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A Molecular Orbital Study on the Effects of Substituents on the Proton Transfer from Ser-195 to His-57 in the Hydrolysis of α-Chymotrypsin

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From the results of molecular orbital study on the effects of substituents on the proton transfer from Ser-195 to His-57 in the "charge relay system" of α-chymotrypsin, the acylation step is thought to be as follows: (1) the carbonyl carbon of the substrate approaches to the oxygen of Ser-195 in the "charge relay system"; (2) after the proton of Ser-195 is transferred to $N^{\delta 2}$ of His-57 by the trigger of the interaction between the oxygen of Ser-195 and the carbonyl carbon of the substrate, the oxygen of Ser-195 covalent-bonds with the carbonyl carbon of the substrate; (3) acyl-Ser-195 rotates by 120 degrees around the serine C^{α}-C^{β} bond. The rotation of acyl-Ser-195 must be accompanied with the deeper movement of the aromatic part of the substrate in the pocket of α-chymotrypsin. Moreover, it was shown that a significant role in accelerating the proton transfer from Ser-195 to His-57 was played by the interaction between the oxygen of Ser-195 and carbonyl carbon of substrate, not by two hydrogen bonds between the carbonyl oxygen and the backbone-NH-groups of Gly-193 and Ser-195.

In an earlier paper we published the results of a molecular orbital study on the mechanism of enzymatic reactions of α-chymotrypsin.[2] The enphasis of the paper was the possible importance of the "charge relay system", delocalization energy and "orbital steering" in the enzymatic reaction of α-chymotrypsin. Those calculations were performed employing the X-ray crystallographic structure of the active site in α-chymotrypsin as determined by Blow, *et al.*,[3] who had satisfactorily clarified the three-dimensional structure of the active site in α-chymotrypsin. The active site or "charge relay system", as it is denoted by Blow, *et al.*,[4] has the following two salient structural features. First the $N^{\delta 1}$ proton of His-57 is in a buried position where it is stabilized by a hydrogen bond to Asp-102, and secondly the hydroxyl group of the serine residue is in a favorable orientation for hydrogen bonding to $N^{\delta 2}$ of His-57. Experimental work by Bender and Nakamura[5] on α-chymotrypsin catalyzed reactions established the

1) Location: *a) 5-9-1, Shirokane, Minato-ku, Tokyo; b) Tsukiji, Chuo-ku, Tokyo.*
2) H. Umeyama, A. Imamura, C. Nagata, and M. Hanano, *J. Theor. Biol.*, **41**, 485 (1973).
3) *a)* P.B. Sigler, D.M. Blow, B.W. Matthews, and R. Henderson, *J. Mol. Biol.*, **35**, 143 (1968); *b)* T.A. Steitz, R. Henderson, and D.M. Blow, *J. Mol. Biol.*, **46**, 337 (1969); *c)* J.J. Birktoft, B.W. Matthews and D.M. Blow, *Biochem. Biophys. Res. Comm.*, **36**, 131 (1969); *d)* R. Henderson, *J. Mol. Biol.*, **54**, 341 (1970).
4) D.M. Blow, J.J. Birktoft, and B.S. Hartley, *Nature*, **221**, 337 (1969).
5) M.L. Bender and K. Nakamura, *J. Amer. Chem. Soc.*, **84**, 2577 (1962).

hammett ρ-constant of acylation for a series of phenyl acetates as 1.8, and the ρ-constant of acylation for a series of phenyl trimethylacetates as 1.4. Bender, *et al.*[6] demonstrated that for both specific and nonspecific substrates of α-chymotrypsin, $k_{H_2O}/k_{D_2O}=2$ to 3 and further that this ratio held for both acylation and deacylation rate constants (2.0 for k_{cat} of N-acetyl-L-tryptophan amide and 2.2 for k_2 of *p*-nitrophenyl trimethyl acetate). They argued that the kinetic isotope effects could not be the results of differences in nucleophilicity and solvation effects but rather reflected a proton transfer in the transition state of both the acylation and deacylation reactions. Substitution effects and the deuterium exchange effects must be explained by any proposed mechanism.

Based on a high-resolution map of tosyl-chymotrypsin,[3a] Steitz, *et al.*[3b] made the following observations: some repositionings of the side chains of His-57 and Ser-195 occur upon the formation of tosyl-chymotrypsin from native enzyme; upon tosylation the oxygen of Ser-195 is rotated about its C^{α}–C^{β} bond, although the magnitude of this rotation remains uncertain due to difficulties in determining the exact position of the serine oxygen in the native enzyme; fixing the indolyl group of a substrate and forming a hydrogen bond between the amide and Ser-214 places the carbonyl carbon in such a position that an acyl intermediate can be formed only after a similar rotation of the oxygen of Ser-195; the extent, timing and function of the serine oxygen movement remain to be determined. Henderson[3d] noted that the serine O^{γ} is displaced by approximately 2.5 Å upon acylation, or a distance which is in almost exact agreement with that due to a rotation of 120 degrees about the serine C^{α}–C^{β} bond. The 1.9 Å displacement of the serine required to bring it within covalent bonding distance of the substrate may also be explained by this rotation. The timing of the rotation of serine oxygen to form a covalent bond with the substrate is, moreover, still open to questions.

