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Molecular Orbital Studies on Serine, Cysteine, and Modified Proteases[1]

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Molecular orbital studies were carried out on α-chymotrypsin, papain, and thiolsubtilisin by using the complete neglect of differential overlap/2 method. By comparison between α-chymotrypsin and papain, the following results were obtained: (1) The proton transfer barrier from Cys-25 (neutral) to His-57 (neutral) is lower than that from Ser-195 (neutral) to His-57 (anion) in the "charge relay system" of α-chymotrypsin. (2) The active site of papain does not have the "charge relay system" and asparagine facilitates the proton transfer from cysteine to histidine. The results for thiolsubtilisin were as follows: (1) The hydrogen bond system structure in the active site is aspartate(neutral)-histidine(neutral)-cysteine(anion), and the "charge relay system" is broken by cysteine in place of serine. (2) Even though the effect of solvent is present, cysteine anion is stable. A "charge relay system" composed of aspartate, histidine, and water dimer was proposed from the calculations by using water dimer in place of serine.

Keywords——molecular orbital; CNDO; proton transfer; charge relay system; papain; chymotrypsin; thiolsubtilisin; cysteine protease; modified protease; serine protease

Serine and cysteine proteases are enzymes which hydrolyze proteins. α-Chymotrypsin, trypsin, elastase, and subtilisin are well known as a serine protease because of serine-residue in the active site, and papain, ficin, and bromelain are cysteine proteases with cysteine in the active site. The structures of the active site of these proteases are clearly clarified by X-ray crystallographic analyses[3] and the reaction mechanisms have been studied by various experiments.[4] It is possible to study the electronic structure of the active sites of enzymes. From the quantum chemical point of view, several papers reported the active site of α-chymotrypsin in recent years.[5] We have also speculated on the active site of α-chymotrypsin using the CNDO/2 (complete neglect of differential overlap/2) method,[6–8] and reported significance of the "charge relay system,"[6] the role of substrate as a trigger,[6] solvolysis of acyl-α-chymotrypsin,[7] the role of hydrogen bonds between the amino acid residues and substrate,

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4) *a*) M.W. Hunkapiller, S.H. Smallcombe, D.R. Whitaker, and J.H. Richards, *Biochemistry*, **12**, 4732 (1973); *b*) J.L. Markley, W.R. Finkenstaft, H. Dugas, P. Leduc, and G.R. Drapeau, *Biochemistry*, **14**, 998 (1975); *c*) G. Robillard and R.G. Shulman, *J. Mol. Biol.*, **86**, 519 (1974); *d*) A.R. Fersht, *J. Am. Chem. Soc.*, **94**, 293 (1972); *e*) M.H. O'Leary, M.D. Kluetz, *J. Am. Chem. Soc.*, **94**, 3585 (1972).
5) *a*) G.L. Amidon, *J. Theor. Biol.*, **46**, 101 (1974); *b*) S. Scheiner, D.A. Kleier, and W.N. Lipscomb, *Proc. Nat. Acad. Sci. U.S.A.*, **72**, 2606 (1975); *c*) S. Scheiner and W.N. Lipscomb, *Proc. Nat. Acad. Sci. U.S.A.*, **73**, 432 (1976); *d*) D. Demoulin and L.C. Allen, private communication.
6) H. Umeyama, A. Imamura, C. Nagata, and M. Hanano, *J. Theor. Biol.*, **41**, 485 (1973).
7) H. Umeyama, *Chem. Pharm. Bull.* (Tokyo), **22**, 2518 (1974).
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and the effect of substrate-substituent on the "charge relay system."[8] On the other hand, active site of cystein protease has a hydrogen bond system similar to that in α-chymotrypsin. The active site is composed of Asn-175, His-159, and Cys-25, instead of Asp-102, His-57, and Ser-195 in the "charge relay system,"[3h,9] the difference being Asn-175 and Cys-25 instead of Asp-102 and Ser-195 in cysteine protease, and the question is why this should be so. Moreover the hydrolysis rate of thiolsubtilisin, in which serine in the "charge relay system" is replaced by cysteine, is lower than that of the "charge relay system" in subtilisin.[10] This is also a doubtful point. In the present work, these points were examined from the quantum chemical point of view.

