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The role of Cys-17 in the pyridoxal 5′-phosphate inhibition of the bovine liver low M_r phosphotyrosine protein phosphatase

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Mammalian tissues contain two low M_r phosphotyrosine protein phosphatase isoforms (type-1 and type-2) that differ in the 40–73 amino-acid sequence. Only one isoform (type-2) is strongly inhibited by pyridoxal 5′-phosphate, whereas the other is poorly inhibited by this compound. The mechanism of pyridoxal 5′-phosphate inhibition of the bovine liver enzyme (a type-2 isoform) has been studied by kinetic methods using a series of pyridoxal 5′-phosphate analogues. These studies indicate that pyridoxal 5′-phosphate interacts with the enzyme in both the phosphate and aldehyde groups. Active site-directed mutagenesis has been used to investigate the sites of pyridoxal 5′-phosphate binding. Our results indicate that Cys-17, essential for enzyme activity, interacts with the phosphate moiety of pyridoxal 5′-phosphate. On the other hand, Cys-12, which is also involved in the catalytic mechanism, does not participate in pyridoxal 5′-phosphate binding.

Introduction

Bovine liver low M_r phosphotyrosine protein phosphatase (PTPase) has previously been known as low M_r acid phosphatase, since this activity was first demonstrated by Heinrikson [1]. Some authors have demonstrated that enzymes from this family possess specific PTPase activity [2–6]. A bovine liver enzyme from this family has been sequenced in our laboratory [7]. An identical enzyme, purified from bovine heart [6], has been recently studied with respect to the reaction mechanism [8,9]. Furthermore, Wo et al. [10] demonstrated that a cysteinyl-phosphate covalent intermediate was formed during the catalysis. Multiple molecular forms of this enzyme were found in various mammalian tissues, such as human erythrocytes [11], chicken muscle [12] and rat liver [13]. The two rat liver isoenzymes (AcP1 and AcP2) have been recently sequenced in our laboratory (14); they (like bovine liver enzyme) consist of a 157 amino-acid chain and possess an identical sequence in the 1–39 and 74–157 zones, whereas they strongly differ in the 40–73 region. In this zone, about 50% of residues are substituted by different amino acids. A very similar picture was also found for human erythrocyte B_{fast} and B_{slow} isoenzymes [15,16]. These results indicate that mammalian tissues contain a couple of low M_r PTPases that probably originated from a single gene by an alternative splicing mechanism [15,16]. The physiological functions of the two isoenzymes are currently investigated in our laboratory; the preliminary results indicate that these isoenzymes have different substrate affinities and sensitivities to activator and inhibitor compounds [14]. The bovine liver enzyme is highly homologous to rat liver AcP2 and human erythrocyte B_{slow} isoenzymes.

Preliminary experiments carried out in our laboratory have demonstrated that rat liver AcP2, human erythrocyte B_{slow} and the bovine liver enzyme are all strongly inhibited by pyridoxal 5′-phosphate, whereas rat AcP1 and human B_{fast} are less sensitive to PLP inhibition. We suggest naming all low M_r PTPases that have high PLP-affinities type-2, and those that have low affinities for this compound type-1.

The present paper reports a study on bovine liver low M_r PTPase PLP inhibition. Site-directed mutagen-

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Abbreviations: PTPase, bovine liver low M_r phosphotyrosine protein phosphatase; MBP, maltose binding protein; PLP, pyridoxal 5′-phosphate; PAL, pyridoxal; PyN, pyridoxamine; PyNP, pyridoxamine 5′-phosphate; PyOH, pyridoxine; AcP1 and AcP2, rat liver phosphotyrosine protein phosphatase isoenzymes.

esis has been used to identify amino-acid residue(s) involved in PLP-enzyme binding.

Materials and Methods

Bovine liver low M_r PTPase was purified by the method described by Lawrence and Van Etten [17] with minor modifications. Pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, pyridoxal, pyridoxamine and pyridoxine were from Sigma. All other reagents used were of the highest purity commercially available.

Enzyme assay. Enzyme activity was determined using *p*-nitrophenylphosphate as substrate, as previously described [5]. The unit was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of *p*-nitrophenylphosphate per min at 37°C. Specific activity was expressed as units per mg of protein.

Protein assay. Protein concentration was determined by the method of Lowry et al. [18].

