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Inhibition and Metal Ion Activation of Pig Kidney Aminopeptidase P

DEPENDENCE ON NATURE OF SUBSTRATE

Georgina S. Lloyd, John Hryszko, Nigel M. Hooper and Anthony J. Turner†*

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, UNIVERSITY OF LEEDS, LEEDS LS2 9JT, U.K.

ABSTRACT. Pig kidney aminopeptidase P (AP-P; EC 3.4.11.9) has been purified to homogeneity after its solubilisation from brush border membranes by phosphatidylinositol-specific phospholipase C. The effects of various activators and inhibitors of AP-P activity have been examined with a number of different substrates for the enzyme. The hydrolysis of bradykinin and ArgProPro is inhibited at Mn^{2+} concentrations above 10^{-5} M, whereas the hydrolysis of other substrates (GlyProHyp, β-casomorphin, substance P) is substantially activated, with 4–10 mM Mn^{2+} being optimal. The thiol reagent, p-chloromercuriphenylsulphonic acid, inhibits the hydrolysis of GlyProHyp but markedly activates the hydrolysis of bradykinin. A number of inhibitors of angiotensin converting enzyme (ACE; EC 3.4.15.1), previously reported to inhibit the hydrolysis of GlyProHyp, have no effect on the hydrolysis of bradykinin except in the presence of Mn^{2+}. Differences were also observed in the degree of inhibition of GlyProHyp and bradykinin hydrolysis by EDTA and their reactivation by divalent cations. The hydrolysis of GlyProHyp follows Michaelis-Menten kinetics with a K_m value of 2.7 mM. Bradykinin inhibits GlyProHyp hydrolysis with an I_{50} of 1.4 μM. The hydrolysis of bradykinin by AP-P reveals anomalous nonlinear kinetics indicative of negative cooperativity or the presence of more than one active site for this substrate. These results indicate that substrates for AP-P can be divided into 2 groups based on their responses to inhibitors and cation activators. BIOCHEM PHARMACOL 52;2:229–236, 1996.

KEY WORDS. aminopeptidase; bradykinin; metalloenzyme; chemical modification

AP-P‡ (EC 3.4.11.9) was first identified in pig kidney as an exopeptidase capable of releasing the N-terminal amino acid residue from peptides with a penultimate proline [1]. Peptides of the X-Pro-Y type were cleaved most readily and a synthetic substrate, glycyl-prolyl-hydroxyproline (GlyProHyp), was originally used to monitor AP-P activity [1]. Other small synthetic peptides, such as ArgProPro, have also been used successfully as assay substrates [2]. The enzyme has been shown to cleave several biologically active peptides containing penultimate N-terminal prolyl residues, such as bradykinin, substance P, β-casomorphin, and peptides of the pancreatic polypeptide family [2–5]. The enzyme may act in concert with ACE (EC 3.4.15.1) in the physiological inactivation of bradykinin [3, 6, 7]. We have previously identified pig kidney AP-P as a cell surface ectoenzyme possessing a glycosyl-phosphatidylinositol (GPI) anchor [8], and have utilised the selective release of AP-P by bacterial phosphatidylinositol-specific PLC (PI-PLC) as an initial step in the purification of the enzyme from pig kidney membranes [9].

During the initial characterisation of AP-P from pig kidney, it was observed that Mn^{2+} ions were required for optimal activity of the enzyme [10], which led others to include Mn^{2+} in enzyme assays [11, 12]. More recent work has confirmed that $MnCl_2$ stimulates the hydrolysis of GlyProHyp, with maximum activity observed at approximately 4 mM $MnCl_2$ [9]. Several groups have reported inhibition of AP-P from different mammalian species and tissues by metal-chelating agents, such as EDTA and 1,10-phenanthroline, suggesting that the peptidase is a metalloenzyme [2, 3, 9, 12] and 1 mol Zn^{2+}/mol of protein has been identified in the purified enzyme [13]. The additional role of Mn^{2+} or other cations in the catalytic process is unclear.

AP-P is unlike a number of other mammalian brush-border aminopeptidases (e.g., aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), and aminopeptidase W (EC 3.4.11.16)) in that it is relatively insensitive to inhibitors such as bestatin, amastatin, actinonin, and puromycin [2, 13]. However, the hydrolysis of GlyProHyp by AP-P can be inhibited by several ACE inhibitors, such as enalaprilat, cilazaprilat, and ramiprilat, all at μM concentrations [13]. Rather than resembling aminopeptidases

* Present address: School of Biochemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

† Corresponding author. Tel. (0113) 233 3131; FAX (0113) 242 3187; E-mail: a.j.turner@leeds.ac.uk

‡ *Abbreviations:* ACE, angiotensin converting enzyme; AP-P, aminopeptidase P; Dip-F, diisopropylfluorophosphate; I_{50}, concentration producing 50% inhibition; NMec, 4-methyl-7-coumarylamide; PLC, phospholipase C; PMPS, p-chlorohydroxymercuriphenylsulphonic acid.

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N, A, and W, AP-P appears to be related to the "proline peptidase" family of hydrolytic enzymes that includes *E. coli* aminopeptidase P, prolidase, methionine aminopeptidase, and creatinase [14, 15]. The amino acid sequence of pig kidney AP-P, obtained by Edman degradation and mass spectrometry of the purified protein, reveals some limited sequence similarities in the C-terminal half of the protein with other members of this family [16]. The amino acid sequence of AP-P does not contain the typical HExxH motif common to many zinc peptidases nor any other zinc binding motifs [17], and residues in the protein important for zinc binding and catalytic activity have not yet been identified.

