Molecular mechanism of uncompetitive inhibition of human placental and germ-cell alkaline phosphatase

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Placental (PLAP) and germ-cell (GCAP) alkaline phosphatases are inhibited uncompetitively by L-Leu and L-Phe. Whereas L-Phe inhibits PLAP and GCAP to the same extent, L-Leu inhibits GCAP 17-fold more strongly than it does PLAP. This difference has been attributed [Hummer & Millán (1991) Biochem. J. **274**, 91–95] to a Glu → Gly substitution at position 429 in GCAP. The D-Phe and D-Leu enantiomorphs are also inhibitory through an uncompetitive mechanism but with greatly decreased efficiencies. Replacement of the active-site residue Arg-166 by Ala-166 changes the inhibition mechanism of the resulting PLAP mutant to a more complex mixed-type inhibition, with decreased affinities for L-Leu and L-Phe. The uncompetitive mechanism is restored on the simultaneous introduction of Gly-429 in the Ala-166 mutant, but the inhibitions of [Ala^{166},Gly^{429}]PLAP and even [Lys^{166},Gly^{429}]PLAP by L-Leu and L-Phe are considerably decreased compared with that of [Gly^{429}]PLAP. These findings point to the importance of Arg-166 during inhibition. Active-site binding of L-Leu requires the presence of covalently bound phosphate in the active-site pocket, and the inhibition of PLAP by L-Leu is pH-sensitive, gradually disappearing when the pH is decreased from 10.5 to 7.5. Our data are compatible with the following molecular model for the uncompetitive inhibition of PLAP and GCAP by L-Phe and L-Leu: after binding of a phosphorylated substrate to the active site, the guanidinium group of Arg-166 (normally involved in positioning phosphate) is redirected to the carboxy group of L-Leu (or L-Phe), thus stabilizing the inhibitor in the active site. Therefore leucinamide and leucinol are weaker inhibitors of [Gly^{429}]PLAP than is L-Leu. During this Arg-166-regulated event, the amino acid side group is positioned in the loop containing Glu-429 or Gly-429, leading to further stabilization. Replacement of Glu-429 by Gly-429 eliminates steric constraints experienced by the bulky L-Leu side group during its positioning and also increases the active-site accessibility for the inhibitor, providing the basis for the 17-fold difference in inhibition efficiency between PLAP and GCAP. Finally, the inhibitor's unprotonated amino group co-ordinates with the active-site Zn^{2+} ion 1, interfering with the hydrolysis of the phosphoenzyme intermediate, a phenomenon that determines the uncompetitive nature of the inhibition.

INTRODUCTION

Alkaline phosphatases (APs, EC 3.1.3.1) are a group of phosphomonoesterases that can catalyse the hydrolytic transfer of phosphate to water or its transphosphorylation to amino alcohols (Coleman & Gettins, 1983). Homologous proteins exist throughout speciation from bacteria to man. However, the physiological function of the eukaryotic molecules has not been elucidated [for review see McComb *et al.* (1979)]. In humans, APs are encoded by a gene family composed of four loci [for review see Harris (1989)]. Three tissue-specific AP (TSAP) genes, i.e. intestinal AP, placental AP (PLAP) and germ-cell AP (GCAP), are each composed of 11 exons occupying less than 5.0 kb of DNA and are co-localized in the long arm of chromosome 2. A single tissue-non-specific AP (TNAP) gene contains an additional differentially spliced exon in the 5′ region, significantly larger introns occupying 40–50 kb of DNA, and resides at the end of the short arm of chromosome 1.

The coding regions of the three TSAP genes are highly conserved and correspond to a 90–98% identity at the protein level (Millán, 1990). The amino acid sequence of the TNAP, on the other hand, is only 50–60% identical with either of the TSAPs. When compared with the AP from *Escherichia coli*, human APs show 33–35% overall sequence similarity. Residues involved in the co-ordination of Zn^{2+} and Mg^{2+} in the active site have been preserved, as well as Asp-91, Ser-92 and Arg-166, which are involved in substrate binding, suggesting that the catalytic reaction in human APs might proceed through steps and intermediates similar to those in the *E. coli* enzyme (Kim & Wyckoff, 1991). However, eukaryotic APs in general, and human APs in particular, display a unique kinetic property not shared by their bacterial ancestors, i.e. that of being inhibited stereospecifically by L-amino acids through an uncompetitive mechanism. Simple amino acids such as phenylalanine and leucine were found to be uncompetitive inhibitors of human TSAPs (Ghosh & Fishman, 1966; Fishman & Sie, 1971). Moreover, the inhibition is stereospecific, i.e. the L-isomer of phenylalanine, but not the D-isomer, was inhibitory (Fishman, 1989). In turn, L-homoarginine is a specific inhibitor of TNAP from a variety of species (Fishman & Sie, 1971). This ability of L-Phe to discriminate between TSAP and TNAP has been used in clinical quantification and identification of AP isoenzymes (Green *et al.*, 1971; Mulivor *et al.*, 1978). In addition, GCAP is inhibited by L-Leu with a 15–20-fold higher efficiency than is PLAP (Doellgast & Fishman, 1976). This difference in inhibitory potential provided, before the availability of specific DNA probes, the only means of discriminating between these closely related gene products. Indeed, although the two isoenzymes are encoded by different genes (Millán, 1986; Millán & Manes, 1988), PLAP and GCAP only differ by 12 amino acid residues and cannot easily be distinguished on the basis of their immunochemical or biophysical properties. Yet distinction of PLAP and GCAP is important because of the enhanced expression of GCAP in well-defined tumours where its determination serves as a useful

Abbreviations used: AP, alkaline phosphatase; PLAP, placental alkaline phosphatase; GCAP, germ-cell alkaline phosphatase; TSAP, tissue-specific alkaline phosphatase; TNAP, tissue-non-specific alkaline phosphatase; wt, wild type; pNPP, *p*-nitrophenyl phosphate.

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marker (Paiva *et al.*, 1983; Jeppsson *et al.*, 1984).

