Selective Alteration of the Regulatory Properties of Fructose 1,6-Diphosphatase by Modification with Pyridoxal 5′-Phosphate†

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ABSTRACT: Treatment of purified pig kidney fructose 1,6-diphosphatase with pyridoxal 5′-phosphate followed by reduction with sodium borohydride leads to the formation of active pyridoxyl phosphate derivatives of the enzyme, showing diminished sensitivities to allosteric AMP inhibition and to high substrate inhibition (Marcus, F., and Hubert, E. (1968), *J. Biol. Chem. 243*, 4923). These alterations were not observed when modification with pyridoxal 5′-phosphate was carried out in the presence of substrate plus AMP. However, when fructose 1,6-diphosphatase was modified with pyridoxal 5′-phosphate in the presence of fructose 1,6-diphosphate, active pyridoxyl phosphate derivatives of the enzyme (containing up to approximately 4 moles of pyridoxyl phosphate/mole of enzyme) were obtained. Under these conditions, only the regulatory properties of fructose 1,6-diphosphatase were altered by modification. The modified enzymes show no cooperative interaction among AMP binding sites (the apparent n value was reduced to 1) and inhibition by AMP was greatly decreased (K_i increased from 0.05 to 0.94 mM when 4 moles of pyridoxal 5′-phosphate was incorporated). Binding studies showed that these results were due to reduced binding of AMP to the modified enzymes. Conversely, catalytic properties (specific activity, high substrate inhibition, substrate binding) were not significantly altered. The evidence presented suggests that the changes induced by selective modification of fructose 1,6-diphosphatase by pyridoxal 5′-phosphate (in the presence of substrate) are the result of the modification of unique lysyl residues of the enzyme, which are necessary structural elements for the regulation of fructose 1,6-diphosphatase by AMP.

The inhibition of fructose 1,6-diphosphatase by AMP is considered to be one of the essential control mechanisms in the regulation of gluconeogenesis (Atkinson, 1966; Horecker *et al.*, 1966). The allosteric nature of this inhibition has been demonstrated kinetically (Taketa and Pogell, 1965), as well as by selective chemical modification of purified fructose 1,6-diphosphatases with several reagents (Rosen and Rosen, 1966; Pontremoli *et al.*, 1966, 1967; Marcus, 1968; Marcus and Hubert, 1968; Krulwich *et al.*, 1969). Among these studies, the specific interaction between pyridoxal-5′-P and a few of the ϵ-aminolysyl residues apparently involved in AMP inhibition appeared to be of particular interest. A previous paper from this laboratory (Marcus and Hubert, 1968) demonstrated that the modification of pig kidney fructose 1,6-diphosphatase by pyridoxal-5′-P permits the obtention of active derivatives containing up to 4 moles of pyridoxyl-5′-P/mole of enzyme, which were no longer sensitive to either the allosteric AMP inhibition or to the high substrate inhibition characteristic of most fructose 1,6-diphosphatases. A somewhat higher incorporation of reagent was obtained by Krulwich *et al.* (1969), who also demonstrated an almost complete desensitization of rabbit liver and kidney fructose 1,6-diphosphatases toward AMP inhibition by modification with pyridoxal-5′-P.

With this background, we have now studied in more detail the modification of pig kidney fructose 1,6-diphosphatase with pyridoxal-5′-P in order to obtain active derivatives with a limited number of modified ϵ-aminolysyl residues, in which only the allosteric properties of the enzyme have been altered.

Experimental Section

Chemicals. The materials used in this study were obtained from the following sources: fructose 1,6-diphosphate (stock no. 750-1), AMP, Tris, EDTA, pyridoxal-5′-P, $NaBH_4$, Sigma Chemical Co.; ammonium sulfate (Art. No. 1211) for biochemical use, Merck AG, Darmstadt; P-cellulose (Cellex-P), Bio-Rad Laboratories; Sephadex G-50 (fine), Sephadex G-200, Pharmacia; uniformly labeled [^{14}C]AMP (diammonium) and [^{14}C]fructose 1,6-diphosphate (sodium), New England Nuclear; all other were proanalysis Merck AG, Darmstadt, Chemicals.