In the present study, we show that the stimulation of pig kidney AP-P activity by Mn^{2+} ions and the inhibition by selected converting enzyme inhibitors appear to be substrate-dependent. Furthermore, EDTA and the thiol reagent PMPS exert distinct effects on GlyProHyp and bradykinin hydrolysis. The kinetics of hydrolysis of bradykinin, but not GlyProHyp, were shown to be anomalous.

MATERIALS AND METHODS

PLC from *Bacillus cereus* was purchased from Fluka Chemie AG, Switzerland and units are in μmol/min. GlyProNMec was obtained from BACHEM U.K. and proline iminopeptidase (EC 3.4.11.5) was from Nacalai Tesque, Kyoto, Japan. The ACE inhibitors, enalaprilat (MK 422) and L155,212 [13] were gifts from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.; cilazaprilat was a gift from Roche Products Ltd., Welwyn Garden City, U.K.; YS980 [13] was a gift from Santen Pharmaceutical Co. Ltd., Osaka, Japan; ramiprilat was from Hoechst Pharmaceutical Research Laboratories, Milton Keynes, U.K. The use of these compounds to inhibit Gly-Pro-Hyp hydrolysis by AP-P has been described previously [13]. EDTA was obtained from BDH Chemical Co., Poole, U.K. Acetonitrile (HPLC grade) was acquired from Fisons, FSA Laboratory Supplies, Loughborough, U.K., and all other materials were from Sigma Chemical Co., Poole, U.K.

Routine Purification of Aminopeptidase P and Antibody Production

AP-P was routinely purified according to the method of Hooper *et al.* [9] and was apparently homogeneous on SDS-PAGE. A polyclonal antibody to AP-P (RP205) was raised in a rabbit using enzyme purified by the above method. Protein A-purified RP205 was, then, coupled to CNBr-activated Sepharose 4B by standard procedures.

Immunoaffinity Purification of Aminopeptidase P

Pig kidney cortex membranes were prepared and solubilised with *B. cereus* PLC, as described for the conventional purification of AP-P. The sample was, then, dialysed against 10 mM Tris/HCl, pH 7.6 and concentrated on a DEAE-cellulose column as above. The eluted fraction was, then, dialysed against PBS and applied to a column of RP205 antiserum coupled to CNBr-activated Sepharose 4B (10 mL bed volume) *via* a precolumn of unmodified Sepharose CL-4B (20 mL bed volume), previously equilibrated in PBS. After washing in PBS, the affinity column was eluted by one of the following: 2 M NaI in PBS, pH 7.5, 0.1 M ethanolamine, pH 10.5, or 0.2 M glycine, pH 2.2, and 1-mL fractions collected in each case. In the case of elution with NaI, fractions containing protein and NaI (as assessed by absorbance at 280 nm) were pooled and desalted using a Sephadex G25 column. Finally, the protein samples were individually concentrated on a column (1 mL bed volume) of DEAE-cellulose and eluted with 0.7 M KCl in 10 mM Tris/HCl, pH 7.6. Fractions containing protein were pooled and dialysed extensively against 10 mM Tris, pH 7.6.

Enzyme Assays

The hydrolysis of GlyProHyp (1 mM) by AP-P was assayed by an HPLC method, as described previously [8]. Preincubation of enzyme samples (65 ng) in 0.1 M Tris/HCl, pH 8.0 containing 4 mM $MnCl_2$ for 5 min was followed by a 30-min incubation with substrate (100 μL total volume), all at 37°C. The reaction was terminated by boiling for 4 min. Under these conditions, the formation of product (ProHyp) was linear with time. Hydrolysis of GlyProHyp was monitored using reverse-phase HPLC [8]. The product, ProHyp, was quantified by calibration from a standard curve.

Hydrolysis of bradykinin, substance P, and β-casomorphin (all at 1 mM) by AP-P was also monitored at A_{214} by reverse-phase HPLC on a C_{18} column, by using a 20-min linear gradient of 4–45% (v/v) acetonitrile in 0.08% H_3PO_4 at a flow rate of 1.5 mL/min, followed by 5-min elution at final conditions. The products (bradykinin (2-9), substance P (2-11) and *des*-Tyr β-casomorphin, respectively) were quantified by calibration from standard curves.

AP-P hydrolysis of GlyProNMec was assayed by a fluorimetric assay modified from the method of Yoshimoto *et al.* [18]. The enzyme sample (200 ng) was incubated with 4 mM $MnCl_2$ and 0.2 mM GlyProNMec in 20 mM Tris/HCl, pH 8.0 (total volume 1 mL) at 37°C for 2 hr and, then, the reaction was terminated by boiling for 4 min and the samples cooled on ice. To release the chromogenic group, 0.1 unit of proline iminopeptidase in 20 mM Tris/HCl, pH 8.0 was added, the samples were incubated at 37°C for 30 min and the reaction terminated by boiling for 4 min. The samples were diluted to 2 mL with water, and fluorescence was read in a Perkin Elmer LS50B fluorimeter at an excitation wavelength of 370 nm and an emission wavelength of 442 nm.