We have shown that Gly-429 is a major determinant of the uncompetitive inhibition of GCAP by L-Leu (Hummer & Millán, 1991). This residue is located in a surface loop, extending from position 400 to 430, that is unique to eukaryotic APs and has no equivalence in the *E. coli* molecule. We have shown that replacement of Glu-429 (found in PLAP) by Gly-429 (present in GCAP) causes a conformational change in the resulting mutant, which was detected by 16 out of 18 anti-PLAP monoclonal antibodies (Hoylaerts & Millán, 1991). The major role played by this amino acid residue in the L-Leu inhibition prompted us to investigate to what extent the surface loop was implicated in establishing the uncompetitive nature of this inhibition. In this paper we have determined that the inhibitor acts primarily by functionally interacting with Arg-166 and Zn^{2+} ion 1 through its carboxy and amino groups respectively, and that the conformation of the unique surface loop located in the vicinity of the active site influences both the positioning of the inhibitor and its accessibility to the active site.

MATERIALS AND METHODS

Wild-type enzymes and PLAP mutants

PLAP (corresponding to an F phenotype) and GCAP used in this study have been described previously (Millán, 1986; Millán & Manes, 1988); they are referred to as a wild-type (wt) PLAP and wt GCAP. A 2.0 kb *Eco*RI–*Kpn*I fragment of PLAP cDNA was used as the source of template DNA to generate a series of PLAP mutants. Site-directed mutagenesis experiments were performed as described by Kunkel (1985) by using the mutagene M_{13} *in vitro* mutagenesis kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The mutants [Gly^{429}]PLAP, [Ser^{429}]PLAP and [His^{429}]PLAP have been described previously (Hoylaerts & Millán, 1991). The sequence of the mutagenesis primers used to generate new mutants described in this report were as follows (underlined bases indicate changes): (a) [Asn^{91}]PLAP: 5′-GGC TCC ACT GTT TGG CAC ATG-3′; (b) [Ala^{91}]PLAP: 5′-GGC TCC ACT GGC TGG CAC ATG-3′; (c) [Ala^{166}]PLAP: 5′-CGA GTA CCA GTT GGC GTT CAC CGT GTG-3′; and (d) [Lys^{166}]PLAP: 5′-CGA GTA CCA GTT TTT GTT CAC CGT GTG-3′. The wt or mutant PLAP cDNAs were subcloned into the vector pSVT7, downstream from the SV40 early promoter (Bird *et al.*, 1987) and transfected by the calcium phosphate procedure (Gorman *et al.*, 1982) into Chinese-hamster ovary cells. Transfected cells were selected as described (Hummer & Millán, 1991) and, at confluency, washed with 20 mM-Tris/HCl buffer, pH 7.5, containing 140 mM-NaCl. The cells were extracted (30 min) with a 1:1 mixture of butan-1-ol and 50 mM-sodium acetate buffer, pH 5.5, containing 100 mM-NaCl, 20 μM-$ZnCl_2$, 1 mM-$MgCl_2$ and 0.05% merthiolate (Sigma). On titrating the pH to 7.5 with 1.0 M-Tris, these extracts were divided into portions and stored at −80 °C.

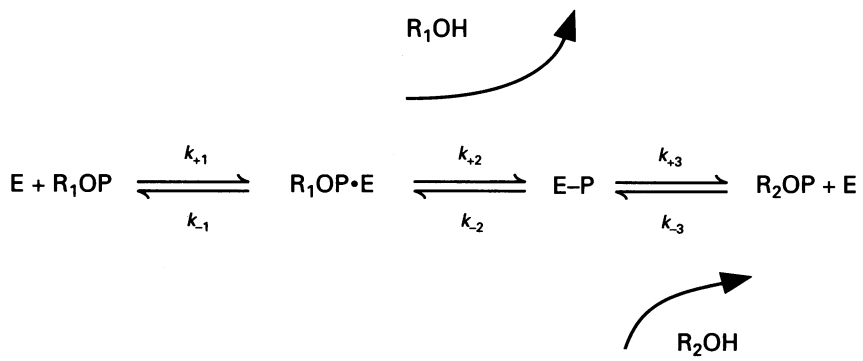
Kinetic measurements

The extracted enzyme samples (wt PLAP, wt GCAP or relevant mutants) were specifically and quantitatively fixed on to microtitre plates precoated with rabbit-(anti-mouse)IgG serum and saturated with the anti-PLAP monoclonal antibody H7 (Millán & Stigbrand, 1983), before kinetic analysis as previously described (Hoylaerts & Millán, 1991). Final concentrations of enzyme ranged from 0.2 to 1 unit/l. The enzymes bound to microtitre plates were incubated in the presence of increasing concentrations of *p*-nitrophenyl phosphate (pNPP, Sigma) (0.1–10 mM final concn.) and amino acid inhibitors (0–200 mM), in some experiments combined with free phosphate (0–50 μM). Substrate and inhibitor dilutions were made in 1 M-diethanolamine buffer, pH 9.8, containing 0.5 mM-$MgCl_2$ and 20 μM-$ZnCl_2$. The formation of *p*-nitrophenol was followed as a function of time for several hours at 37 °C and the ΔA_{405}/min was calculated from the linear part of the time-curve. The pH-dependence of L-Leu sensitivity was investigated in 50 mM-Bistris-propane buffer (Sigma), containing 0.1 M-NaCl, and adjusted to different pH values ranging from 5.5 to 9.5 in steps of 0.5 unit. Ethylamine (50 mM) buffer containing 0.1 M-NaCl was used for the higher pH values (10.0, 10.5 and 11.0).

Michaelis–Menten constants (K_m) were graphically determined from double-reciprocal plots of initial rates v (calculated from ΔA_{405}/min and from the molar absorption coefficient for *p*-nitrophenol formed, $\epsilon = 10080$ litre·mol^{-1}·cm^{-1}) versus substrate concentration (pNPP). Statistically weighted numerical values for K_m and for $V_{max.}$ were calculated as described by Wilkinson (1961), including the determination of standard errors of the mean (S.E.M.). Mean $V_{max.}$ values (±S.D.) were calculated by averaging different experiments, and catalytic rate constants ($k_{cat.}$) were calculated as the ratio ($V_{max.} \pm$S.D.)/([E]° ±S.D.), the mean enzyme concentration [E]° ±S.D. being determined by double-sandwich e.l.i.s.a. modified from that described by Hummer & Millán (1991). For this purpose, microtitre plates were precoated with polyclonal anti-PLAP antibodies (Dakopatts) and the anti-PLAP monoclonal antibodies H7 and C2 were chosen as second antibodies, wt PLAP being used as a reference. Graphically determined inhibition constants (K_i) were derived from double-reciprocal plots of v versus [pNPP] in the presence of various concentrations of inhibitor and from replots of the ordinate intercepts versus [inhibitor]. The numerical mean values for $K_i \pm$S.D. were obtained by calculation of the ordinate intercepts (apparent $V_{max.}$) for each line (Wilkinson, 1961) and by fitting this number and the calculated $V_{max.}$ into the proper expression for v at infinite [pNPP].

