Biochem. J. (1991) 274, 91–95 (Printed in Great Britain)

Gly^{429} is the major determinant of uncompetitive inhibition of human germ cell alkaline phosphatase by L-leucine

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The catalytic activity of human placental alkaline phosphatase (PLAP) and germ cell alkaline phosphatase (GCAP) can be inhibited, through an uncompetitive mechanism, by L-Phe. GCAP is also selectively inhibited by L-Leu. Site-directed mutagenesis of five of the 12 residues which are different in PLAP and GCAP revealed that Gly^{429} is the primary determinant of GCAP inhibition by L-Leu, and Ser^{84} and Leu^{297} play a modulatory role in the inhibition.

INTRODUCTION

Eukaryotic alkaline phosphatases (APs, EC 3.1.3.1) are universally inhibited by certain amino acids and peptides (Fishman, 1974). This inhibition occurs by an uncompetitive mechanism (Ghosh & Fishman, 1966; Byers *et al.*, 1972), it is stereospecific to L- but not D-enantiomers (Fishman & Sie, 1971) and it is organ-specific (Fishman & Sie, 1971).

In humans, AP isoenzymes constitute a gene family composed of four genetic loci (for reviews see Millán, 1988; Harris, 1989). The tissue non-specific AP (TNAP) gene is expressed in the liver, bone, kidney, fibroblasts, leukocytes and early placenta, and in a variety of other tissues at low levels (McComb *et al.*, 1979). This isoenzyme is inhibited by L-homoarginine, L-His, levamisole and bromotetramizole, but not by L-Phe, L-Leu or the related peptides L-Leu-Gly-Gly or L-Phe-Gly-Gly (Fishman & Sie, 1971; Lin & Fishman, 1972; Mulivor *et al.*, 1978). The three other human isoenzymes are referred to as tissue-specific APs (TSAPs) and comprise the intestinal AP (IAP), placental AP (PLAP) and germ cell (GCAP) isoenzymes. Whereas the three TSAP isozymes display more than 90% sequence identity, they share only 50% similarity with the TNAP isoenzyme. The three TSAP isoenzymes are equally inhibited by L-Phe but not by L-homoarginine or by the other TNAP-specific inhibitors (Fishman & Sie, 1971; Fishman *et al.*, 1963; Mulivor *et al.*, 1978). Furthermore, GCAP is selectively inhibited by L-Leu (Doellgast & Fishman, 1976). The organ-specific sensitivity of AP isoenzymes to the different uncompetitive inhibitors has provided the basis for the specific quantification of AP isoenzymes in clinical chemistry (Green *et al.*, 1971; Van Belle *et al.*, 1977; Millán *et al.*, 1980).

PLAP and GCAP are useful tumour markers in the management of testicular cancer and ovarian cancer patients (for review see Millán, 1990), and inhibition by L-Leu has provided a powerful means of distinguishing these closely related gene products (Haije *et al.*, 1987).

The cloning of PLAP and GCAP revealed that the amino acid sequences of these two isoenzymes are 98% identical, displaying only 12 amino acid substitutions. It is clear that one or more of these 12 residues must be responsible for the specific inhibition of GCAP by L-Leu. These closely related isoenzymes provide an experimentally accessible system with which to attempt to understand the organ-specific inhibition of APs by amino acids and peptides, and to elucidate the mechanism underlying the kinetics of uncompetitive inhibition. In this report we present site-directed mutagenesis results that conclusively point to residue 429 of the primary GCAP sequence as the major determinant of the specific inhibition by L-Leu. We report also that the full spectrum of inhibition of GCAP by L-Phe, L-Leu and L-Phe-Gly-Gly requires the co-operative interaction of Ser^{84} and Gly^{429}.