Hydrolysis of ArgProPro and substance P (1-4) (both at 0.5 mM) by AP-P was assayed by using a fluorimetric assay adapted from the method of Simmons and Orawski [2]. After preincubation in 0.1 M HEPES, pH 8.0 for 5 min at 37°C, the enzyme was incubated with ArgProPro (20 ng) or substance P (1-4) (250 ng; 0.1 mL total volume) for 30 min or 4 hr, respectively, at 37°C. Aliquots (10 μL) were then

mixed with o-phthalaldehyde, 2-mercaptoethanol reagent (3 mL) [19], and the fluorescence read using a Perkin Elmer LS50B fluorimeter at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The released Arg was quantified by calibration from a standard curve.

Incubations were performed in triplicate under each set of conditions, and the enzyme was preincubated for 30 min at 37°C with $MnCl_2$ and/or inhibitor, as required. For each assay, the amount of AP-P included was such that there was maximally 20% substrate breakdown observed with each substrate, in the absence of inhibitor, under optimal conditions.

SDS/PAGE

SDS/PAGE was performed with a 7–17% polyacrylamide gradient as described previously [20].

Determination of Cobalt

All buffers were made up with deionised water. Dialysis tubing was boiled in the presence of EDTA and washed extensively with deionised water. All glassware was acid-washed in 6 M HCl and rinsed with deionised water before use. The enzyme sample (0.1–0.2 mg protein/mL) was dialysed against two changes of 5 mM Tris/HCl buffer, pH 7.5 at 4°C to remove any nonspecifically bound metal ion. After determining the protein concentration and enzyme activity of the dialysed sample, duplicate 20-μL aliquots were analyzed by atomic absorption spectrometry. $CoCl_2$ (0–1 μM) was used as standard.

Protein Determination

Protein concentrations were determined using the bicinchoninic acid (BCA) assay [21], using BSA as the standard. The method was adapted for use with 96-well microtitre plates [22].

RESULTS

Purification of Aminopeptidase P

Two procedures were compared for purification of AP-P from pig kidney cortex; both were effective in producing homogeneous enzyme as assessed by SDS-PAGE (M_r approx. 91,000). In both cases, AP-P activity was solubilised from the brush-border membranes by the action of phospholipase C from *Bacillus cereus*. 0.35 units PLC/mg of membrane protein was found to be optimal, although each batch of PLC needs to be checked because the PI-specific activity is a minor and variable component in the overall PLC preparation. The conventional method for purification of AP-P involved a series of chromatography steps and has previously been described in detail by Hooper *et al.* [9]. The availability of a polyclonal antibody (RP205) against enzyme purified by this method allowed us to assess the efficacy of an alternative immunoaffinity purification procedure. In this case, PLC-solubilised AP-P was adsorbed to immobilised antibody. The affinity resin was washed with PBS and, in preliminary experiments, bound protein was eluted by 1 of 3 methods: 2 M NaI in PBS, pH 7.5, 0.1 M ethanolamine, pH 10.5, or 0.2 M glycine, pH 2.2. Ethanolamine was the most efficient eluant, producing AP-P of highest specific activity (6.7 μmol ProHyp/min/mg). In contrast, purification from an equivalent amount of starting material by the conventional chromatographic procedure produced AP-P with a final specific activity of 11.1 μmol ProHyp/min/mg. Although the procedure of Hooper *et al.* [9] was considerably more time-consuming than the immunoaffinity procedure, it was used for all the characterization of AP-P reported below because of the advantages of overall yield and specific activity. Where comparisons were made in properties (e.g., in Mn^{2+} activation effects and inhibitor effects), no differences were seen in enzyme purified by the 2 methods. When 100 ng of aminopeptidase P purified by either method was incubated with the appropriate substrate (GlyProNMec) and buffer for 30 min at 37°C, no detectable dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) activity was observed with any of the preparations. The hydrolysis of bradykinin by purified AP-P was not affected by preincubation with 1 mM Dip-F, an inhibitor of DPPIV.

Effect of $MnCl_2$ on Activity of Aminopeptidase P

The effect of a range of concentrations of Mn^{2+} ions on the activity of purified AP-P towards several potential substrates was examined (Figs. 1 and 2). In agreement with previous results [9], hydrolysis of GlyProHyp was stimulated by $MnCl_2$ with optimal activity observed in the range 4–10 mM $MnCl_2$, and with inhibition occurring at higher concentrations of the bivalent cation. In contrast, AP-P hydrolysis of both ArgProPro and the natural substrate, bradykinin, was not stimulated by Mn^{2+} ions. In both cases, activity was optimal in the absence of $MnCl_2$, with inhibition occurring with increasing concentrations of the bivalent cation. The hydrolysis of substance P, substance P (1-4), and β-casomorphin by AP-P was also stimulated by $MnCl_2$ and was optimal at 10 mM Mn^{2+} for substance P and β-casomorphin, and 4 mM for substance P (1-4) (Fig. 2).