Method

All the SCF (self-consistent-field) calculations were carried out within the closed shell LCAO (linear-combination-of-atomic-orbital)-SCF approximation with the CNDO/2 method of Pople and Segal.[11] Calculations were carried out using a HITAC 8700 and 8800 computer in the Tokyo University Computer Center. Stability of the energy was employed as a check for convergence in the iteration calculation. Since it is not feasible to perform calculations for an enzyme, only the active site was explicitly considered. In order to approximate the behavior of Asp-102, His-57, and Ser-195, acetic acid, imidazole and methanol were used as described in our previous papers.[6-8] For papain, acetamide, imidazole, and methanethiol were used to approximate the behavior of Asn-175, His-159, and Cys-25, respectively. Since the barrier of proton transfer depends on the hydrogen-bond distance, the distance dependency of the barrier was checked by the variation of ±0.2 Å of the hydrogen bond distance. In order to perform calculations on the enzyme system, it is necessary to know the coordinates of all the atoms during the enzymic reaction. The coordinates of the "charge relay system" are the same as those described in our previous papers.[6-8] The coordinates of methanethiol and acetamide are presented in Table I. In the calculation of water effect on cysteine-

Table I. Coordinates of Methanethiol and Acetamide

Atom	X	Y	Z
Methanethiol			
S	4.30987	0.81759	0.00000
C	4.09059	−0.88522	−0.59698
H	3.04763	1.23382	0.00000
H	4.87141	−1.52067	−0.17910
H	3.11458	−1.25719	−0.28532
H	4.15410	−0.90034	−1.68502
Acetamide			
O	−3.54969	1.04955	0.00000
N	−4.28251	3.25814	0.00000
C	−4.50909	1.89687	0.00000
C	−5.99817	1.64635	0.00000
H	−3.32742	3.61618	0.00000
H	−5.07014	3.90625	0.00000
H	−6.52744	2.59922	0.00000
H	−6.27174	1.07936	0.88982
H	−6.27174	1.07936	−0.88983

modified Asp-102, His-57, and cysteine system, the coordinates of water are presented in Table II(a). The coordinates of water in a created "charge relay system" are presented in Table II(b).

9) *a*) J. Drenth, J.N. Jansonius, R. Koekoek, L.A.A. Sluyterman, and B.G. Wolthers, *Philos. Trans. R. Soc. London, Ser. B*, **257**, 231 (1970); *b*) G. Lowe, *Philos. Trans. R. Soc. London, Ser. B*, **257**, 237 (1970).
10) K.E. Neet, A. Nanci, and D.E. Koshland, Jr., *J. Biol. Chem.*, **243**, 6392 (1968).
11) J.A. Pople and G.A. Segal, *J. Chem. Phys.*, **44**, 3289 (1966).

TABLE II-a. Coordinates of Water in the Calculation of Water Effect on Cysteine-modified Asp-102, His-57, and Cysteine System

Atom	X	Y	Z
Water 1			
O^1	6.21796	1.98493	−2.28834
H	5.64673	1.63545	−1.60327
H	6.42151	2.90154	−2.09822
Water 2 (the distance of 2.42 Å between O^1 and O^2)			
O^2	8.26909	0.70278	−2.36174
H	8.84032	1.05225	−3.04681
H	7.45711	1.21034	−2.33268

TABLE II-b. Coordinates of Water in the Created "Charge Relay System"

Atom	X	Y	Z
Water 3			
O^3	3.92999	0.94285	0.00000
H	3.02208	1.24224	0.00000
H	3.89570	0.03847	−0.30796
Water 4			
O^4	6.00952	2.68948	−0.92828
H	5.31683	2.10768	−0.61907
H	6.54146	2.87811	−0.15666

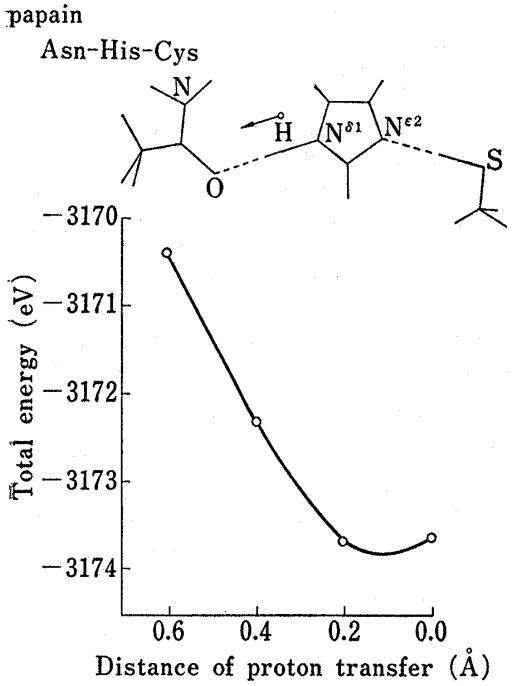

Fig. 1. Structure and Potential Curve of the Proton Transfer from Histidine (neutral) to Asparagine (neutral) in the Hydrogen Bond System Composed of Asparagine (neutral), Histidine (neutral), and Cysteine (neutral) of the Active Site of Papain