MATERIALS AND METHODS

Site-directed mutagenesis

A 2.0 kb *Eco*RI–*Kpn*I fragment of the PLAP cDNA (Millán, 1986) was used as the source of template DNA for the mutagenesis experiments. Site-directed mutagenesis experiments were performed by the procedure of Kunkel (1985) using the Mutagene M_{13} *in vitro* mutagenesis kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Three subfragments of the PLAP cDNA, i.e. a 600 bp *Bam*HI–*Sst*I, a 359 bp *Sst*I–*Sst*I and a 731 bp *Sst*I–*Kpn*I fragment were subcloned into M_{13} to be used as single-stranded DNA templates for *in vitro* mutagenesis (Fig. 1). Oligonucleotide primers were synthesized on an Applied Biosystems DNA Synthesizer at our institution. The sequences of the mutagenesis primers were as follows [overscored base(s) indicate changes]: (*a*) [Phe^{68}]PLAP: GGGGCCTGAGATATT-CCTGGCCATGGAC′, (*b*) [Ser^{84}]PLAP: GTCTACAC-TGTATGTCTT,(*c*)[Val^{133}]PLAPCGAGGTCATCTCCGTGG-TGAATCGGGCC, (*d*) [Leu^{254}]PLAP: GGAAGCCTGCAG-GAGCTC′, (*e*) [Leu^{297}]PLAP: GCTCAGCAGGAGCAGGGC and (*f*) [Gly^{429}]PLAP: GGTCTCTCCGTCCAGGGG′. The mutagenized fragments were fully sequenced to confirm that no other alterations had been introduced into the template cDNA, cut from the M_{13} double-stranded replicative form DNA and subcloned into Bluescript KS plus vector (Stratagene, San Diego, CA, U.S.A.). To reassemble a functional mutagenized PLAP cDNA, four-way ligations were performed with the pSVT7 vector and either of the following combinations of fragments: (*a*) the 600 bp *Bam*HI–*Sst*I + 154 bp *Sst*I + *Sac*II + 396 bp *Sac*II–*Kpn*I fragments, or (*b*) the 754 bp *Bam*HI–*Sac*II + 205 bp *Sac*II–SstI + 731 bp SstI–KpnI fragments, as depicted in Fig. 1. A 2.8 kb *Eco*RI PLAP cDNA was subcloned into the vector pSVT7, downstream from the simian virus 40 early promoter (Bird *et al.*, 1987) and used as a wild-type PLAP control for the transfection experiments. A promoterless GCAP gene sequence was obtained by *Aha*III–*Hin*dIII digestion of the GCAP genomic clone (Millán & Manes, 1988), subcloned into the *Sma*I–*Hin*dIII-cut pSVT7 vector, and used in the transfection experiments as a

Abbreviations used: AP, alkaline phosphatase; TNAP, tissue-non-specific AP; TSAP, tissue-specific AP; IAP, intestinal AP, PLAP, human placental AP; GCAP, germ cell AP; CHO cells, Chinese hamster ovary cells; DMEM, Dulbecco's modified essential Eagle's medium.

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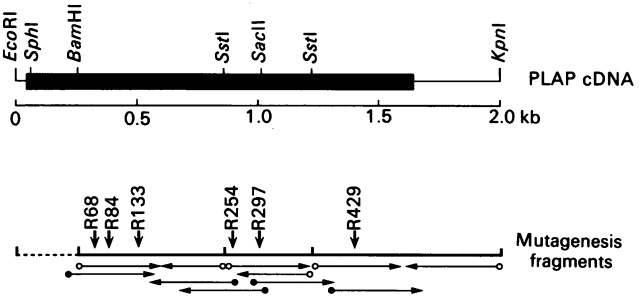

Fig. 1. Partial restriction endonuclease map of the PLAP cDNA

The map (Millán, 1986) displays those restriction sites used for the generation of mutagenesis fragments and in the subcloning strategy used to reassemble the mutant cDNAs. The fragments *Bam*HI–*Sst*I, *Sst*I–*Sst*I and *Sst*I–*Kpn*I are indicated underneath the cDNA map, as well as the relative positions of the mutagenized residues 68, 84, 133, 254, 297 and 429. The sequencing strategy is represented underneath this scheme. The use of commercially available M_{13} and/or Bluescript 17-mer primers is indicated by ○, whereas 17-mer or 18-mer oligonucleotide primers synthesized at our institution are represented by ●. Some oligonucleotide primers were designed to sequence through *Bam*HI and *Sst*I boundaries to confirm the correct assembly of mutant cDNAs.

wild-type GCAP control. A full-length mouse TNAP cDNA, kindly provided by Ann Hahnel, Department of Medical Biochemistry, University of Calgary, Alberta, Canada (Hahnel & Schultz, 1989), was subcloned in the *Eco*RI site of the pSVT7 vectors and served as the TNAP wild-type control.