Effects of ACE Inhibitors on Activity of Aminopeptidase P

The effects of a selection of ACE inhibitors, previously shown to inhibit AP-P [13] when GlyProHyp was used as substrate, were examined on the activity of the purified enzyme using other substrates. With GlyProHyp as substrate, all 5 of the selected ACE inhibitors caused complete inhibition of enzyme activity, either in the presence of $MnCl_2$ (Table 1) or in its absence (data not shown). With GlyProNMec, substance P and β-casomorphin, the inhibition data indicate that all of the inhibitors studied caused more than 75% inhibition of activity. However, with bradykinin as substrate, only YS980 showed any significant inhibition of AP-P activity. The hydrolysis of ArgProPro was, also, largely resistant to the action of the ACE inhibitors, with the exception of YS980.

Because the sulphydryl compound, YS980, was the only ACE inhibitor selected that significantly inhibited all the

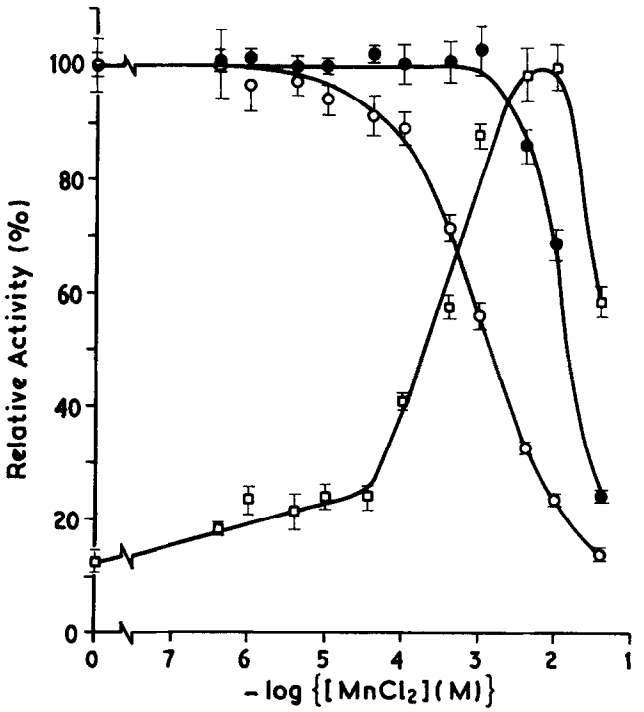

FIG. 1. Effect of $MnCl_2$ concentration on the activity of aminopeptidase P towards GlyProHyp, ArgProPro, and bradykinin. Purified aminopeptidase P (16.5 ng, ArgProPro or bradykinin as substrate, or 65 ng, GlyProHyp as substrate) was incubated with the appropriate substrate at 37°C in the presence of various concentrations of $MnCl_2$, and the products quantified as in Materials and Methods. Each point is the mean ± SEM of 4 separate determinations. □, GlyProHyp; ●, ArgProPro; ○, bradykinin.

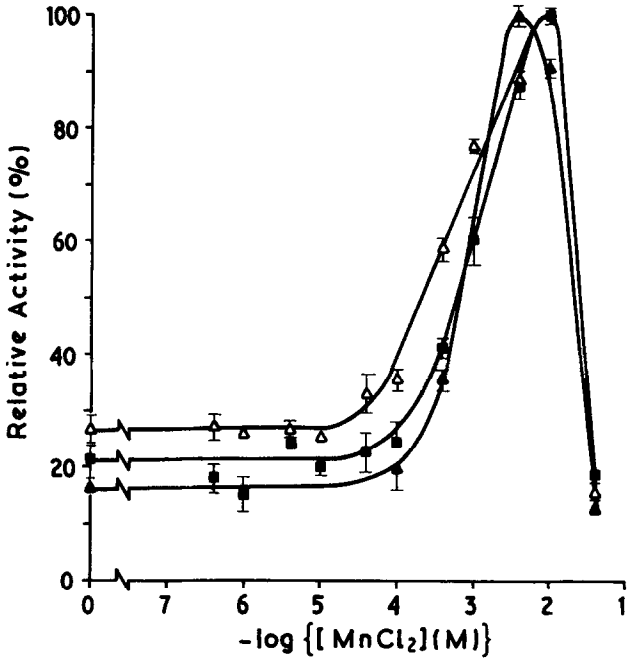

FIG. 2. Effect of $MnCl_2$ concentration on the activity of aminopeptidase P towards substance P, substance P (1-4), and β-casomorphin. Purified aminopeptidase P (65 ng, β-casomorphin as substrate, or 250 ng, substance P and substance P (1-4) as substrate) was incubated with the appropriate substrate at 37°C in the presence of various concentrations of $MnCl_2$, and the products quantified as in Materials and Methods. Each point is the mean ± SEM of 4 separate determinations. ▲, substance P; △, substance P (1-4); ■, β-casomorphin.

substrates studied, I_{50} values were obtained for 4 of the substrates with this compound. I_{50} values with the different substrates were as follows: ArgProPro, 23.3 μM; GlyProHyp, 23.4 μM; β-casomorphin, 285 μM, and bradykinin, 1.63 mM.

The effects of enalaprilat and cilazaprilat on the hydrolysis of bradykinin by AP-P were further examined in the presence of 0.4 mM and 4 mM Mn^{2+}. Although these concentrations of Mn^{2+} are themselves inhibitory to bradykinin hydrolysis (see Fig. 1), further inhibition was observed in the presence of the two ACE inhibitors (Table 2).

