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A Molecular Orbital Study on the Zinc-Water-Glu 270 System in Carboxypeptidase A

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Many investigations on the reaction mechanism of carboxypeptidase A(CPA) have shown that the zinc ion and Glu 270 in the active site are important in the catalytic reaction with a peptide substrate. X-Ray crystallographic studies of native CPA showed that the zinc ion is coordinated to His 69, Glu 72, His 196, and one water molecule and that the Zn-coordinated water molecule forms a hydrogen bond with Glu 270. The zinc-water-Glu 270 system of native CPA was analyzed by the *ab initio* SCF-LCAO-MO method. Some ligands of zinc are included in the MO calculations as point fractional charges. The results show that the Zn-coordinated water molecule acts as a proton donor to Glu 270, and that the electrostatic effect of Zn^{2+} and its ligands and the electron delocalization between Zn^{2+} and the water play a significant role in lowering the barrier height of proton transfer. We consider that the carbonyl group of the substrate, without breaking the hydrogen bond between Glu 270 and the Zn-coordinated water molecule, points towards a fifth coordination site slightly away from Zn^{2+}, and that the water molecule itself is modified by its connection to Glu 270 in a way that favors the reaction.

Keywords——carboxypeptidase A; *ab initio*; zinc; proteolytic enzyme; structure; catalysis; mechanism; molecular orbital (MO); enzyme

Carboxypeptidase A (CPA) is a zinc-containing proteolytic enzyme. X-Ray crystallographic analyses of native CPA and its complex with the dipeptide glycyl-L-tyrosine(Gly-Tyr) showed that zinc, Glu 270 and Tyr 248 were important in catalysis of the reaction with the peptide substrate.[1] Zinc is coordinated to His 69, Glu 72, His 196 and one water molecule in native CPA. The Zn-coordinated water molecule forms a hydrogen bond with Glu 270, which is significant in the catalytic reaction of CPA, and His 69 forms a hydrogen bond with Asp 142. In the CPA complex with Gly–Tyr, the Zn-coordinated water molecule is displaced by the carbonyl group of the scissile peptide bond of the substrate, since Glu 270 is moved towards the N-terminal of Gly-Tyr.[1]

A "Zn-hydroxide mechanism" and a "Zn-carbonyl mechanism" have been proposed for CPA catalysis.[1a] In the "Zn-hydroxide mechanism," the Zn-coordinated water molecule, in its dissociated form, acts as an attacking nucleophile. In "Zn-carbonyl mechanism" the carbonyl group of the peptide substrate binds so as to displace the water molecule from Zn. Zinc polarizes the carbonyl group,[2] and Glu 270 acts as a nucleophile to form an anhydride intermediate or a general base to deliver nucleophilic water. Kaiser and Kaiser supported

the Glu 270 nucleophilic mechanism in the "Zn-carbonyl mechanism" on the basis of kinetic studies.[3] Although evidence has been presented in the case of ester substrates that Glu 270 acts as a nucleophile leading to the formation of anhydride species,[4] no anhydride intermediate has been detected in the hydrolysis of a peptide substrate. Breslow and Wernic supported the concept of general base catalysis by Glu 270 on the basis of oxygen-18 exchange experiments.[5] Recently Sugimoto and Kaiser reported the enolization of a ketone substrate of CPA, and concluded that Glu 270 acts as a proton donor and acceptor for the 3 position of the ketone substrate.[6]

Several approaches to the catalytic mechanism of CPA had been made by means of molecular orbital calculations. Hayes and Kollman studied the electrostatic environment effect on the scissile peptide bond of a substrate.[7] Scheiner and Lipscomb showed that the action of an electrophile at the carbonyl group of a substrate was effective in enhancing the hydrolysis.[2b] Nakagawa and Umeyama showed that three arginine residues were important for the binding of a substrate.[8] The proton-labilizing properties of Zn^{2+} were elucidated by an analysis of the bonding between Zn^{2+} and some molecules including water and imidazole in connection with the catalysis by carbonic anhydrase.[9] To investigate the mechanism of the enzymatic reaction, in the present work, we performed *ab initio* MO(molecular orbital) calculations on model systems of native CPA, consisting of a zinc ion, a Zn-coordinated water molecule and three amino acid residues (His 69, Asp 142 and Glu 270). The potential energy curves were computed for two types of proton transfer, namely, the proton transfer from His 69 to Asp 142 and from the Zn-coordinated water molecule to Glu 270.