First we will discuss an intermediate structure based on the following steps. First the proton of Ser-195 is transferred to the $N^{\delta 2}$ of His-57. This is followed by a 120 degrees rotation of the ionized C–O^- bond of the Ser-195 and subsequent attack on the carbonyl carbon of the substrate to form an acyl enzyme intermediate. This mechanism does not appear to be compatible with the experimental results cited above. If the reaction were to proceed through such an intermediate, then the rate constant should not have any dependence on isotopic substitution. As noted earlier, biochemical experiment have indicated that this is not true. Considering this, it might be meaningful to test, from the quantum chemical point of view, the reliability of the following two structures. The first is the X-ray crystalographic structure which we used in our previous paper;[2] employing phenylacetates in place in the model substrates (N-methylacetamide and methylacetate). Second is a slight modification of the rejected structure such that the isotopic dependence may be explained. Instead of allowing rotation of the ionized C–O^- bond in Ser-195, one attacks the carbonyl carbon of the substrate immediately after the proton is transferred from the Ser-195 to the $N^{\delta 2}$ of His-57. Our results indicate that this structure, coupling the deuterium effects of the proton transfer from Ser-195 to His-57 with substituent effects, is consistent with the biochemical experiments. Finally, we consider, from a quantum chemical point of view, the kinetic significance of two hydrogen bonds between the carbonyl oxygen and the backbone-NH-groups of Gly-193 and Ser-195 in the enzymatic reaction. In this report, the calculated values of activation energies are actually activation enthalpies.

Method

The method used in our calculations is the CNDO/2 (Complete Neglect of Differential Overlap/2) method, developed by Pople and Segal,[7] details of which need not be described here. Calculations were carried out using a HITAC 8350 in the National Cancer Center and a HITAC 8700 and 8800 in the Computer Center,

6) M.L. Bender, G.E. Clement, F.J. Kezdy, and H. D'A Heck, *J. Amer. Chem. Soc.*, **86**, 3680 (1964).
7) J.A. Pople and G.A. Segal, *J. Chem. Phys.*, **44**, 3289 (1966).

the University of Tokyo. The stability of the electronic energy was employed as a check for convergence in the iteration calculation.

Since it is not feasible to perform a calculation for the entire α-chymotrypsin molecule, only the active site was explicitly considered. As described in our previous paper,[2] acetic acid, imidazole and ethanol (or methanol in the case of larger substrates such as phenyl acetates) were used to approximate the behavior of Asp-102, His-57 and Ser-195 respectively. The aromatic parts of actual substrates, such as N-acetyl-L-tryptophan methyl ester and substituted N-acetyl-L-tyrosin anilides were found to interact with the pocket part of α-chymotrypsin, being stabilized as a result of Van der Waals forces. However, since this stabilization energy may reasonably be considered to be constant throughout all steps of the enzymatic reaction, it was not taken into account in the present calculation. Calculations were performed on the structure containing

Table I(a). Coordinates of Methanol Corresponding to Ser-195 as shown in Fig. 2(a), 2(b), 3(a), 3(b) and 4.

	X	Y	Z
Methanol			
4H (initial)	3.01828	1.24349	0.00000
4H (transition)	2.41047	1.44392	0.00000
5H	4.85545	−0.95423	0.00963
6H	3.08795	−0.98278	−0.19598
7H	4.13281	−0.47992	−1.54599
6O	3.92999	0.94285	0.00000
7C	4.00658	−0.46061	−0.46350

Table I(b). Coordinates of *p*-Nitrophenyl and *m*-Acetylphenyl Acetates under the Conditions Uncoupling the Substituent Effects with the Proton Transfer as shown in Fig. 2(a) and 2(b).

	X	Y	Z
p-Nitrophenyl acetate			
8H	2.30764	−3.46172	1.58144
9H	0.86934	−2.41795	1.67617
10H	1.53964	−3.14571	3.15543
11H	1.33574	2.20884	2.83366
12H	2.89766	4.11193	3.14026
13H	4.67172	−0.48770	2.57678
14H	6.23364	1.41539	2.88338
8C	2.68003	−1.50354	2.37607
9O	3.89265	−1.63591	2.39702
10C	1.78617	−2.71780	2.18372
11O	2.04272	−0.31035	2.51658
12C	2.90520	0.74053	2.68588
13C	2.40573	2.03551	2.84453
14C	3.28522	3.10710	3.01717
15C	4.66417	2.88371	3.03117
16C	5.16364	1.58873	2.87252
17C	4.28416	0.51714	2.69988
18N	5.53295	3.94226	3.20171
19O	5.06119	5.04841	3.33577
20O	6.71996	3.70759	3.20804
m-Acetylphenyl acetate			
15H	5.34661	3.71521	3.16513
16H	8.58038	2.27834	3.07483
17H	7.30658	3.06342	4.03827
18H	7.36298	3.30113	2.27547
21C	6.62452	1.35207	2.88735
22O	7.09801	0.21440	2.74909
23O	7.53602	2.59030	3.08348