K_m, V_{max} and K_i measurements. The initial velocity measurements were carried out in duplicate. At least three inhibitor concentrations were used in K_i measurements. K_m and V_{max} were calculated by plotting the reciprocal of initial velocities measured at various substrate concentrations vs. the reciprocal of substrate concentrations according to Lineweaver and Burk [19]. K_i was calculated by replotting the apparent K_m or K_m/V_{max} values measured at different inhibitor concentrations.

Amino-acid analysis. Amino-acid analysis of the enzyme after treatment with PLP and sodium borohydride or sodium cyanoborohydride was carried out by the method of Spackman et al. [20] using a Carlo Erba 3A29 amino-acid analyzer equipped with a Spectra Physics SP 4100 computing integrator. Synthesis of ϵ-pyridoxyllysine for use as chromatographic standard was achieved by the method of McKinley-McKee and Morris [21], starting from *N*-carbobenzoxylysine.

Preparation of recombinant PTPase. The preparation of a synthetic gene coding for bovine liver low M_r PTPase has been recently described in our laboratory [22]. Mutants in Cys-12 to Ala and Ser and in Cys-17 to Ser were obtained as recently described by Chiarugi et al. [23].

Polyacrylamide gel electrophoresis. PAGE was performed by the method of Laemmli [24].

Equilibrium dialysis. Equilibrium dialysis was used to study the binding and dissociation constants between inactive mutants and PLP and P_i. The very simple apparatus described by Reinard and Jacobsen [25] was used to measure PLP and P_i concentrations after equilibrium dialysis was achieved. Protein concentrations in the range 5–25 μM for PLP and 100–200 μM for P_i binding experiments were used. [^{32}P]P_i was used in the P_i binding experiments. PLP concentrations were determined by HPLC analysis performed on a reverse phase C_{18} column (Supelco, 4.6 × 150 mm, 3 μm), using fluorescence detection. The column was equilibrated and eluted with 20% methanol containing 10 mM trifluoroacetic acid. The fluorimeter excitation wavelength was set at 290 nm and the emission wavelength was set at 400 nm. A linear calibration curve was obtained in the 25–800 pmol range. Pyridoxal was well separated from PLP and was found to be present at much lower concentrations with respect to PLP, after the equilibrium dialysis was achieved. The Scatchard plot was used to calculate the PLP-enzyme and P_i-enzyme complex dissociation constants. At least three inhibitor concentrations were used.

Results

Natural enzyme inhibition

All kinetic measurements were performed in 0.1 M sodium acetate buffer, (pH 5.5), containing 1 mM EDTA. PLP inhibition type and inhibition constants were determined by initial velocity measurements at a series of *p*-nitrophenylphosphate concentrations. The results were plotted according to Lineweaver and Burk [19] (see Fig. 1). The common intercept on the $1/v$ axis clearly indicates that the PLP inhibition is purely competitive with respect to *p*-nitrophenylphosphate. The K_i obtained from the linear plot of apparent K_m values vs. PLP concentrations was 14 μM. Fig. 2A reports the results for pyridoxal and Fig. 2B reports those for pyridoxamine 5′-phosphate. Both pyridoxal and pyridoxamine 5′-phosphate showed a competitive type inhibition. The K_i values were 3.8 mM for pyridoxal and 5.7 mM for pyridoxamine 5′-phosphate. Furthermore, Fig. 2C and D show the effect of pyridoxine

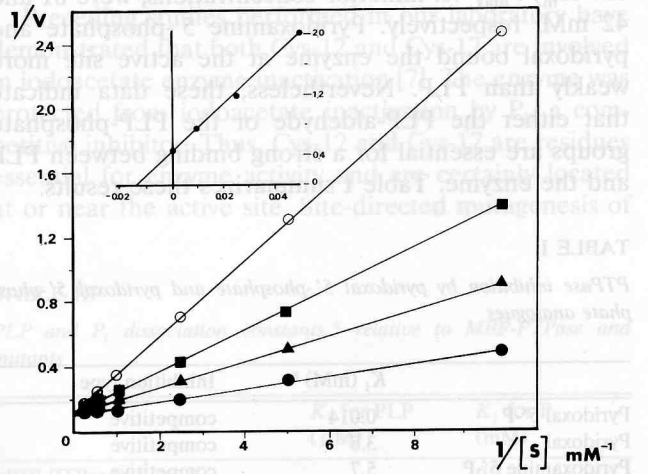

Fig. 1. Pyridoxal 5′-phosphate competitive inhibition of the bovine liver low M_r PTPase activity on *p*-nitrophenylphosphate. (●), without PLP; (▲), 0.01 mM PLP; (■), 0.025 mM PLP; (○), 0.05 mM PLP. Inset: secondary plot of the apparent K_m (ordinate) measured at different PLP concentrations against PLP concentration (abscissa).