Tissue culture

The pSVT7 vector containing the DNA constructions described above was transfected by the calcium phosphate procedure (Gorman *et al.*, 1982) into Chinese hamster ovary (CHO) cells (ATCC CCL 61). A pSV2-dhfr plasmid (Schimke, 1982) was co-transfected to allow selection of transfectants in nucleoside-free medium and possible amplification of the transfected sequences upon methotrexate treatment. The cells were plated on 150 mm dishes and cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM). They were subsequently divided (1:15 ratio) and plated in selection medium, consisting of high-glucose DMEM, deprived of hypoxanthine and thymidine and supplemented with 10% dialysed fetal bovine serum. The cells were fed and observed every 48 h. After 12 days, surviving foci were collected by aspiration with a Pasteur pipette and expanded in selection medium. Each colony was plated in duplicate 24-well plates and the cells in one plate were stained for total AP activity as described (Narisawa *et al.*, 1988). The positive cells from the duplicate plate were expanded and collected by trypsin treatment.

Inhibition assays

Cell pellets were extracted with butanol as described (Narisawa *et al.*, 1988). Microtitre-well plates were coated with 10 μg of H7 monoclonal antibody/ml (specific for PLAP and GCAP) as described (Millán & Stigbrand, 1983). Butanol extracts (200 μl) were added to the microtitre wells appropriately diluted in Dulbecco's phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 to a concentration of antigen of 10–20 ng/ml. Butanol extracts of control TNAP transfectants were added to the different substrate solutions without previous binding to antibody-coated microtitre wells. The inhibitors L-homoarginine, L-Leu and L-Phe and the non-inhibitory enantiomers D-Leu and D-Phe were purchased from Sigma (St. Louis, MO, U.S.A.). The peptides L-Phe-Gly-Gly and L-Leu-Gly-Gly were purchased from Worthington Biochemicals. The inhibition assays were performed as previously described (Millán *et al.*, 1982). Total AP activity was measured using, as substrate, 10 mM-*p*-nitrophenylphosphate in 1 M-diethanolamine buffer, pH 9.8, containing 0.5 mM-$MgCl_2$. Inhibition results were obtained from absorbance measurements (at 405 nm) by adding the different inhibitors to the substrate buffer, at the concentrations indicated in the Figures and Tables.

Kinetic measurements

Changes in A_{405} were measured at 37 °C. The molar absorption coefficient of *p*-nitrophenol in the microtitre-plate system was determined to be 10080 $M^{-1} \cdot cm^{-1}$. Protein concentrations of PLAP, GCAP and the mutants were determined by a sandwich e.l.i.s.a. as previously described (Millán & Stigbrand, 1983).

RESULTS

Selection of residues for mutagenesis

The cloning of the PLAP cDNA and the GCAP gene has revealed that the predicted primary structure of these isoenzymes are 98% identical, displaying only 12 amino acid substitutions (Millán & Manes, 1988). Additional differences found in the signal peptide region were not considered, since they are not reflected in the mature protein. Two of the 12 substitutions reside in the predicted *C*-terminal end of the proteins, downstream from the Asp^{484} residue, which is known to be the site of phosphatidylinositolglycan attachment (Micanovic *et al.*, 1988). These

Table 1. Amino acid differences between PLAP and GCAP

'Comments' refer to the identity of the residue in GCAP.