Inhibition by EDTA and Reactivation by Divalent Metal Ions

The effect of the metal-chelator EDTA on AP-P activity was investigated. I_{50} values for the hydrolysis of GlyProHyp and of bradykinin were obtained and the inhibition curves are shown in Fig. 3. I_{50} values were as follows: GlyProHyp, 1.9 μM, and bradykinin, 2.4 μM. Although hydrolysis of GlyProHyp was completely inhibited by 10^{-5} M EDTA, a maximum of only 75% of bradykinin hydrolysis was inhibited even at 10^{-3} M EDTA. The residual 25% of AP-P activity towards bradykinin was not inhibited by 1 mM Dip-F, indicating that it was not attributable to contamination with DPP-IV activity, and the only product detectable by HPLC was bradykinin (2-9) consistent with the action of AP-P.

To explore further the differences in behaviour between GlyProHyp and bradykinin hydrolysis, reactivation by divalent cations after EDTA inactivation was compared (Table 3). As above, with GlyProHyp as substrate, AP-P was completely inactivated by extensive dialysis against EDTA. The metal-free enzyme was reactivated by $MnCl_2$ and $CoCl_2$, and optimal activity was recovered at 4 mM of each cation. $MgCl_2$ and $CaCl_2$ at 4 mM were capable of reactivating AP-P, but to a lesser extent than either $MnCl_2$ or $CoCl_2$. $ZnCl_2$ at 0.04 mM reactivated the enzyme to some extent, but higher concentrations were inhibitory.

Marked differences were seen when bradykinin was used as substrate. None of the cations studied at any of the concentrations restored AP-P activity to its control levels. Mn^{2+} (0.04 mM) had the most significant effect on bradykinin hydrolysis by AP-P, increasing activity to 87% of control values, and 0.04 mM Co^{2+} had a somewhat lesser effect (68% of control).

Estimation of Co^{2+} Content

The possible presence of Co^{2+} in purified pig kidney AP-P was examined by atomic absorption spectroscopy. Less than

TABLE 1. Effects of a selection of ACE inhibitors on AP-P activity

Inhibitor (1 mM)	Activity (%) GlyProHyp (1 mM)	GlyProNMec (0.2 mM)	Substance P (1 mM)	β-casomorphin (1 mM)	ArgProPro (0.5 mM)	Bradykinin (0.05 mM)
None	100	100	100	100	100	100
Enalaprilat	0	1.6 ± 0.8	0	0	78.1 ± 6.5	93.6 ± 2.2
Cilazaprilat	0	1.9 ± 1.7	0	0	76.3 ± 0.6	117.9 ± 8.3
Ramiprilat	0	10.7 ± 1.0	0	25 ± 1.6	86.6 ± 7.0	112.4 ± 3.1
L155,212	0	3.0 ± 0.5	0	3 ± 1.0	35.7 ± 7.3	89.2 ± 8.4
YS980	0	25 ± 8.0	0	0	10.4 ± 0.5	21.6 ± 1.7

Purified aminopeptidase P was incubated with GlyProHyp, GlyProNMec, substance P and β-casomorphin in the presence of $MnCl_2$, as described in Materials and Methods, both in the absence and presence of inhibitors (1 mM). For ArgProPro and bradykinin, no $MnCl_2$ was present. Results are the mean ± SEM of at least 2 separate triplicate determinations. In the absence of any inhibitors, approximately 20% substrate breakdown was observed.

0.05 mol Co^{2+}/mol of enzyme protein was detectable in a fully active preparation of the enzyme.

Kinetic Studies on Aminopeptidase P

The effects of bradykinin on GlyProHyp hydrolysis by AP-P, and GlyProHyp on the hydrolysis of bradykinin were investigated. Bradykinin was capable of inhibiting GlyProHyp hydrolysis with an approximate I_{50} of 1.4 μM (Fig. 4), whereas 10 mM GlyProHyp did not appear to inhibit bradykinin hydrolysis significantly (result not shown). Kinetic studies were carried out with bradykinin and GlyProHyp. The result obtained with GlyProHyp is shown in Fig. 5A, and the K_m value obtained for GlyProHyp hydrolysis was 3.06 ± 0.1 mM. The hydrolysis by AP-P of bradykinin over a wide range of concentrations revealed a non-linear double reciprocal plot (Fig. 5B).

Effect of PMPS on Aminopeptidase P Activity

The effect of the thiol inhibitor PMPS on AP-P activity was examined using either bradykinin or GlyProHyp as substrate. With GlyProHyp as substrate, inhibition was observed with complete inactivation by 0.01 mM PMPS following a 30-min preincubation. However, with bradykinin as substrate, stimulation of enzyme activity by PMPS was observed in the presence of Mn^{2+} (Table 4), and no inhibition in its absence.

TABLE 2. Effects of Mn^{2+} on sensitivity of bradykinin hydrolysis to ACE inhibitors

Inhibitor	% Activity (Mn^{2+}, mM) (0)	(0.4)	(4)
None	100	70	30
Enalaprilat	104	28	7.7
Cilazaprilat	110	39.9	12.9

Purified aminopeptidase P was incubated with bradykinin in the absence and presence of $MnCl_2$ and inhibitors, as described in Materials and Methods. Results are the means of triplicate determinations that did not differ by more than 6%. In the absence of any inhibitors, approximately 20% substrate breakdown was observed.