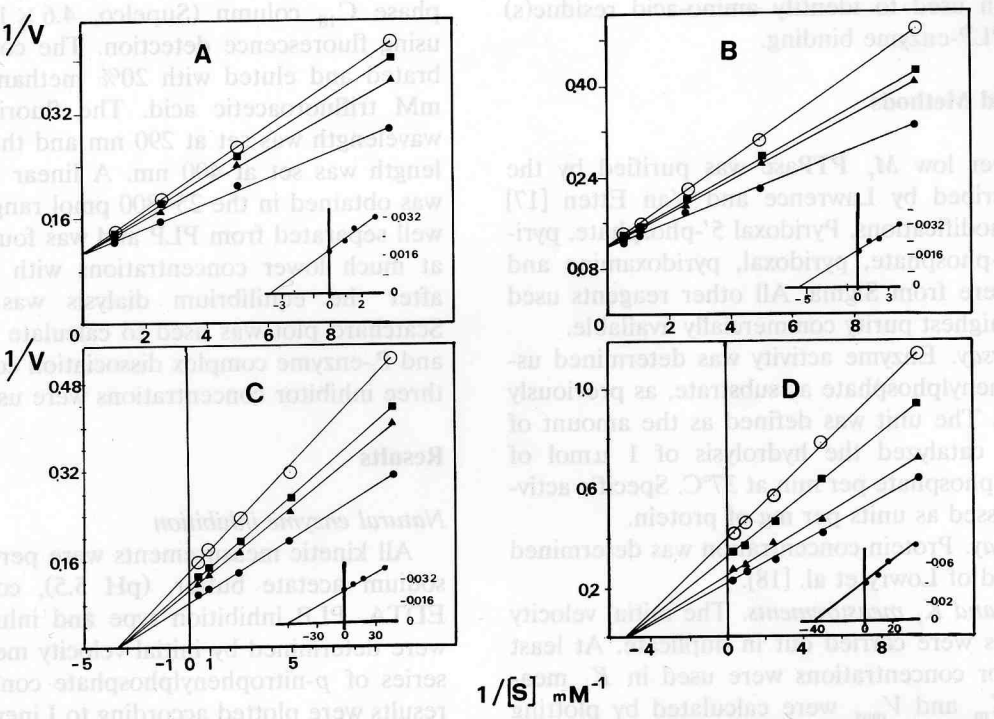

Fig. 2. Inhibition of low M_r PTPase by PLP-analogues. (A): pyridoxal competitive inhibition; (●), without PAL; (▲), 1 mM PAL; (■), 1.5 mM PAL; (○), 3 mM PAL. (B): pyridoxamine 5′-phosphate competitive inhibition; (●), without PyNP; (▲), 1 mM PyNP; (■), 2 mM PyNP; (○), 4 mM PyNP. (C): pyridoxine non-competitive inhibition; (●), without PyOH; (▲), 10 mM PyOH; (■), 20 mM PyOH; (○), 40 mM PyOH. (D): pyridoxamine non-competitive inhibition; (●), without PyN; (▲), 10 mM PyN; (■), 20 mM PyN; (○), 40 mM PyN. Inset: secondary plots of the apparent K_m (A and B, ordinate) or K_m / V_{max} (C and D, ordinate) vs. inhibitor concentrations (mM, abscissa).

and pyridoxamine on the enzyme activity. It can be seen that both these PLP analogues inhibit the enzyme by a non-competitive mechanism, since the curves obtained at different inhibitor concentrations have a common intercept on the $1/[S]$ axis of the Lineweaver-Burk plot, whereas they have different intercepts on the $1/v$ axis. The K_i values, calculated by replotting the apparent K_m/V_{max} vs. inhibitor concentrations, were 61 and 42 mM, respectively. Pyridoxamine 5′-phosphate and pyridoxal bound the enzyme at the active site more weakly than PLP. Nevertheless, these data indicate that either the PLP-aldehyde or the PLP-phosphate groups are essential for a strong binding between PLP and the enzyme. Table I summarizes these results.