Residue	PLAP	GCAP	Comments
15	Glu (GAG)	Gln (CAG)	Exists in human IAP and all TNAP isoenzymes
38	Ile (ATC)	Met (ATG)	Exists in all TNAP isoenzymes. Possible allelic polymorphism*
67	Ile (ATA)	Thr (ACC)	Exists in all TSAP and TNAP isoenzymes
68	Pro (CCC)	Phe (TTC)	Unique
84	Asn (AAT)	Ser (AGT)	Unique
133	Met (ATG)	Val (GTG)	Unique. Possible allelic polymorphism*
241	Arg (CGC)	His (CAC)	Exists in some TSAP and TNAP isoenzymes
254	Met (ATG)	Leu (CTG)	Exists in all TNAP isoenzymes
297	Arg (CGC)	Leu (CTC)	Unique. Possible allelic polymorphism*
429	Glu (GAA)	Gly (GGA)	Unique

* Residues 38, 133 and 297 were found to contain a different substitution by Watanabe *et al.* (1989) while sequencing a choriocarcinoma cell line GCAP cDNA.

Table 2. Effects of AP mutations on inhibition of activity by L-amino acids

The values are means ± 2 S.D. for each sample, calculated from a minimum of three independent determinations.

AP	Inhibition (%)		
	L-hArg (5 mM)	L-Phe (5 mM)	L-Leu (1 mM)
Wild-type PLAP	5.7±12.9	78.0±4.0	22.7±15.1
Wild-type GCAP	10.7±8.3	82.5±1.7	65.5±0.8
Wild-type TNAP	66.7±8.5	12.3±16.2	8.6±14.8
[Phe^{68}]PLAP	6.3±14.2	76.3±0.2	16.0±20.3
[Ser^{84}]PLAP	4.7±9.0	52.0±11.1	10.7±21.0
[Val^{133}]PLAP	4.7±11.4	74.3±4.2	18.7±17.5
[Leu^{254}]PLAP	3.7±1.2	75.3±3.9	18.0±7.2
[Leu^{297}]PLAP	5.3±9.5	54.3±5.0	17.0±6.1
[Gly^{429}]PLAP	31.0±8.0	88.7±4.6	81.0±5.3
[Ser^{84},Gly^{429}]PLAP	14.0±14.0	85.0±2.0	68.3±11.4
[Ser^{84},Leu^{297}]PLAP	3.2±11.1	54.5±6.8	12.5±2.0
[Leu^{297},Gly^{429}]PLAP	7.6±18.1	83.5±0.9	60.6±4.6
[Ser^{84},Leu^{297},Gly^{429}]PLAP	6.5±6.6	84.1±0.7	63.6±5.5

Table 3. Kinetic parameters of the wild-type and mutant enzymes

AP	K_m (mM)	$V_{max.}$ (nM/s)	$[E_o]$ (nM)	$k_{cat.}$ (s^{-1})	$10^{-6} \times k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$)
Wild-type PLAP	0.29	9.17	0.024	382	1.38
Wild-type GCAP	0.10	8.27	0.111	75	0.75
[Phe^{68}]PLAP	0.26	8.62	0.024	359	1.38
[Ser^{84}]PLAP	0.32	10.40	0.034	306	0.96
[Val^{133}]PLAP	0.30	12.60	0.048	263	0.88
[Leu^{254}]PLAP	0.63	4.13	0.016	260	0.41
[Leu^{297}]PLAP	0.32	12.60	0.041	263	0.82
[Gly^{429}]PLAP	0.09	9.83	0.066	149	1.65
[Ser^{84},Gly^{429}]PLAP	0.10	11.00	0.192	57	0.57

two substitutions were not expected to result in differences in the mature proteins. Therefore 10 positions were considered as possible candidates to provide the structural basis for the differential inhibition of GCAP by L-Leu. Table 1 shows the residues occupying the 10 positions in PLAP and GCAP and indicates whether they represent unique residues or substitutions shared by other AP isoenzymes.