Assays. Fructose 1,6-diphosphatase concentration was determined by its absorbancy at 280 nm using a value of 0.755 for absorbancy per mg per ml (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). Reaction mixtures have been modified, introducing the presence of potassium ions in the assay system (Hubert *et al.*, 1970). 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 $MgSO_4$, 150 mM K_2SO_4, 0.5 mM fructose 1,6-diphosphate, 0.1 mM EDTA, and 0.10 ml of a solution containing fructose 1,6-diphosphatase. 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.

A unit of fructose 1,6-diphosphatase activity is defined as that amount of enzyme which catalyzes the formation of 1 μmole of inorganic phosphate/min at 30° under the conditions described. Specific activity is expressed in terms of units per milligram of protein.

Purification of Pig Kidney Fructose 1,6-Diphosphatase. The enzyme was purified by a modification of the original procedure of Marcus (1967). Since the modified version in-

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cludes several important changes, which simplifies the purification method, it is described herein with some detail. Unless otherwise stated, the manipulations were carried out at 2–4°.

EXTRACTION AND ACID FRACTIONATION. Approximately 350 g of pig kidney cortex was homogenized for 2 min in a Waring Blendor with two volumes of cold 1.0 mM EDTA (pH 8.0). The homogenate was then adjusted to pH 4.5 with 1.7 M CH_3COOH. The solution was stirred for 5 min and the precipitate was discarded by centrifuging for 30 min at 5000*g* (fraction I).

AMMOMIUM SULFATE FRACTIONATION. This step was carried out as described in step 3 of our earlier procedure (Marcus, 1967), except that the precipitate finally obtained (0.33–0.52 ammonium sulfate saturation) was redissolved with 30–40 ml of 10 mM potassium malonate buffer (pH 6.6) containing 0.1 mM EDTA, and dialyzed three times (for at least 8 hr each) against 1000 ml of the same buffer (fraction II).

FRACTIONATION ON P-CELLULOSE. The chromatographic separation described here was carried out at room temperature. Fraction II was applied to a P-cellulose column (1.9 × 40 cm) which had been equilibrated with 10 mM malonate buffer (pH 6.6) containing 0.1 mM EDTA. Once the enzyme solution had passed into the resin, the column was washed with 20 mM malonate buffer (pH 6.6) containing 0.1 mM EDTA to remove a large peak of protein without fructose 1,6-diphosphatase activity. After 700 ml, absorbancy at 280 nm was approximately 0.100; elution of fructose 1,6-diphosphatase was carried out with 20 mM malonate buffer (pH 6.6) containing 0.1 mM EDTA, 0.5 mM fructose 1,6-diphosphate, and 0.5 mM AMP (Pogell, 1962; Carminatti *et al.*, 1969). Fractions of 5 ml were collected at a flow rate of 0.8 ml/min. A peak, with fructose 1,6-diphosphatase activity, appeared with the front of the eluent containing fructose 1,6-diphosphate and AMP. Fructose 1,6-diphosphatase activity was measured and those fractions having more than 20 units/ml were collected and precipitated by the addition of solid ammonium sulfate to 0.90 saturation. After 1-hr equilibration, the precipitate was collected by centrifugation for 1 hr at 35,000*g* and redissolved with 6–7 ml of a buffer (pH 7.5) containing 20 mM Tris-HCl, 2 mM $MgSO_4$, and 0.1 mM EDTA. Finally it was desalted on a column (1.9 × 34 cm) of Sephadex G-50 fine. Fractions of 1 ml were collected, absorbancy at 280 nm was measured, and those fractions having an absorbance higher than 1.00 were pooled (fraction III).

HEAT FRACTIONATION. Fraction III was heated to 60° in a water bath and maintained at this temperature for 8 min. The precipitate was discarded by centrifuging for 30 min at 27,000*g*, and the supernatant obtained was stored frozen until used (fraction IV).

The results of the purification procedure are summarized in Table I. The purified fructose 1,6-diphosphatase was shown to be homogeneous by analytical centrifugation, and except for the presence of a trace band, it was also homogeneous on disc gel electrophoresis.