TABLE I

PTPase inhibition by pyridoxal 5′-phosphate and pyridoxal 5′-phosphate analogues

	K_i (mM) [a]	Inhibition type
Pyridoxal 5′-P	0.014	competitive
Pyridoxal	3.8	competitive
Pyridoxamine 5′-P	5.7	competitive
Pyridoxamine	42	non-competitive
Pyridoxine	61	non-competitive

[a] Mean values of three different determinations.

Schiff-base formation testing

Several enzymes are found to be inhibited or inactivated by PLP. In most of, the reported cases [26], a Schiff-base formation between PLP and the enzyme was demonstrated. Thus, we searched for a Schiff-base formation between the bovine liver low M_r PTPase and PLP. The enzyme (22 nmol) was incubated with 250 nmol of PLP in a total volume of 0.5 ml of 0.1 M 3,3-dimethylglutarate buffer (pH 7.4) for 10 min at room temperature and then reduced by adding a 200-fold molar excess of sodium borohydride or sodium cyanoborohydride. During the addition of these reagents, the pH was adjusted to 6.0–6.5 with 2 M acetic acid. The reduced mixture was tested for enzyme activity: more than 80% of activity was recovered after reduction. Generally, a complete loss of enzyme activity after reduction of PLP-enzyme complexes to form a secondary amine was observed [26–30]. We have purified the enzyme from the reduced mixture using HPLC on a reverse-phase Aquapore RP300 column. The ultraviolet absorption spectrum showed no bands in the 325 nm region (Fig. 3), demonstrating that no secondary amine was formed in the reduced PLP-enzyme mixture. In addition, the enzyme thus purified was analyzed for amino-acid composition. ϵ-pyridoxyllysine was not found in the hydrolysate.

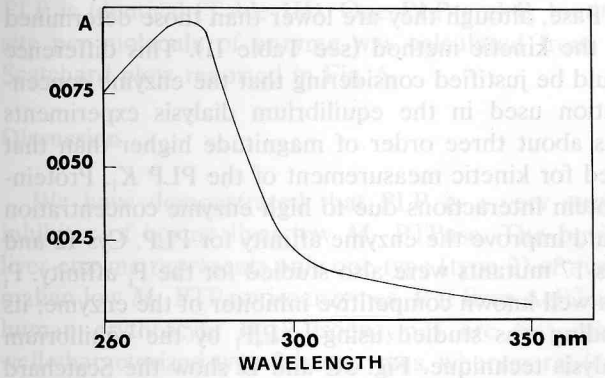

Fig. 3. Ultraviolet absorption spectrum of HPLC-purified PLP and borohydride-treated PTPase in 0.2 M ammonium bicarbonate solution.

Preparation and kinetic properties of the MBP-PTPase fusion protein

The synthetic gene coding for bovine liver low M_r PTPase was expressed in *Escherichia coli* (using the pMALc expression vector) as a fusion protein in which the enzyme is linked to the maltose binding protein through a peptide chain linker. The fusion protein was purified by affinity chromatography as described [23]. Polyacrylamide gel electrophoresis demonstrated that a single band of 60 kDa was present in the purified fusion protein preparation as expected, considering that the maltose binding protein was 42 kDa and the low molecular mass PTPase was 18 kDa. The protein was analyzed for its kinetic properties. Fig. 4A reports the Lineweaver-Burk plot from which a K_m of 0.3 mM and a V_{max} of 1517 units per μmol of fusion enzyme were calculated. These data agree with the K_m and V_{max} values of the natural enzyme, indicating that the fusion PTPase retained most of its original conformation and active site. Fig. 4A and B also report the Lineweaver-Burk plots of initial velocity at different *p*-nitrophenylphosphate and PLP or P_i concentrations. From these figures, it is evident that the PLP and P_i inhibitions of the recombinant fusion enzyme are purely competitive with respect to *p*-nitrophenylphosphate and that K_i values of 13 μM and 1.0 mM were calculated for PLP and P_i, respectively, by replotting the apparent K_m against PLP or P_i concentrations. The K_m, V_{max}, PLP and P_i K_i data for the fusion enzyme indicate that the active site is not greatly changed by the presence of the linked maltose binding protein (Table II).