Method

All the calculations were carried out within the closed shell LCAO-SCF approximation by the *ab initio* MO method. The IMSPACK program[10] was used for the calculations. The Gaussian basis set used for Zn was the (11s7p5d) set modified from the (12s6p4d) set of Roos *et al.*[11a] The two most diffuse s type functions were replaced by one s type function with exponent 0.2 and a set of p and d type functions with exponents 0.25 and 0.2, respectively, was added to the original set.[11b] The basis functions were then contracted to (6221/4111/311). The 4-31G basis set[12] was used for the hydrogen and first-row atoms.

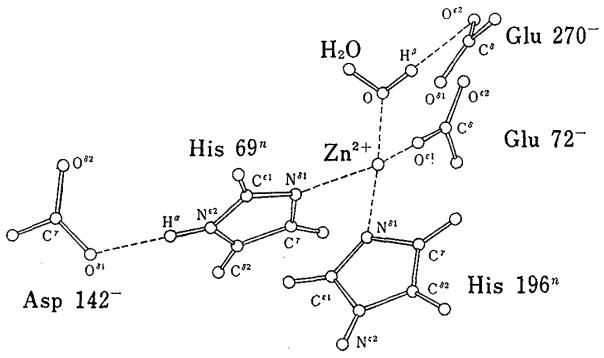

Fig. 1(a). Calculated Structure composed of Zn^{2+}, H_2O, His 69^n, Glu 72$^-$, Asp 142$^-$, His 196^n, and Glu 270$^-$

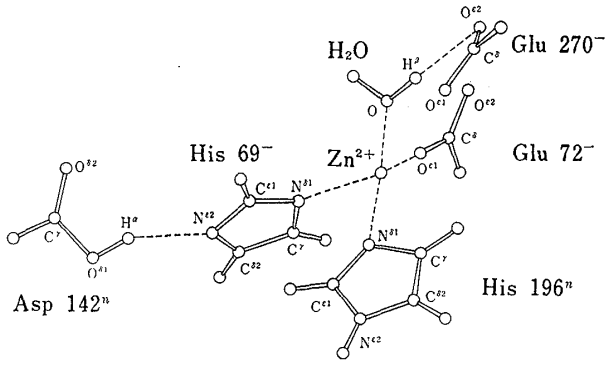

Fig. 1(b). Calculated Structure composed of Zn^{2+}, H_2O, His 69$^-$, Glu 72$^-$, Asp 142^n, His 196^n, and Glu 270$^-$

Geometries——The coordinates for native CPA were taken from the work of Quiocho and Lipscomb.[1b] Figure 1 shows the structures used for calculations. His 69 and His 196 are replaced by imidazole molecules, and Glu 72, Asp 142 and Glu 270 by formic acid molecules superposed at appropriate positions. In Fig. 1(a), a formic acid anion is positioned on each of Glu 72, Asp 142 and Glu 270. A neutral imidazole is positioned on each of His 69 and His 196. In Fig. 1(b), a neutral formic acid is positioned on Asp 142 and an imidazolate anion is positioned on His 69. Glu 72, His 196, Glu 270, and the Zn-coordinated water molecule have the same geometries as in Fig. 1(a). In this paper, amino acid residues will be symbolized as Glu 72$^-$, His 69$^-$, His 69^n, Asp 142$^-$, Asp 142^n, His 196^n, and Glu 270$^-$. The superscripts "n" and "–" indicate the neutral

and anion forms, respectively. The position of the Zn-coordinated water molecule has not been published so far. However, metal-ligand bond angles in native CPA are available from Table IX of ref. 1b. The internuclear distance between Zn and the oxygen atom of the water molecule was taken to be 2.0 Å in our assumed structure. The distance of 2.0 Å seems to be reasonable when Zn^{2+} has several ligands, though it is somewhat longer than the optimized value of 1.89 Å for the Zn^{2+}-water system.[9] The distance between the oxygen atom of the Zn-coordinated water molecule and $O^{\varepsilon 2}$ of Glu 270 is 3.19 Å in Fig. 1. The coordinates of the oxygen of the water are $x=-4.1214$, $y=28.4964$, and $z=-5.2876$. The ORTEP program was used for drawing the molecular structures.[13]