In deciding our experimental strategy, we assumed that the residues involved in L-Leu inhibition would have to be specific for GCAP and not shared by any of the other known TSAP sequences, and should also be different from those occupying equivalent positions in the TNAP isoenzymes. This criterion clearly identified residues 68, 84, 133, 297 and 429 as candidates for mutagenesis experiments. Residue 254 was included as a residue not expected to have any effect.

Inhibition properties of mutants

The six candidate positions in PLAP were mutagenized to those residues present in the wild-type GCAP, i.e. residues 68 (Pro to Phe), 84 (Asn to Ser), 133 (Met to Val), 254 (Met to Leu), 297 (Arg to Leu) and 429 (Glu to Gly). CHO cells stably transfected with the three control gene constructions (wild-type PLAP, GCAP and TNAP) and the six mutant PLAP constructions were extracted and tested in enzyme immunoassays with the inhibitors L-Phe, L-Leu and L-homoarginine, as described in the Materials and methods section, at the concentrations that previously have been found to be discriminatory (Millán *et al.*, 1985). As the trapping antibody we used the H7 monoclonal antibody that has been thoroughly characterized (Millán & Stigbrand, 1983; Millán *et al.*, 1985) as reacting with all common phenotypes of PLAP and all known phenotypes of GCAP.

Initial screening

The results of the initial screening (Table 2) revealed that three positions, residues 84, 297 and 429, had affected the quantitative response to specific inhibitors. The [Ser^{84}]PLAP and [Leu^{297}]PLAP mutants were less inhibited by L-Phe than was wild-type PLAP. [Gly^{429}]PLAP showed a greatly enhanced inhibition by L-Leu, even higher than that observed with wild-type GCAP. [Gly^{429}]PLAP also displayed a slightly higher percentage inhibition by L-Phe compared with wild-type GCAP. These preliminary results suggested that Gly^{429} could, by itself, confer the full phenotype of L-Leu sensitivity characteristic of the GCAP molecule.

When the mutations at residues 84 and 297 were combined in the same construction with the mutation at residue 429 i.e. [Ser^{84},Gly^{429}]PLAP or [Leu^{297},Gly^{429}]PLAP, the inhibitory effects of L-Leu and L-Phe corresponded to those on the GCAP control. The combination of Ser^{84} plus Leu^{297} did not further affect sensitivity to L-Phe as compared with the single Ser^{84} or Leu^{297} substitutions. On the other hand, the combination of Ser^{84} + Leu^{297} + Gly^{429} did not change sensitivity to L-Leu by any more than the respective double mutations including Gly^{429}. The K_m, $V_{max.}$ and $K_{cat.}$ values and the catalytic efficiencies ($k_{cat.}/K_m$) of the different mutants (Table 3) were comparable with those of