Table I(c). Coordinates of *p*-Nitrophenyl and *m*-Acethylphenyl Acetates under the Conditions Coupling the Substituent Effects with the Proton Transfer as shown in Fig. 3(a) and 3(b). The Value used as the Distance between the Oxygen of Ser-195 and the carbonyl carbon of substrate was 2.0. Å

	X	Y	Z
p-Nitrophenyl acetate			
8H	3.70949	−0.85523	1.80774
9H	2.64070	0.52083	2.17009
10H	3.76423	−0.08401	3.41066
11H	4.28801	5.00418	1.78205
12H	6.10598	6.46921	0.94299
13H	6.70726	1.54392	0.98206
14H	8.52524	3.00896	0.14300
8C	4.69449	1.05081	1.84496
9O	5.75740	0.61928	1.42976
10C	3.62700	0.09042	2.34349
11O	4.37908	2.37266	1.89830
12C	5.38296	3.18164	1.43498
13C	5.21368	4.56830	1.42398
14C	6.23734	5.39323	0.95153
15C	7.43029	4.83150	0.49007
16C	7.59957	3.44484	0.50107
17C	6.57591	2.61990	0.97352
18N	8.44149	5.64639	0.02336
19O	8.25627	6.84211	0.03014
20O	9.45921	5.12154	−0.36764
m-Acetylphenyl acetate			
15H	8.22460	5.47160	0.12347
16H	9.55881	4.40817	−1.33175
17H	10.82454	3.26450	−0.82471
18H	10.23908	4.50424	0.30993
21C	8.86340	2.84974	0.01220
22O	9.04335	1.62287	0.00944
23C	9.95195	3.82908	−0.49617

Table I(d). Coordinates of Methyl Acetate and two Ammonia Corresponding to Two Peptide Chains as shown in Fig. 4.

	X	Y	Z
Methyl acetate			
8H	6.34488	3.08399	2.00670
9H	5.13707	4.27699	1.47277
10H	5.66461	2.98793	0.36501
11H	3.70949	−0.85523	1.80774
12H	2.64070	0.52083	2.17009
13H	3.76423	−0.08401	3.41066
8C	4.69449	1.05081	1.84496
9O	5.75740	0.61928	1.42976
10C	3.62700	0.09042	2.34349
11O	4.37908	2.37266	1.89830
12C	5.44891	3.23479	1.40454
Ammonia			
14H	6.14833	−0.90250	2.26628
15H	7.43020	1.22591	1.38331
16H	5.63322	−1.99592	3.41278
17H	6.45021	−2.52817	2.06272
18H	8.98984	0.88093	0.91015
19H	8.71565	1.74405	2.30773
13N	6.37234	−1.77453	2.74563
14N	8.38877	1.57353	1.35669

methyl acetate in place of N-acetyl-L-tryptophan methyl ester, as well as on structures containing phenyl acetates which are actual substrates. The details of the enzymatic reactions (acylation and deacylation) of α-chymotrypsin were presented in the previous paper.[2]

In order to perform calculations on the enzyme-substrate system, it is necessary to know the coordinates of all the atoms during each stage of the enzymatic reaction. The coordinates of the "charge relay system" are the same as those described in the previous paper.[3] The coordinates of methanol are presented in Table I(a). In this report, *p*-nitrophenyl and *m*-acetylphenyl acetates were used as the substrates of phenylacetate derivatives. Their substrates were used in the experiments by Bender and Nakamura.[5] The coordinates of *p*-nitrophenyl and *m*-acetylphenyl acetates under conditions where the substitution effects are uncoupled are shown in Table I(b). The corresponding coordinates under coupled conditions are given in Table I(c). The coordinates of the two ammonia molecules were used to simulate the backbone-NH-groups. The study of hydrogen bond effects are given in Table I(d). Hubbard and Kirsch[8] reported that the Hammett ρ values obtained for many *meta*- and *para*-substituted *p*-nitrophenyl benzoates and 2,4-dinitrophenyl benzoates in the reactions of hydrolysis by α-chymotrypsin were 0.97 and 1.6, respectively. He noted that the transition state in which O^{γ} of Ser-195 directly attacks the carbonyl carbon of the substrate is consistent with the observed acyl-chymotrypsin, and on the other hand the transition state in which $N^{\delta 2}$ of His-57 attacks the carbonyl carbon of the substrate is supported by the close similarity of the ρ values for acylation by imidazole and by α-chymotrypsin. In this report, based on X-ray diffraction data, we consider only the transition state in which O^{γ} of Ser-195 directly attacks the carbonyl carbon of the substrate.

Results and Discussion

Substituent Effects for the Proton Transfer from Ser-195 to His-57

In the "charge relay system" composed of Asp-102, His-57 and Ser-195, it should be impossible for the proton transfer from $N^{\delta 1}$ of His-57 to Asp-102 to be synchronous with the transfer from the Ser-195 to the His-57. Since such an occurrence would result in a destabilization corresponding to two covalent bonds. Thus, in order to decide the rate-determining step, the potential energies of the proton transfer from Ser-195 to $N^{\delta 2}$ of His-57 were compared with

Table II. Potential Energies of the Proton Transfers from Ser-195 to His-57 and from His-57 to Asp-102

Molecules concerned	Distances from initial place of the proton (Å)	Total energies (eV)
(a)	0.0	−2741.69
	0.3	−2741.6
	0.4	−2741.5
	0.5	−2741.5
	0.6	−2741.7
(b)	0.0	−2307.14
	0.24	−2306.5
	0.34	−2304.6
	0.44	−2303.7
	0.54	−2303.1
	0.64	−2302.6
(c)	0.0	−2284.6
	0.4	−2283.2
	0.57	−2282.7
	0.64	−2282.6
	0.74	−2283.0
	1.04	−2283.32

(a): The proton transfer from His-57 to Asp-102.
(b): The proton transfer from Ser-195 to His-57.
(c): The proton transfer from Ser-195 to His-57 anion.