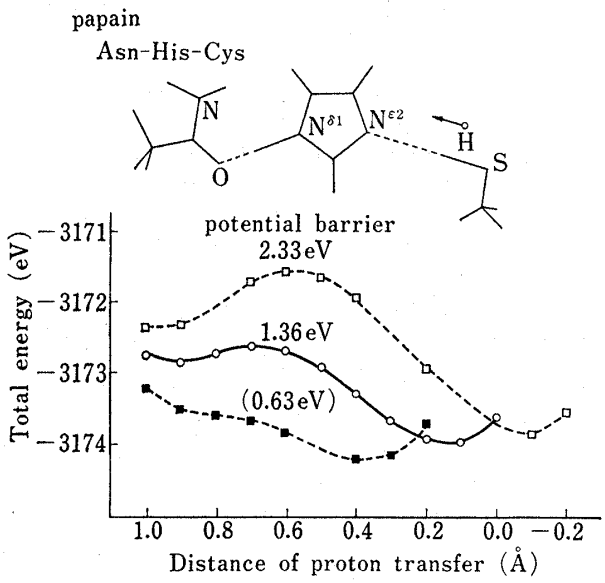

Fig. 2. Structure and Potential Curve of the Proton Transfer from Cysteine (neutral) to Histidine (neutral) in the Hydrogen Bond System Composed of Asparagine (neutral), Histidine (neutral), and Cysteine (neutral) of the Active Site of Papain

distance between $N^{\varepsilon 2}$ and S
□-----□: 3.6 Å ○—○: 3.4 Å ■-----■: 3.2 Å

Results and Discussion

Cysteine and Serine Proteases

In order to study the reaction mechanism of cysteine proteases, proton transfers were calculated using Asn-175, His-159, and Cys-25 system in papain. Figure 1 shows the structure and the potential curve of the proton transfer from the nitrogen ($N^{\delta 1}$) of histidine to the oxygen of asparagine. The distance between $N^{\delta 1}$ and O is 2.6 Å from the X-ray crystallographic analysis.[9a] The increase of the distance between the proton and $N^{\delta 1}$ of histidine makes the total energy very unstable. This result indicates that the proton transfer is not easy. Figure 2 shows the structure and the potential curve of the proton transfer from S of cysteine to $N^{\varepsilon 2}$ of histidine. This proton trasfer has a potential barrier. At the distance of 3.4 Å obtained from X-ray crystallographic analysis, the barrier is 1.36 eV. In order to calculate the distance dependency, barrier calculations at the distances of 3.2 Å and 3.6 Å were carried out additionally. These barriers were calculated to be 0.6 and 2.3 eV at the distance of 3.2 and 3.6 Å, respectively. On the other hand, we had already made a few calculations for the barrier of the charge relay system in α-chymotrypsin.[6] For comparison of reaction mechanisms between papain and α-chymotrypsin, however, calculations for α-chymotrypsin reported in the previous paper are not sufficient.

Figure 3 shows the structure of the proton transfer from $N^{\delta 1}$ of histidine to O^- of aspartate in the "charge relay system" of α-chymotrypsin. The barrier is about 0.2 eV.[8] The proton

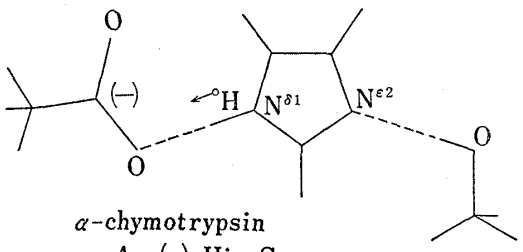

Fig. 3. Structure of the Proton Transfer from Histidine (neutral) to Aspartate (anion) in the "Charge Relay System" Composed of Aspartate (anion), Histidine (neutral), and Serine (neutral) of the Active Site of α-Chymotrypsin

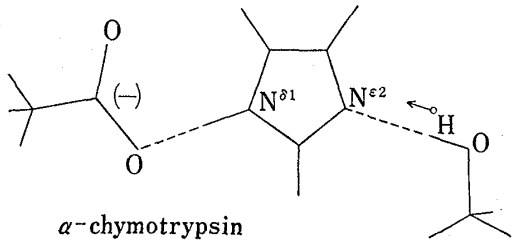

Fig. 4. Structure of the Proton Transfer from Serine (neutral) to Histidine (neutral) in the "Charge Relay System" Composed of Aspartate (anion), Histidine (neutral), and Serine (neutral) of the Active Site of α-Chymotrypsin

