Aminopeptidase A (APA) is a type II membrane-bound protein implicated in the regulation of blood pressure in the brain renin-angiotensin system. In this study, a recombinant soluble form of APA was expressed in a baculovirus system, purified to homogeneity, and characterized. By using synthetic substrates, it was shown that although the enzyme has a rather broad substrate specificity in the absence of Ca$^{2+}$, the preferential release of acidic amino acid residues was observed in the presence of Ca$^{2+}$. Moreover, Ca$^{2+}$ up- or down-regulated the enzymatic activity depending on the substrate. By searching for natural substrates of APA, we found that peptides having acidic amino acids at their N terminus (angiotensin II, neurokinin B, cholecystokinin-8, and chromogranin A) were cleaved by the enzyme efficiently in the presence but not in the absence of Ca$^{2+}$. Moreover, kallidin (Lys-bradykinin) was converted to bradykinin efficiently in the presence of Ca$^{2+}$, the preferential release of acidic amino acid residues was observed in the presence of Ca$^{2+}$. These results suggest that Ca$^{2+}$ increases the preference of the enzyme for the peptide substrates having N-terminal acidic amino acids. In addition, we found that angiotensin IV could bind to APA both in the presence and absence of Ca$^{2+}$ and inhibited the enzymatic activity of APA competitively, suggesting that angiotensin IV acts as a negative regulator of the enzyme once generated from angiotensin II by the serial actions of aminopeptidases. Taken together, these results suggest that there exists a complex regulation of the enzymatic activity of APA, which may contribute to homeostasis such as regulation of blood pressure, maintenance of memory, and normal pregnancy by controlling the concentrations of peptide substrates.

Aminopeptidases hydrolyze N-terminal amino acids of proteins and peptide substrates. They are distributed widely in animal and plant tissues as well as in bacteria and fungi, suggesting that they play important roles in protein maturation, activation, modulation, the degradation of bioactive peptides, and the determination of protein stability (1). The zinc-containing M1 family of aminopeptidases (gluzincins) shares the consensus GAMEN and HEXXH(X)$_{18}$E zinc-binding motifs essential for enzymatic activity (2). So far, 11 enzymes belonging to this family have been identified, although the enzymatic activity of laeuniform has not been reported (3).

Growing evidence indicates the biological significance of aminopeptidases belonging to the M1 family. For instance, placentallucine aminopeptidase (P-LAP)$^2$/insulin-regulated aminopeptidase (IRAP) plays a role in controlling the concentration of utoeroton and vasoactive hormones, such as oxytocin and vasopressin, possibly derived from the fetus at the interface between the fetus and mother and thus preventing premature delivery and pre-eclampsia (4). It has been reported that adipocyte-derived leucine aminopeptidase (A-LAP)/endoplastic reticulum aminopeptidase is a multifunctional aminopeptidase with roles in the regulation of blood pressure, angiogenesis, the shedding of cytokine receptors, and the presentation of antigens to major histocompatibility complex class I molecules (3). In addition, several physiological and/or pathological functions in the brain, including the regulation of blood pressure and apoptosis, are carried out by aminopeptidases such as aminopeptidase A (APA), aminopeptidase N, and puromycin-sensitive aminopeptidase (5–8). It was also reported that puromycin-sensitive aminopeptidase gene-deficient mice exhibited dwarfism and increased anxiety and analgesia (9). Because of the pathophysiological significance of M1 family members, it is important to characterize their enzymatic properties and regulatory mechanisms in detail.

Aminopeptidase A (EC 3.4.11.7) is a homodimeric membrane-spanning zinc-metallopeptidase that preferentially cleaves N-terminal acidic amino acids from peptide substrates such as angiotensin (Ang) II and cholecystokinin-8 (CCK8) in vivo (5, 10). Reports by several groups showed that Ca$^{2+}$ up-regulates the enzymatic activity of APA toward synthetic substrates (such as Glu-β-naphthylamide (NA)) (11–13). APA is expressed in many areas, including the brush border of intestinal and renal epithelial cells and the vascular endothelium. It has also been identified as a murine B lymphocyte differentiation antigen (BP-1/6C3) and a human kidney (gp160) differentiation antigen (12, 14, 15). By employing highly selective APA and aminopeptidase N inhibitors, Reaux et al. (6) elucidated the...
**Characterization of Aminopeptidase A**

important roles of these two enzymes in the brain renin-angiotensin system (RAS). APA is responsible for the conversion of brain Ang II to Ang III and Ang III then mediates the increase in blood pressure. These results indicate that brain APA is a potential therapeutic target for the treatment of hypertension.

The structural features and mode of enzymatic action of APA have been extensively characterized by molecular modeling and mutational analysis (16–20). In addition, the extracellular domain of porcine APA was divided into two subdomains by proteolytic fragmentation, a 107-kDa domain containing an HEXXXH(X)_18E motif and a 45-kDa C-terminal domain (21). It was shown that the C-terminal domain is required for the dimerization of the enzyme and acts as an intramolecular chaperone necessary for the correct folding, intracellular trafficking, and activity of the enzyme (22, 23). On the other hand, the enzymatic properties of APA have been poorly characterized because of the limited availability of purified enzyme.