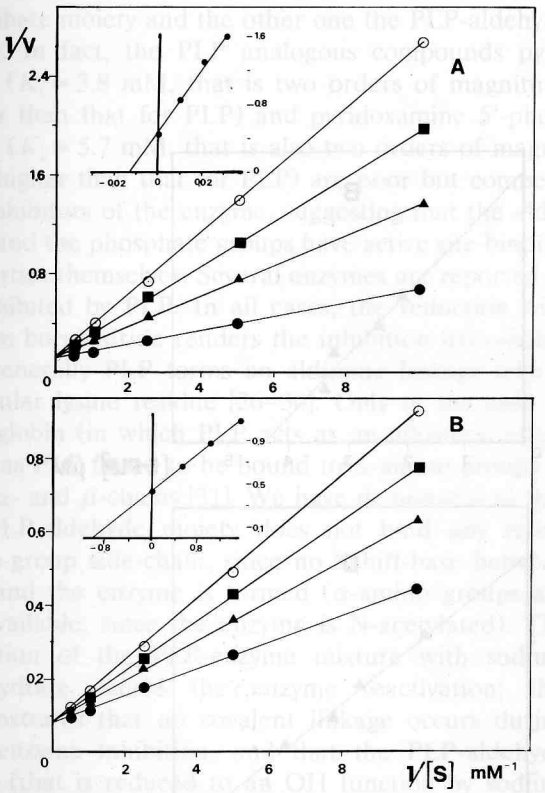

Fig. 4. PLP and P_i inhibition of MBP-PTPase fusion protein. A; (●), without PLP; (▲), 0.01 mM PLP; (■), 0.02 mM PLP; (○), 0.03 mM PLP. (B): (●), without P_i; (▲), 0.5 mM P_i; (■), 1.0 mM P_i; (○), 1.5 mM P_i. Inset: secondary plots of apparent K_m (ordinate) vs. inhibitor concentrations (mM, abscissa).

TABLE II

PTPase and MBP-PTPase kinetic properties comparison

Results are mean values of three different determinations.

	PTPase	MBP-PTPase
K_m on PNPP (mM)	0.2	0.3
Specific activity (units/μmol)	1795	1517
K_i for PLP (μM)	14	13
K_i for P_i (mM)	0.6	1.0

Site-directed mutagenesis

Preceding studies performed in our laboratory have demonstrated that both Cys-12 and Cys-17 are involved in iodoacetate enzyme inactivation [7]. The enzyme was protected from iodoacetate inactivation by P_i, a competitive inhibitor. Thus, Cys-12 and Cys-17 are residues essential for enzyme activity and are certainly located at or near the active site. Site-directed mutagenesis of

TABLE III

PLP and P_i dissociation constants [a] relative to MBP-PTPase and mutants

	K_i for PLP (μM)	K_i for P_i (mM)
MBP-PTPase		0.3
MBP-PTPase Cys-12 → Ala	1.0	
MBP-PTPase Cys-12 → Ser	2.0	0.2
MBP-PTPase Cys-17 → Ser	not bound	not bound

[a] Determined by the equilibrium dialysis method.

Cys-12 to Ala and to Ser and of Cys-17 to Ser was achieved as described [23]. The mutated genes were then expressed in *E. coli* using the pMALc expression vector and the mutated proteins were purified by affinity chromatography as described for the non mutated protein [23]. Both Cys-12 and Cys-17 mutants lost the enzyme activity, demonstrating their direct involvment in the active site [23]. Thus, we decided to test if these mutations have some effect on PLP-enzyme binding. The mutated fusion enzymes were directly used for this study, since, as we have demonstrated above, the non-mutated fusion enzyme retains most of the kinetic properties of the natural enzyme. The PLP-mutant binding was studied by the equilibrium dialysis technique. All experiments were performed in 0.1 M sodium acetate buffer (pH 5.5). Our results demonstrate that the mutation of Cys-17 to Ser (that produces an inactive enzyme form) results in the complete loss of PLP-binding capacity, whereas the mutations of Cys-12 to Ala or Ser (that also give inactive enzymes) produce proteins that are already able to bind PLP. Fig. 5A and B show the Scatchard plots that enabled us to calculate the dissociation constants for the enzyme-PLP complexes of 2.0 and 1.0 μM, for Cys-12 → Ser and Cys-12 → Ala mutants, respectively. These values are near to those found for the native enzyme and for MBP-PTPase, altough they are lower than those determined by the kinetic method (see Table II). This difference could be justified considering that the enzyme concentration used in the equilibrium dialysis experiments was about three order of magnitude higher than that used for kinetic measurement of the PLP K_i. Protein-protein interactions due to high enzyme concentration could improve the enzyme affinity for PLP. Cys-12 and Cys-17 mutants were also studied for the P_i affinity. P_i is a well known competitive inhibitor of the enzyme; its binding was studied using [^{32}P]P_i by the equilibrium dialysis technique. Fig. 5C and D show the Scatchard plots (relative to P_i binding) of the non-mutated fusion enzyme and of the Cys-12 → Ser mutant, respectively. Dissociation constants of 0.3 and 0.2 mM were calculated for the non-mutated fusion enzyme-P_i and for Cys-12 → Ser mutant-P_i complexes, respectively. A lowering of the K_i values for P_i with respect to that found by the kinetic method (see Table II) was probably due to the very high enzyme concentration used in the equilibrium dialysis experiments (about four order of magnitude higher than that used for the kinetic measurements). This effect agrees with that observed above for PLP. On the other hand, the Cys-17 → Ser mutant did not bind the inorganic phosphate. It is evident that the binding behavior of PTPase vs. P_i and