8) C.D. Hubbard and J.F. Kirsh, *Biochemistry*, **11**, 2483 (1972).

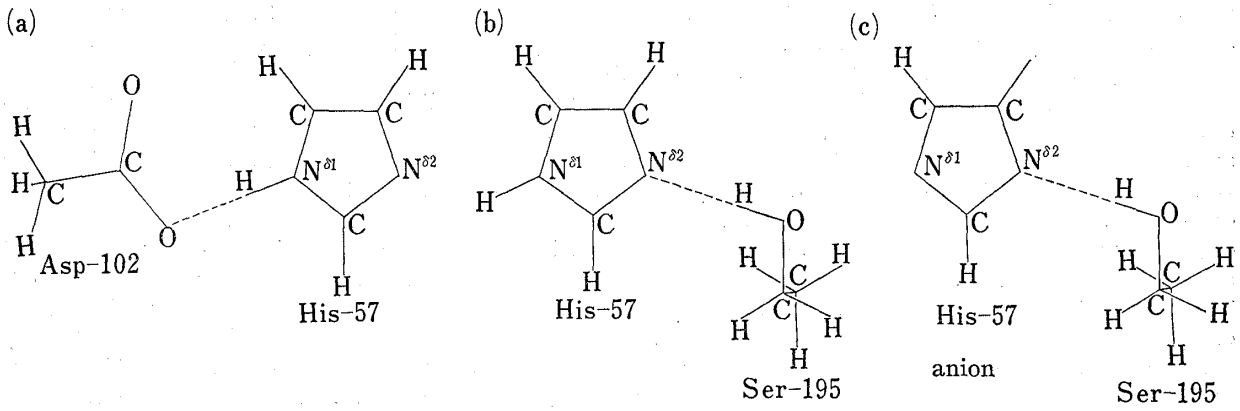

Fig. 1.

(a): structure composed of Asp-102 and His-57
(b): structure composed of His-57 and Ser-195
(c): structure composed of His-57 anion and Ser-195

those of the proton transfer from $N^{\delta 1}$ of His-57 to Asp-102. The results are evidenced in Table II, while the coordinates of the "charge relay system" were described in the previous paper[2] and structures for the two proton transfers are shown in Fig. 1(a) and Fig. 1(b). The height of the potential barrier of the proton transfer from $N^{\delta 1}$ of His-57 to Asp-102 was 0.2 eV at displacements of both 0.4 Å and 0.5 Å, from the covalent bond distance of a proton to the $N^{\delta 1}$ of His-57 (Table II(a)). On the other hand, the height of the potential barrier of the proton transfer from Ser-195 to $N^{\delta 2}$ of His-57 were 2.5 eV, 3.4 eV, and 4.0 eV at respective displacement of 0.34 Å, 0.44 Å, and 0.54 Å, from the equilibrium bond distance in Ser-195 (Table II(b)). Accordingly, it was shown 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, and that the proton transfer from Ser-195 to $N^{\delta 2}$ of His-57 is the rate-determining step.

In order to determine which of two proton transfers occurrs first the potential energy of the proton transfer from Ser-195 to His-57 was calculated on the hydrogen bond system composed of Ser-195 and His-57, assuming that the transfer from $N^{\delta 1}$ of His-57 to Asp-102 had already occurred. The height of potential barrier was 2.0 eV at the distance, 0.64 Å, from the equilibrium position of the proton of Ser-195 as listed in Table II(c). Comparing this number to the corresponding barrier assuming that no prior proton transfer has occurred (Table II(b)), it is clear that it is energetically more favorable for the proton of Ser-195 to transfer to $N^{\delta 2}$ of His-57 after the $N^{\delta 1}$ proton of His-57 has transferred to Asp-102. This indicates that, in the transition state, the proton transfer of Ser-195 occurrs when the proton between Asp-102 and $N^{\delta 1}$ of His-57 is covalently-bonded to Asp-102. Accordingly, it should be pointed out that Asp-102 plays an important role in the extraction of the proton from His-57.

For the sake of comparison on the results of the proton transfer on the structure composed of Ser-195 and His-57 lacking the proton of $N^{\delta 1}$, calculations were performed on the "charge relay system" (Asp-102 (neutral), His-57 (anion) and Ser-195). The potential barrier was 1.98 eV at a proton displacement of 0.64 Å, in close agreement with the value of 2.0 eV attained at the corresponding distance in the Ser-His system. It is reasonable, therefore, to use the system lacking Asp-102 (neutral) of the "charge relay system" for the purpose of performing calculations on the energetics of proton transfer in the active site containing the "charge relay system" and large substrates such as phenyl acetates.