The geometries of formic acids (Asp 142$^-$ and Glu 270$^-$) and H_2O were optimized by using the 4-31G basis set. For $HCOO^-$, r(CH) is 1.1126 Å, r(CO) is 1.2506 Å, and $\angle$HCO is 114.8°. For H_2O, r(OH) is 0.9505 Å and $\angle$HOH is 111.2°. The geometry of the imidazole ring was that obtained experimentally.[14] r(CH) and r(NH) of imidazoles (His 69^n and His 196^n) were optimized with 4-31G basis set, on the assumption that the three r(CH) are the same. r(NH) is 0.9892 Å, and r(CH) is 1.0622 Å.

Table I. Calculated Models[a]

Models		Proton transfer
I	Zn^{2+}, His 69^n, Asp 142$^-$, *H_2O, *Glu 72$^-$, *His 196^n, *Glu 270$^-$	α
II	His 69^n, Asp 142$^-$, *Zn^{2+}, *H_2O, *Glu 72$^-$, *His 196^n, *Glu 270$^-$	α
III	His 69^n, Asp 142$^-$	α
IV	Zn^{2+}, H_2O, Glu 270$^-$, *His 69$^-$, *Glu 72$^-$, *Asp 142^n, *His 196^n	β
V	H_2O, Glu 270$^-$, *Zn^{2+}, *His 69$^-$, *Glu 72$^-$, *Asp 142^n, *His 196^n	β
VI	H_2O, Glu 270$^-$	β

a) The fragments labeled * are approximated by point fractional charges in the calculations.

The calculated model systems for the catalytic site of native CPA are summarized in Table I. Some molecules or Zn were not included in the MO calculations in these model systems, but were replaced by point fractional charges. Such approximated parts are labeled "*" in Table I. Point fractional charges were determined from the Mulliken populations[15] calculated with the STO-3G basis set for each isolated molecule.[16] The geometries were taken from ref. 17. The last column in Table I shows the types of proton transfer calculated in the models. The proton transfer from His 69 to Asp 142 is called α proton transfer and that from the Zn-coordinated water molecule to Glu 270 is called β proton transfer. The α proton was moved along a line between H($N^{\varepsilon 2}$) of His 69^n and $O^{\delta 1}$ of Asp 142$^-$. When α proton transfer occurred, the conformations of His 69 and Asp 142 were assumed to be unchanged in our calculations. The β proton was moved along a line between the oxygen of the water molecule and $O^{\varepsilon 2}$ of Glu 270$^-$.[18] Similarly, when β proton transfer occurred, the conformations of the Zn-coordinated water molecule and Glu 270 were assumed to be unchanged.

Results and Discussion

Proton Transfer in the Zinc-His 69-Asp 142 System

Calculations of α proton transfer in the Zn–His 69-Asp 142 system were performed. In the calculations on Model I (see Table I and Fig. 1 (a)), H_2O, Glu 72$^-$, His 196^n, and Glu 270$^-$ were represented by point fractional charges. The calculated results for α proton transfer are shown as potential curve I in Fig. 2. The curve gave two energy minima at transfer distances of the α proton of 0.1 and 1.0 Å corresponding to the forms Zn^{2+}–His 69^n–Asp 142$^-$ and Zn^{2+}–His 69$^-$–Asp 142^n, respectively. The minimum energy of the former is higher than that of the latter by 3 kcal/mol. The barrier for α proton transfer was calculated to be 15 kcal/mol. Hence, the proton may transfer from His 69 to Asp 142 in native CPA.

In order to analyze the role of Zn in the Zn–His 69-Asp 142 system, calculations were performed on Models II and III. In Model II, two positive charges are positioned instead of Zn^{2+} in Model I. The potential curve for Model II is very similar to that for Model I, as shown in Fig. 2. Therefore the electrostatic interaction energy among Zn^{2+}, His 69 and Asp 142 plays a significant role. Model III consists of just His 69 and Asp 142 and does not include Zn or any other ligand. The minimum energy of the form His 69$^-$–Asp 142^n is higher than that of the form His 69^n–Asp 142$^-$ by 24 kcal/mol in Model III. The barrier height at a distance

of 0.6 Å was calculated to be 30 kcal/mol. From a comparison between I and III, it can be seen that the electrostatic field due to Zn together with its neighboring ligands greatly lowers the barrier height. Accordingly, Zn and its ligands have a significant effect on proton transfer from His 69 to Asp 142.

