a shift in the pH optimum of the reaction, nor to association or dissociation of the enzyme. (c) *Specific protection against inactivation.* From the complete protection by either the substrate fructose 1,6-diphosphate or the inhibitor fructose-6-P against inactivation of the enzyme by pyridoxal-P (Table II), it may be also inferred that the modification occurs at the active site of fructose-1,6-diphosphatase.

Although assigning the action of a modifier to a catalytic site, binding site or conformation-controlling site is often very difficult and sometimes impossible (Cohen, 1970), three types of observations suggest that the site of reaction of pyridoxal-P with fructose-1,6-diphosphatase (in the presence of AMP) is a lysyl residue at (or near) the 6-phosphate substrate binding site, rather than another specificity residue or a catalytic residue. (a) The complete protection afforded by either substrate fructose 1,6-diphosphate or the inhibitor fructose 6-phosphate against inactivation produced by modification with pyridoxal-P. Conversely, the substrates (fructose 1-phosphate or β-glycerophosphate) afforded no protection. (b) The changes in K_m and V_{max} for fructose 1-phosphate, but only in V_{max} for fructose 1,6-diphosphate, after modification. (c) The loss of fructose 6-phosphate inhibition of the enzyme reaction with fructose 1-phosphate which occurs after modification.

General Considerations on Modification of Enzymes with Pyridoxal-P. The results presented herein provide another example of the usefulness of pyridoxal-P as a means of specifically labeling lysyl residues in or near phosphate binding sites of proteins (Means and Feeney, 1971a; Raetz and Auld, 1972). To the author's view, there are already in the increasing literature in the field a number of conclusive examples which fall in this category (Table IV). In addition to the examples given in Table IV, there is suggestive evidence, but not as conclusive as those of the examples included in Table IV, for modification of the same type in several other reports. These suggest the pyridoxal-P modification of reactive lysyl residues at the fructose 6-phosphate binding site of phosphofructokinase (Uyeda, 1969; Setlow and Mansour, 1972), near the FAD binding site of kidney D-amino acid oxidase (Miyake and Yamano, 1970), at lys-

TABLE IV: Modification of Proteins with Pyridoxal-P at Amino Groups in or Near Phosphate Binding Sites.

Protein (Source)	Proposed Site of Modification	Ref
P-gluconate dehydrogenase (*C. utilis*)	P-binding site of P-gluconate	*b*
Fructose-1,6-P_2 aldolase (rabbit muscle)	6-P binding site of Fructose-1,6-P_2	*c*
P-glucose isomerase (rabbit muscle)	Substrate binding site	*d*
Ribonuclease A (bovine pancreas)	P-binding site (Lys-7 and Lys-41)	*e*
Deoxyhemoglobin (human blood)	2,3-Diphosphoglycerate binding site[a]	*f*
Aspartate transcarbamylase (*E. coli*)	Active site of the catalytic subunit	*g*
Fructose-1,6-diphosphatase (pig kidney)	6-P binding site of fructose-1,6-P_2	This report

[a] Modification occurs at the N-terminal amino group of Val 1 β. In all others, at ϵ-amino lysyl residues. [b] Rippa *et al.* (1967). [c] Shapiro *et al.* (1968). [d] Schnackerz and Noltmann (1971). [e] Means and Feeney (1971b); Raetz and Auld (1972). [f] Benesch *et al.* (1972). [g] Greenwell *et al.* (1973).

ines-191 and -212, a suggested binding site for anions in rabbit muscle glyceraldehyde-3-P dehydrogenase (Forcina *et al.*, 1971), at the coenzyme binding site of liver alcohol dehydrogenase (McKinley-McKee and Morris, 1972), and at the nucleoside phosphate binding site of *Escherichia coli* RNA polymerase (Venegas *et al.*, 1973). On the other hand, it should be also pointed out that there are several reports of pyridoxal-P modification which probably do not involve necessarily the modification of lysyl residues at or near phosphate binding sites, since preferential modification by pyridoxal-P can also occur as a consequence of the increased reactivity of an amino group of abnormally low pK_a (*i.e.*, as in glutamate dehydrogenase, Piszkiewicz and Smith, 1971a,b; Blumenthal and Smith, 1973) without involvement of a phosphate binding site.

Finally, it is interesting to remark that the four enzymes utilizing fructose 6-phosphate or fructose 1,6-diphosphate (P-glucose isomerase, P-fructokinase, fructose-1,6-diphosphate aldolase, and fructose-1,6-diphosphatase) appear to be reactive to pyridoxal-P at their respective substrate binding sites. It would be of interest from an evolutionary point of view to compare the amino acid sequence around the reactive lysyl residue of these four enzymes, one of which (the pyridoxal-P binding site of rabbit muscle aldolase) has been already determined (Anai *et al.*, 1973).

Acknowledgments

The authors thank Professor H. Peña (Department of English) for his critical reading of this manuscript. We are also indebted to Mr. R. Bustos and Mr. R. Vela for their help in the preparation of illustrations, and to Mrs. M. Angélica Espinoza for excellent secretarial assistance.

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