In this paper, we established a system for the production of a recombinant soluble form of APA and elucidated the enzymatic properties of the protein in detail. Our data indicate that depending on the substrates tested Ca\(^{2+}\) up-or down-regulates the enzymatic activity of APA and increases the specificity of the enzyme for acidic amino acids. By searching for natural substrates, we found that peptidases having acidic amino acids at their N terminus, such as Ang II, neuropeptide B, CCK8, and chromogranin A, were good substrates of APA in the presence of Ca\(^{2+}\), whereas kallidin, a peptide hormone with N-terminal nonacidic amino acid, was converted to bradykinin efficiently only in the absence of Ca\(^{2+}\). Moreover, we found that Ang IV can act as a competitive inhibitor of the enzyme. The results presented in this study provide new insight into the regulation of APA that plays an important role in brain functions such as the regulation of blood pressure and pain perception.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of a Soluble Form of Human APA in a Baculovirus System**—cDNA encoding human APA starting at Ala\(^{72}\) with the human trypsin II signal peptide (MNLLLILT-FVAAVAA) at its N terminus and a His\(_8\) tag at its C terminus was generated by PCR (24, 25). The amplified fragment was cloned into the vector pDONR (Invitrogen) by recombination with BP Clonase\textsuperscript{TM} (Invitrogen). After confirmation of the nucleotide sequence with an ABI 377 DNA sequencer, the cDNA for a soluble form of APA was transferred into a baculovirus transfer vector, pDEST8 (Invitrogen). The plasmid was then introduced into DH10Bac cells to produce recombinant bacmid DNA containing the cDNA for the soluble form of APA. Next Sf9 insect cells were transfected with the bacmid using Cellfectin reagent (Invitrogen), and after 72 h, recombinant baculoviruses were harvested. For the expression of the soluble form of APA, Sf9 cells (2.0 \times 10^\text{6}/ml) infected with the recombinant baculovirus (multiplicity of infection = ~1–3) were cultured for 72 h in 650 ml of SFM-900 II medium (Invitrogen) at 27 °C.

The conditioned medium was collected by centrifugation, concentrated in an ultrafiltration system, dialyzed against 50 mM Tris/HCl buffer (pH 8.0), and then applied to a DEAE-Toyopearl (TOSOH, Tokyo, Japan) column (2.5 \times 10 cm) equilibrated in the same buffer and eluted with a linear gradient of 0–0.25 M NaCl. The APA-containing fractions obtained from the DEAE-Toyopearl column were pooled, applied to a chelating-Toyopearl (TOSOH, Tokyo, Japan) column (1.0 \times 10 cm) preloaded with Co\(^{2+}\), and then eluted with 200 mM imidazole. The active fractions were collected, concentrated, and subjected to further characterization.

**De-glycosylation of sAPA**—Purified sAPA (100 \mu g/ml) was incubated with either peptide:N-glycosidase F (30 \mu g/ml) in 50 mM sodium phosphate buffer (pH 7.5) containing aprotonin (0.1 TIU/\mu l) or leupeptin (100 \mu M) at 4 °C for 2 h. Cleavage of the sugar moiety was monitored by periodic acid-Schiff staining using GelCode glycoprotein staining kit (Pierce). Heat-denatured proteins were prepared by boiling for 5 min.

**Measurement of Aminopeptidase Activity of APA**—The aminopeptidase activity of the recombinant human APA was determined with various fluorogenic substrates, aminoacyl-4-methylcoumaryl-7-amides (aminoacyl-MCAs). The reaction mixture containing various concentrations of aminoacyl-MCA and the enzyme in 0.5 ml of 25 mM Tris/HCl buffer (pH 7.5) with or without 1.0 mM Ca\(^{2+}\) ion was incubated at 37 °C for 5 min. The amount of 7-amino-4-methylcoumarin released was measured by spectrofluorophotometry (F-2000; Hitachi) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The kinetic parameters were calculated from Lineeweaver-Burk plots. The results are represented by $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values. All measurements were performed in triplicate.

**Cleavage of Peptide Hormones by APA**—Peptide hormones (Peptide Institute, Osaka, Japan) (25 \mu M) were incubated with the enzyme (1 \mu g/ml) at 37 °C in 25 mM Tris/HCl buffer (pH 7.5) containing 50 mM NaCl. The reaction was terminated by adding 2.5% (v/v) formic acid. The peptides generated were separated by reverse-phase HPLC (AT-10; Shimazu) on a COS-MOSIL (4.6 \times 250 mm) column (Nacalai Tesque, Kyoto, Japan) at a flow rate of 0.5 ml/min. The buffers used for the isocratic separation of the peptides were as follows: for the peptides from Ang II or kallidin, 19% acetonitrile containing 0.086% trifluoroacetic acid; for the peptides from CCK8 or neuropeptide B, 35% acetonitrile containing 0.083% trifluoroacetic acid; and for the peptides from chromogranin A, 21% acetonitrile containing 0.086% trifluoroacetic acid. The molecular masses of peptides were determined by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS with a REFLEX mass spectrometer (Bruker-Franzen Analytik) using 2-mercaptobenzothiazole as the matrix.