In order to examine the substituent effects of phenyl acetates on the proton transfer from Ser-195 to $N^{\delta 2}$ of His-57, two substrates, *p*-nitrophenyl acetate and *m*-acetylphenyl acetate, were selected for study. They were chosen because of the difference in the rate constant of the acylation reaction between two phenyl acetates (shown by Bender and Nakamura[5]). In calculations on the system containing large substrates such as phenyl acetates, methanol was used in place of Ser-195. The coordinates of methanol are shown in Table I(a). In the case of

methanol, the potential barrier for proton transfer is 1.89 eV, which is 0.09 eV lower than the corresponding barrier (1.98 eV) obtained using ethanol. In discussing substituent effects on energy difference between the two substituted phenyl acetates. It is expected that using methanol, in place of ethanol, will have little effect on this difference.

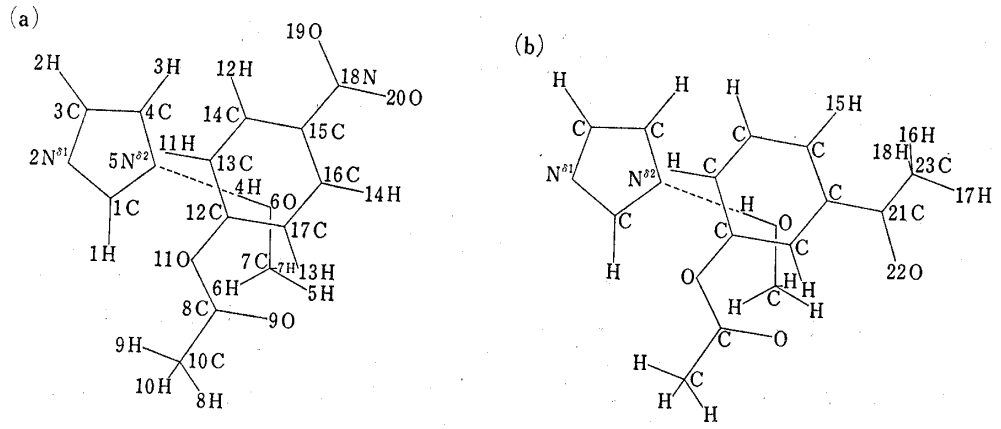

Fig. 2. Structures under the Conditions Uncoupling the Substituent Effects with the Proton Transfer

(a): structure composed of His-57 anion, Ser-195 and *p*-nitrophenyl acetate
(b): structure composed of His-57 anion, Ser-195 and *m*-acetylphenyl acetate

TABLE III. Total Energies of the Structures under the Conditions Uncoupling the Substituent Effects with the Proton Transfer

Structure	Total energies		Activation energy (eV)
	Initial (eV)	Transition (eV)	
Structure with *p*-nitrophenyl acetate	−6061.15	−6059.23	1.92
Structure with *m*-acetylphenyl acetate	−5688.37	−5686.40	1.97

First we will consider the acyl-enzyme intermediate structures in which the ionized C–O⁻ bond of Ser-195 is rotated by 120 degrees prior to attack on the carbonyl carbons of the substrates, *p*-nitrophenyl and *m*-acetylphenyl acetates. The coordinates of Ser-195, His-57 anion and the substrate obtained from the X-ray diffraction data were described in the previous paper.[2] The carbonyl groups of *p*-nitrophenyl acetate and *m*-acetylphenyl acetate were positioned in the same place as in the model substrate, methyl acetate (Fig. 2). The coordinates of *p*-nitrophenyl acetate and *m*-acetylphenyl acetate are shown in Table I(b). Under these conditions the calculations of the proton transfer from Ser-195 to His-57 anion were carried out on the structures containing *p*-nitrophenyl acetate and *m*-acetylphenyl acetate in place of methyl acetate. The total energy of the initial and transition structures containing *p*-nitrophenyl acetate and *m*-acetylphenyl acetate is given in Table III. The transition state structures reflect a 0.64 Å displacement of the Ser-195 proton from its initial position in the direction of the His-57 anion. These calculations yield activation energies of 1.92 eV (44.3 kcal/mole) and 1.97 eV (45.4 kcal/mol) for the complexes containing *p*-nitrophenyl acetate and *m*-acetylphenyl acetate respectively. Experimental activation energies (ΔH^{*}) determined from deacylation reactions similar to the acylation reactions considered here are 10.3 kcal/mole and 12.0 kcal/mole for L-tyrosyl-α-chymotrypsin and N-acetyl-L-tryptophanyl-α-chymotrypsin, respectively.[9] The calculated results are seen to be about four times as large as experimental values.

9) M.L. Bender, F.J. Kezdy, and C.R. Gunter, *J. Amer. Chem. Soc.*, **86**, 3714 (1964).

Using this manner as a scaling factor one expects that the difference in activation energy for the two systems is approximately 0.01 eV (0.05 eV/4). This scaled value for the difference between proton transfer from Ser-195 to His-57, one is interested only in the activation energies, 0.01 eV, is within the thermal energy, 0.026 eV, at a temperature of 298° Kelvin. Under such uncoupling conditions, therefore, the experimental difference of the activation energies of the proton transfers from Ser-195 to His-57 anion is not satisfactorily explained.