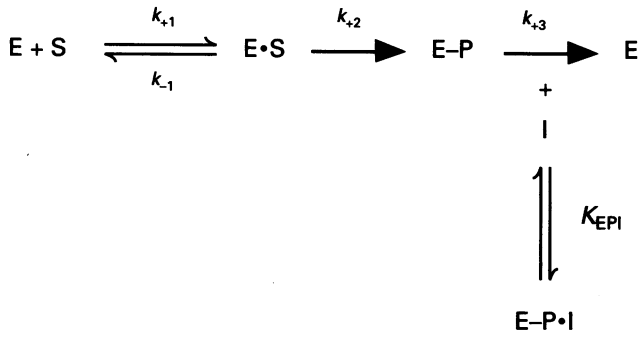
Kinetic mechanism of inhibition

By omitting the hydrolysis step, in the presence of an excess of a transphosphorylating alcohol, the general reaction for AP (Coleman & Gettins, 1983) can be simplified to Scheme 1:



Scheme 1

with R_1OP representing the substrate pNPP and R_2OH the second alcohol diethanolamine. The apparent first-order rate constant k_3 would primarily describe the rate of transphosphorylation at a given $[R_2OH]$. Owing to the large excess of diethanolamine (1 M, constant thoughout this study), this reaction can be simplified further by omitting the last two reverse reactions. In this case, the uncompetitive inhibition of APs by amino acids can be represented in accordance with Byers *et al.* (1972) as Scheme 2:



Scheme 2

in which the uncompetitive inhibitors prevent dephosphorylation of the phosphoenzyme intermediate. Scheme 2 can kinetically be described by the classical rate expression for uncompetitive inhibition (Cleland, 1970):

$$v = \frac{d[E\text{–}P]}{dt} = \frac{V_{max.}}{1+\frac{K_m}{[S]^\circ}+\frac{[I]^\circ}{K_i}} = \frac{k_{+2}[E]^\circ}{1+\frac{k_{-1}+k_{+2}}{k_1[S]^\circ}+\frac{k_{+2}}{k_{+3}}\left(1+\frac{[I]^\circ}{K_{EPI}}\right)} \quad (1)$$

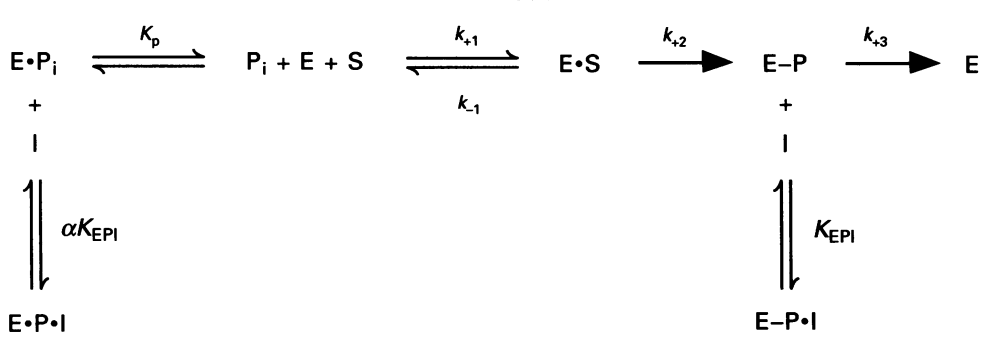
with $$k_{cat.} = \frac{k_{+2}}{1+k_{+2}/k_{+3}}; \quad K_m = \frac{k_{-1}+k_{+2}}{k_{+1}(1+k_{+2}/k_{+3})};$$

$$k_{cat.}/K_m = \frac{k_1}{1+k_{-1}/k_{+2}}$$

and $$K_{EPI} = \frac{[E\text{–}P][I]^\circ}{[E\text{–}P\cdot I]}, \text{ with } K_i = K_{EPI}(1+k_{+3}/k_{+2})$$

The dissociation constant K_{EPI} describes the affinity of reversible inhibitor binding (Byers *et al.*, 1972). The Michaelis constant K_m and the catalytic rate constant $k_{cat.}$ (determined in the absence of inhibitor) are complex functions of k_{+1}, k_{+2} and k_{+3} (Chaidaroglou & Kantrowitz, 1989). Because K_i also involves rate constants for both phosphorylation (k_{+2}) and dephosphorylation (k_{+3}), deductions regarding accessibility based on K_i values need to be approached with caution.

The co-inhibition of AP by L-Leu (I) in the presence of free phosphate (P_i) is represented by Scheme 3:



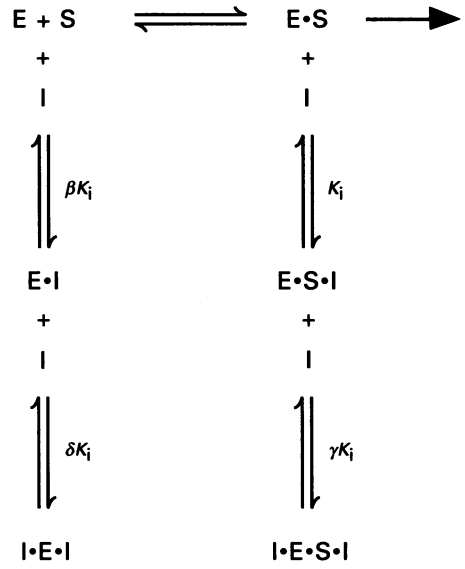
Scheme 3

with the corresponding rate equation:

$$v = \frac{k_{+2}[E]^\circ}{1+\frac{k_{+2}}{k_{+3}}\left(1+\frac{[I]^\circ}{K_{EPI}}\right)+\frac{k_{-1}+k_{+2}}{k_{+1}[S]^\circ}\left[1+\frac{[P_i]^\circ}{K_p}\left(1+\frac{[I]^\circ}{\alpha K_{EPI}}\right)\right]} \quad (2)$$

Co-inhibition studies of AP have been performed for mixtures of uncompetitive inhibitors (NADH) and mixed-type inhibitors (NAD^+), from which analysis it was concluded that NAD^+ and NADH bind to a common site despite their differential inhibition mechanisms (Ramasamy & Butterworth, 1973). Therefore, in agreement with Yonetani & Theorell (1964), who defined an interaction constant α to investigate to what extent two inhibitors compete for the same sites on the enzyme, we define α as a constant that relates the stability of the $E\cdot P_i\cdot I$ and the $E\text{–}P\cdot I$ complexes. For experiments carried out at a fixed $[S]^\circ$, a finite value for α implies an intersection in a plot of $1/v$ versus $[I]^\circ$ at $-\alpha K_{EPI}$, because at this concentration eqn. (2) would become independent of $[P_i]^\circ$. On the contrary, if phosphate, reversibly bound in the active site, is not capable of binding L-leucine, i.e. α is infinite, parallel lines are to be expected in the plot of $1/v$ versus $[I]^\circ$ with slopes equal to $1/K_{EPI}$.