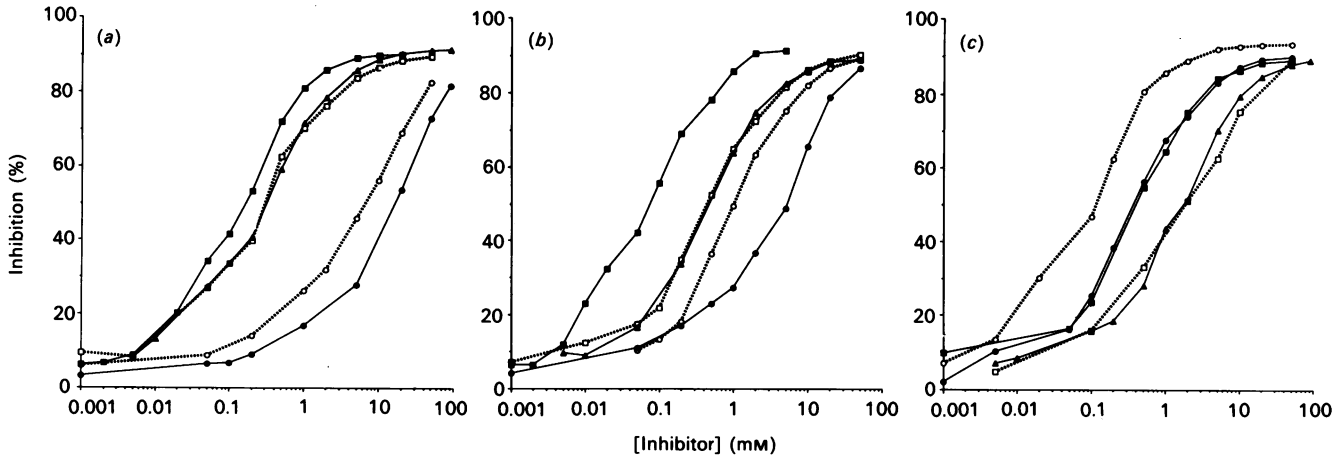

Fig. 2. Inhibitor concentration curves of inhibition of wild-type and mutagenized APs by L-amino acids

APs used were wild-type PLAP (○) wild-type GCAP (□), and the mutants [Ser^{84}]PLAP (●) [Gly^{429}]PLAP (■) and the combination mutant [Ser^{84},Gly^{429}]PLAP (▲). They were incubated with various concentrations of L-Leu (*a*), L-Phe (*b*) or L-Phe-Gly-Gly (*c*).

the wild-type PLAP and wild-type GCAP, indicating that their overall structure was not affected by the introduced mutations.

Inhibition curves

To extend the initial observations based on a single concentration of L-amino acids, AP inhibition was further analysed dose-dependently by using a wide range of inhibitor concentrations. Fig. 2 shows the AP inhibition curves of wild-type PLAP and GCAP, and of the PLAP mutants [Ser^{84}]PLAP, [Gly^{429}]PLAP and [Ser^{84},Gly^{429}]PLAP by the discriminating inhibitors L-Leu, L-Phe and L-Phe-Gly-Gly. For the clarity of representation, all data involving the Leu^{297} constructions (alone or in combination with Gly^{429}) have been omitted from Fig. 2, since the curves, in all cases, correspond to those obtained for the Ser^{84} constructions.

The L-Leu inhibition curve (Fig. 2) of the [Gly^{429}]PLAP is shifted slightly, but reproducibly, towards lower concentrations of inhibitor compared with the wild-type GCAP. The inhibition curve of [Ser^{84},Gly^{429}]PLAP, however, exactly superimposes that of the GCAP control curve. It is clear that the Gly^{429} residue by itself is the major determinant of L-Leu inhibition of GCAP, with Ser^{84} playing a minor modulating role on this effect.

The L-Phe inhibition phenotype for GCAP (Fig. 2) is clearly conferred by the simultaneous presence of two PLAP gene substitutions, Ser^{84} and Gly^{429}. The inhibition curves of wild-type GCAP and PLAP, although similar, show, as we have previously reported (Millán *et al.*, 1982), a clear and reproducible displacement. The [Gly^{429}]PLAP mutant is even more efficiently inhibited by L-Phe than is wild-type PLAP. The [Ser^{84}]PLAP mutant displays an inhibition curve that is shifted to approx. 10-fold higher concentrations of L-Phe. The simultaneous appearance of Ser^{84} and Gly^{429} in the PLAP cDNA results in an inhibition curve that exactly overlaps that of wild-type GCAP, but not that of wild-type PLAP.

In the case of inhibition by L-Phe-Gly-Gly (Fig. 2) the [Ser^{84}]PLAP and the [Gly^{429}]PLAP mutants give superimposed inhibition curves that are different from those of both wild-type GCAP and wild-type PLAP. However, once again the combination of these two mutations in a single construction shifts the response curve to coincide exactly with that of wild-type GCAP. However, unlike for the inhibition curves with L-Leu and L-Phe, the L-Phe-Gly-Gly inhibition curve for the [Ser^{84},Gly^{429}]PLAP combination mutant did not occupy an intermediate position between those of the two single mutants.

