Aminopeptidase A (EC 3.4.11.7, APA) is a homodimeric membrane-bound glycoprotein that contains the consensus sequence HEXXH (385–389) found in zinc metallopeptidases such as thermolysin. The x-ray structure of the latter enzyme revealed that the two histidines of this motif are two of the three zinc-coordinating ligands and that the glutamate is a crucial amino acid involved in catalysis. Alignment of the sequence of mouse APA with those of the already characterized metallopeptidases showed the presence of several conserved amino acids such as a glutamate residue in position 408 which may constitute the putative third zinc ligand. The functional implication of this residue and the role of Glu-386 in the HELVH (385–389) motif are two of the three zinc-coordinating ligands and confirms the presence of conserved zinc ligands, and the glutamate acts as a catalytic base. The overall amino acid homology of APA with other metallopeptidases such as aminopeptidase N (APN), angiotensin-converting enzyme, and leukotriene A4 hydrolase reaches 25–35%, and the conservation is higher in the region surrounding the zinc binding motif (Fig. 1). The x-ray crystal structure of thermolysin showed the zinc ion to play a catalytic role. It is coordinated to three amino acid side chains and a water molecule that, upon ionization/polarization by the glutamate, initiates the nucleophilic attack of the substrate scissile peptide bond (13).

Site-directed mutagenesis studies have allowed the identification of corresponding residues in some members of the gluzincin family, including neutral endopeptidase (EC 3.4.24.11, NEP) (14–16), angiotensin-converting enzyme (ACE) (17); (18), neutral metalloprotease from Streptomyces cacaoi (19), and LTA4 hydrolase (20), which has an aminopeptidase activity in addition to its epoxide hydrolase activity (21). In APA, replacement of His-389 by a phenylalanine in the zinc binding motif of APA abolished the enzymatic activity, suggesting that this residue is probably one of the zinc ligands (22). Nevertheless, the presence or absence of the zinc atom in the protein and the demonstration of its role in catalysis were not directly demonstrated and remain to be established by a direct approach.

Alignment of APA with other metallopeptidases (Fig. 1) in the region surrounding the zinc binding motif reveals that several residues known to be involved in catalysis or zinc binding are conserved. Interestingly, the sequence surrounding the glutamate zinc ligand in LTA4 hydrolase (20), WLXEG, is conserved among the different aminopeptidases presented in Fig. 1 (APA, APN, LTA4 hydrolase, and thryotropin-releasing hormone-degrading enzyme). Furthermore, the amino acid sequence distance between glutamates of the HEXXH and WLXEG motifs (21 residues) is also conserved.

We therefore hypothesized that Glu-408 located in the WLXEG (405–409) motif of APA constitutes the third zinc ligand and that Glu-386 located in the zinc binding motif HELVH (385–389) plays a crucial role in catalysis as observed for other gluzincins. Another conserved motif, EXIXD, where the glutamate is the third zinc ligand, has been identified in thermolysin, NEP, and ACE (23). Kinetic analysis and binding experiments performed with NEP, ACE, and various mutants indicate that the aspartate is involved in the precise positioning of the first histidine zinc ligand in the HEXXH motif, via a...
Glutamate Residues in the Active Site of APA

Site-directed Mutagenesis and Construction of Expression Plasmids—Site-directed mutagenesis was performed on the mouse cDNA encoding APA (22), contained in the expression vector SRalpI, using the polymerase chain reaction as described previously (25). Two overlapping regions of the cDNA were amplified separately using two flanking oligonucleotides A and B and one variable internal oligonucleotide C (Table I). Primer C, containing the mutation and a non-unique restriction site (TaqI), was used with primer A to amplify the upstream fragment. The downstream fragment was obtained after amplification with primer D and primer B followed by digestion with the restriction enzyme TaqI and purification from a 2% agarose gel (GeneClean 2, BIO 101). The upstream fragment containing the mutation and the downstream fragment, digested with TaqI that cut in the overlapping sequence, were ligated. The resulting fragment was amplified with the 5′- and 3′-flanking primers A and B, which were chosen to cover a sequence including unique restriction sites. DNA polymerase from Pyrococcus furiosus (Pfu) (1 unit) was used for 30 cycles: 94°C, 30 s; 55°C, 30 s; 72°C, 1 min. After gel purification of the fragment, the 350-base pair polymerase chain reaction DNA insert containing the mutation was substituted for the corresponding nonmutated region (NarI–EcoRV) of the full-length APA cDNA. The presence of the mutations and the absence of nnspecific mutations were confirmed by sequencing the 350-base pair mutated region by the dideoxy chain termination method (Sequenase 2, U.S. Biochemical Corp.).