We next consider the transition state structures, speculated from the biochemical experiments, in which the C–O$^-$ bond of Ser-195 does not rotate, but rather attacks the carbonyl carbon of the substrate immediately following the proton transfer from Ser-195 to His-57. In this case the positions of the substrates, *p*-nitrophenyl acetate and *m*-acetylphenyl acetate, were displaced a little to satisfy the conditions described earlier. The coordinates of *p*-nitrophenyl acetate and *m*-acetylphenyl acetate are shown in Table I(b). The structure composed of His-57 anion, Ser-195 and either *p*-nitrophenyl acetate or *m*-acetylphenyl acetate are shown in Fig. 3 (a) and Fig. 3 (b), respectively. In treating organic chemical reactions, the distance between the substrate and the reagent in the transition state is conventionally taken as 1.5 times that in the covalent-bond state. On this basis we tentatively assumed a value of 2.0 Å for the distance between the O$^\gamma$ of Ser-195 and the carbonyl carbon of the substrate. As before we have assumed that there is a 0.64 Å displacement of the Ser-195 proton in going from the free enzyme to the transition state. Using those configulation the total energy of initial and transition state structures were calculated. The results are given in Table IV. These calculations yield activation energies of 1.18 eV (27.2 kcal/mole) and 1.30 eV (30.0 kcal/mole) for the

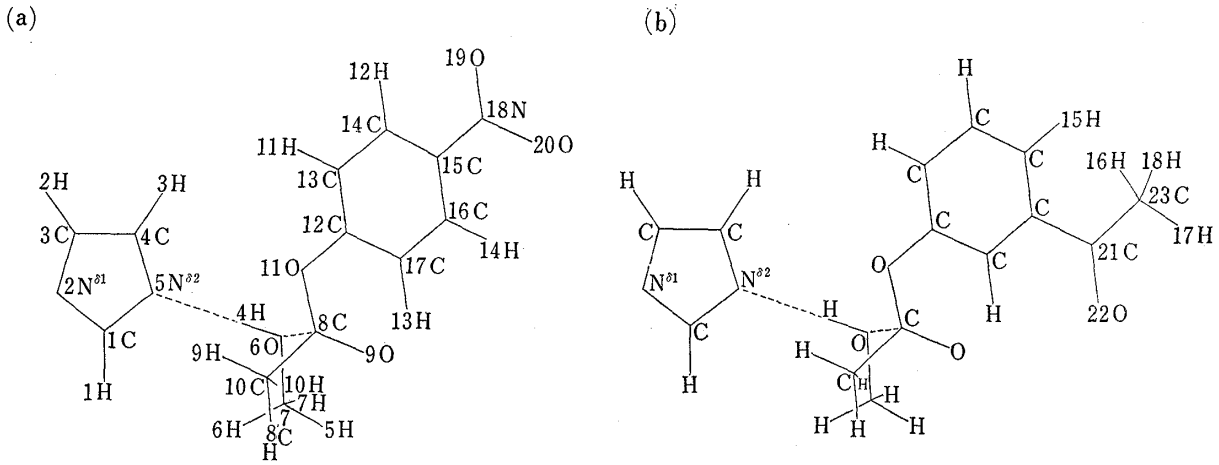

Fig. 3. Structures under the Conditions Coupling the Substituent Effects with the Proton Transfer

(a): structure composed of His-57 anion, Ser-195 and *p*-nitrophenyl acetate
(b): structure composed of His-57 anion, Ser-195 and *m*-acetylphenyl acetate

TABLE IV. Total Energies of the Structures under the Conditions Coupling the Substituent Effects with the Proton Transfer

Distance (Å)	Substituent contained	Total energies	
		Initial (eV)	Transition (eV)
1.8	*p*-NO_2	−6062.48	−6061.85
	m-$COCH_3$	−5689.67	−5688.91
2.0	*p*-NO_2	−6061.99	−6061.81
	m-$COCH_3$	−5689.17	−5687.87
2.2	*p*-NO_2	−6061.57	−6060.04
	m-$COCH_3$	−5688.78	−5687.19

TABLE V. Activation Energies and Differences between Them of the Structures Containing *p*-Nitrophenyl and *m*-Acetylphenyl Acetates at the Distances 1.8, 2.0, 2.2Å and ∞, between O^r of Ser-195 and the carbonyl carbon of the Substrate. Acceleration Energies of the Proton Transfer from Ser-195 to His-57 due to the Approach of the Substrate.

Distance (Å)	Substituent contained	Activation energies (eV)	Difference between activation energies (eV)	Acceleration energies of the proton transfer (eV)
1.8	*p*-NO_2	0.63	0.13	1.26
	m-$COCH_3$	0.76		1.13
2.0	*p*-NO_2	1.18	0.12	0.71
	m-$COCH_3$	1.30		0.59
2.2	*p*-NO_2	1.53	0.06	0.37
	m-$COCH_3$	1.59		0.30
∞	*p*-NO_2	1.89		
	m-$COCH_3$	1.89		

formation of the intermediate complexes containing *p*-nitrophenyl acetate and *m*-acetylphenyl acetate respectively (Table V). The rate of acylation with *p*-nitrophenyl acetate as the substrate. is, therefore, energetically more favorable than the corresponding reaction where *m*-acetylphenyl acetate is the subtrate.