Preparation of Pyridoxyl-5′-P–Fructose 1,6-Diphosphatases. Fructose 1,6-diphosphatase as obtained in fraction IV of the purification procedure was dialyzed against 1000 ml of 0.5 M KCl containing 0.1 mM EDTA. The enzyme (1.25×10^{-5} M) was then incubated with pyridoxal-5′-P at 30° for 15 min in 100 mM sodium borate buffer (pH 8.0). The solution was then cooled to 0° and 2 drops of octyl alcohol was added to avoid foaming. A few milligrams of solid $NaBH_4$ (just enough to produce decoloration of the reaction system) was then added and after 5 min at 0°, 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 0.8 ml were collected, absorbancy was measured at 280 and 325 nm, and only those fractions having an absorbance higher than 1.00 at 280 nm and a constant ratio of absorbancies (absorbance at 325 nm:absorbance at 280 nm) were pooled and stored frozen until used. The number of P-pyridoxylamino groups present in pyridoxyl-5′-P–fructose 1,6-diphosphatases was estimated from the absorbance at 325 nm by using the molar extinction coefficient of 10,150 for pyridoxyl-5′-P–lysine (Fischer *et al.*, 1963), and by assuming that pyridoxyl-5′-P–fructose 1,6-diphosphatase has a comparable absorption. The molecular weight of pig kidney fructose 1,6-diphosphatase was taken as 130,000 (Mendicino *et al.*, 1968).

TABLE I: Modified Preparation of Pig Kidney Fructose 1,6-Diphosphatase.[a]

Fraction[b]	Total Units	Total Protein (mg)	Sp Act.[c]	Yield (%)
I	4650	9796	0.5	100
II	2380	2746	0.9	51
III	1157	65	17.8	25
IV	945	52	18.2	20

[a] The initial amount of pig kidneys was 365 g. [b] The fraction numbers corresponds to those described in the text. [c] Specific activity is expressed in units per mg.

Binding Studies. With minor modifications, the binding of AMP and fructose 1,6-diphosphate was measured by gel filtration as described by Sarngadharan *et al.* (1969). All these studies were carried out at 3°. Fructose 1,6-diphosphatase or pyridoxyl-5′-P–fructose 1,6-diphosphatase was previously desalted on a column of Sephadex G-50 fine, equilibrated with 50 mM Tris-HCl buffer (pH 7.5 at 3°) containing 0.1 mM EDTA.

For AMP binding studies, columns of Sephadex G-50 fine (1.3 × 24 cm) were equilibrated with 50 mM Tris-HCl buffer (pH 7.5 at 3°) containing 0.1 mM EDTA, 0.2 mM fructose 1,6-diphosphate and [^{14}C]AMP as indicated. The gel surface was stabilized by using a disc of glass filter paper. Enzyme samples (1 ml) in the solution used for column equili bration were applied to the top of the column, and allowed to enter the gel. The column was eluted with more of the same solution and fractions of 1.2–1.3 ml were collected at a flow rate of 12 ml/hr; 0.5 ml of each fraction was counted in a 6801 Nuclear-Chicago scintillation spectrometer employing 14 ml of a scintillation solution prepared with 500 ml of absolute ethanol and 1000 ml of toluene containing 4 g of 2,5-diphenyloxazole and 0.1 g of 2,2-*p*-1,4-bis[2-(5-phenyloxazolyl)]benzene. Calculations were made as indicated by Kemp and Krebs (1967).

The same procedure mentioned above was used for measuring the binding of [^{14}C]fructose 1,6-diphosphate, except that Tris-HCl buffers did not contain EDTA.