The glutamate residue at position 386 was substituted for either an aspartate (Asp-386), or an alanine (Ala-386); glutamate 408 was mutated either to an aspartate (Asp-408) or to an alanine (Ala-408), and serine 412 was replaced by an alanine (Ala-412). The primers used for the constructions of the mutant cDNAs are shown in Table I.

The ACE mutants were a generous gift from Dr. L. Wei (ACE Lys-361, 365) and Dr. T. Williams (ACE Lys-361, 365 Val-987). Transfection of COS-7 Cells—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) complemented with 10% fetal calf serum and were transfected with 30 μg of plasmid by electroporation (200 V, 1,800 microfarads, Euromedic electroporator). Each pool of transfected cells was divided into two 35-mm Petri dishes, one for enzyme assay and one for metabolic labeling.

Metabolic Labeling and Immunoprecipitation—Transfected COS-7 cells were incubated 48 h after transfection in methionine- and serum-free medium (Ham’s F-12) containing 30 μCi/ml [35S]methionine for 5 h. Proteins were solubilized for 2 h in 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 10 mM EDTA, and 1% Triton X-100 and then centrifuged at 15,000 × g for 5 min at 4°C. The supernatants were recovered, and immunoprecipitation was performed by incubating these cell extracts overnight at 4°C with protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) (50 μl of 50% suspension in solubilization buffer) and 3 μl of a polyclonal anti-rat APA rabbit antisera (final dilution: 1:200). The immune complexes were collected by centrifugation and washed three times with solubilization buffer and once with 20 mM Tris-HCl, pH 6.8. Proteins were eluted (by boiling in 20 mM Tris-HCl, pH 6.8, containing 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and resolved by SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide) according to Laemmli (26). The dried gel was then exposed to autoradiography.

To account for small variations in APA synthesis due to differences in the efficiency of electroporation or in the number of surviving cells, the APA activity was determined for the wild type and the mutant protein. The APA activity of the cell extracts was determined in a microtitr plate by following the rate of hydrolysis of a synthetic substrate, ω-glycyl-L-α-naphthylamide (GluNA, Bachem) (4). The kinetic parameters (initial velocity, V0, and Michaelis constant, Km) were determined from Lineweaver-Burk plots using final concentration ranging from 0.025 to 0.4 mM GluNA.

The sensitivity of APA and some of the mutants to glutamate-β-hydantoin (GluSH), an inhibitor of APA (27), was determined by establishing dose-dependent inhibition curves and calculating their IC50 values (analyzed using a weighted nonlinear least squares regression program (S. Urien; MICROPHARM, Faculté de Médecine, Department de Pharmacologie, Creteil, France)).

Expression of Recombinant APA in COS-7 Cells and Metabolic Labeling—To study the role of the amino acids proposed to be essential in the APA active site, we constructed a series of APA cDNAs encoding mutants of glutamates 386, 408, and serine 412. Equivalent amounts of mutated APA cDNAs were transiently transfected into COS-7 cells. After transfection, the cells were labeled by incubation with [35S]methionine, harvested, and the proteins from cell extracts were solubilized, immunoprecipitated with an anti-APA polyclonal antiserum, resolved on SDS-polyacrylamide gel electrophoresis, and detected by autoradiography. Fig. 2 shows the presence of a major band migrating with an apparent molecular mass of 130 kDa for the wild type APA and for the different mutants, indicating that each mutant was expressed and similarly glycosylated.
Muscle-specific alkaline phosphatase (APA) is a key enzyme in the degradation of the muscle-specific phosphatase inhibitor GluSH. The enzyme is involved in the regulation of muscle contraction and relaxation. The active site of APA is rich in glutamate residues, which are essential for the enzyme's activity.