Identical calculations to those described above were performed where the distance between the O^r of Ser-195 and the carbonyl carbon of the substrate was taken to be 1.8 Å or 2.2 Å. At the distance, 1.8 Å, the activation energy of the structures containing *p*-nitrophenyl acetate and *m*-acetylphenyl acetate are respectively 0.63 eV (14.5 kcal/mole) and 0.76 eV (17.5 kcal/mole). At the distance, 2.2 Å, they were 1.53 eV (35.3 kcal/mole) and 1.59 eV (36.7 kcal/mole), respectively. The acylation of the structures containing *p*-nitrophenyl acetates at the distances, 1.8 Å and 2.2 Å, energetically favored over the acylation containing *m*-acetylphenyl acetate by 0.13 eV (3.0 kcal/mole) and 0.06 eV (1.4 kcal/mole) respectively. The qualitative conclusions obtained at 2.0 Å seem to be reliable over a range of distances between the O^r of the Ser-195 and the carbonyl carbon of the substrate.

The ratio of the rate constants for the structure containing a *p*-nitrophenyl group and that containing a *m*-acetylphenyl group was determined by Bender and Nakamura[5] to be 1.22×10^2. The corresponding ratio may be determined theoretically by letting the difference in activation energies of the *p*-nitrophenyl acetate and the *m*-acetylphenyl acetate represent the V^* in the equation given below

$$V_{p\text{-}NO_2}/V_{m\text{-}COCH_3} = \exp(V^*/kT).$$

The theoretical ratios were 1.48×10^2, 9.95×10 and 9.97 at the distances, 1.8 Å, 2.0 Å, and 2.2 Å, respectively. The theoretical ratio at the distance, 1.8 Å, is in good agreement with the experimental ratio, 1.22×10^2.

Activation enthalpies ($\Delta H^{\neq}$), as determined by Bender, *et al.*[9] from the reactions of deacylations, were 10.3 and 12.0 kcal/mole for acyl-α-chymotrypsins.[9] Considering these experimental results the activation energies we calculated at 1.8 Å, 14.5 kcal/mole and 17.5 kcal/mole seem to be reasonable values.

On the basis of this evidence we conclude that structures coupling the substituent effects with the effects of the proton transfers are capable of explaining the biochemical experiments. Calculations on the energetics of the proton transfer are shown in Table V. The potential barrier without substrate was 1.89 eV. At the distance, 1.8 Å, the values of acceleration of the proton transfer in question were 1.26 eV (1.89 eV—0.63 eV) and 1.13 eV (1.89 eV—0.76 eV) for hydrolysis of *p*-nitrophenyl acetate and *m*-acetylphenyl acetate, respectively. Accordingly, it was pointed out that, after the interaction between the aromatic part of a actual substrate

and the pocket part of α-chymotrypsin, the approach of the carbonyl carbon of the substrate to O^{γ} of Ser-195 of α-chymotrypsin made it very easy to transfer the proton of Ser-195.

Despite the apparent successes of this last structure, there still remains an alternate structure which needs to be considered. This structure simultaneously explains the rotation of 120 degrees of Ser-195, the substituent effects, and the deuterium effects in the transition state of hydrolysis of the ester substrate. The transition state is thought to involve in the following manner. The oxygen of Ser-195 is rotated by 120 degrees about C^{α}–C^{β} bond, such that its distance to the carbonyl carbon of the substrate is approximately 2.0 Å. As the oxygen of Ser-195 rotates, its hydrogen atom gradually migrates to the $N^{\delta 2}$ of His-57. This shift occurrs in such a manner as to always maintain the linearity of the hydrogen bond with the oxygen of Ser-195. The exact positioning of the hydrogen is determined by the requirement that it must always be at a point of minimum potential energy. But, from the X-ray diffraction data of Henderson[3d] it is known that in the process of acylation the imidazole of His-57 moves by 0.3 Å outwards towards the solvent region, and hydrogen-bonds a water which hydrogen-bonds with the carbonyl oxygen of the substrate, without hydrogen-bonding with the oxygen of Ser-195; that is the imidazole of His-57 moves to the successful and essential place in order to draw the hydrogen of the active water in the process of deacylation without hydrogen-bonding with the oxygen of acyl-Ser-195. Accordingly, in this report we did not consider the structure which simultaneously explained the rotation of 120 degrees of Ser-195, the substituent effects and the deuterium effects in the transition state.