Results

Modification of Fructose 1,6-Diphosphatase with Pyridoxal 5′-Phosphate. In a previous paper from this laboratory (Mar-

TABLE II: Effect of Substrate and AMP on Modification of Fructose 1,6-Diphosphatase by Pyridoxal-5′-P.[a]

Enzyme	Sp Act.[b] (Units/mg)	Inhibn by Excess Substrate (%)	Inhibn by AMP (%)	Pyridoxyl-5′-P Incorp (Moles/Mole)
1. Native	15	58	96	
2. Treated with $NaBH_4$[c]	16.2	57	96	
3. Modified	6.9	25	10	5.3
4. Modified in the presence of 10 mM fructose 1,6-diphosphate	13.6	53	17	4.0
5. Modified in the presence of 2.5 mM AMP	4.7	23	39	3.7
6. Modified in the presence of 10 mM fructose 1,6-diphosphate plus 2.5 mM AMP	14.7	52	94	2.5

[a] Modification by pyridoxal-5′-P (expt 3–6) was carried out as described in the preparation of pyridoxyl-5′-P–fructose 1,6-diphosphatases using 5 mM pyridoxal-5′-P and other additions as indicated. Specific activity was measured at pH 7.5 as described in the text. For substrate inhibition the same method was used, but at 10 mM fructose 1,6-diphosphate. AMP inhibition was measured at 0.5 mM fructose 1,6-diphosphate plus 0.2 mM AMP. [b] Fructose 1,6-diphosphatase concentration was determined by its absorbancy at 280 nm (Marcus and Hubert, 1968). No corrections were made for the changes in absorbance due to incorporation of pyridoxyl-P into the protein. [c] Control enzyme treated with $NaBH_4$ in the absence of pyridoxal-5′-P.

cus and Hubert, 1968) we have shown that modification of pig kidney fructose 1,6-diphosphatase with pyridoxal-5′-P can lead to the formation of pyridoxyl-5′-P derivatives of the enzyme with altered sensitivities to the allosteric AMP inhibition and to the high substrate inhibition characteristic of most fructose 1,6-diphosphatases. In expt 3 of Table II such a result is shown, together with experiments showing the effect exerted by fructose 1,6-diphosphate, AMP, or both, on the modification of fructose 1,6-diphosphatase by pyridoxal-5′-P.

As it can be seen, fructose 1,6-diphosphate selectively protects against loss of high substrate inhibition by pyridoxal-5′-P treatment, but does not protect against desensitization toward AMP inhibition (Table II, expt 4). Conversely, AMP exerts no protection against the loss of high substrate inhibition but partially protects against the loss of AMP inhibition (Table II, expt 5). That AMP only affords partial protection against desensitization can be explained on the basis of the reported requirement of fructose 1,6-diphosphate for AMP binding (Watanabe *et al.*, 1968; Sarngadharan *et al.*, 1969). This explanation is confirmed in Table II (expt 6) showing that when both fructose 1,6-diphosphate and AMP are present during pyridoxal-5′-P treatment, complete protection from the effects of pyridoxal-5′-P is observed.

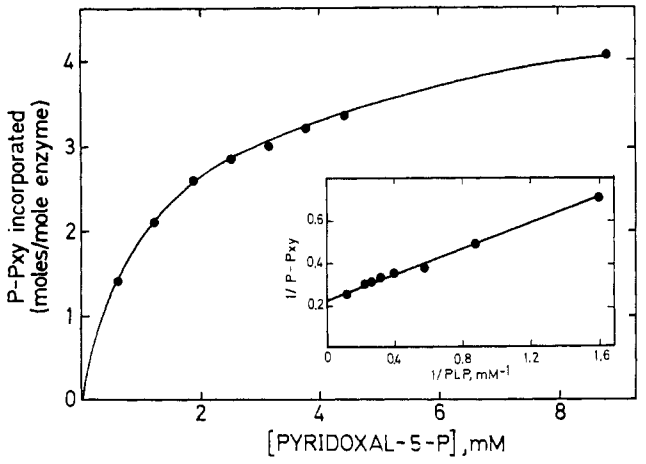

FIGURE 1: Incorporation of pyridoxal-5′-P into fructose 1,6-diphosphatase in the presence of substrate, as a function of pyridoxal-5′-P concentration. Enzyme derivatives were prepared as described in the Experimental Section, excepting that modification was carried out in the presence of 10 mM fructose 1,6-diphosphate and pyridoxal-5′-P as indicated. The *inset* shows a double reciprocal plot of data from this figure, in which theab breviations PLP and P-Pxy are used for pyridoxal-5′-P and pyridoxyl-5′-P, respectively.