**Analytical Ultracentrifugation**—The molecular mass of recombinant APA was measured by the sedimentation equilibrium method using a Beckman-Coulter Optima XL-A analytical ultracentrifuge. Prior to the analysis, the sample was dialyzed against 25 mM Tris/HCl buffer (pH 7.5) containing 100 mM KCl. The enzyme solution (absorbance at 280 nm = 0.6) and reference buffer were loaded into double-sector centerpieces and mounted in an An-50 Ti rotor. Samples were centrifuged at 10,000 rpm and at 20 °C with the absorbance of the sample monitored at 280 nm every hour for 12 h. The data obtained were analyzed with Origin 6.0 software. The partial specific volume of the soluble form of APA calculated by the SEDNTERP program is 0.7345.
Materials—Asp-, Gln-, Glu-, Gly-, Ile-, Val-, and S-benzyl-Cys-MCAs were purchased from Bachem AG (Bubendorf, Switzerland). Ala-, Arg-, Leu-, Lys-, Met-, and Phe-MCAs were from the Peptide Institute (Osaka, Japan). Aprotinin and p-(4-amidinophenyl)-methanesulfonyl fluoride were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Amastatin, bestatin, and E64 were purchased from Sigma. Leupeptin, pepstatin, and all peptide hormones were from the Peptide Institute. Peptide:N-glycosidase F was purchased from New England Biolabs (Beverly, MA).

RESULTS

Production of a Recombinant Soluble Form of APA—To characterize the enzymatic properties of APA in detail, we established a method to produce a recombinant form of the protein using a baculovirus system. To facilitate purification, a His tag sequence was attached at the C-terminal end of the enzyme. The soluble form of APA (sAPA) was transiently expressed in the baculovirus system and purified to homogeneity from the culture medium by serial chromatography on columns of DEAE-Toyopearl and Co2+/H11001-chelating Toyopearl. From 650 ml of culture medium, 1.46 mg of sAPA was obtained with 40% recovery and 22-fold purification. As shown in Fig. 1A, the purified protein gave a single band with a molecular mass of 115 kDa on SDS-PAGE under both reducing and non-reducing conditions. The purified sAPA was sensitive to peptide:N-glycosidase F and shifted to 103 kDa, after treatment, which was exactly expected from the amino acid sequence of sAPA (expected molecular weight is 103,079.3). De-glycosylation of the enzyme by peptide:N-glycosidase F had little effect on the enzymatic activity of sAPA toward Glu-MCA (Fig. 1B).

Because it was shown that native APA forms a dimer via a disulfide bond (15, 22), we next performed an ultracentrifuge-based analysis to measure the molecular weight of purified sAPA exactly. The molecular weight of sAPA measured by the sedimentation equilibrium method was calculated as 237 kDa (data not shown). These results indicate that sAPA is a non-sulfide-linked homodimeric protein.

Fig. 1C shows the pH profile of the enzymatic activity. The optimal pH of the enzyme was 7.0 with relatively broad pH dependence.

Characterization of Aminopeptidase Activity of APA—We then measured the aminopeptidase activity of sAPA toward various synthetic substrates with special reference to the effects of Ca2+ on the enzymatic activity because it was reported that Ca2+ up-regulated the activity of APA toward acidic amino acids (Fig. 2) (11–13). As expected, in the presence of 1.0 mM Ca2+, the enzyme hydrolyzed 2.3- and 5.4-fold more Glu-MCA and Asp-MCA, respectively. In addition, we found that the sub-

![Figure 1](image-url)
Characterization of Aminopeptidase A

![Graph](image)

**FIGURE 2. Specificity of sAPA toward synthetic substrates.** Purified sAPA (1 μg/ml) was incubated with various aminoacyl-MCA substrates (50 μM) in the presence (closed bar) or absence (open bar) of 1.0 mM calcium chloride. Each bar shows the mean ± S.D. (n = 3). The hydrolytic activity toward Glu-MCA measured in the absence of Ca$^{2+}$ was taken as 100%.

**TABLE 1**

| Substrate | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ × 10$^4$ |
|-----------|------------|---------------------|-----------------------|
| Glu-MCA   | 1243 ± 114 | 13.3 ± 0.2          | 10.7 ± 1.0            |
| Asp-MCA   | 2291 ± 201 | 2.8 ± 0.2           | 0.9 ± 0.1             |
| Gln-MCA   | 601 ± 257  | 9.5 ± 0.1           | 15.8 ± 1.2            |

*The values are mean ± S.D. (n = 3).*

strate specificity of sAPA measured in the presence of Ca$^{2+}$ is indeed fairly restricted and limited to Glu-MCA and Asp-MCA. Little activity was detected toward other substrates tested with the exception of Gln-MCA. On the other hand, in the absence of Ca$^{2+}$, the substrate specificity was rather broad, and Gln-MCA and Glu-MCA were hydrolyzed equally by the enzyme. Moreover, although at fairly low levels, the cleavage of neutral amino acids (i.e. Ala, Leu and Met) and basic amino acids (i.e. Lys and Arg) was clearly detected.

Intriguingly, Ca$^{2+}$ modulated the hydrolytic activity of sAPA differently depending on the substrate. In contrast to the Ca$^{2+}$-mediated up-regulation of the enzymatic action toward acidic amino acids (i.e. Glu and Asp), Ca$^{2+}$ caused a 76.7% decrease in the hydrolytic activity toward Gln-MCA. Hydrolytic activities toward Ala-, Leu-, Met-, Lys-, and Arg-MCA were barely detectable in the absence of Ca$^{2+}$. Taken together, these results indicate that Ca$^{2+}$ modulates the substrate specificity of sAPA and increases its preference for the acidic amino acids.