On the basis of our results, the acylation step is thought to be as follows: (1) the carbonyl carbon of the substrate approaches to the oxygen of Ser-195, which hydrogen-bonds with $N^{\delta 2}$ of His-57; (2) after the proton of Ser-195 is transferred to $N^{\delta 2}$ of His-57 by the trigger of the interaction between the oxygen of Ser-195 and the carbonyl carbon of the substrate, the oxygen of Ser-195 covalent-bonds with the carbonyl carbon of the substrate; (3) acyl-Ser-195 rotates by 120 degrees about the serine C^{α}—C^{β} bond. The rotation of acyl-Ser-195 must be accompanied with the deeper movement of the aromatic part of the substrate in the pocket of α-chymotrypsin. Evidence of this movement might actually exist in the following X-ray diffraction data:[3d] the indole group of the indoleacryloyl-chymotrypsin which has the structure composed of the hydrogen bond system between Asp-102 and His-57 and the rotating acyl-Ser-195 lies about 0.5 to 1.0 Å deeper into the pocket than the corresponding indole group of N-formyl-L-tryptophan in the active site of α-chymotrypsin, which has the "charge relay system" composed of Asp-102, His-57 and Ser-195; in addition there are small movements of 1.0 Å of S^{γ} (Met-192) and 0.35 Å of the disulphide of Cys—191—220 by the rotation of Ser-195, the residues of which are some parts of the pocket in α-chymotrypsin, and above-mentioned movements reflect small movements (0.2 to 0.4 Å) of the polypeptide backbone in this pocket of α-chymotrypsin. The reaction path that, after the oxygen of Ser-195 hydrogen-bonding with $N^{\delta 2}$ of His-57 reacted with the carbonyl carbon of the substrate, the oxygen of acylating Ser-195 rotates by 120 degrees about the C^{α}—C^{β} bond is good agreement with the discussion byHenderson[3d] that movements of some parts of the pocket in α-chymotrypsin as above-mentioned appear to occur as a result of acylation since in the tosyl-enzyme very similar movements occur. On the other hand, Henderson[3d] discussed on the mechanism for acylation that, during acylation, the rotation of 120 degrees about the C^{α}–C^{β} bond of Ser-195 moves the oxygen atom almost directly towards the carbonyl carbon of the substrate. Against the discussion, we reported that, after the oxygen of Ser-195 covalent-bonded with the carbonyl carbon of the substrate, the acyl-Ser-195 rotated by 120 degrees about the C^{α}–C^{β} bond of Ser-195.

Effects of Hydrogen Bonds of the Backbone-NH-groups

In order to consider the effects of the hydrogen bonds on the backbone-NH-groups of Gly-193 and Ser-195 on the enzymatic reaction,[3b] calculations were carried out on the structures containing two hydrogen bonds between the backbone-NH-groups and the carbonyl oxygen

of the substrate.[10] For the purpose of the simplicity of calculations, methanol and methylacetate was used in place of Ser-195 and the substrate. Fig. 4 shows the structure composed of His-57 anion, Ser-195, the substrate (methylacetate) and the backbone-NH-groups (two ammonia). The total energy of the structure, composed of His-57 anion, Ser-195 and the substrate (methylacetate), without and with the backbone-NH-groups is given in Table VI. The activation energies were 1.30 eV and 1.39 eV with and without the backbone-NH-groups, respectively. Hence, inclusion of the hydrogen-bonding to the backbone lowers the energy requirement of the reaction by 0.09 eV. In comparison the approach of the substrate (methylacetate) lowers the activation energy of the proton transfer by 0.59 eV.[11] It therefore appears that the hydrogen bonds between the substrate and the backbone-NH-groups do not play as large a role in facilitating the proton transfer from Ser-195 to His-57 as does the approach of the substrate to Ser-195.

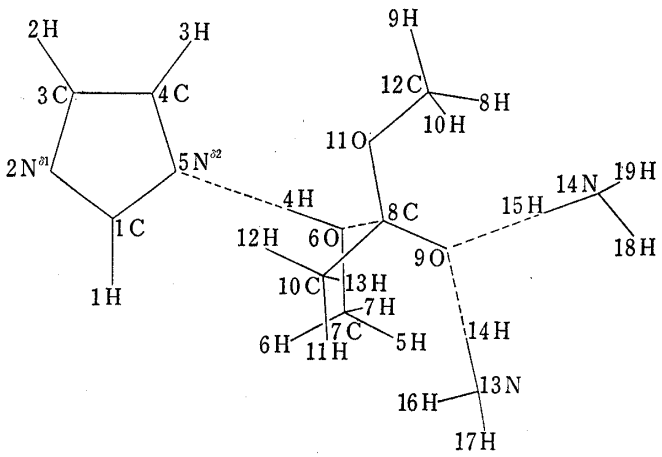

Fig. 4. Structure composed of His-57 anion, Ser-195, the Substrate (Methylacetate) and the Backbone-NH-groups

TABLE VI. Total Energies of the Structures used in the Calculations of the Effects of Hydrogen Bonds of the Backbone-NH-groups and Activation Energies

Structure	Total energies		Activation energy (eV)
	Initial (eV)	Transition (eV)	
Structure without –NH–groups	−3754.46	−3753.07	1.39
Structure with –NH–groups	−4509.95	−4508.65	1.30

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10) In this previous section, it was noted that the relative values of the activation energies for the substrates at the distance, 2.0Å, are concordant with those of the activation energies at the distance, 1.8Å. It therefore seems reasonable in the present case to also use the distance, 2.0Å, between O^{γ} of Ser-195 and the carbonyl carbon of the substrate for the purpose of the discussion of relative values.

11) The values, 0.59 eV, was obtained as the difference between 1.89 eV and 1.30 eV (the value, 1.89 eV, was shown in Table V).