DISCUSSION

The purification of AP-P was much facilitated by the discovery that it is linked to the plasma membrane by a GPI anchor, allowing its selective release in a hydrophilic form by treatment with PI-PLC [8, 9]. This procedure allowed

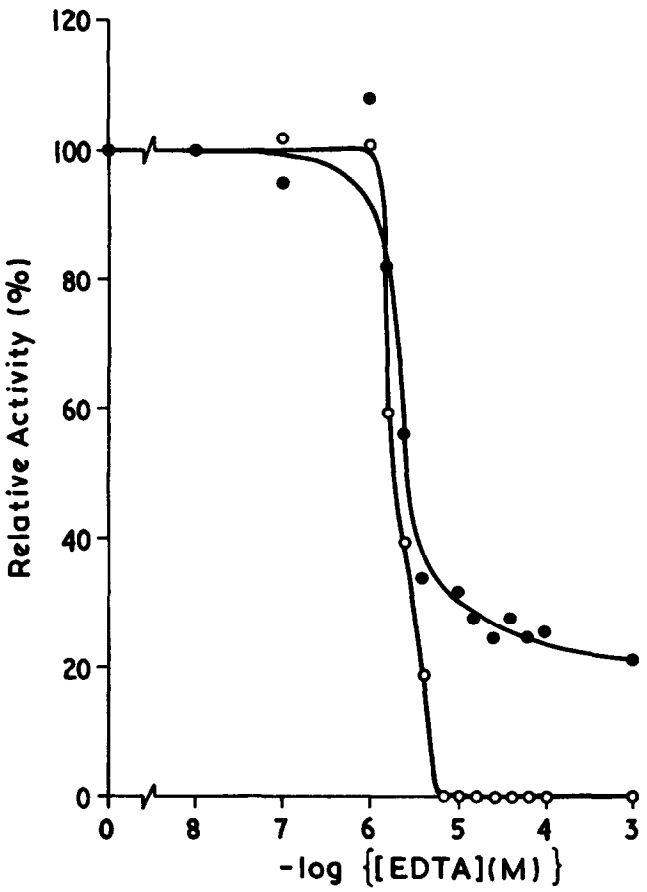

FIG. 3. Inhibition of aminopeptidase P by EDTA. Purified aminopeptidase P (5 ng, bradykinin as substrate, or 65 ng, GlyProHyp as substrate) was preincubated for 60 min with EDTA and, then, incubated with substrate for 30 min at 37°C. Substrate and product peaks were separated and quantified by HPLC. Each point is the mean of triplicate determinations that did not differ by more than 5%. The result shown is typical of 2 separate experiments. ○, GlyProHyp; ●, bradykinin.

TABLE 3. Reactivation of EDTA-dialysed aminopeptidase P by divalent cations

Enzyme	Bivalent cation	Conc. (mM)	Relative Activity (%) GlyProHyp (1 mM)	Relative Activity (%) Bradykinin (0.05 mM)
Control AP-P	None		100	100
EDTA-dialysed AP-P	None		0	40.0
	Mn^{2+}	4	565.3	26.7
		0.4	162.2	56.0
		0.04	114.7	86.6
	Co^{2+}	4	586.9	10.9
		0.4	507.8	56.0
		0.04	142.2	68.4
	Zn^{2+}	4	0	3.4
		0.4	78.4	18.2
		0.04	158.8	56.2
	Mg^{2+}	4	146.6	41.6
		0.4	70.3	44.9
		0.04	0	44.0
	Ca^{2+}	4	161.6	40.2
		0.4	62.4	55.7
		0.04	0	49.7

Purified AP-P was dialysed overnight against 1 mM EDTA. The inactivated AP-P (65.5 ng, for GlyProHyp as substrate, or 5 ng, bradykinin as substrate) was, then, preincubated in the presence of various bivalent cations for 15 min at 37°C. The results are the means of duplicate determinations for each concentration of cation that did not differ by more than 9%, and are representative of 3 sets of data. Results are expressed relative to values for control AP-P assayed in the absence of bivalent cations; activities were as follows: with GlyProHyp, 1.6 mmol ProHyp/min/mg; with bradykinin, 5.9 mmol bradykinin (2-9)/min/mg.

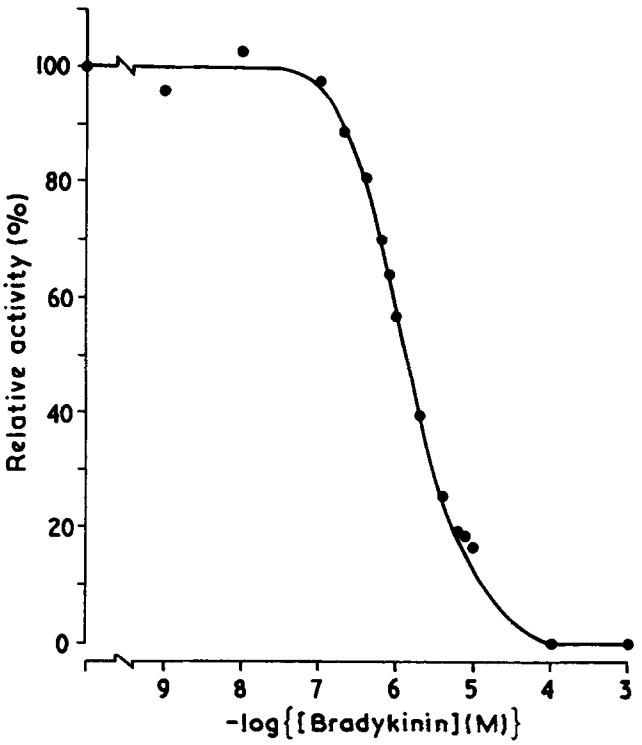

FIG. 4. Inhibition of GlyProHyp hydrolysis by bradykinin. Purified aminopeptidase P (65 ng) was incubated in the presence of 4 mM $MnCl_2$ and various concentrations of bradykinin, and the products quantified as in Materials and Methods. Each point is the mean ± SEM for 2 separate triplicate determinations.