transfer from $N^{\delta 1}$ of histidine to O^- of aspartate is very easy compared to papain. Figure 4 shows the structure of the proton transfer from O of serine to $N^{\varepsilon 2}$ of histidine in α-chymotrypsin, where Asp-102, His-57, and Ser-195 are anionic, neutral, and neutral, respectively. The proton transfer energy from the covalently-bonded structure for Ser-195 to covalently-bonded structure for His-57 was 4.0 eV.[6] In the "charge relay system" of α-chymotrypsin, the proton transfer from His-57 (neutral) to Asp-102 (anion) is easier than that from Ser-195 (neutral) to His-57 (neutral). In the first process of the reaction, accordingly, the proton transfer from His-57 (neutral) to Asp-102 (anion) occurs. Figure 5 shows the structure and potential curve of the proton transfer from Ser-195 (neutral) to His-57 (anion). Our previous papers did not report the distance dependency of serine residue between $N^{\varepsilon 2}$ of His-57 and O of Ser-195.[6] The barriers of the proton transfer from Ser-195 (neutral) to His-57 (anion) were calculated as 1.36, 2.46, and 3.81 eV at the distances of 2.8, 3.0, and 3.2 Å, respectively, between $N^{\varepsilon 2}$ and O. The proton transfer barriers obtained from papain were calculated as 0.59, 1.36, and 2.33 eV at the distances of 3.2, 3.4, and 3.6 Å between N and S. The distances of 3.0 and 3.4 Å for α-chymotrypsin and papain, respectively, were obtained from X-ray crystallographic analysis, and it indicated that the proton transfer for papain is rather easy.

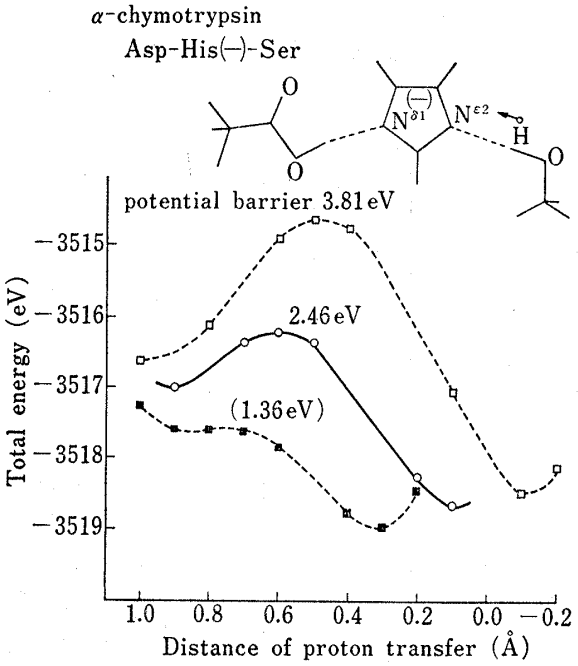

Fig. 5. Structure and Potential Curve of the Proton Transfer from Serine (neutral) to Histidine (anion) in the "Charge Relay System" Composed of Aspartate (neutral), Histidine (anion), and Serine (neutral) of the Active Site of α-Chymotripsin

distance between $N^{\varepsilon 2}$ and O
□-----□: 3.2 Å ○—○: 3.0 Å
■-----■: 2.8 Å

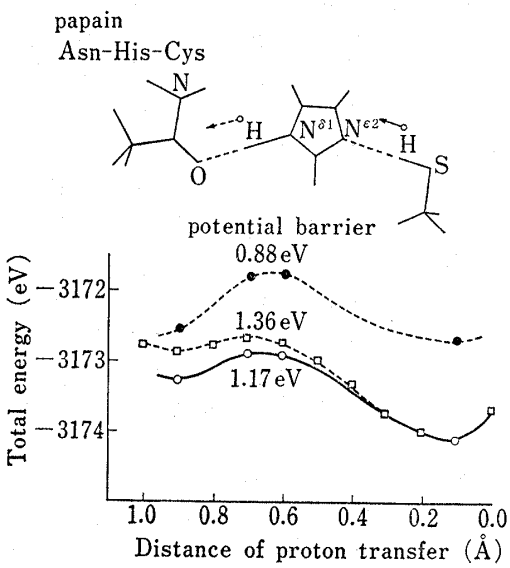

Fig. 6. Structure and Potential Curve of the Proton Transfer from Histidine (neutral) to Asparagine (neutral) at the Various Separations of 1.0, 1.2, and 1.4 Å between $N^{\delta 1}$ and H in the Hydrogen Bond System Composed of Asparagine (neutral), Histidine (neutral), and Cysteine (neutral) of the Active Site of Papain

distance between $N^{\delta 1}$ and H
□-----□: 1.00 Å ○—○: 1.20 Å
●-----●: 1.40 Å

In the hydrogen bond system of papain composed of Asn-175, His-159, and Cys-25, the proton transfer occurs from Cys-25 (neutral) to His-159 (neutral). Accordingly, it was found that papain does not have the charge relay system. It is important to clarify the role of asparagine in the hydrogen bond system. The distance between the hydrogen covalently bonded to $N^{\delta 1}$ and $N^{\delta 1}$ in Fig. 6 is increased by 0.2 and 0.4 Å without changing the distance between O of asparagine and $N^{\delta 1}$. This result is shown in Fig. 6. At the increased distance of 0.2 Å between H and $N^{\delta 1}$, the proton transfer from S to $N^{\varepsilon 2}$ is easier than that without this increase. This shows that asparagine plays a role in facilitating the proton transfer from S to $N^{\varepsilon 2}$ by pulling the proton by about 0.2 Å from $N^{\delta 1}$ of histidine.