The enzymes that have been described in previous studies include wild-type APA and its mutants, all of which have the same activity. The mutants of interest are those that lack the negative charge at position 408, which is essential for the enzyme's activity. This lack of charge is due to the substitution of alanine (Ala) for aspartate (Asp).

The results show that the inhibition of APA activity by GluSH is about four times lower for Asp-408 compared with the wild type enzyme. This indicates that the decrease in activity is likely due to a lower efficiency of hydrolysis. In contrast, the inhibitory potency of GluSH for Asp-408 is comparable to the wild type enzyme. The apparent affinities of the wild type enzyme and Asp-408 for GluNA are comparable, suggesting that the decrease in activity is likely due to a lower efficiency of hydrolysis. In contrast, the inhibitory potency of GluSH is about four times lower for Asp-408 compared with the nonmutated enzyme.

To characterize further the properties of the mutant Asp-408, we studied the $K_m$ of this mutant and its response to the APA inhibitor GluSH. Asp-408 had a $K_m$ of 98 ± 2 μM, close to that of wild type APA (60 ± 15 μM). $IC_{50}$ values for GluSH were 4.2 ± 0.18 × 10^{-7} M for Asp-408 and 1.0 ± 0.3 × 10^{-7} M for the wild type enzyme. The apparent affinities of the wild type enzyme and Asp-408 for GluNA are comparable, suggesting that the decrease in activity is likely due to a lower efficiency of hydrolysis. In contrast, the inhibitory potency of GluSH is about four times lower for Asp-408 compared with the nonmutated enzyme.

65Zn Binding to Recombinant APA—To test for the presence or absence of the zinc ion in the active site of the wild type APA and its mutants, COS-7 cells were transfected with the different mutated cDNAs and incubated with the medium containing 65Zn. Cell extracts were then immunoprecipitated and the immune complexes counted. In parallel, an equivalent number of transfected cells was subjected to metabolic labeling with [35S]methionine. Autoradiography and densitometric scanning of immune complexes were performed after SDS-polyacrylamide gel electrophoresis, and allowed us, as for the enzymatic activity measurements, to calibrate the 65Zn measurements.

The sensitivity was improved by counting the radioactivity of the immunoprecipitated fractions. To estimate the nonspecific labeling in these immunoprecipitated fractions, a control of nontransfected COS-7 cells was treated in parallel to estimate the background level, which was then subtracted from the previously obtained 65Zn labeling values of the mutants.
resulting specific labeling values were then corrected with the corresponding enzyme expression levels ($^{35}$S labeling values).

To validate the method, this protocol was applied to determine the zinc content of the ACE (Fig. 4). ACE is a zinc metalloprotease that has two homologous domains, each bearing a catalytically functional active site (17) able to bind a zinc atom (28, 29). Previous data demonstrated that mutation of either both histidines of the zinc binding motif of one domain or of Glu-987, the amino acid identified as the third zinc binding residue of the C domain, abolished the ability of this domain to bind the zinc ion (18). In addition to the wildtype ACE, we used a mutant ACE Lys-361,365 containing an intact C domain and an N domain inactivated by the replacement of both histidines by lysines in the zinc binding motif. The second mutant (ACE Lys-361,365 → Val-987) had the same mutations in the N domain and was also mutated on the third zinc binding residue of the C domain. The experiments were performed as described previously for APA, except that immunoprecipitation was performed with an ACE antiserum.