Table 1 shows the enzymatic parameters of sAPA measured in the presence or absence of 1.0 mM Ca$^{2+}$. With Glu-MCA or Asp-MCA as the substrate, Ca$^{2+}$ caused an enhancement of the hydrolytic activity by decreasing the $K_m$ value and increasing the $k_{cat}$ value. Conversely, when Gln-MCA was used, Ca$^{2+}$ caused a decrease in the hydrolytic activity by increasing the $K_m$ value and decreasing the $k_{cat}$ value.

We next examined the effects of various divalent cations on the enzymatic activity of sAPA (Fig. 3). When Glu-MCA was employed as a substrate, it was observed that although Ca$^{2+}$ enhanced the hydrolytic activity in a dose-dependent manner as expected, Zn$^{2+}$, which is known to be an effective inhibitor of various M1 aminopeptidases, suppressed the activity dose-dependently. Cu$^{2+}$ and Co$^{2+}$ also suppressed the activity although moderately, whereas Mg$^{2+}$ and Mn$^{2+}$ had little effect up to 2.5 mM. However, when Gln-MCA was employed, Zn$^{2+}$ suppressed the enzymatic activity most effectively. Other cations, including Ca$^{2+}$, moderately suppressed the activity. Even Mg$^{2+}$ and Mn$^{2+}$ exerted suppressive effects on the Gln-MCA-hydrolyzing activity of the enzyme. These results indicate that among the cations tested, Ca$^{2+}$ is unique in its ability to enhance the hydrolytic activity of sAPA toward Glu-MCA and Asp-MCA. On the other hand, consistent with its effects on other M1 family members (24, 26, 27), Zn$^{2+}$ was the most effective inhibitor of the hydrolytic actions of sAPA on all synthetic substrates.

Fig. 4 shows the effects of various inhibitors of proteolytic enzymes on the hydrolytic activity of sAPA measured in the presence of 1.0 mM Ca$^{2+}$. As for known aminopeptidase inhibitors, whereas amastatin was effective at a concentration as low as 1.0 μM (IC$_{50}$ = 0.157 ± 0.057 μM), bestatin had little activity up to 1 mM. As is the case with other aminopeptidases, 1,10-phenanthroline, a metal chelator, inhibited sAPA activity at 0.1–1 mM (IC$_{50}$ = 60.2 ± 5.2 μM). Leupeptin (serine/cysteine protease inhibitor), pepstatin A (aspartic acid protease inhibitor), p-(4-amidinophenyl)methanesulfonfonyl fluoride (serine...
protease inhibitor), and E64 (cysteine protease inhibitor) had little inhibitory effects at 1 mM. Nearly the same results were obtained when enzymatic activity was measured in the absence of Ca$^{2+}$ (data not shown). Although an inhibitory effect of bestatin was barely detectable, we concluded that the overall inhibitor profile of sAPA is consistent with that of the M1 aminopeptidases characterized to date (7, 24, 26, 27).

Cleavage of Natural Peptides by APA—We then tested for sAPA-mediated degradation of natural peptides. To identify possible substrates of the enzyme, the binding of various peptides was assessed by measuring the competition from each peptide for the hydrolysis of Glu-MCA because sAPA hydrolyzes Glu-MCA both in the presence and in the absence of Ca$^{2+}$. Table 2 shows the effects of various peptides on the sAPA-mediated hydrolysis of Glu-MCA. Based on previous works (11–13), we initially estimated that only peptides having N-terminal acidic amino acids could bind to the enzyme. Quite unexpectedly, however, Ang III, Ang IV, kallidin, and substance P, which have N-terminal nonacidic amino acids, inhibited (>50%) the hydrolysis of the substrate in the absence of Ca$^{2+}$ at 10 μM, suggesting the binding of these peptides to the enzyme. These peptides (with the exception of Ang IV) were less inhibitory at the same concentration in the presence of Ca$^{2+}$. At 100 μM, a variety of peptides could inhibit the hydrolytic activity of the enzyme. On the other hand, in the presence of Ca$^{2+}$, >50% inhibition was only observed at 100 μM of peptides such as Ang II, Ang IV, neurokinin B, CCK8, and chromogranin A. These peptides (with the exception of Ang IV) have acidic amino acids at their N-terminal ends. From these results, we speculated that peptides with N-terminal acidic amino acids that inhibited the enzyme efficiently in the presence of Ca$^{2+}$ might be cleaved and therefore are candidates for the substrates. On the other hand, in the absence of Ca$^{2+}$, a relatively wide range of peptides may bind to the enzyme without cleavage. However, it should be noted that in contrast to other peptides having N-terminal acidic amino acids, chromogranin A prevented the hydrolysis of Glu-MCA only in the presence of Ca$^{2+}$ in this assay. Because of its cluster of N-terminal glutamic acids, chromogranin A may be unique and bind to the enzyme only in the presence of Ca$^{2+}$.

It is also noteworthy that although parathyroid hormone has glutamic acid at its N-terminal end, it showed only a marginal inhibitory effect on the hydrolysis, suggesting that not all peptides with N-terminal amino acids are good substrates of the enzyme. Taken together, it is conceivable from these results that Ca$^{2+}$ modulates the binding and hydrolytic activities of the enzyme depending on the peptides tested and thus may regulate its enzymatic action. However, as shown below, although Ang IV clearly inhibited the hydrolytic activity of the enzyme both in the presence and absence of Ca$^{2+}$, its cleavage by the enzyme was detectable only after a long incubation period. In addition, despite an efficient inhibitory activity (especially in the absence of Ca$^{2+}$), Ang III was a poor substrate of the enzyme. Therefore, we concluded that it is difficult to estimate the possible physiological substrates by this inhibition assay, which is often performed for the estimation (12, 28). Table 3 shows the IC$_{50}$ values of several hormones with efficient inhibitory activities.