The inhibition of [Ala[166]]PLAP by L-Leu (and by L-Phe) and of [Ala[166],Gly[429]]PLAP by D-Leu (and by D-Phe) can be represented by a model which is a mixture of a pure uncompetitive and pure competitive type of inhibition (Segel, 1975). In this model the existence of a third binding site is postulated, explaining the parabolic nature of the inhibition. This complex type of inhibition is represented in Scheme 4.



Scheme 4

It is described by an uncompetitive (K_i) and by a competitive (βK_i) inhibition constant. In this model an intersection in double-reciprocal plots of v versus [S]° is only obtained when $\gamma = \delta$ (Segel, 1975), whereas the system will formally reduce to a non-competitive inhibition type when $\beta = 1$. Values for β were estimated from the position of the intersection observed in the double-reciprocal plots. No further attempts were made to clarify the meaning of γ or to estimate its value.

Because the rate equation for the parabolic inhibition:

$$v = \frac{V_{max.}}{\left(1+\frac{[I]^\circ}{K_i}+\frac{([I]^\circ)^2}{\gamma K_i^2}\right)+\frac{K_m}{[S]^\circ}\left(1+\frac{[I]^\circ}{\beta K_i}+\frac{([I]^\circ)^2}{\beta\gamma K_i^2}\right)} \quad (3)$$

contains quadratic terms, no linear relation will be found in replots of $1/v$ (y intercepts) versus [I]°. Yet K_i can be estimated from such replots by drawing the slope to the exponentially regressed line at [I]° = 0.

RESULTS

The inhibition of wt PLAP and wt GCAP by L-Leu and L-Phe follows an uncompetitive mechanism. Inhibition by L-Leu (but not L-Phe) is approx. 17-fold more effective for wt GCAP than for wt PLAP (Table 1), and this difference has been attributed primarily to the substitution of Gly-429 for Glu-429 in GCAP (Hummer & Millán, 1991). The two amino acids inhibit [Gly^{429}]PLAP (Table 1) to a comparable extent, but to a higher degree than wt PLAP. Fig. 1(*a*) shows the uncompetitive nature of the inhibition of [Gly^{429}]PLAP by L-Leu. Our analysis surprisingly indicates that, although with considerably decreased efficiencies, D-Leu and D-Phe are also uncompetitive inhibitors of [Gly^{429}]PLAP (Fig. 1*b*) and, to a somewhat lower degree, also of wt PLAP and wt GCAP (Table 1). Mutagenesis of Glu-429 in wt PLAP for Ser-429 (present in intestinal AP) or His-429 (found in tissue non-specific AP) produces mutants with K_i values for L-Leu inhibition comparable with those of [Gly^{429}]PLAP, i.e. for [Ser^{429}]PLAP $K_i = 0.25$ mM, and for [His^{429}]PLAP $K_i = 0.3$ mM, in agreement with previous data (Hoylaerts & Millán, 1991).

Clearly, mutations at position 429 in wt PLAP have very pronounced effects on the inhibition efficiency by amino acids. Although these mutations were found to affect the conformation of the surface loop of PLAP greatly (Hoylaerts & Millán, 1991), we here find that they do not introduce any major structural changes in the active-site pocket, as revealed experimentally by inhibition kinetic experiments with free phosphate as a competitive inhibitor. P_i displays very similar affinities for the different enzymes, i.e. K_i (wt PLAP) = 40 μM, K_i ([Gly^{429}]PLAP) = 40 μM and K_i (wt GCAP) = 25 μM. Also, the Glu-to-Gly substitution has no major effects on the $k_{cat.}$ (Table 1), indicating that the

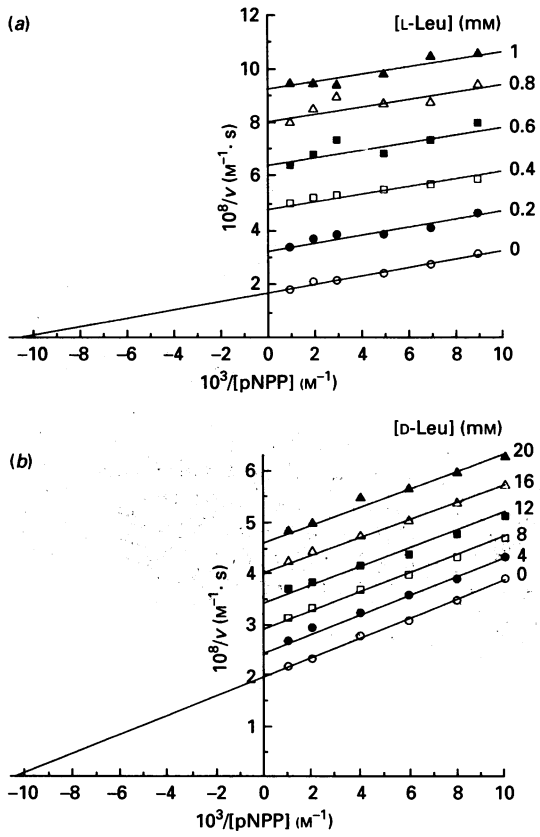


Fig. 1. Double-reciprocal plot of the rate of *p*-nitrophenol formation (v) versus substrate concentration (pNPP), during the inhibition of [Gly^{429}]PLAP (0.5 unit/l) by increasing [L-Leu] (0–1 mM) (*a*) and by increasing [D-Leu] (0–20 mM) (*b*)

Table 1. Kinetic parameters of wt PLAP, wt GCAP and PLAP mutants

Our present estimates of $k_{cat.}$ for wt GCAP and for [Gly^{429}]PLAP are 2–3-fold higher than previously reported (Hummer & Millán, 1991). These differences are due to a more accurate determination of the enzyme concentrations by the presently applied e.l.i.s.a., in which we take into account differences in immunoaffinity for various PLAP variants and mutants (Hoylaerts & Millán, 1991).