Counting measurements presented in Fig. 4 indicated that Zn incorporation of the ACE Lys-361,365 mutant represents 40% of that of wild type ACE, showing that suppression of one zinc binding motif of ACE allowed the enzyme to bind about half the zinc content compared with wild type ACE. The ACE Lys-361,365 → Val-987 mutant exhibited 13% of radiolabelling compared with the wild type ACE, indicating that mutating the third zinc ligand of the other domain, thus both ACE active sites are inactivated, resulted in an almost complete abolishment of isotope incorporation.

In Fig. 5, the radioactivity of the different mutant immune complexes are compared with that of wild type APA. The immune complex radioactivity of Ala-386 was identical to wild type APA, indicating that this mutation did not impair $^{65}$Zn binding. In contrast, Ala-408 was not able to incorporate the zinc isotope. When the negative charge was restored (Asp-408), the mutant bound the zinc ion, but the affinity for the metal appeared lower since only 60% was recovered in the immunoprecipitated $^{65}$Zn-labeled enzyme.

**DISCUSSION**

APA was first described as a calcium-stimulated aminopeptidase (30). In this study, we were able to show by metabolic labeling with $^{65}$Zn that this protein could bind the zinc isotope, confirming directly that APA is a zinc-dependent enzyme in agreement with the presence of the signature HEXXH in the sequence (11). Our biochemical characterization and zinc content analysis of the expressed mutants demonstrate that both glutamates residues 386 and 408 are essential for APA enzymatic activity and function differently in the catalytic mechanism. Glutamate 386 is directly involved in the catalytic activity, whereas glutamate 408 functions as the third zinc coordinating residue. In contrast to the aspartate of the consensus sequence of other thermolysin-related metallopeptidases (thermolysin, NEP, ACE), Ser-412 is not involved in the precise positioning of the active-site zinc ion. This is consistent with the fact that this residue is not conserved as an Asp in thermolysin-related peptidases.

The constructions were expressed in a way similar to the nonmutated cDNA and indicate that the different mutations do not affect the biosynthesis, the folding, or the stability of the resulting proteins. The loss of activity of Ala-386 could be due to the replacement of a charged amino acid (Glu) by a hydrophobic alanine, thus causing structural modifications of the active site. As no residual activity was detected when the charge was restored with an Asp residue (Asp-386), we deduced that the loss of activity was not due to structural changes, considering the minimal structural difference between Glu and Asp.

The glutamate in position 408 was also shown to be essential since the mutant Ala-408 was inactive, but in contrast to the mutant Glu-386, the substitution for an aspartate at the same position partially restored 5% of activity. The similar apparent affinity for the substrate of Asp-408 and wild type APA suggests that the loss of activity is due to an alteration of the ability of this mutant to efficiently cleave the peptide bond of the substrate, rather than an alteration in substrate binding.

The lack of activity of the different mutants (with the exception of Ala-412) might consequently be due to a direct participation of the mutated residue in catalysis or an indirect in-
volvement of the residue in the binding of the catalytically essential zinc ion. To distinguish between these possibilities, we determined directly the presence or absence of the zinc ion in the active site of APA mutants by metabolic labeling of transfected cells with $^{65}$Zn. This approach has been recently used on the zinc-dependent insulin-degrading enzyme (31).

Validation of the methodology was achieved by studying the zinc content of previously characterized ACE mutants (17, 18). The results are in agreement with previous work in which the presence of zinc in ACE mutants was indirectly estimated by testing the sensitivity to $[^{3}H]$trandolaprilat, a high affinity inhibitor that interacted with the zinc atom, demonstrating that this residue is essential for catalytic activity but does not coordinate with the zinc ion.

Furthermore, the replacement of glutamate 408 by an aspartate leads to a reduction in the hydrolysis efficiency and zinc affinity. This conclusion is further supported by a second approach using the APA inhibitor GluSH. The thiol group of GluSH interacts strongly with the zinc ion (4, 27). Therefore, the affinity of the enzyme for this competitive inhibitor depends on the presence of the zinc ion in the active site, and a difference in the inhibitory potency for the mutated or nonmutated enzyme would be consistent with an alteration in zinc ion binding. The difference observed between the inhibitory potency of GluSH for the wild type APA and Asp-408 indicates that the zinc ion is still present in the active site of Asp-408 and allows the binding of the inhibitor, but its lower affinity suggests a decrease in the interaction with the inhibitor, probably due to a modification of the zinc ion position in the active site of the mutant, as proposed for the NEP-related mutant (16).