**TABLE 2**

Inhibitory effects of various human peptide hormones on Glu-MCA hydrolytic activity of sAPA

| Peptide | Concentration (%) | CaCl$_2$ |
|---------|-------------------|-----------|
| Ang II  | +                 | 64.6 ± 1.3|
| Ang IV  | +                 | 10.4 ± 0.3|
| CCK8    | +                 | 24.2 ± 5.7|
| Neurokinin B | +     | 60.2 ± 5.2|
| Chromogranin A | +     | 48.9 ± 2.9|
| Ang IV  | −                 | 1.1 ± 0.1|
| Kallidin| −                 | 4.4 ± 0.6|

* The values are the means ± S.D. (n = 3).

**TABLE 3**

The IC$_{50}$ values of human peptide hormones toward sAPA

| Peptide | CaCl$_2$ |
|---------|-----------|
| Ang II  | +         |
| Ang IV  | +         |
| CCK8    | +         |
| Neurokinin B | +     |
| Chromogranin A | +     |
| Ang IV  | −         |
| Kallidin| −         |
To search the substrate peptides exactly, we next examined the cleavage of peptides both in the presence and absence of Ca\(^{2+}\) (Fig. 5). Fig. 5A shows the sAPA-mediated cleavage of Ang II, a well known substrate of the enzyme. In the presence of Ca\(^{2+}\), Ang II (Fig. 5A, peak a) was cleaved effectively, and 62.1% of the hormone was converted to Ang III (peak b) within 20 min in this assay system. Unexpectedly, further degradation to Ang IV (Fig. 5A, peak c) did occur, although it was marginal. When the degradation was examined in the absence of Ca\(^{2+}\), a significant (~70%) reduction in the conversion to Ang III was observed. It is worth noting that more Ang IV was accumulated in the absence of Ca\(^{2+}\), suggesting that the conversion of Ang III to Ang IV by the enzyme without Ca\(^{2+}\) occurs faster than with it. Furthermore, after a 60-min incubation, de-[Val]Ang IV (Fig. 5A, peak d) was detected in the absence of Ca\(^{2+}\). These results indicated that peptides having nonacidic amino acids at the N terminus could be substrates of the enzyme especially in the absence of Ca\(^{2+}\). We then examined whether Ang III (peak a) could be a substrate as well (Fig. 5B). We found that although more time was required, the conversion of Ang III to Ang IV (Fig. 5B, peak b) (and further to de-[Val]Ang IV (peak c) was observed. Although 18.7% of Ang III was converted to Ang IV (and subsequently to de-[Val]Ang IV) in the absence of Ca\(^{2+}\) within 5 h, only 11.6% was converted in its presence, indicating that although Ang III is a poor substrate, the enzyme cleaves Ang III slightly faster in the absence of Ca\(^{2+}\) than in its presence. Of note, we observed this phenomenon repeatedly.

Because peptides having N-terminal nonacidic amino acids could be substrates of the enzyme, we next examined the cleavage of kallidin (Lys-bradykinin) (Fig. 5C). In the absence of Ca\(^{2+}\), it inhibited the hydrolytic activity of sAPA effectively, whereas only a little activity was detectable in the presence of Ca\(^{2+}\) (Table 2). Consistent with the results of the inhibition assay, we found that kallidin (Fig. 5C, peak a) was effectively cleaved and converted to bradykinin (peak b) in the absence but not in the presence of Ca\(^{2+}\). Although 76.5% of kallidin was hydrolyzed by the enzyme within 20 min in the
absence of Ca\(^{2+}\), only 9.0% was cleaved with Ca\(^{2+}\). Kinetic parameters measured in the absence of Ca\(^{2+}\) is shown in Table 4, indicating that \(k_{cat}/K_m\) value is comparable with Ang II. These results suggest that, as is the case with synthetic substrates, the specificity of the enzyme toward natural substrates is relatively broad in the absence of Ca\(^{2+}\). Ca\(^{2+}\) increases the specificity for acidic amino acids at the N-terminal end of the substrate.

We then measured the enzymatic activity of the peptides that have acidic amino acids at their N terminus. In the case of chromogranin A (Fig. 6A, peak a), sequential cleavage of five glutamic acids at the N terminus was clearly observed in the presence of Ca\(^{2+}\) (peaks b–f), but cleavage was barely detectable in the absence of Ca\(^{2+}\). As shown in Fig. 6B, CCK8 (peak a) was inactivated within 10 min in the presence of Ca\(^{2+}\), whereas in the absence of Ca\(^{2+}\), a significant decrease in the cleavage was observed. As for neurokinin B (Fig. 6C, peak a), it was inactivated and converted to de-[Asp]neurokinin B (peak b) by the efficient cleavage of N-terminal Asp, and the subsequent release of Met was also observed both in the presence and in the absence of Ca\(^{2+}\). Considering the rate of decrease for neurokinin B, the enzyme was less active in converting the hormone to de-[Asp]neurokinin B in the absence of Ca\(^{2+}\). However, judging from the accumulation of peak c, further cleavage to de-[Asp-Met]neurokinin B seems to occur quickly, which may reflect the broad substrate specificity of the enzyme in the absence of Ca\(^{2+}\). Table 4 shows the kinetic parameters of sAPA for the natural peptides examined above. It was observed that CCK8 and neurokinin B are better substrates of sAPA than Ang II, a well known physiological substrate, supporting the notion that APA indeed mediates some pathophysiological functions by regulating the activities of these peptides. These results again suggest that the enzyme prefers peptides with N-terminal acidic amino acids in the presence of Ca\(^{2+}\). Table 4 summarizes the kinetic parameters of sAPA toward several hormones that were identified as good substrates of the enzyme.