With these preliminary observations, our interest was centered on those conditions (presence of fructose 1,6-diphosphate during pyridoxal-5′-P treatment) which selectively alter only the AMP inhibition. Figure 1 shows the number of moles of pyridoxyl-5′-P incorporated per mole of enzyme, when variable amounts of pyridoxal-5′-P are used for modification of fructose 1,6-diphosphatase in the presence of fructose 1,6-diphosphate. The result shows a typical saturation curve, from which a maximum value of 4.35 moles of pyridoxyl-5′-P incorporated per mole of enzyme is obtained from the vertical intercept of a double-reciprocal plot (inset of Figure 1).

Properties of Pyridoxyl-5′-P–Fructose 1,6-Diphosphatases. Some properties of modified fructose 1,6-diphosphatase containing variable amounts of pyridoxyl-5′-P per mole of enzyme are shown in Table III, demonstrating that the introduction of pyridoxyl-5′-P into the enzyme molecule only changes the response to the inhibitor AMP, while specific activity and inhibition by excess substrate are not significantly affected. Furthermore, no significant alteration of the Mg saturation curves was observed after modification of fructose 1,6-diphosphatase with pyridoxal-5′-P.

A summary of a detailed study of the AMP inhibition of fructose 1,6-diphosphatase derivatives containing different amounts of pyridoxyl-5′-P is given in Figure 2 and Table IV. The data included in Figure 2 show that a significant change occurred in the response of the modified enzymes to AMP inhibition as compared to the native fructose 1,6-diphos-

TABLE III: Properties of Pyridoxyl-5′-P–Fructose 1,6-Diphosphatases.[a]

Expt	Enzyme Derivative (Moles of Pyridoxyl-5′-P/Mole)	Sp Act.	Inhibn by Excess Substrate (%)	Inhibn by AMP (%)
1	0	14.6	44	91
2	1.42	15.6		52
3	2.06	13.9	46	37
4	2.60	15.5	45	32
5	2.86	14.7	41	28
6	3.01	12.5	40	22
7	3.21	13.1	40	22
8	3.36	13.5	38	21
9	4.06	12.5	40	19

[a] Enzyme derivatives were those prepared in the experiment described in Figure 1. Assays were carried out as described in the legend of Table II.

phatase. Table IV summarizes K_i and n values obtained for pyridoxyl-5′-P–fructose 1,6-diphosphatases, when the inhibition data were analyzed according to Taketa and Pogell (1965) as $\log (V_0 - v)/v$ *vs.* log AMP. It may be seen in Table IV that the change in AMP sensitivity is due to increases in the K_i value, which increased from a value of 0.05 mM for the native enzyme up to 0.94 mM of the derivative containing 4.06 moles of pyridoxyl-5′-P/mole of enzyme. Furthermore, modification of fructose 1,6-diphosphatase by pyridoxal-5′-P abolished cooperative interaction among AMP binding sites, since n equals approximately 1.0 in all the modified enzymes compared to the value of 1.47 obtained for the native enzyme.

AMP binding studies were then carried out to see whether

TABLE IV: K_i and n Values of Pyridoxyl-5′-P–Fructose 1,6-Diphosphatases.[a]

Expt	Enzyme Derivative (Moles of Pyridoxyl-5′-P/Mole)	K_i (mM)	n
1	0	0.05	1.47
2	1.42	0.24	0.98
3	2.06	0.52	0.92
4	2.60	0.57	0.91
5	2.86	0.70	0.90
6	3.01	0.69	0.91
7	3.21	0.77	0.91
8	3.36	0.87	0.99
9	4.06	0.94	0.90

[a] Hill plots according to Taketa and Pogell (1965) were used. K_i and n were obtained by a least-squares fit for a straight line with a program for a Hewlett-Packard 9100 A calculator.