AP (mutant)	$K_m \pm$S.E.M. (mM)	$k_{cat.} \pm$S.D. (s^{-1})	Inhibition constant $K_i \pm$S.D. (mM)			
			L-Leu	D-Leu	L-Phe	D-Phe
wt PLAP	0.35±0.03	460±11	9.2±1.2	53±12	0.61±0.072	48±15
wt GCAP	0.11±0.005	277±16	0.54±0.01	37±2.1	0.6±0.005	36±1.9
[Gly^{429}]PLAP	0.091±0.006	344±14	0.2±0.01	16±0.5	0.15±0.006	9.2±0.34
[Asn^{91}]PLAP	0.62±0.09	30±15	11±0.44	–	0.44±0.06	–
[Ala^{91}]PLAP	1.1±0.07	39±4	8.5±1.6	–	1.8±0.24	–
[Ala^{166}]PLAP	4.4±0.41	20±9	40*	–	69†	–
[Ala^{166},Gly^{429}]PLAP	0.7±0.05	18±4	3.1±0.3	80*	20±3.8	90†
[Lys^{166},Gly^{429}]PLAP	0.53±0.09	7.8±2.7	5.6±0.9	–	17±2.9	–

* Inhibition mechanism is a mixture of uncompetitive type (K_i determined graphically) and competitive type (calculated from β and K_i as βK_i = 66 mM for L-Leu inhibition, βK_i = 140 mM for D-Leu inhibition), compounded by the existence of additional Leu interactions, which results in a parabolic inhibition.

† Inhibition mechanism is formally non-competitive ($\beta = 1$), but parabolic.

rates of phosphorylation (k_{+2}) and dephosphorylation (k_{+3}) are not affected in [Gly^{429}]PLAP. However, [Gly^{429}]PLAP displays three to five times lower K_m and K_i values (measured with D-Leu, D-Phe and L-Phe) than wt PLAP, indicating an increased accessibility of substrate (higher k_{+1} and/or lower k_{-1}) and inhibitors (lower K_{EPI}) to the active site of the mutant enzyme.

Replacement of the active-site residue Asp-91 (equivalent to Asp-101 in *E. coli* AP) by Asn-91 or Ala-91 generates PLAP mutants that are still active, but display considerably decreased turnover numbers. Whereas K_m values for [Asn^{91}]PLAP and [Ala^{91}]PLAP only rose 2–3-fold compared with that for wt PLAP, catalytic rate constants for [Asn^{91}]PLAP and [Ala^{91}]PLAP fell by a factor of 13–15 (Table 1). Both [Asn^{91}]PLAP and [Ala^{91}]PLAP are inhibited by L-Leu according to an uncompetitive inhibition (results not shown), and their K_i values are comparable with that of wt PLAP, indicating that Asp-91 is not involved in stabilizing L-Leu during enzyme inhibition. The inhibition of [Ala^{91}]PLAP by L-Phe, although uncompetitive, consistently revealed a slight elevation of K_i, a phenomenon not found for the inhibition of [Asn^{91}]PLAP by L-Phe.

Very different effects were observed on mutagenesis of Arg-166. In the *E. coli* active site, residue Arg-166 has been attributed a stabilizing role during the positioning of the phosphorylated substrate in the active site of the enzyme (Sowadski *et al.*, 1985; Chaidaroglou *et al.*, 1988; Butler-Ransohoff *et al.*, 1988). We have here replaced Arg-166 by Ala-166 in wt PLAP. Michaelis–Menten kinetics with [Ala^{166}]PLAP showed a 12-fold decrease in K_m and a 23-fold decrease in turnover (Table 1), indicating that this mutation causes the catalytic efficiency ($k_{cat.}/K_m$) to fall by a factor of almost 300. More importantly, the replacement of the positively charged Arg-166 residue by a neutral Ala-166 residue had a major effect on the mechanism of inhibition by L-Leu (Fig. 2*a*) and L-Phe (results not shown). Inhibition is observed only at higher concentrations, and, in the case of L-Leu, the inhibition mechanism is changed from an uncompetitive to a more complex mixed-type one, with double-reciprocal plots that intersect below the x axis. The position of this intersection indicates that, in addition to weak uncompetitive L-Leu binding (K_i = 40 mM), at high [L-Leu] a second competitive binding site with lower affinity (βK_i = 66 mM) is being saturated. Moreover, as judged from the secondary replot of the ordinate intercepts versus the inhibitor concentration, it is obvious that no linear relationship exists (Fig. 2*b*), indicative of a parabolic mechanism and suggestive of the existence of a third binding site, which was not further investigated in this study. The switch in inhibition mechanism suggests that, at high concentrations, L-Leu can directly bind to the enzyme, most probably by chelation of active-site Zn^{2+} ions (Gasser & Kirschner, 1987). The inhibition of [Ala^{166}]PLAP by L-Phe was likewise parabolic (results not shown), but because $\beta = 1$, the inhibition reduced to a non-competitive mechanism. The replacement, in PLAP, of Arg-166 by Ala-166 causes the inhibition constant for L-Phe to rise 100-fold, in contrast with the inhibition constant for L-Leu, which rises about 5-fold only (Table 1). Although k_{+2} and k_{+3} change when Arg-166 is replaced by Ala-166, these findings indicate that the observed shifts in K_i mainly reflect affinity changes (i.e. of K_{EPI}).

Because of the profound changes in inhibition properties induced by replacing Arg-166, we have analysed the importance of position 166 further in the context of Gly-429, which constitutes the major determinant of the differential inhibition of PLAP and GCAP by L-Leu. Compared with [Gly^{429}]PLAP, the double mutant [Ala^{166},Gly^{429}]PLAP displays an 8-fold lower substrate affinity, a factor comparable with that relating the substrate affinities of wt PLAP and [Ala^{166}]PLAP. Although in [Lys^{166},Gly^{429}]PLAP position 166 was mutagenized in a charge-conservative fashion, substrate affinity is also affected by a factor