**Effect of Ang IV on the Enzymatic Activity of APA**—Although Ang IV binds to the enzyme, its cleavage was barely detectable both in the presence and absence of Ca\(^{2+}\). Therefore, we assumed that the hormone could inhibit the enzymatic activity.

### Table 4

| Substrate | CaCl\(_2\) | \(K_m^a\) | \(k_{cat}^a\) | \(k_{cat}/K_m \times 10^{12}\) |
|-----------|-----------|-----------|-------------|-----------------|
| Ang II    | +         | 29 ± 1    | 7.1 ± 0.1   | 243 ± 8         |
| CCK8      | +         | 149 ± 96  | 48.9 ± 12.1 | 430 ± 10        |
| Neurokinin B | +        | 141 ± 34  | 41.8 ± 8.9  | 297 ± 8         |
| Kallidin  | −         | 23 ± 8    | 3.6 ± 0.5   | 164 ± 38        |

*The values are mean ± S.D. (n = 3).*

**FIGURE 6.** Effects of calcium on the enzymatic activity of sAPA toward natural peptides with N-terminal acidic amino acids. A, cleavage of chromogranin A. Chromogranin A (25 \(\mu\)g/ml) was incubated with purified sAPA (1 \(\mu\)g/ml) at 37 °C for 20 min in the presence or absence of 1 mM calcium chloride. The peptides generated were loaded onto an HPLC column and separated, and their amino acid sequences were then estimated by MALDI-TOF MS. B, cleavage of CCK8. CCK8 (25 \(\mu\)g/ml) was incubated with purified sAPA (1 \(\mu\)g/ml) at 37 °C for 10 min in the presence or absence of 1 mM calcium chloride. C, cleavage of neurokinin B. Neurokinin B (25 \(\mu\)g/ml) was incubated with purified sAPA (1 \(\mu\)g/ml) at 37 °C for 10 min in the presence or absence of 1 mM calcium chloride.
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and we analyzed its inhibitory action using Lineweaver-Burk plots (Fig. 7). It was observed that Ang IV competitively inhibited the enzymatic action of sAPA toward Glu-MCA in the presence of Ca\(^{2+}\) efficiently with a \(K_i\) of 1.03 \(\mu\)M. Then the effect of Ang IV on the sAPA-mediated cleavage of Ang II was examined (Fig. 8). As expected, only a small portion of the hormone was degraded to de-[Val]Ang IV during the incubation period (Fig. 8D). On the other hand, Ang IV inhibited the conversion of Ang II to Ang III in a dose-dependent manner with 72.3% inhibition observed in the presence of 25 \(\mu\)M Ang IV (Fig. 8, F–H). The cleavage of CCK8 and of neurokinin B by the enzyme was also inhibited (59.5 and 86.7%, respectively) by 25 \(\mu\)M Ang IV (data not shown). It is noteworthy that Ang III had less inhibitory activity than Ang IV, and only a 20% inhibition of sAPA-mediated cleavage of Ang II was observed in the presence of 25 \(\mu\)M Ang IV (data not shown). These results indicate that Ang IV binds to and can act as a competitive inhibitor of the enzyme and suggest that there is a negative feedback mechanism for the regulation of APA in the RAS.

DISCUSSION

APA has been recognized as an important factor that regulates blood pressure (29). Indeed, APA gene-deficient mice suffer from hypertension (30, 31). However, its enzymatic properties have been poorly characterized because of the lack of a system to produce APA. In this study, we have established a baculovirus system for the production of a recombinant sAPA and purified the enzyme to homogeneity. The availability of milligrams of sAPA quantities per liter of culture medium made it possible to characterize the enzymatic properties of sAPA.

The formation of a homodimer is one of the characteristic features of the membrane-bound M1 family of aminopeptidases (15). It was shown that the membrane-bound form of native APA is a homodimeric protein with an intermolecular disulfide bond. A cysteine residue (Cys-43 in human and murine APAs) located in the vicinity of the transmembrane domain is responsible for the dimer (15, 22). Judging from the molecular weight measured by SDS-PAGE and ultracentrifuga-

tion, sAPA lacking Cys-43 still forms a homodimer in a nonsulfide-linked manner.

Because native APA is not available to us at present, we cannot exclude the possible difference of the tertiary structures between sAPA and the membrane-bound form of native enzyme. The difference may cause their different enzymatic properties. However, several characteristic features of native APA reported to date will give rationales to use recombinant sAPA for the characterization of enzymatic properties of APA. It was reported that the C-terminal domain but not the interchain disulfide is required for the correct folding and function of APA (22, 23). In addition, besides the membrane-bound form, several groups purified APA from retro-placental serum (32, 33), indicating that native APA also exists as a soluble form. Available data indicate that \(K_m\) values of sAPA toward Glu-NA and Asp-NA measured in the presence of Ca\(^{2+}\) are comparable with those of the membrane-bound form of APA (18). Kinetic parameters \((K_m, k_{cat}, k_{cat}/K_m)\) toward Ang II and the EC\(_{50}\) values of calcium ion for sAPA (Table 4 and Fig. 3) are also comparable with those of membrane-bound form of APA reported in Refs. 17 and 18.