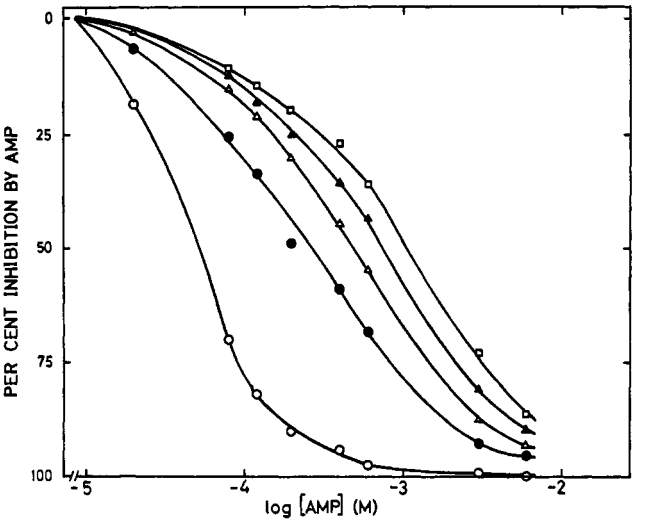

FIGURE 2: Percentage inhibition of native- and pyridoxyl-5′-P–fructose 1,6-diphosphatases at different AMP concentrations. The assays were performed at pH 7.5 as described in the Experimental Section, except that AMP was added as indicated. (○) Native fructose 1,6-diphosphatase, (●) pyridoxyl-5′-P–fructose 1,6-diphosphatase (1.42 moles/mole of enzyme), (△) pyridoxyl-5′-P–fructose 1,6-diphosphatase (2.06 moles/mole of enzyme), (▲) pyridoxyl-5′-P–fructose 1,6-diphosphatase (3.01 moles/mole of enzyme), and (□) pyridoxyl-5′P–fructose 1,6-diphosphatase (4.06 moles/mole of enzyme).

the decreased AMP inhibition of pyridoxyl-5′-P–fructose 1,6-diphosphatases were due to reduced binding of AMP to the modified enzymes. As shown in Figure 3, this was the case, since under conditions in which the native enzyme binds 3.48 moles of AMP/mole of enzyme, modified enzyme containing 2.6 moles of pyridoxyl-5′-P/mole of enzyme binds only 0.83 mole of AMP. Similar results were obtained in AMP binding studies carried out at pH 8.6 and 3°. Under these conditions, AMP binding of modified fructose 1,6-diphosphatases containing 2.1 and 4.1 moles of pyridoxyl-5′-P per mole of enzyme was less than 10% of the binding value obtained with the native enzyme.

As a control, substrate binding studies showed no significant differences of fructose 1,6-diphosphate binding between the native and modified fructose 1,6-diphosphatases.

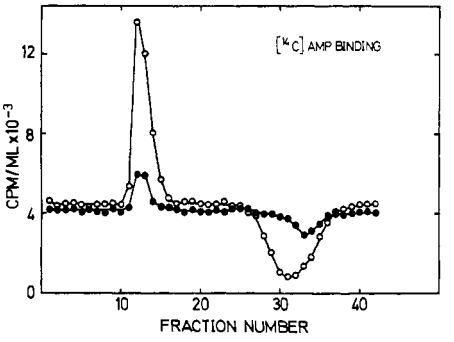

FIGURE 3: Elution profiles for the binding of AMP by native and modified fructose 1,6-diphosphatase. Conditions are described in the Experimental Section. The buffer contained 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 mM fructose 1,6-diphosphate, and 6 μM [^{14}C]AMP (specific radioactivity, 6×10^5 cpm/μmole). (○) 1.5 mg of fructose 1,6-diphosphatase and (●) 1.2 mg of fructose 1,6-diphosphatase containing 2.6 moles of pyridoxyl-5′-P/mole of enzyme.