Proton Transfer in the Zinc-Water-Glu 270 System

Calculations of β proton transfer in the Zn–water–Glu 270 system were performed. The model systems employed were IV, V, and VI in Table I. Model IV (Fig. 1 (b)) consists of Zn–water–Glu 270 along with His 69$^-$, Glu 72$^-$, Asp 142^n and His 196^n, which are included as point fractional charges. His 69 and Asp 142 were assumed to be anionic and neutral in this system, respectively. The calculated results for β proton transfer are shown as potential curve IV in Fig. 3. The calculations gave two energy minima. Since the internuclear distance between the oxygen of H_2O and $O^{\epsilon 2}$ of Glu 270 was 3.19 Å, the minima were obtained at transfer positions of 0.1 and 1.2 Å. The atomic population on Zn changed from +1.8 to +1.6 upon proton transfer. The barrier height for β proton transfer was calculated to be 23 kcal/mol. The minimum energy of the form Zn^{2+}–OH^-–Glu 270^n was lower than that of the form Zn^{2+}–water–Glu 270$^-$ by 12 kcal/mol. The results suggest that β proton transfer may occur in the active site of CPA. The energy of the proton affinity of Zn^{2+}–water system was calculated to be 85.5 kcal/mol.[9] Accordingly, the deprotonation of the Zn-coordinated water molecule is greatly promoted by the existence of the proton acceptor (Glu 270).

The role of Zn and its ligands in β proton transfer was studied in the same way as in the preceding section, with Models V and VI. The results are shown in Fig. 3. The potential curves were also found to be double well in form for both Models V and VI. In Model V, the minimum value of the form OH^-–Glu 270^n was higher than that of the form H_2O–Glu 270$^-$ by 8 kcal/mol. The potential barrier in this model was calculated to be 33 kcal/mol at a distance of 0.7 Å. By comparison between Models IV and V, it can be seen that the form OH^-–Glu 270^n is stabilized by the electron delocalization between the deprotonated water and Zn. In Model VI, the minimum value of the form OH^-–Glu 270^n was much higher than that of the form H_2O–Glu 270$^-$ (higher by 65 kcal/mol). This result shows that β proton