In addition, the relative hydrolytic activities of two forms of the enzyme toward synthetic substrates (i.e. to Glu and Asp) measured in the presence of Ca\(^{2+}\) are also comparable with other preparations reported so far (13), further suggesting that the N-terminal cytosolic and transmembrane domains of the enzyme are not essential for the enzymatic activity, and soluble enzyme retains the characteristic enzymatic features of the membrane-bound enzyme (32, 34). Moreover, as discussed below, hydrolytic activity of sAPA toward Glu- and Asp-MCA is up-regulated comparably to native enzymes reported to date (11, 13, 17, 35, 36). The overlapping nature suggests that the substrate pocket of the enzymes was conserved among native and recombinant enzymes. It was also noteworthy that sAPA retains the C-terminal domain that acts as an intramolecular chaperone required for the correct folding of the protein (21, 23). Finally, although sAPA was glycosylated, de-glycosylated sAPA retained the enzymatic activity, strongly suggesting that sugar chains had little effect on the structural features of the substrate-binding site of the enzyme. Taken together, we conclude that it is reasonable to speculate that the sAPA produced in our system retains the principal enzymatic properties of both membrane-bound and soluble forms of native enzymes characterized to date.

In our previous work (24, 38, 39), we purified and characterized native membrane-bound and soluble forms as well as a recombinant soluble form of P-LAP/IRAP. When comparing the enzymatic properties toward synthetic and natural hormone substrates, little characteristic difference was observed between these enzymes tested so far. By analogy, these results support the notion that sAPA retains the enzymatic properties of APA and maintains integrity of the substrate pocket, thus allowing detailed analyses of the enzyme employing sAPA.

In addition to the sAPA shown in this study, soluble forms of both native and recombinant P-LAP/IRAP are also nonsulfide-linked dimers (24). At present it is difficult to speculate the mechanism of dimer formation of the enzymes, because A-LAP/endoplasmic reticulum aminopeptidase 1, another M1
aminopeptidase, is monomeric, although it shows significant homology to both APA and P-LAP/IRAP (25). It is necessary to determine and compare the three-dimensional structures of the enzymes to elucidate the mechanism of dimer formation.

The initial characterization of the activity of sAPA toward synthetic substrates revealed that the enzymatic actions are regulated by divalent cations in a complex manner. It was reported that Ca\(^{2+}\)/H\(_{11001}\) up-regulates the hydrolytic activity of APA toward Glu-NA (11–13, 17, 35, 36). In this study, we have shown that Ca\(^{2+}\)/H\(_{11001}\) enhances the release of acidic amino acids (Glu and Asp) from synthetic peptides, confirming previous results. Consequently, because the enzyme was less active toward Asp-MCA than Glu-MCA, it hydrolyzed Glu-MCA preferentially in the presence of Ca\(^{2+}\). On the other hand, in the absence of Ca\(^{2+}\), the substrate specificity of the enzyme was rather broad, and it exerted nearly the same level of activity toward Glu- and Gln-MCAs. In addition, the enzyme also exerted the hydrolytic activity toward nonacidic substrates such as Ala-, Leu-, Met-, Lys-, and Arg-MCAs. The hydrolysis of substrates other than Glu- and Asp-MCAs was down-regulated by Ca\(^{2+}\) (35). These results indicate that Ca\(^{2+}\) modulates the substrate specificity of the enzyme, increasing its preference for acidic amino acids, and thus might regulate the distinctive pathophysiological functions of APA by restricting the type of peptides with which it reacts. We measured CD spectra to detect possible structural changes induced by Ca\(^{2+}\). However, we could detect no gross change, indicating that the effect of Ca\(^{2+}\) on the conformational modulation of APA is local rather than global (data not shown).

Among the divalent cations tested, Zn\(^{2+}\) was the most effective inhibitor of the enzyme. In contrast to Ca\(^{2+}\), Zn\(^{2+}\) suppressed the hydrolysis of both Glu- and Gln-MCAs. Zn\(^{2+}\) is one of the most effective inhibitors of the M1 family of aminopeptidases to be tested (24, 26, 27). It is conceivable that the balance between Ca\(^{2+}\) and Zn\(^{2+}\) is important to the biological function of APA in vivo.