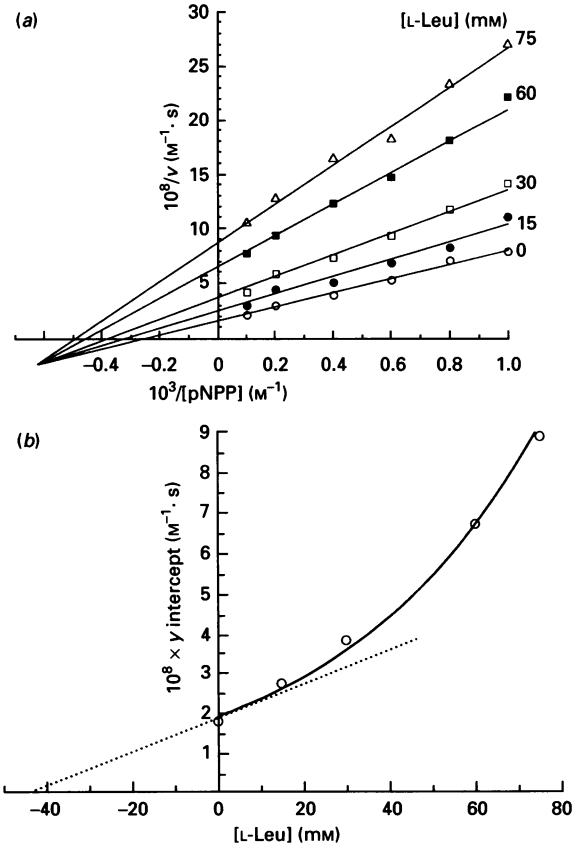


Fig. 2. Double-reciprocal plot of the rate of *p*-nitrophenol formation (v) versus substrate concentration (pNPP), during the inhibition of [Ala^{166}]PLAP (0.5 unit/l) by increasing [L-Leu] (0–75 mM) (*a*) and secondary replot of the *y*-axis intercepts versus [L-Leu] (*b*)

The dotted line in (*b*) represents the slope to the exponentially regressed curve at [L-Leu] = 0.

of 6 (Table 1). Turnover rates were calculated as 18 s^{-1} and 8 s^{-1} for [Ala^{166},Gly^{429}]PLAP and [Lys^{166},Gly^{429}]PLAP respectively (Table 1). Analysis of the mechanisms of [Ala^{166},Gly^{429}]PLAP and [Lys^{166},Gly^{429}]PLAP inhibition by L-Phe and L-Leu clearly shows an uncompetitive type of inhibition for both amino acids, with K_i values 15–25-fold (L-Leu) to 120-fold (L-Phe) higher than the corresponding values determined for the inhibition of [Gly^{429}]-PLAP (Table 1). The greater inhibition of the double mutant, compared with that of [Ala^{166}]PLAP, is in agreement with the increase in active-site accessibility that accompanies the Glu-429 to Gly-429 substitution, and it explains why the mixed-type inhibition of [Ala^{166}]PLAP reduces to an uncompetitive type in the double mutants. Inhibition of [Ala^{166},Gly^{429}]PLAP by D-Leu occurs only at high inhibitor concentrations, as expected (K_i = 80 mM). Similarly to the inhibition of [Ala^{166}]PLAP by L-Leu, this inhibition is slightly parabolic and of a mixed type (Fig. 3), yielding an intersection below the x axis in double-reciprocal plots, confirming the existence of a second competitive binding site with lower affinity for Leu (βK_i = 140 mM). In contrast, although slightly parabolic, the inhibition of [Ala^{166},Gly^{429}]PLAP by D-Phe appeared to be non-competitive (not shown), as was found for the inhibition of [Ala^{166}]PLAP by L-Phe, and to be comparable with the inhibition by D-Leu (Table 1).

The previous findings suggest interaction of Arg-166 with the inhibitory amino acid. Since the carboxy group of L-Leu (or L-Phe) is the most likely candidate for this interaction involving the

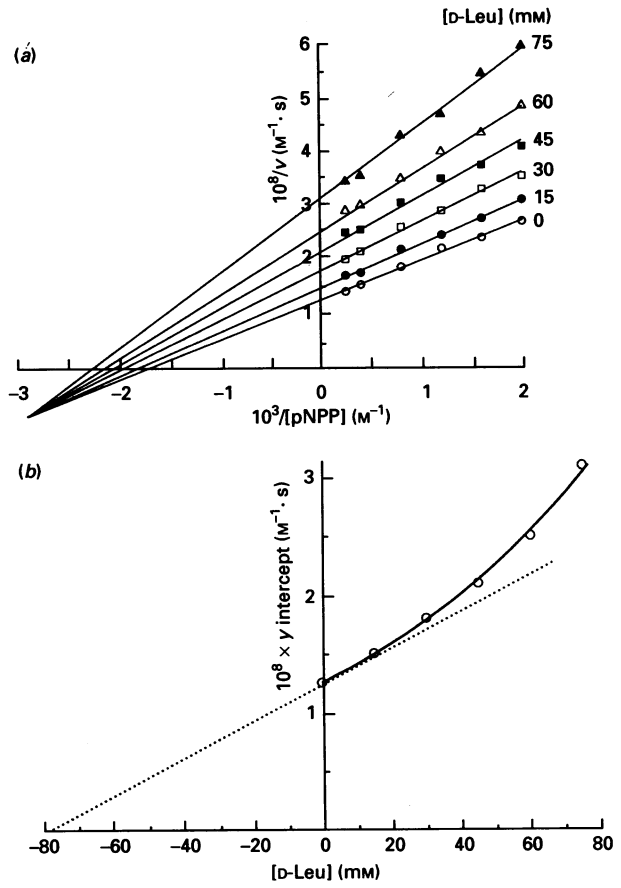


Fig. 3. Double-reciprocal plot of the rate of *p*-nitrophenol formation (v) versus substrate concentration (pNPP), during the inhibition of [Ala^{166},Gly^{429}]PLAP (0.7 unit/l) by increasing [D-Leu] (0–75 mM) (*a*) and secondary replot of the *y*-axis intercepts versus [D-Leu] (*b*)

The dotted line in (*b*) represents the slope to the exponentially regressed curve at [D-Leu] = 0.