the purification of AP-P to homogeneity for the first time by using a series of chromatographic steps [9]. To facilitate purification of AP-P, we have examined the utility of an immunoaffinity procedure. Purification of serum AP-P has previously been reported by use of an immobilised monoclonal antibody [23]. In the present work, AP-P was adsorbed to the immunoaffinity matrix and elution was effected most efficiently with ethanolamine. No heterogeneity of AP-P protein was seen on SDS gels, in contrast to the two differentially glycosylated forms reported in [16]; although, in the latter case, the initial solubilisation step involved butanol treatment, rather than the milder and more selective procedure of PI-PLC release. The procedure of immunoaffinity purification is a much more rapid technique for AP-P purification than that previously described [9], but the latter does provide a higher overall recovery and final yield than the immunoaffinity procedure. No difference was seen in the kinetic or inhibitor properties of the enzyme when purified by the 2 methods, suggesting the differences seen in substrate behaviour were unlikely to be due to any contaminating peptidase activity. The development of a specific inhibitor of AP-P suitable for affinity chromatography is likely to provide the most efficient purification procedure for the enzyme but, at present, no potent and selective inhibitor of the enzyme is available.

Aminopeptidase P was initially identified in pig kidney [1] and it was observed that Mn^{2+} was required for optimal activity when GlyProHyp was used as substrate [9]. Subsequent studies have yielded a confusing picture of the effects of cations on enzyme activity, partly because of the use of different substrates as well as tissue and species source for the enzyme [2, 3, 9, 12, 24]. In the present work, we have used a range of substrates to explore the cation dependence on a homogeneous preparation of pig kidney AP-P, and establish that the effect appears to be a substrate-dependent phenomenon that has implications for the catalytic mechanism of the enzyme. Substrates can be divided into two groups on the basis of Mn^{2+}-dependence. The hydrolysis of GlyProHyp, β-casomorphin, substance P, and substance P (1-4) (Group 1 substrates) were all substantially stimulated by $MnCl_2$, whereas $MnCl_2$ has no effect on the metabolism of ArgProPro and bradykinin (Group 2 substrates) at low concentrations and inhibits at higher levels. Orawski *et al.* [3] previously observed that, with the bovine lung enzyme, GlyProHyp hydrolysis was stimulated by Mn^{2+} but hydrolysis of ArgProPro was not affected.

Our attention was first drawn to the nature of the N-terminal residue as a possible factor in explaining these differences, Mn^{2+} aiding the binding of those substrates with a neutral side-chain (e.g., Gly, in GlyProHyp, or Tyr, in β-casomorphin), perhaps through interaction with a carboxylate group in the active site of the enzyme. Mn^{2+} would, then, inhibit binding of substrates such as bradykinin and Arg-Pro-Pro, with a basic (arginyl) side-chain at their N-terminus. However, substance P and substance P

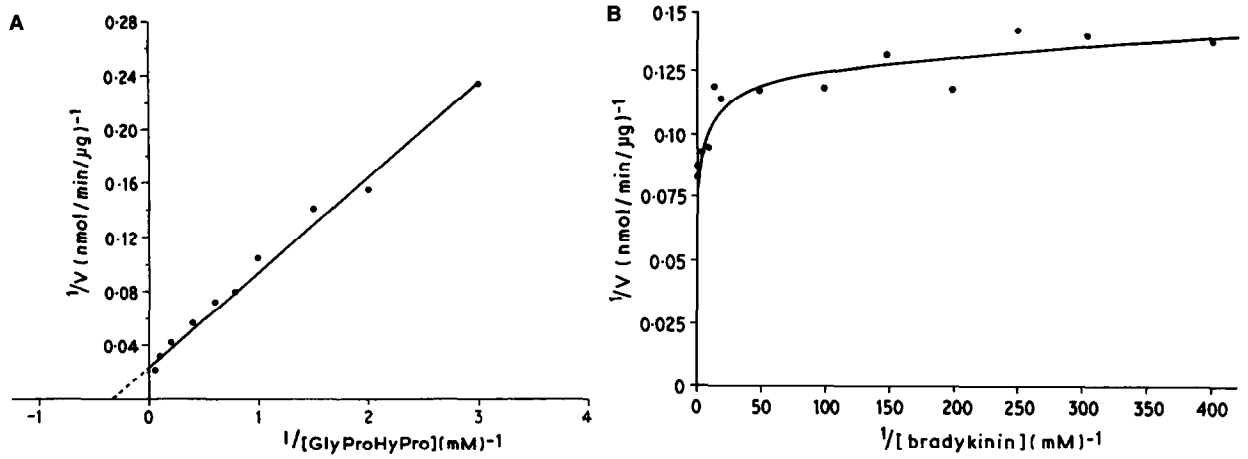

FIG. 5. Kinetics of substrate hydrolysis by aminopeptidase P. A. Purified aminopeptidase P (17.5–131 ng, as appropriate) was incubated with various concentrations of GlyProHyp for 30 min at 37°C in the presence of 4 mM $MnCl_2$. The results shown were typical of 3 experiments. B. Purified aminopeptidase P (0.26–52 ng, as appropriate) was incubated with various concentrations of bradykinin for 15 min at 37°C in the absence of $MnCl_2$. The results shown were typical of 4 experiments.