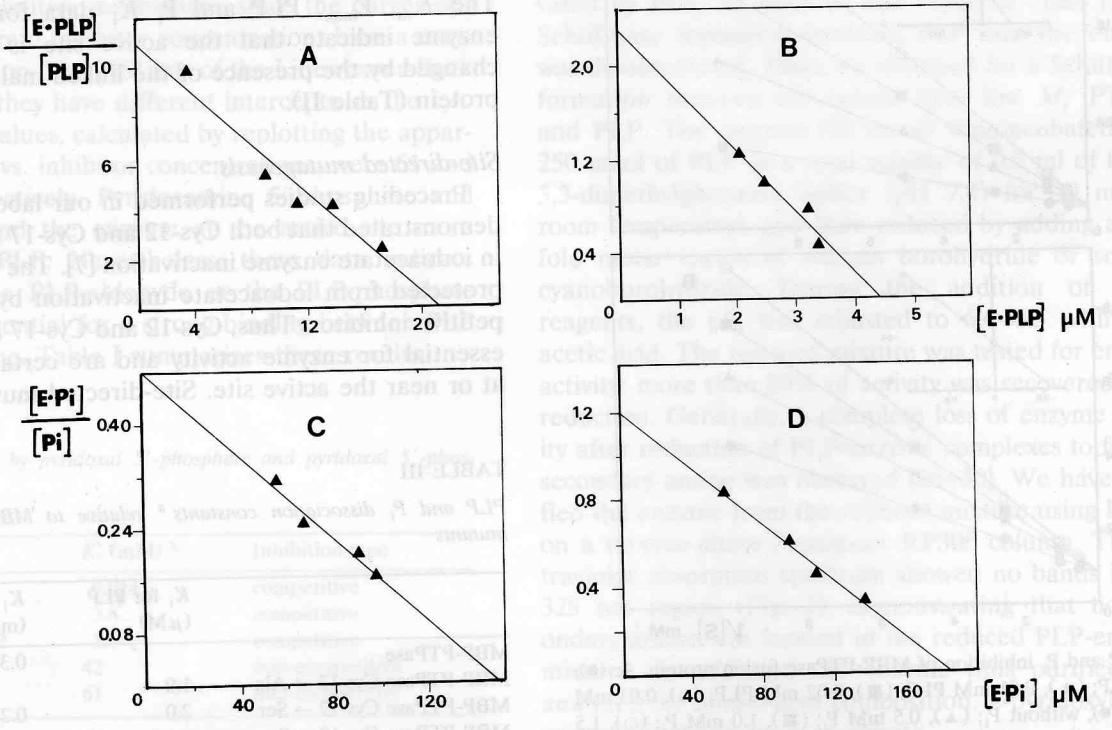

Fig. 5. PLP and P_i binding of MBP-PTPase and MBP-PTPase mutants (Scatchard plots). (A), (PLP), Cys-12 → Ser; (B), (PLP), Cys-12 → Ala; (C), (P_i), MBP-PTPase fusion protein; (D), (P_i), Cys-12 → Ser mutant.

PLP is identical (Table III). One PLP and P_i binding site per molecule of enzyme was calculated from all Scatchard plots reported in Fig. 5.