Fig. 5. Dose-dependence (0–1.6 mM) for the inhibition at pH 10.5 of [Gly^{429}]PLAP activity by L-Leu (*a*) and pH-dependence (pH 7.5–10.5) for the inhibition of [Gly^{429}]PLAP activity by 0.8 mM L-Leu

The degree of inhibition is expressed relative to the enzyme activity measured for a constant [pNPP] (10 mM) at the corresponding pH in the absence of inhibitor.

guanidinium group of Arg-166, we have investigated the inhibitory properties of carboxy-group derivatives of L-Leu. Inhibition of wt PLAP and [Gly^{429}]PLAP by two L-Leu analogues, i.e. L-leucinamide and leucinol, is uncompetitive (results not shown). However, inhibition of wt PLAP by L-leucinamide ($K_i = 1.9 \pm 0.1$ mM) is even slightly more effective than inhibition by L-Leu. In addition, unlike [Gly^{429}]PLAP, which is inhibited by L-Leu to a higher degree than is PLAP, the Glu to Gly replacement is almost entirely silent in the case of inhibition by L-leucinamide, even causing a slight loss in inhibitor affinity ($K_i = 3.3 \pm 0.14$ mM) for [Gly^{429}]PLAP inhibition. Although still uncompetitive, the more modified leucinol is a weaker inhibitor of wt PLAP ($K_i = 11 \pm 0.9$ mM) and an even weaker inhibitor of [Gly^{429}]PLAP ($K_i = 15 \pm 1.7$ mM).

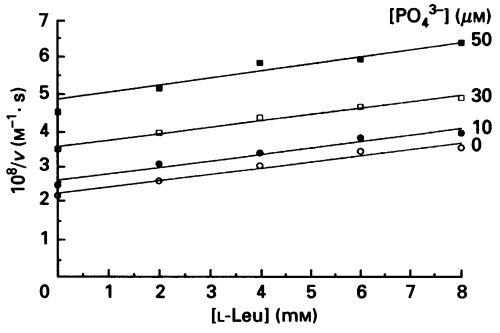


Fig. 4. Reciprocal Yonetani–Theorell plot of the rate of *p*-nitrophenol formation (v) versus L-Leu concentration, during the co-inhibition of wt PLAP (0.5 unit/l) by increasing [phosphate] (0–50 μM) and simultaneously increasing [L-Leu] (0–8 mM), at constant [pNPP] (0.3 mM, equal to K_m)

Stabilization of the inhibitory amino acid in the active site by interaction between its carboxy group and the guanidinium group of Arg-166 is likely to occur consecutively to substrate binding (Byers *et al.*, 1972), during which Arg-166 is rendered available for interaction with other ligands (Kim & Wyckhof, 1991). We investigated whether in the relevant range of L-Leu concentrations a ternary complex is formed in the active site between non-covalently bound P_i and the inhibitory amino acid. The parallel lines in Fig. 4, reflecting the co-inhibition of wt PLAP by increasing concentrations of free phosphate and L-Leu, do not provide any evidence for the existence of a ternary complex between the enzyme, phosphate and L-Leu, i.e. α is infinite. Identical results were obtained in co-inhibition experiments of wt GCAP or [Gly^{429}]PLAP with phosphate and L-Leu (results not shown). These findings corroborate the fact that inhibition occurs at a later stage in the enzyme cycle than the reversible substrate positioning (Byers *et al.*, 1972) and involves an additional interaction related to substrate catalysis. In agreement with Byers *et al.* (1972), we also found that inhibition is greatly dependent on pH. For each pH, residual [Gly^{429}]PLAP activities in the presence of 0.8 mM-L-Leu were expressed relative to the enzyme activity at that pH in the absence of inhibitor, from which percentage inhibition was calculated. Fig. 5(*b*) shows that the inhibitory capacity of L-Leu rapidly diminishes with decreasing pH, from a maximum around pH 10.5 to complete disappearance at pH 7.5. These results suggest the involvement

of a primary amino function in the inhibition mechanism; on lowering the pH it is protonated, thus losing its inhibitory capacity.

DISCUSSION

Inhibition of APs by amino acids can proceed via either a non-competitive or an uncompetitive mechanism. The first mechanism is usually explained by chelation of an essential Zn^{2+} in the active site (Gasser & Kirschner, 1987). The uncompetitive mechanism has been described kinetically as a trapping of the phosphorylated intermediate of the enzyme by the inhibitor (Byers *et al.*, 1972). These authors suggested that all three functional groups of the inhibitor, i.e. carboxy, amino and side groups, would participate in the process. We have identified Gly-429 as the major determinant of the L-Leu sensitivity of GCAP (Hummer & Millán, 1991) and attributed this phenotype to a considerable conformational change accompanying a Glu-429 → Gly-429 transition between PLAP and GCAP (Hoylaerts & Millán, 1991). We now present evidence for the involvement of residue Arg-166 and Zn^{2+} ion 1 during the inhibition and define the role of the loop containing Gly-429 in terms of accessibility and side-chain positioning of the inhibitor.

Targeting to Arg-166

It seems clear from our present findings that residues Asp-91 and Arg-166 in PLAP and GCAP serve analogous active-site functions to their counterparts, Asp-101 and Arg-166 in *E. coli* AP (Chaidaroglou & Kantrowitz, 1989). In our experimental setting, i.e. in the presence of transphosphorylating alcohol, different substitutions of Asp-91 have little impact on K_m but cause a 15-fold fall in turnover rate. In contrast, substitutions of Arg-166 have a dramatic effect, not only on $k_{cat.}$, which decreases at least 20-fold, but also on K_m, which increases by a factor of 6–10, whether Arg-166 is subjected to mutagenesis alone or in combination with Glu-429. The large fall in $k_{cat.}$ can be explained for [Ala^{91}]PLAP and [Asn^{91}]PLAP by a defective positioning of the active-site Ser-92, possibly linked to a change in the configuration of the peptide backbone in the region of the active site. For [Ala^{166}]PLAP, [Ala^{166},Gly^{429}]PLAP and [Lys^{166},Gly^{429}]PLAP, a defective positioning of phosphate in the active site may be postulated, and this can be related to the necessity of charge neutralization of the phosphate group to facilitate nucleophilic attack by serine during phosphorylation and by water or R_2OH during dephosphorylation.

Consecutively to substrate binding in the active site of *E. coli* AP, the ionic Asp-101–Arg-166 bond dissociates, releasing the Arg-166 guanidinium group to participate in substrate positioning through interactions with the phosphate moiety of the substrate (Kim & Wyckoff, 1991). It is clear from our inhibition studies involving [Asn^{91}]PLAP and [Ala^{91}]PLAP that the Asp-91 residue is not involved in stabilization of L-Leu inside the active-site pocket during enzyme inhibition. However, both L-Leu and L-Phe inhibition kinetics involving [Ala^{166}]PLAP, [Ala^{166},Gly^{429}]PLAP and [Lys^{166},Gly^{429}]PLAP identified an important role for Arg-166 during inhibition. Consistently, its substitution led to an increase in inhibition, up to 100-fold for [Ala^{166},Gly^{429}]PLAP and [Lys^{166},Gly^{429}]PLAP by L-Phe. From these findings we conclude that the guanidinium group of Arg-166 is involved in an interaction with the carboxy group of the inhibitory amino acid. The weak inhibition of [Gly^{429}]PLAP by D-Leu and by D-Phe is even further compromised in [Ala^{166},Gly^{429}]PLAP, indicating that, even in the case of a differentially positioned side group, the interaction between the amino acid carboxy group and the Arg-166 guanidinium group still contributes to stabilization of the inhibitor in the active site. The more complex types of inhibition observed at high concentrations of L-Leu necessitate definition of additional binding sites, probably related to chelation of active-site metal ions (Gasser & Kirschner, 1987).