Using thermolysin and NEP as an active-site model for APA (Fig. 6) (13, 16), replacement of the third zinc ligand Glu-408 with an aspartate increases the distance between the zinc ion and the carbonyl group of the substrate and/or the zinc-bound water molecule. The consequence of such a modification would be a reduction in the nucleophilicity of the water molecule, thus producing a drastic effect on catalysis.

The negative charge in position 408 is crucial for retention of the zinc ion in the active site of the enzyme, and the length of the Glu-408 side chain allows a precise positioning of the zinc ion essential for catalysis (33).

Many zinc metalloproteases have been shown to share conserved zinc-coordinating residues in their active sites. Mutation of the histidines of the zinc binding motif or the conserved glutamate identified as the third zinc-coordinating residue by a hydrophobic or positively charged residue led to the loss of catalytic activity and zinc binding for APA (22), NEP (15, 16), ACE (17, 18), LTA4 hydrolase (20), and neutral metalloprotease (19) gluazincins.

In summary, the present data show the importance of Glu-386 and Glu-408 in the catalysis and zinc binding of APA, respectively. These results are consistent with the catalytic mechanism of APA being similar to that proposed for thermolysin for which the three-dimensional structure is known. This suggests that the APA exopeptidase is functionally more closely related to metalloendopeptidases such as thermolysin or NEP than to other related metalloexopeptidases such as carboxypeptidases. The binding motif of the catalytic zinc atom is shared by many zinc metalloproteases (35), and the amino acids of this motif appear to have a conserved function since their mutation leads to similar characteristics with regard to enzymatic activity and zinc binding. This assumed similarity in the catalytic site of the endopeptidase NEP and the exopeptidase APN was at the basis of the development of dual NEP/APN inhibitors (33). Nevertheless, the loss of enzymatic activity and zinc affinity in the APA mutant Asp-408 seems to be slightly lower than that of the corresponding aspartate NEP and ACE mutants (16, 18). This suggests the occurrence of some differences in the spatial disposition of zinc ligand and the glutamate involved in catalysis in APA and thermolysin, NEP or ACE. This hypothesis is supported by the absence of a functional role for Ser-412 in the catalytic process of APA.
contrary to what was shown for the corresponding aspartate of the consensus sequence EXIXD in thermolysin, NEP and ACE.

In the absence of any structural information on APA or the related zinc aminopeptidases, the generation of functional knowledge of the APA catalytic site may aid in the design of specific and potent APA inhibitors, tools that are essential for exploring the physiological role of APA.

Acknowledgments—We are grateful to Drs. Bernard Roques and Marie-Thérèse Chauvet for helpful discussions during the course of this work and critical reading of this manuscript. We also thank Dr. Sherwin Wilk for providing the APA antiserum, Dr. Marie-Claude Fournié-Zaluski for the gift of the APA inhibitor, and Drs. Lei Wei and Tracy Williams for the gift of ACE mutants.