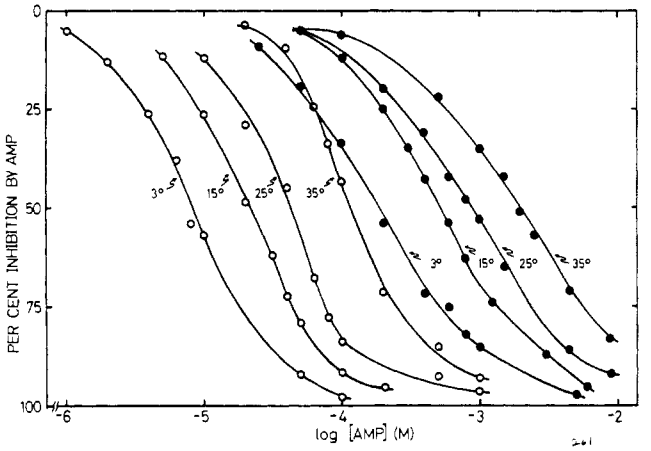

FIGURE 4: Percentage inhibition of native and pyridoxyl-5′-P–fructose 1,6-diphosphatase by varying concentrations of AMP at different temperatures. The assays were performed at pH 7.5 at the selected temperatures, as described in the Experimental Section, except that AMP was added as indicated. (○) Native fructose 1,6-diphosphatase and (●) pyridoxyl-5′-P–fructose 1,6-diphosphatase (4.1 moles/mole of enzyme).

Under similar experimental conditions, the native enzyme and a modified enzyme containing 2.6 moles of pyridoxyl-5′-P/mole of enzyme bind 1.54 and 1.47 moles of fructose 1,6-diphosphate per mole of enzyme, respectively.

The alteration of the regulatory properties of fructose 1,6-diphosphatase after modification with pyridoxal-5′-P was not due to dissociation of the enzyme into subunits as studied by gel filtration. A single protein peak of V/V_0 of approximately 1.57 was obtained when a mixture of native enzyme (6.6 mg) and fructose 1,6-diphosphatase containing 3.0 moles of pyridoxyl-5′-P per mole of enzyme (6.6 mg) was subjected to gel filtration on a 1.8 × 58 cm column of Sephadex G-200.

Since the modulation of fructose 1,6-diphosphatase activity by AMP has been shown to be highly dependent on temperature (Taketa and Pogell, 1965) it seemed important to see whether this property was also present in the enzyme after modification with pyridoxal-5′-P. As shown in Figure 4, a derivative containing 4.1 moles of pyridoxyl-5′-P/mole of enzyme, although much less sensitive to AMP inhibition, still shows temperature-dependent AMP sensitivity. K_i[1] and n obtained from the data of Figure 4 are summarized in Table V, which also includes a measure of the dependence of K_i on temperature (relative K_i), showing no large alteration of this parameter after modification of fructose 1,6-diphosphatase by pyridoxal-5′-P, although cooperativity is abolished at all temperatures.

TABLE V: K_i and n of Native- and Pyridoxyl-5′-P–Fructose 1,6-Diphosphatase as a Function of Temperature.[a]

Temp (°C)	Native Enzyme K_i (mM)	Rel K_i[b]	n	Pyridoxyl-5′-P–Enzyme K_i (mM)	Rel K_i[c]	n
3	0.008	(1)	1.44	0.188	(1)	1.05
15	0.020	2.5	1.45	0.525	2.8	1.15
25	0.038	4.6	1.62	0.852	4.4	1.04
35	0.104	16.0	1.54	1.950	10.4	1.00

[a] The data of Figure 4 were used to calculate K_i and n according to Taketa and Pogell (1965). [b] A relative value of 1 was given to the K_i obtained for the native enzyme at 3°. [c] A relative value of 1 was given to the K_i obtained for the pyridoxyl-5′-P–enzyme at 3°.

Discussion

The highly selective reaction of pyridoxal 5′-phosphate with only a few reactive ε-aminolysyl residues in enzymes that do not require this compound as a coenzyme makes of this reagent an extremely useful tool for the identification of ε-aminolysyl groups participating in enzyme function (Anderson *et al.*, 1966; Rippa *et al.*, 1967; Shapiro *et al.*, 1968; Ronchi *et al.*, 1969; Johnson and Deal, 1970; Miyake and Yamano, 1970). The best studied example is that of bovine liver glutamate dehydrogenase in which the reactive lysyl residue essential for catalytic activity has been located in the amino acid sequence (Piskiewicz *et al.*, 1970; Smith *et al.*, 1970).