(1-4) which, like bradykinin, possess an N-terminal arginyl residue, behave like GlyProHyp. Length of peptide substrate, also, does not appear to be a critical factor in determining Mn^{2+} sensitivity.

AP-P has some limited similarities with members of the proline peptidase family [14, 15]. Within this family, *E. coli* methionine aminopeptidase is a dual-metal ion enzyme containing a pair of closely linked metal ions at the active site (reportedly Co^{2+} for optimal activity) [25]. The crystal structure of the *E. coli* enzyme has revealed that the two metal ions are coordinated to two Asp, two Glu, and a His residue. The eukaryotic methionyl aminopeptidases are also cobalt-dependent, but only distantly related to the prokaryotic enzyme [26]. Prolidase is, also, proposed to require the action of two metal ions in the active site [27]. We have previously shown that pig kidney AP-P contains one mol Zn^{2+}/mol protein [13] and that Co^{2+}, like Mn^{2+}, can further activate the enzyme [9]. However, here we have failed to detect the presence of significant amounts of cobalt in the purified, active enzyme. Any additional metal ion is, therefore, unknown at present. The binding of a number of ACE inhibitors to AP-P appears to require the presence of Mn^{2+} as shown here and elsewhere [24], particularly in the case of bradykinin as substrate. Because millimolar manganese is required to observe this effect, it is unlikely that these inhibitors affect the metabolism of bradykinin by AP-P *in vivo*.

TABLE 4. Effect of PMPS on aminopeptidase P activity

	Relative activity (%)	
PMPS (mM)	**No $MnCl_2$**	**4 mM $MnCl_2$**
0	100	100
0.01	138 ± 32	524 ± 3
0.1	129 ± 4	478 ± 13
1.0	116 ± 2.5	207 ± 1

Purified AP-P (5 ng) was preincubated with PMPS at the concentrations shown and with $MnCl_2$ as required, for 30 min at 37°C, then incubated for a further 20 min in the presence of bradykinin (0.05 mM final concentration). Results are expressed relative to control values in the absence of inhibitor with specific activities as follows: with no $MnCl_2$, 11.4 ± 0.4 μmol bradykinin (2-9)/min/mg; with 4 mM $MnCl_2$, 2.2 ± 0.3 μmol bradykinin (2-9)/min/mg, and are the means ± SD for 2 sets of triplicate determinations.

The hydrolysis of bradykinin by AP-P exhibits other features that distinguish it from Group 1 substrates. For example, the marked thiol activation by PMPS, the partial resistance to EDTA inhibition, and the nonlinear nature of the double reciprocal plot. The latter is suggestive of negative cooperativity or, possibly, the presence of two catalytic sites with differing affinities for bradykinin (K_m values of approximately 1 μM and 100 μM). The IC_{50} for inhibition of GlyProHyp hydrolysis by bradykinin of 1.4 μM is consistent with the presence of a high-affinity site corresponding to the GlyProHyp site and with a reported K_m for guinea pig AP-P of 1 μM [23]. A survey of the literature reveals wide variations in the reported K_m of AP-P for bradykinin (e.g., 21 μM in rat lung [24] and 76 μM in bovine lung [2]). This wide variation may reflect failure to recognise the nonlinear nature of the response.

In our original characterization of pig kidney AP-P [9], we pointed out that the enzyme showed little similarity in properties to other zinc-containing mammalian aminopeptidases, such as aminopeptidases N, A, and W, and was unlikely to resemble them structurally. The present data extend these studies and support the concept that pig kidney AP-P is related to other dual-metal ion-containing peptidases of the proline peptidase family, as suggested elsewhere [14, 15]. An additional complexity is that bradykinin, and to a lesser extent ArgProPro, showed marked differences in behaviour from other substrates for AP-P, such as substance P and GlyProHyp. The two groups of substrates may bind in distinct ways to the active site. Alternatively, the presence of a second active site for bradykinin cannot be excluded. There are analogies with the

major bradykinin degrading enzyme, ACE. ACE is a dual domain enzyme possessing 2 functional active sites [28, 29]. The kinetics of anion activation of ACE have been shown to be complex and dependent on substrate and pH [30]. The size of the AP-P polypeptide chain is substantially larger than its mammalian relatives, prolidase and methionyl aminopeptidase [26, 27], and the common regions of these enzymes are restricted to the C-terminal half of AP-P. Unfortunately, knowledge of the complete amino acid sequence of AP-P [16] fails to indicate likely catalytic residues or domains within the protein. cDNA cloning of the enzyme coupled with site-directed mutagenesis should provide a route to understanding this problem, and may provide further insight into the little-understood chemical mechanisms underlying this new class of proteolytic enzymes.

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