DISCUSSION

The uncompetitive inhibition of APs by certain amino acids and peptides is an established phenomenon in enzyme kinetics, although the exact mechanism is unclear (Fersht, 1985). This unique characteristic of APs has been exploited successfully to aid in the specific quantification and differentiation of AP isoenzymes (Green *et al.*, 1971; Van Belle *et al.*, 1977; Millán *et al.*, 1980; Haije *et al.*, 1987). The biological significance of this phenomenon is far from clear, as is the function of APs. It is striking to us that the AP inhibitors are naturally occurring L-amino acids and peptides containing L-amino acids. L-Homoarginine is an exception, since it was empirically identified as a better inhibitor than its natural homologue L-Arg (Fishman & Sie, 1971). It is possible that free amino acids in the intimacy of the tissues where AP isoenzymes are expressed, through their action as inhibitors, modulate the activity and function of APs. Alternatively, the AP inhibition site may represent a binding site able to recognize these L-amino acids in the context of a peptide or protein molecule. The isoenzyme specificity of AP function could, therefore, be provided by the interaction of this binding site (not necessarily an inhibitory site) with its cognate sequence built into the substrate molecules that would allow certain phosphorylated peptides or proteins, and not others, to be acted upon by the catalytic site of an AP isoenzyme.

In order to understand the function of APs and the physiological significance of the uncompetitive inhibition phenomenon of APs, a detailed structural/functional analysis must be carried out. In this report we present conclusive evidence that implicates Gly^{429} of GCAP as the primary determinant for the inhibition of this isoenzyme by L-Leu. Gly^{429} is found in a loop region that is unique to eukaryotic APs and absent from the *Escherichia coli* AP molecule. Although a comparison of GCAP with the *E. coli* AP sequence and its three-dimensional structure (Millán, 1988, 1990; Sowadski *et al.*, 1985) would predict that the location of this residue lies in proximity to the entrance to the active site, we will have to await the elucidation of the crystallographic structure of human GCAP or PLAP to gain an understanding of the topology of the inhibitory site.

It is also clear from our results that Ser^{84}, distant in the primary sequence from Gly^{429}, is able to influence (either by contributing structurally to the inhibitory site or by conformationally modifying this site) the response of GCAP, in the presence of substrate, to L-Leu, L-Phe and L-Phe-Gly-Gly.

A third mutation, [Leu^{297}]PLAP, was also found to have a modulatory effect on the inhibition. However, this effect was not additive to those seen with [Ser^{84}]PLAP and [Gly^{429}]PLAP, and mutations containing all three substitutions did not behave differently from those including either Ser^{84} and Gly^{429} or Leu^{297} and Gly^{429} alone. Leu^{297} has been found by Watanabe *et al.* (1989) to be an allelic position in GCAP, and so it is not surprising that the effects of this substitution do not contribute substantially to the general properties of GCAP. However, the effects of Leu^{297} are entirely comparable with those of Ser^{84}. If position 84 were also found to be polymorphic, Ser^{84} and Leu^{297} could play interchangeable roles.

None of the mutations tested conferred a specific inhibition to L-homoarginine. The [Gly^{429}]PLAP mutant displayed a 5-fold increase in sensitivity to L-homoarginine that was not accompanied by a simultaneous decrease in inhibition by L-Phe. Mutagenesis of residue 429 to the amino acid found in TNAP isoenzymes, i.e. His^{429}, did not confer L-homoarginine inhibition properties (results not shown). Considering the lesser degree of sequence similarity between TNAP and TSAP isoenzymes (50 %), identification of the site(s) responsible for inhibition by L-homoarginine of TNAP isoenzymes will most likely have to be approached in the future by domain swapping until putative candidates are identified for site-directed mutagenesis experiments.

The results presented in this paper have clearly identified the structural residues in GCAP that underlie its inhibition by L-Leu. These data will allow the generation of heterodimers of known structure that will enable us to clarify the mechanism of uncompetitive inhibition and study the biological significance of this phenomenon.

We thank Dr. William H. Fishman and Dr. Marc F. Hoylaerts for critically reading this manuscript. This work was supported in part by grant CA42595 from the National Institutes of Health and by a training stipend from Teknologirådet, Denmark.

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Received 25 September 1990/17 October 1990; accepted 16 November 1990