For the reaction mechanism of papain, it is widely accepted in the literature that, during acyl-enzyme formation, the thiol group reacts in its non-dissociated form, neutral His-159 and neutral Cys-25, assisted by general base catalysis.[12] On the other hand, the presence of mercaptide ion and histidine cation in catalytically active papain was supported from the following evidences: the alkylation of papain by haloacetamides displays double sigmoid pH-rate profiles with similar pK_a values (4.0 and 8.4)[13]; D_2O effect could not be observed[13]; fluorescence of free papain and SS papain as a function of pH;[14] difference spectroscopy of papain in the absence and presence of a thiolalkylating agent as a function of pH.[15] Calcu-

12) *a*) E.C. Lucas and A. Williams, *Biochemistry*, **8**, 5125 (1969); *b*) G. Lowe and Y. Yuthavong, *Biochem. J.*, **124**, 117 (1971); *c*) K. Brocklehurst and G. Little, *Biochem. J.*, **128**, 471 (1972); *d*) P. Campbell and E.T. Kaiser, *J. Am. Chem. Soc.*, **95**, 3735 (1973); *e*) A.R. Fersht, *J. Am. Chem. Soc.*, **93**, 3504 (1971); *f*) A. Williams, E.C. Lucas and K.T. Douglas, *J. Chem. Soc. Perkin Trans., II*, **1972**, 1493.
13) L. Polgàr, *Eur. J. Biochem.*, **33**, 104 (1973).
14) *a*) L.A. AE. Sluyterman and M.J.M. De Graaf, *Biochem. Biophys. Acta*, **200**, 595 (1970); *b*) G. Lowe and A.S. Whitworth, *Biochem. J.*, **141**, 503 (1974).
15) L. Polgàr, *FEBS Lett.*, **38**, 187 (1974).

lation for the possibility of ion-ion pair was carried out. Figure 2 and Fig. 6 show its results. The structure composed of Asn-175 (neutral), His-159 (cation), and Cys-25 (anion) is more labile than the structure of Asn-175 (neutral), His-159 (neutral) and Cys-25 (neutral). By using the structure obtained from X-ray crystallographic analysis, the ion-ion pair structure is more unstable by 1.14 and 0.79 eV than neutral-neutral structure at the increased distance of 0.0 and 0.2 Å, respectively, of the covalently-bonded hydrogen from $N^{\delta 1}$ of histidine.

Figure 7 shows the structure and the potential curve composed of His-159 and Cys-25 hydrogen-bonded by water. The native papain without the substrate may have hydrogen-bonded water as a Ser-195 in α-chymotrypsin.[3d] The proton transfer energy was calculated to be 1.42 eV. The hydrogen bond system of papain with water does not become to have the nature of ion-ion pair structure. Accordingly, the barrier of proton transfer in the hydrogen bond system of papain is smaller than in the case of α-chymotrypsin, and in our calculations the non-dissociated form in the active site of papain is more stable than the ion-ion pair structure, though this problem is still under investigation.

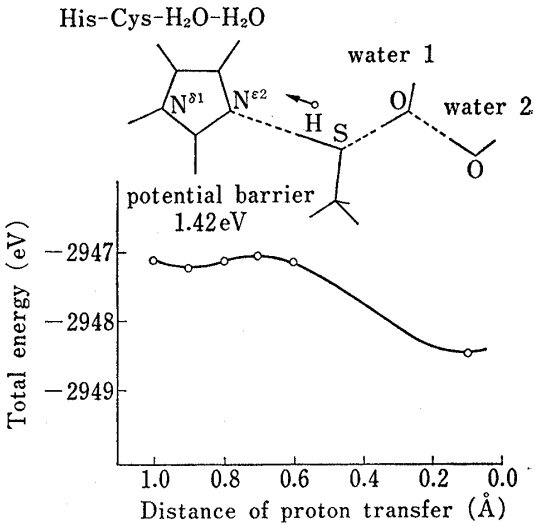

Fig. 7. Structure and Potential Curve of the Proton Transfer from Cysteine (neutral) to Histidine (neutral) in the Hydrogen Bond System Composed of Histidine (neutral), Cysteine (neutral), and Water Dimer of the Active Site of Papain