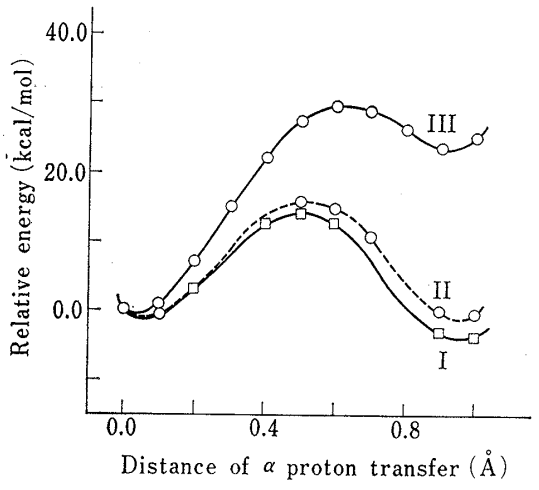

Fig. 2. Potential Curves for α Proton Transfer in Models I to III

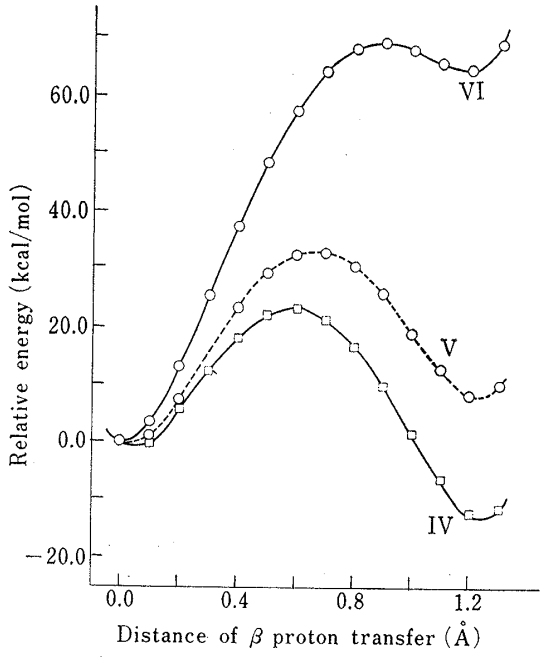

Fig. 3. Potential Curves for β Proton Transfer in Models IV to VI

transfer does not occur in Model VI. By comparison between Models V and VI in Fig. 3, it can be seen that the form OH^-–Glu 270^n is strongly stabilized by the electrostatic field due to Zn and its ligands. The calculated results mentioned above showed that Zn and its ligands play a significant role in proton transfer from the water molecule to Glu 270. Accordingly, Zn and its ligands are necessary for the general base catalysis of Glu 270 in CPA.

In the general base catalysis of CPA, Lipscomb *et al.* proposed that the Zn-coordinated water molecule was displaced by the carbonyl group of the peptide substrate and that the placement of a water molecule necessitated a reorientation of the side-chain of Glu 270 from its native conformation.[1)] However our calculated results show that Zn and its ligands are involved in the deprotonation of the water molecule in the general base catalysis. From the standpoint of the CPA active site structure, therefore, we considered carefully whether the carbonyl group of a peptide substrate would be able to approach Zn without displacing the Zn–coordinated water molecule. Figure 4 shows the structure in which Gly–Tyr inhibitor and Zn–coordinated water molecule are superposed on the active site of native CPA. Gly–Tyr is fixed by the hydrophobic pocket and the side chain of Arg 145 in the complex structure, and a real substrate should also be bound in a similar position. The carbonyl oxygen(O^I) of Gly–Tyr is in a fifth coordination site of Zn^{2+} as shown in Fig. 4. $\angle O^I$–Zn–$O(H_2O)$ is 45.3°, $\angle O^I$–Zn–$N^{\delta 1}$(His 69) is 77.4°, $\angle O^I$–Zn–$O^{\varepsilon 1}$(Glu 72) is 122.3°, and $\angle O^I$–Zn–$N^{\delta 1}$(His 196) is 94.9°. The carbonyl carbon(C^I) of Gly–Tyr is in just the position that is attacked by the oxygen of the Zn–coordinated water molecule. $\angle O(H_2O)$–C^I–O^I is 73.1°, $\angle O(H_2O)$–C^I–$C^{\alpha I}$ is 72.1°, and $\angle O(H_2O)$–C^I–N^I is 127.4°. The distance between the oxygen of the Zn–coordinated water molecule and C^I is 1.56 Å. Since this distance corresponds to the length of a covalent bond, it will be possible to form a tetrahedral intermediate on Zn^{2+}. From the structural viewpoint, accordingly, it seems reasonable to think that the carbonyl group of the substrate, without breaking the hydrogen bond between Glu 270 and the Zn–coordinated water molecule, points towards a fifth coordination site slightly away from Zn and that the water molecule itself is affected by its connection to Glu 270 in a way that favors the catalysis.

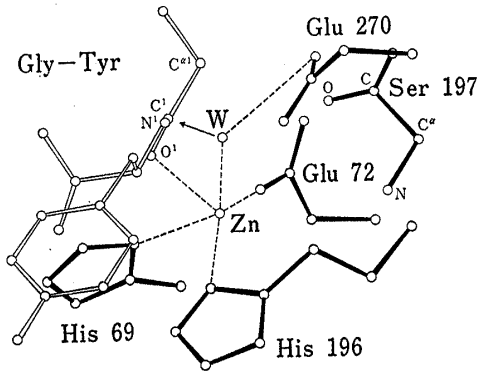

Fig. 4. The Structure of the Active Site of CPA composed of Zn, the Zn-coordinated Water Molecule, Gly–Tyr, and Amino Acid Residues Present within 5 Å of the Oxygen of the Zn-coordinated Water Molecule

It is concluded that the β proton of the Zn–coordinated water molecule in the structure shown in Fig. 4 transfers easily to $O^{\varepsilon 2}$ of Glu 270. The γ-carboxylic acid group of Glu 270 acts as the proton acceptor and Zn^{2+} plays a significant role in lowering the barrier height of proton transfer.