The experiments presented here make use of pyridoxal-5′-P to confirm and extend previous studies of modification of fructose 1,6-diphosphatase with pyridoxal-5′-P, which suggested the participation of lysine residues in allosteric regulation of fructose 1,6-diphosphatase activity (Marcus and Hubert, 1968; Krulwich *et al.*, 1969). The modification by pyridoxal-5′-P has now been made selective for amino groups related to allosteric AMP inhibition by performing the reaction in the presence of the substrate, fructose 1,6-diphosphate. As a result of modification in these conditions, the enzyme derivatives obtained show reduced AMP binding, and a complete loss of cooperativity among AMP binding sites. Conversely, none of the catalytic properties tested (activity measured in the absence of AMP, high substrate inhibition, Mg saturation, substrate binding) were significantly altered. Since the modification leading to the loss of the regulatory properties follows a typical saturation curve approximating a maximum incorporation of 4 moles of pyridoxyl-5′-P/mole of fructose 1,6-diphosphatase (Figure 1), these findings can be related with those demonstrating the existence of four AMP binding sites per mole of enzyme (Pontremoli *et al.*, 1968; Sarngadharan *et al.*, 1969), as well as with recent preliminary evidence which is postulating for fructose 1,6-diphosphatase a model of four identical subunits (Black *et al.*, 1971). This suggests that the changes in regulatory properties of fructose 1,6-diphosphatase are the result of the modification of a single lysyl residue per subunit. Experiments are in progress to prove this hypothesis, and then identify the lysine residues involved in allosteric regulation of fructose 1,6-diphosphatase activity.

The most significant conclusions arising from the present work are the following. (a) From a total number of approximately 100 lysyl residues present in fructose 1.6-diphosphatase (Mendicino *et al.*, 1968; Krulwich *et al.*, 1969) there is only a limited number, 4, which are modified by pyridoxal-5′-P in the presence of substrate. This can be taken as an indication of a set of lysyl residues of unusual reactivity. (b) These reactive lysyl residues are necessary structural elements for cooperative interaction between subunits, since cooperativity is abolished by their modification with pyridoxal-5′-P. As

[1] K_i denotes concentration of AMP necessary for 50% inhibition.

a consequence, affinity for AMP is greatly decreased. Important to mention is the fact that modification of less than two residues per mole of enzyme is sufficient to abolish cooperativity. (c) The reactivity of these lysyl residues diminishes in the presence of substrate plus AMP, since no changes in the properties of the enzyme are observed when modification with pyridoxal-5′-P is carried out in such conditions (Table II, expt 6). Furthermore, the number of moles of pyridoxyl-5′-P incorporated decreases. (d) The chemical modification is probably introducing only slight structural changes in localized regions of the enzyme molecule, since no alterations in catalytic properties are observed and the modified enzyme still shows temperature-dependent AMP sensitivity.

Whether these reactive lysyl residues, which are necessary structural elements for cooperativity interaction between subunits, are located at, close to, or distant from the AMP binding site cannot be established with the present data without entering into speculation. The first interpretation appears to be the less plausible, in view of the results obtained in the temperature-dependence studies of the AMP inhibition, which showed that although cooperativity has been abolished, the K_i of the modified enzyme for AMP at 3° is of the same order as that obtained in the native enzyme at 35° (Table V). As a working hypothesis, and by analogy with the so well studied model of allosteric enzymes, hemoglobin, it could be suggested that modification occurs at specially reactive lysyl residues located at a subunit interface which is required for retention of cooperativity. The analogy is made considering the role of the sulfhydryl group at position 93 in the β chain of hemoglobin. As elegantly shown recently by following directly the effects of chemical modification of this highly reactive residue (Simon *et al.*, 1971; Moffat, 1971; Moffat *et al.*, 1971), whose reactivity depends on the degree of oxygenation of hemoglobin (Antonini and Brunori, 1969), its environment is involved in the structural changes which accompany cooperativity.

Since the question about the number of subunits of fructose 1,6-diphosphatase, and their identity or not, has not been definitely resolved, no considerations are made here about this point. However, this discussion would be equally valid if the enzyme were composed of four regulatory and four catalytic subunits (Sia *et al.*, 1969) or of four identical subunits (Black *et al.*, 1971), in which case each subunit should contain one catalytic and one regulatory site.

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