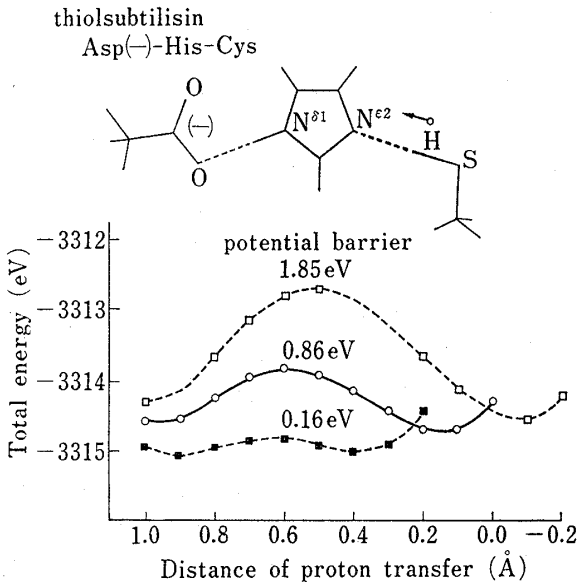

Fig. 8. Structure and Potential Curve of the Proton Transfer from Cysteine (neutral) to Histidine (neutral) in the Hydrogen Bond System Composed of Aspartate (anion), Histidine (neutral), and Cysteine (neutral) of the Active Site of Thiolsubtilisin

distance between $N^{\varepsilon 2}$ and S
□-----□: 3.6 Å ○—○: 3.4 Å
■-----■: 3.2 Å

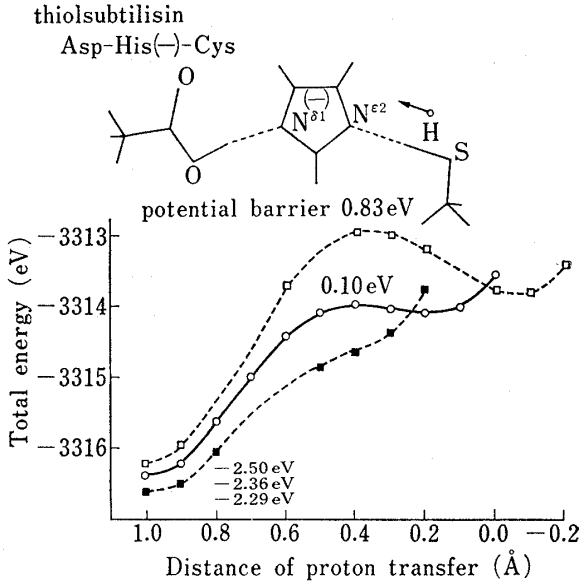

Fig. 9. Structure and Potential Curve of the Proton Transfer from Cysteine (neutral) to Histidine (anion) in the Hydrogen Bond System Composed of Aspartate (neutral), Histidine (anion), and Cysteine (neutral) of the Active Site of Thiolsubtilisin

distance between $N^{\varepsilon 2}$ and S
□-----□: 3.6 Å ○—○: 3.4 Å
■-----■: 3.2 Å

Thiolsubtilisin Mechanism

Thiolsubtilisin is subtilisin modified by cysteine in place of serine in the "charge relay system". Subtilisin has the same "charge relay system" as α-chymotrypsin. Koshland, *et al.*[10] reported that the hydrolysis rate of thiolsubtilisin is very slow, and Polgàr reported that the SH-group of thiolsubtilisin is mainly present as a mercaptide ion in the pH-range of 6.0—8.5.[16] He implied that the equilibrium is shifted to the formation of the mercaptide ion. These problems were examined from the quantum chemical point of view and are described in this section. It was found already that the proton covalently-bonded to $N^{\delta 1}$ of His-57 may move in a nearly unrestricted manner between $N^{\delta 1}$ of His-57 and Asp-102.[8] Figure 8 shows the structure and the potential curve of the proton transfer from cysteine to histidine in the hydrogen bond system, aspartate (anion)-histidine (neutral)-cysteine (neutral). At the distance of 3.0, 3.2, and 3.6 Å between S and $N^{\epsilon 2}$, the potential barriers were calculated as 0.16, 0.86, and 1.85 eV. These results suggest that the proton transfer from cysteine to histidine without the proton transfer from histidine (neutral) to aspartate (anion) is possible. Figure 9 shows the structure and the potential curve of the proton transfer from cysteine to histidine after a very easy proton transfer from histidine (neutral) to aspartate (anion).[8] The potential barrier is only 0.1 eV at the distance of 3.4 Å between $N^{\epsilon 2}$ and S. The total energy after the proton transfer from cysteine to histidine anion is more stable by 2.36 eV than that before the proton transfer. Similar conclusions were obtained at the distance of 3.2 and 3.6 Å. Accordingly, the most stable structure of the hydrogen bond system is aspartate (neutral), histidine (neutral), and cysteine (anion). This result is consistent with the result (mercaptide ion) of experiments by Polgàr.[16] In the case of α-chymotrypsin, approach