By searching for natural substrates of APA, we observed a complicated effect of Ca\(^{2+}\) on the regulation of the enzymatic activity. To identify possible substrates, we measured the hydrolytic activity of the enzyme toward Glu-MCA in the presence of various naturally occurring peptides. This approach is often employed for the screening of natural peptide substrates of aminopeptidases (12, 28) and provided us with several candidates. We indeed confirmed that the enzyme could cleave Ang II, neurokinin B, CCK8, and chromogranin A effectively in the presence of Ca\(^{2+}\), indicating its preference for N-terminal acidic amino acids where Ca\(^{2+}\) is available. Because Ang II, Ang III, neurokinin B, and CCK8 are expressed in the brain and involved in the regulation of blood pressure, pain perception, anxiety, and memory, these results support the notion that the enzymatic action of APA is important for the regulation of brain functions (29, 40, 41). It was also shown that Ang II and neurokinin B were present in high concentrations in the plasma of women with pre-eclampsia (42). Because placental APA can inactivate neurokinin B also derived from the placenta and regulate its level in plasma, a deficiency of APA in the placenta may cause pre-eclampsia (43). On the other hand, kallidin, which has lysine at its N-terminal end, was preferentially hydrolyzed in the absence of Ca\(^{2+}\). In the presence of Ca\(^{2+}\), the conversion of kallidin to bradykinin was significantly reduced, further supporting a role for Ca\(^{2+}\) in the regulation of the substrate specificity of the enzyme. In addition, this activity also suggests that the enzyme plays a role in the regulation of blood pressure. It is noteworthy here that several peptides, including...
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Ang III and substance P, whose N terminus does not have acidic amino acids, can bind to the enzyme in the absence of Ca\(^{2+}\). Moreover, Ang IV bound to the enzyme in both the presence and absence of Ca\(^{2+}\), although it is not cleaved by the enzyme. It is plausible that the substrate pocket of the enzyme is flexible in the absence of Ca\(^{2+}\), allowing more peptides to enter it (17). To test this hypothesis, we will have to await the determination of the three-dimensional structure of the enzyme by x-ray crystallography.

APA is expressed in many tissues, including kidney, intestine, placenta, and brain (13, 44). It was suggested that APA is first synthesized in the kidney and placenta as a type II membrane-bound protein, some of which is then proteolytically cleaved and secreted into extracellular fluids (21). Therefore, the catalytic domain of native enzyme is expected to be exposed to and function in extracellular fluids. It is well known that extracellular concentration of calcium ion is strictly regulated and, in general, maintained approximately at 1.0–2.0 mM, suggesting strongly that the data presented in this study have physiological and/or pathological relevance. It is conceivable that strict regulation of APA activity by the calcium ion is important for the blood pressure regulation by maintaining the rather low level of Ang II. Dereregulation of the calcium concentration (for example in the case of hypothyroidism) may induce the undesired activity of APA, causing deterioration of the disease. In general, APA activity is measured in the presence of 1–10 mM Ca\(^{2+}\) to reflect its pathophysiological relevance (44).

Ang IV inhibited the activity of sAPA toward Glu-MCA. Moreover, Ang IV inhibited the sAPA-mediated conversion of Ang II to Ang III as well as the cleavage of both CCK8 and neurokinin B. These results indicate that Ang IV can act as a competitive inhibitor of the enzyme. Through cleavage by APA and then aminopeptidase N, Ang IV is generated from Ang II in the RAS (29). Another enzyme, A-LAP, which may regulate blood pressure, also generates Ang IV by acting directly on Ang II (25, 45). It is conceivable that Ang IV generated by these aminopeptidases, in turn, acts as a negative regulator of APA by binding to the enzyme. Our data suggest an additional role for Ang IV in the regulation of blood pressure through inhibition of APA in the RAS. Of note, it was also reported that Ang IV inhibits the activity of angiotensin-1-converting enzyme (46), further suggesting a role for Ang IV in the regulation of Ang II levels.

Ang IV is a newly recognized hormone that facilitates memory retention and retrieval (29, 47). In a model of amnesia using rats, Ang IV reversed memory deficits detected by the Morris water maze paradigm. The Ang IV receptor was isolated from bovine adrenal membranes and identified as P-LAP/IRAP, a member of the M1 family of aminopeptidases (48). Because ligands for the Ang IV receptor, including Ang IV analogues and the structurally unrelated LVV-hemorphin-7, are competitive inhibitors of P-LAP/IRAP (37), it is speculated that Ang IV exerts its actions by inhibiting the activity of the enzyme. It is conceivable that Ang IV also inhibits the activity of APA and thus elongates the life span of some unidentified substrates responsible for memory retention and retrieval. It is important to elucidate the pathophysiological functions that Ang IV achieves through regulation of the enzymatic activity of APA and P-LAP/IRAP.

In this study, we have established a large scale production system for a recombinant sAPA and characterized its enzymatic properties. We have identified several natural peptides as possible substrates of APA, which might have relevance to its pathophysiological functions. Our data indicated that Ca\(^{2+}\) actually modulates the substrate specificity of APA. Preferential cleavage of acidic amino acid MCAs by the enzyme was observed only in the presence of Ca\(^{2+}\). Although Ca\(^{2+}\) enhanced the release of acidic amino acids, Zn\(^{2+}\) acted as a general inhibitor of the enzyme for all the substrates tested. As for natural substrates, the specificity measured in the presence of Ca\(^{2+}\) was strict and peptides having acidic amino acids but not other amino acids at the N terminus were efficiently cleaved by the enzyme. But in the absence of Ca\(^{2+}\), kallidin was preferentially hydrolyzed. These results suggest that Ca\(^{2+}\) increases the preference of the enzyme for N-terminal acidic amino acids in vivo and thus regulates blood pressure by facilitating the inactivation of Ang II. In addition, we showed the possibility that Ang IV is a negative feedback regulator of the enzyme. The data presented in this study therefore indicate that there might be a unique and complex mechanism for the regulation of blood pressure involving modulation of the enzymatic activity of APA in the RAS. It is necessary to elucidate the pathophysiological relevance of the regulation by calcium and Ang IV of APA activity in future studies.

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