Side-chain positioning

L-Leu and L-Phe are considered to be stereospecific inhibitors. We have here shown that D-Leu and D-Phe can also act as uncompetitive inhibitors, with the D-amino acids displaying comparably weak reactivity with wt PLAP. Since L-Phe is a 70-fold better inhibitor of wt PLAP, wt GCAP and [Gly^{429}]PLAP than D-Phe, we conclude that the sterospecificity of L-Phe inhibition depends on steric hindrance experienced by the D-Phe side group during positioning of the inhibitor in the active site. This conclusion supports earlier predictions of the nature of the stereospecificity (Fishman & Ghosh, 1967) and the existence of a hydrophobic pocket that stabilizes L-amino acid side groups (Fishman & Sie, 1971). Our data further indicate that this pocket is mainly formed by the loop that harbours residue 429, and is capable of adopting different conformations depending on the identity of that residue (Hoylaerts & Millán, 1991). Replacement of the spacious side chain of Glu-429 (in wt PLAP) by a proton (Gly-429 in wt GCAP) dramatically increases the accessibility for the L-Leu side chain, facilitating its positioning. [Gly^{429}]PLAP is also inhibited three to five times more readily by D-amino acids than is wt PLAP, indicating that the conformational change is accompanied by a facilitated entrance of the inhibitor to the active site. Both events contribute to the 17-fold decrease in K_i for L-Leu inhibition of GCAP. Because the planar ring of the L-Phe side group does not interfere with Glu-429 during its positioning in the pocket, only the second event contributes to the decrease in the K_i for this inhibition.

Whereas modifications to the side chains of the inhibitory amino acids can influence their positioning in the pocket, modifications of the α-carboxy group of these inhibitors can influence the positioning of the amino acid derivatives in the active site. Indeed, L-leucinamide and leucinol are both molecules that can inhibit wt PLAP and [Gly^{429}]PLAP uncompetitively. Obviously, these inhibitors cannot interact with Arg-166 identically with L-Leu itself, leading to their different positioning. The amide group of leucinamide and the alcohol group of leucinol produce less stability than the carboxy group of L-Leu, which translates into progressively increasing inhibition of [Gly^{429}]PLAP by L-Leu, L-leucinamide and leucinol. Although residual active-site stabilization of these inhibitor derivatives causes the inhibition mechanism to be uncompetitive, the slightly different positioning of the inhibitor (as compared with L-Leu) already produces reorientation of the side chain in the pocket away from Glu-429. Indeed, the Glu-429 to Gly-429 substitution is almost entirely silent during the inhibition by these derivatives since wt PLAP is no longer inhibited to a lower degree than [Gly^{429}]PLAP.

Co-ordination with Zn^{2+} ion 1

The activity of AP depends on the presence of Zn^{2+} and Mg^{2+} ions in the catalytic site. Several studies have identified Zn^{2+} ion 1 as of primary importance for catalysis, its removal causing a complete loss of enzyme activity (Trotman & Greenwood, 1971). On the basis of a series of crystal structures, Kim & Wyckoff (1991) have demonstrated not only that Zn^{2+} ion 1 is involved in the positioning of the phosphate moiety of the substrate, but that it participates in the formation and hydrolysis of the phosphoenzyme complex during catalysis. Inhibition by L-Leu occurs to the same degree in the presence or absence of transphosphorylating substances, and we have demonstrated that the presence of phosphate reversibly bound in the active site does not produce an

enzyme-L-Leu complex. Therefore, in agreement with its uncompetitive nature, L-Leu only interferes during the catalytic step. We have found, in agreement with Byers *et al.* (1972), that L-Leu inhibition is strongly pH-dependent, the activity being maximal at pH 10.5 and virtually non-existent at pH 7.5. This behaviour correlates with the protonation state of the amino group of the inhibitor. In additional experiments, we have shown that primary amines (ethylamine, 2-amino-5-methylhexane) are capable of inhibiting AP activity at concentrations exceeding 100 mM. Other more complex molecules display the opposite pH profile; e.g. from the inhibition of pig kidney TNAP by NADH, Ramasamy & Butterworth (1975) concluded that binding of NADH to the enzyme is favoured by protonation of an enzymic group with a pK of about 9. These data suggest that more complex inhibitors may be stabilized by additional interaction points. It is also noteworthy that the inhibition of TNAP by L-homoarginine is hampered by substitutions in the guanidinium side arm of the inhibitor (Fishman & Sie, 1971), supporting the existence of such interactions.

In summary, we conclude that the observed uncompetitive AP inhibition by L-Leu or L-Phe primarily requires that the carboxy group of the inhibitor be targeted to Arg-166, during which process its side chain needs to be positioned in an accessible pocket consisting of the unique eukaryotic AP top loop (residues 400–430). In wt PLAP this loop contains Glu-429, the side chain of which points towards the active site, sterically interfering with the L-Leu side group during its positioning. Replacement of Glu-429 by Gly-429 (present at that position in wt GCAP) removes steric constraints during L-Leu positioning and causes a conformational change in the top loop that also enhances the active-site accessibility for both substrate and inhibitor. These characteristics provide the basis for the differences in inhibition of PLAP and GCAP by L-Leu. The true AP inhibition during catalysis is exerted by the unprotonated amino group of the inhibitor which is able to co-ordinate with Zn^{2+} ion 1 as soon as this residue becomes available for interaction, i.e. on completion of the phosphorylation of the active-site Ser-92. Binding of the amino group to Zn^{2+} ion 1 impairs hydrolysis (or transphosphorylation) of the phosphoenzyme, thereby arresting the catalytic cycle. We conclude that the uncompetitive nature of AP inhibition depends on contributions from each functional group of the amino acid and that modification of just one of these three functions suffices to influence inhibition strongly.

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