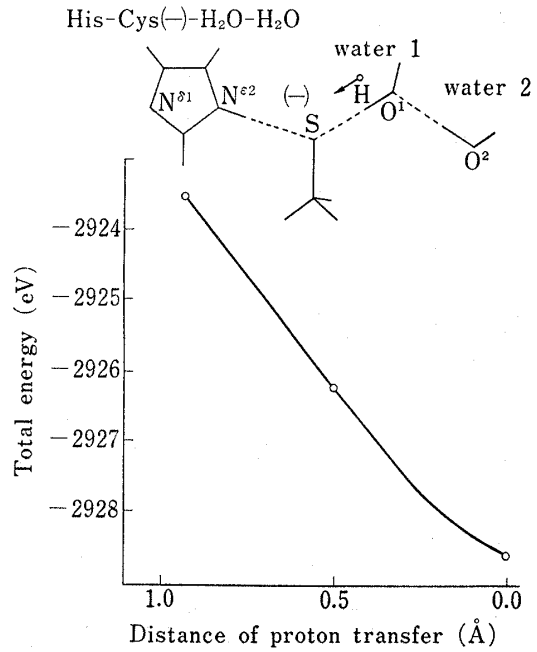

Fig. 10(a). Structure and Potential Curve of the Proton Transfer from Water to Cysteine (anion) in the Hydrogen Bond System Composed of Histidine (neutral), Cysteine (anion), and Water Dimer of the Active Site of Thiolsubtilisin

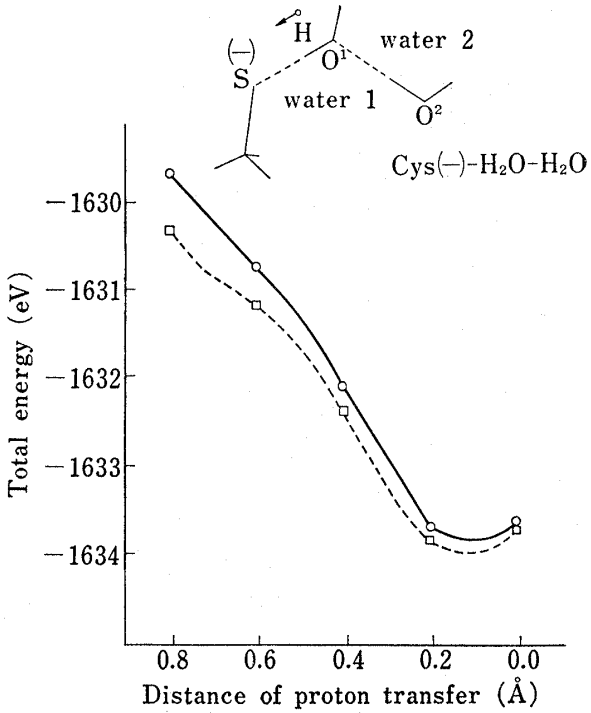

Fig. 10(b). Structure and Potential Curve of the Proton Transfer from Water to Cysteine (anion) in the Hydrogen Bond System Composed of Cysteine (anion) and Water Dimer of the Active Site of Thiolsubtilisin

distance between O^1 and O^2
○—○: 2.80 Å □-----□: 2.42 Å

16) L. Polgàr, P. Halasez, and E. Moravcsik, *Eur. J. Biochem.*, **39**, 421 (1973).

of the substrate became a trigger of the charge relay.[6] In the hydrogen bond system of thiolsubtilisin, however, the charge relay was finished before approach of the substrate. In other words, the "charge relay system" is broken by the substitution of cysteine in place of Ser-195 in α-chymotrypsin.

Effect of Water on the Hydrogen Bond System of Thiolsubtilisin

Calculations were carried out for solvent effect on the hydrogen bond system in the active site of papain. Figure 10(a) shows the structure and the potential curve of the proton transfer from O^1 of water to S of cysteine. The distance between S and O^1 of water was assumed to be 3.2 Å. The proton transfer may be almost impossible. Fugure 10(b) shows the structure and the potential curve without histidine. Results were the same at the distances of 2.8 and 2.42 Å between O^1 of water and O^2 of water. Conclusion similar to Fig. 10(a) was obtained. Accordingly, even though the effect of water on the hydrogen bond system is considered, the cysteine residue is anion structure. Additionally, the interaction energy between the cysteine anion and the substrate may be larger due to the electrostatic interaction, than that between the "charge relay system" of α-chymotrypsin and the substrate. The inactivity of thiolsubtilisin will be attributed to the processes after acylation step. Our next paper will treat this problem.