Discussion

We have demonstrated that PLP is a very strong inhibitor of bovine liver low M_r PTPase. The bovine liver enzyme represents only one type (type-2) of mammalian low M_r PTPase isoenzymes. Rat liver AcP2 and human erythrocyte B_{slow} isoenzymes are two other well-characterized type-2 isoenzymes, whereas rat liver AcP1 and human erythrocyte B_{fast} are type-1 isoenzymes. Only type-2 PTPases are strongly inhibited by PLP, whereas type-1 are poorly inhibited by this compound. There is a structural difference between type-1 and type-2 low M_r PTPases, since they have an alternative sequence in the 40–73 region. They are produced from a single gene through an alternative splicing mechanism (unpublished results). We have studied the PLP inhibition mechanism and have found that both the PLP-phosphate and the PLP-aldehyde groups are essential for strong binding at the active site. On the other hand, the PLP-analogues pyridoxamine and pyridoxine bind the enzyme very weakly with a non-competitive mechanism. PLP probably binds the enzyme at two distinct sites: the first one links the PLP-phosphate moiety and the other one the PLP-aldehyde group. In fact, the PLP analogous compounds pyridoxal ($K_i = 3.8$ mM, that is two orders of magnitude higher than that for PLP) and pyridoxamine 5′-phosphate ($K_i = 5.7$ mM, that is also two orders of magnitude higher than that for PLP) are poor but competitive inhibitors of the enzyme, suggesting that the aldehyde and the phosphate groups have active site binding properties themselves. Several enzymes are reported to be inhibited by PLP. In all cases, the reduction with sodium borohydride renders the inhibition irreversible and generally PLP forms an aldimine linkage with a particular lysine residue [26–30]. Only in the case of hemoglobin (in which PLP acts as an allosteric effector) was PLP found to be bound to α-amino groups of both α- and β-chains [31]. We have demonstrated that the PLP-aldehyde moiety does not bind any lysine amino-group side-chain, since no Schiff-base between PLP and the enzyme is formed (α-amino groups are not available, since the enzyme is N-acetylated). The reduction of the PLP-enzyme mixture with sodium borohydride causes the enzyme reactivation; this demonstrates that no covalent linkage occurs during PLP enzyme inhibition, and that the PLP-aldehyde group (that is reduced to an OH function by sodium borohydride) is essential for inhibition to occur. Site-directed mutagenesis experiments have demonstrated that the Cys-17 sulfur atom is essential for PLP binding, since the mutation of this residue to Ser abolishes the PLP binding capacity. Cys-17 was found to participate in the active site of the enzyme [7,23] and, thus, it is likely that this residue is directly involved in the PLP binding. A study on the iodoacetate inhibition mechanism has demonstrated that Cys-12 is also necessary for enzyme catalysis [7]. Site-directed mutagenesis has confirmed its participation in the active site.

Nevertheless, the mutation of Cys-12 either to Ser or Ala does not suppress the binding power of the enzyme for PLP. Although both Cys-12 and Cys-17 are located in the active site, only Cys-17 binds strongly to PLP whereas Cys-12 is not involved in the binding. Heinrikson et al. [32] reported that the enzyme rhodanese (that possesses an active-site Cys-247) was inactivated by phenylglyoxal (without modification of any Arg) through a redox reaction that involved the formation of an S-S bridge between the essential Cys-247 and either Cys-254 or Cys-263. We exclude that the PLP-aldehyde group could participate in enzyme inactivation of this PTPase through a redox reaction, since the inhibition was completely reversed by dialysis. Site-directed mutagenesis of Cys-12 and Cys-17 enables us to investigate the mechanism of PLP-enzyme binding. In particular, the equilibrium dialysis experiments with P_i demonstrate that the Cys-17 to Ser mutant loses binding power against the enzyme, suggesting that it is the PLP-phosphate moiety that binds Cys-17. The mutation of Cys-12 to Ser does not suppress the P_i binding, in agreement with PLP-binding experiments (see Table III). This suggests that this active site residue is not involved in the PLP binding. We underline that a Cys-17–Arg-18 sequence is present in this PTPase and that preceding experiments [33,34] have shown that arginine residue(s) are essential for the enzyme catalysis.

Arginine is a positively-charged residue that is generally involved in the binding of anionic groups of substrates. Finally, we can consider that type-1 PTPases also possesses a Cys-17–Arg-18 sequence. These isoenzymes bind PLP weakly. We think that the 40–73 zone (which is type-specific) may play a critical role in the PLP strong inhibition and probably contributes to the creation of a better PLP three-dimensional binding site.

Acknowledgements

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