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References and Notes

1) *a*) W.N. Lipscomb, J.A. Hartsuck, G.N. Reeke, Jr., F.A. Quiocho, P.H. Bethge, M.L. Ludwig, T.A. Steitz, H. Muirhead, and J.C. Coppola, *Brookhaven Symp. Biol.*, **21**, 24 (1968); *b*) F.A. Quiocho and W.N. Lipscomb, *Adv. Protein Chem.*, **25**, 1 (1971); *c*) J.A. Hartsuck and W.N. Lipscomb, "The Enzyme," Vol. III, P.D. Boyer, Ed., Academic Press, New York, N.Y., 1971, Chapter 1.

2) *a*) R. Hoffmann and W.N. Lipscomb, *J. Chem. Phys.*, **37**, 2872 (1962); *b*) S. Scheiner and W.N. Lipscomb, *J. Am. Chem. Soc.*, **99**, 3466 (1977).
3) E.T. Kaiser and B.L. Kaiser, *Acc. Chem. Res.*, **5**, 219 (1972).
4) T. Sugimoto and E.T. Kaiser, *J. Org. Chem.*, **43**, 331 (1978).
5) *a*) R. Breslow and D.E. McClure, *J. Am. Chem. Soc.*, **98**, 258 (1976); *b*) R. Breslow and D.L. Wernick, *J. Am. Chem. Soc.*, **98**, 259 (1976); *c*) R. Breslow and D.L. Wernick, *Proc. Natl. Acad. Sci. USA*, **74**, 1303 (1977).
6) *a*) T. Sugimoto and E.T. Kaiser, *J. Am. Chem. Soc.*, **100**, 7750 (1978); *b*) T. Sugimoto and E.T. Kaiser, *J. Am. Chem. Soc.*, **101**, 3946 (1979).
7) D.M. Hayes and P.A. Kollman, *J. Am. Chem. Soc.*, **98**, 7811 (1976).
8) S. Nakagawa and H. Umeyama, *J. Am. Chem. Soc.*, **100**, 7716 (1978).
9) *a*) D. Demoulin, A. Pullman, and B. Sarkar, *J. Am. Chem. Soc.*, **99**, 8498 (1977); *b*) D. Demoulin and A. Pullman, *Theoret. Chim. Acta (berl.)*, **49**, 161 (1978); *c*) A. Pullman and D. Demoulin, *Int. J. Quant. Chem.*, **XVI**, 641 (1979).
10) *a*) An *ab initio* program package consisting of GAUSSIAN 70, HONDO, and many of our own routines; *b*) K. Morokuma, S. Kato, K. Kitaure, I. Ohmine, and S. Sakai, unpublished.
11) *a*) B. Roos, A. Veillard, and G. Vinot, *Theoret. Chim. Acta (berl.)*, **20**, 1 (1971); *b*) A. Veillard and J. Demuynck in "Application of electronic Structure Theory," H.F. Schaefer, Ed., Plenum Press, New York, N.Y., 1977, Chapter 5.
12) R. Ditchfield, W.J. Hehre, and J.A. Pople, *J. Chem. Phys.*, **54**, 724 (1971).
13) C.K. Johnson, ORNL-3794 Oak Ridge National Laboratory, Oak Ridge, Tenn., 1971.
14) L.E. Sutton, Table of interatomic distances, *Chem. Soc. Spec. Publ.*, **18** (1965).
15) R.S. Mulliken, *J. Chem. Phys.*, **23**, 1833 (1955).
16) Since the value of the dipole moment obtained from the calculations for H_2O with an STO-3G basis set is close to that obtained experimentally, the net charges with an STO-3G basis were used as point fractional charges.
17) H. Umeyama and S. Nakagawa, *Chem. Pharm. Bull.*, **27**, 1524 (1979).
18) Since the distance between the oxygen of the Zn-coordinated water molecule and $O^{\varepsilon 2}$ of Glu 270 was shorter than the distance between the oxygen of the water and $O^{\varepsilon 1}$ of Glu 270 by 0.13 Å, the β proton was transferred towards $O^{\varepsilon 2}$ of Glu 270.