Created "Charge Relay System"

In a biological system, there may be a "charge relay system" other than serine protease. We created a "charge relay system" composed of aspartate, histidine, and water dimer. In the "charge relay system" of α-chymotrypsin, water or water dimer was used in place of Ser-195. Figure 11(a) shows the structure and potential curve of the proton transfer from water to histidine anion at the distance of 3.0 Å between $N^{\varepsilon 2}$ of histidine and O^3 of water. The

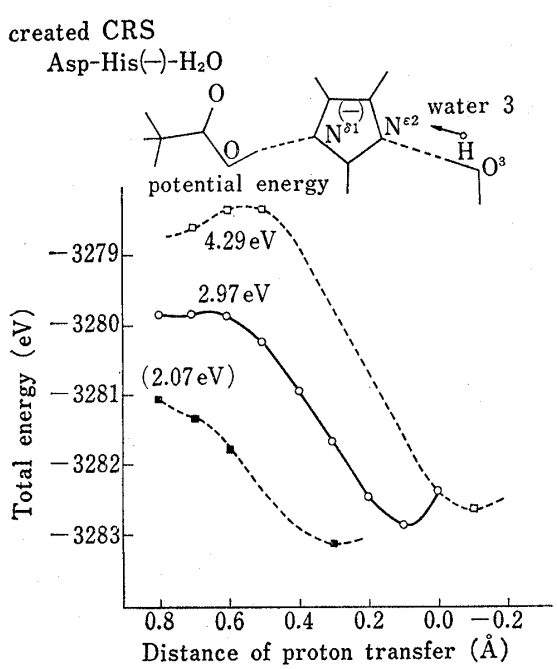

Fig. 11(a). Structure and Potential Curve of the Proton Transfer from Water (neutral) to Histidine (anion) in the Created "Charge Relay System" Composed of Aspartate (neutral), Histidine (anion) and Water

distance between O^3 and $N^{\varepsilon 2}$
□-----□: 3.2 Å ○—○: 3.0 Å
■-----■: 2.8 Å

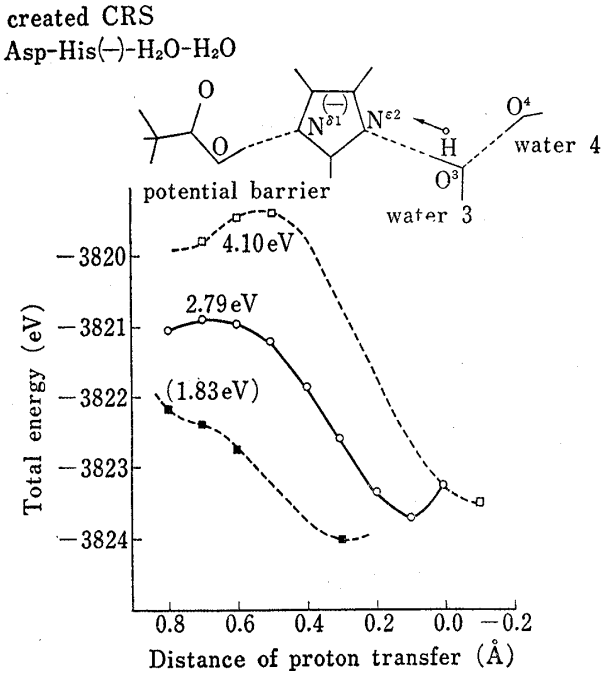

Fig. 11(b). Structure and Potential Curve of the Proton Transfer from Water (neutral) to Histidine (anion) in the Created "Charge Relay System" Composed of Aspartate (neutral), Histidine (anion), and Water Dimer

distance between O^3 and $N^{\varepsilon 2}$
□-----□: 3.2 Å ○—○: 3.0 Å
■-----■: 2.8 Å

proton transfer barrier is a little higher than that from Ser-195 to His-57 anion in α-chymotrypsin at the distance of 3.0 Å. Figure 11(b) shows the structure and potential curve of the proton transfer from water dimer to histidine anion. The barrier was near the potential barrier from Ser-195 to His-57 anion in α-chymotrypsin. The subtle charge of the distance between $N^{\varepsilon 2}$ of His-57 and O^3 of water will give a lower barrier than the "charge relay system" in α-chymotrypsin. At the distance of 2.8 Å between $N^{\varepsilon 2}$ and O^3, the potential barrier was calculated as 1.83 eV. Accordingly, the hydrogen bond system, aspartate, histidine, and water dimer may be a "charge relay system" in a biological system, though it has not been found yet.

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