REFERENCES

1. Wilk, S., and Healy, D. (1993) Adv. Neuroimmunol. 3, 195-207
2. Nagatsu, I., Nagatsu, T., Yamamoto, T., Glenner, G. G., and Muhl, J. W. (1970) Biochim. Biophys. Acta 196, 255-270
3. Lodi, Z., and Gossrau, R. (1980) Histochemistry 67, 267-290
4. Chauvel, E. N., Llorens-Cortés, C., Coric, P., Wilk, S., Roques, B., and Fournié-Zaluski, M. C. (1994) J. Med. Chem. 37, 2950–2956
5. Chai, S. Y., Allen, A. M., Adam, W. R., and Mendelsohn, F. A. O. (1986) J. Cardiovasc. Pharmacol. 10, 533–539
6. Bottari, S. P., de Gasparo, M., Steckel, U. M., and Levens, N. R. (1993) Front. Neuroendocrinol. 14, 123–171
7. Danielsen, E. M., Norén, O., Sjöström, H., Ingram, J., and Kenny, A. J. (1980) Biochem. J. 189, 591–603
8. Wu, Q., Lahti, J. M., Air, G. M., Burrows, D. P., and Cooper, M. D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 993–997
9. Nanus, D. M., Engerstein, D., Gartl, G. A., Gluck, L., Vidal, M. J., Morrison, M., Finstad, C. L., Band, N. H., and Albino, A. P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7069–7073
10. Li, L., Wang, J., and Cooper, M. D. (1993) Genomics 17, 657–664
11. Jongeneel, C. V., Bouvier, J., and Bairoch, A. (1989) FEBS Lett. 242, 211-214
12. Hooper, N. M. (1994) FEBS Lett. 354, 1–6
13. Matthews, B. W. (1988) Acc. Chem. Res. 21, 333–340
14. Devault, A., Nault, C., Zollinger, M., Fournié-Zaluski, M. C., Roques, B. P., Crine, P., and Bouleau, G. (1980) J. Biol. Chem. 263, 4033–4040
15. Devault, A., Sales, V., Nault, C., Beaumont, A., Roques, B., Crine, P., and Bouleau, G. (1988) FEBS Lett. 231, 54–58
16. LeMoual, H., Devault, A., Roques, B., Crine, P., and Bouleau, G. (1989) J. Biol. Chem. 266, 15670–15674
17. Wei, L., Alhenc-Gelas, F., Corvol, P., and Clauser, E. (1991) J. Biol. Chem. 266, 9002–9008
18. Williams, T. A., Corvol, P., and Soubrier, F. (1994) J. Biol. Chem. 269, 29430–29434
19. Chang, C. P., and Lee, Y. H. W. (1992) J. Biol. Chem. 267, 3952–3958
20. Medina, J. F., Wetterholm, A., Radmark, O., Shapiro, R., Hagegström, J. Z., Vallee, B. L., and Samuelsson, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7620–7624
21. Orning, L., Krivi, G., and Fitzpatrick, F. A. (1991) J. Biol. Mol. 266, 1375–1378
22. Wang, J. Y., and Cooper, M. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1222–1226
23. Roques, B. P. (1993) Biochem. Soc. Trans. 21, 678–685
24. LeMoual, H., Dion, N., Roques, B., Crine, P., and Bouleau, G. (1994) Eur. J. Biochem. 221, 475–480
25. Herlitze, S., and Keenen, M. (1990) Gene (Amst.) 91, 143–147
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Wilk, S., and Thurston, L. S. (1989) Neuropeptides 16, 163–168
28. Ehlers, M. R. W., and Riordan, J. F. (1991) Biochemistry 30, 7118–7126
29. Williams, T. A., Barnes, K., Kenny, A. J., Turner, A. J., and Hooper, N. M. (1992) Biochem. J. 288, 875–881
30. Glonek, G. G., McMillan, P. J., and Folk, J. E. (1962) Nature 194, 867–868
31. Periman, R. K., and Rosner, M. R. (1994) J. Biol. Chem. 269, 33140–33145
32. Wei, L., Clauser, E., Alhenc-Gelas, F., and Corvol, P. (1992) J. Biol. Chem. 267, 13398–13405
33. Roques, B. P., Noble, F., Daugé, V., Fournié-Zaluski, M. C., and Beaumont, A. (1993) Pharmacol. Rev. 45, 87–145
34. Wetterholm, A., Medina, J. F., Radmark, O., Shapiro, R., Hagegström, J. Z., Vallee, B. L., and Samuelsson, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9141–9145
35. Vallee, B. L., and Auld, D. S. (1990) Biochemistry 29, 5647–5658