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Polymer Effects under Pressure. IV. The Hydrolysis of Phenyl Esters Catalyzed by α-Chymotrypsin up to 2000 bar[1)]

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The rates of the hydrolysis of 2-valeryloxy(C_5)- and 2-heptanoyloxy(C_7)-benzoic acids catalyzed by α-chymotrypsin (α-CHT) were measured at pressures up to 2000 bar at 30 °C and pH 7.8 in a 0.05 M Tris buffer solution. The apparent Michaelis constant, K_m^{app}, was estimated to vary from 5.9 to 9.9 mM, and k_{cat} from 11×10^{-3} to 52×10^{-3} s^{-1}, for the C_5 ester, and K_m^{app}, from 4.7 to 10 mM, and k_{cat} from 37×10^{-3} to 200×10^{-3} s^{-1}, for the C_7 ester, between 1 and 2000 bar. From the pressure dependences of K_m^{app} and k_{cat}, the volume changes accompanying the dissociation of the enzyme–substrate complex and the activation volume for the process of the product formation were calculated to be -6.3 ± 2 and -20 ± 2 cm^3/mol for the C_5 ester and -9.5 ± 2 and -21 ± 2 cm^3/mol for the C_7 ester. The effects of the pressure on the hydrolysis of *p*-nitrophenyl acetate (PNPA) catalyzed by α-CHT have also been measured up to 3000 bar at pH 7.8 in a 0.05 M Tris buffer solution and at 25 °C and 35 °C. The activation volumes were -3 cm^3/mol at 25 °C and -4 cm^3/mol at 30 °C and 25 °C. These results were discussed on the basis of the X-ray study of the enzyme–substrate complex.

Werbin and McLaren[2)] have studied the effect of the pressure on the rate of the α-chymotrypsin-catalyzed hydrolysis of casein and of the L-tyrosine ethyl ester over a limited range of substrate concentrations. The activation volume of the former was -13.8 cm^3/mol for the apparent first-order rate constant, while that of the latter was -13.5 cm^3/mol for the zeroth-order rate constant. Recently Lockyer *et al.*[3)] have studied the pseudo-first-order rate of the hydrolysis of *p*-nitrophenyl esters. The activation volumes for the deacylation of the corresponding acyl-enzymes, were -6, -3, and -2 cm^3/mol for the *p*-nitrophenyl esters of acetic, isobutyric, and pivalic acid respectively. The relationship between these negative volume changes and the mechanism of the enzyme reaction was not clarified in detail. While there was no loss of activity of α-CHT in the pH 7.8 Tris buffer solution after pressure treatment for 1 h at 2000 bar and at 22 °C (this result was also supported by Miyagawa and Suzuki[4)]), it is not clear whether or not α-CHT undergoes reversible inactivation.

In the present study, the enzyme activity was first measured in the hydrolysis of PNPA catalyzed by α-CHT up to 3000 bar at three temperatures. The hydrolysis rate of 2-valeryloxy(C_5)- and 2-heptanoyloxy-(C_7)-benzoic acids catalyzed by α-CHT was measured up to 2000 bar at 30 °C. The results were compared with those of PNPA in order to discuss the reaction mechanism of the α-CHT-catalyzed hydrolysis.

Experimental

Materials. The 2-valeryloxy(C_5)- and 2-heptanolyoxy-(C_7)-benzoic acids were obtained by the method of Hofstee.[5)] The products, recrystallized five times from ethanol, were freed from unchanged 2-hydroxybenzoic acid, as checked by the absorption at 300 nm of 2-hydroxybenzoic acid. C_5 ester: mp 84—85 °C (lit,[5)] mp 86 °C); MS (75 eV); Found: *m/e* 222.0888. Calcd for $C_{12}H_{14}O_4$: M, 222.1082. C_7 ester: mp 72.5—73.0 °C (lit,[5)] 70 °C); MS (75 eV); Found: *m/e* 250.1200. Calcd for $C_{14}H_{18}O_4$: M, 250.1398. The PNPA (Aldrich Chemical Co., Inc.) was recrystallized twice from hexane; mp 79—80 °C (lit,[6)] mp 79.5—80 °C). The α-CHT was obtained from the Nutritional Biochemicals Co. (3× crystallized, Lot #6373). The methanol and acetonitrile were distilled before use.

Apparatus and Procedure. The high-pressure apparatus and procedure have been reported in detail in a previous paper.[1a)] The hydrolysis was performed at pH 7.8 in a 0.05 M Tris-HCl buffer up to 3000 bar for PNPA and up to 2000 bar at 30 ± 0.1 °C for 2-acyloxybenzoic acids. The hydrolysis was monitored by observing the change in the optical density at 400 nm due to the *p*-nitrophenolate ion and at 300 nm due to 2-hydroxybenzoic acid by means of a Hitachi-Perkin Elmer EP 139-type spectrophotometer directly under pressure. The sample cell for the measurement of the UV region was made up of a quartz window, and the pressure-transmitting fluid was silicone oil (Shinetsu Chemicals Co.), which was found to be free from absorbance near 300 nm. The enzyme stock solutions were assayed using PNPA before each experiment. The data used from all the enzyme hydrolyses were taken during the first 30 min of each run.

Results

Hydrolysis of C_5 and C_7 Esters. These esters are hydrolyzed spontaneously in the absence of the enzyme. The rate of the spontaneous hydrolysis was proportional to the ester concentration. Therefore, the rate of hydrolysis in the presence of the enzyme, V_{app}, is separated into two terms, the uncatalyzed rate, V_w, and the enzyme-catalyzed rate, V_{cat}:

$$V_{app} = V_{cat} + V_w. \quad (1)$$

At each pressure, the hydrolysis rates, V_{cat}, for the enzyme reaction obtained by means of Eq. 1 increase with the substrate concentration, then level off at higher concentrations, thus following the Michaelis-Menten kinetics as is shown in Eq 2:

$$E + S \underset{K_m^{app}}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P, \quad (2)$$

where E is the enzyme; S, the substrate; ES, the Michaelis complex, and P, the product. When $[E]\ll[S]$, the rate of the product formation is expressed as:

$$V_{cat} = \frac{k_{cat}\cdot[E]\cdot[S]}{K_m^{app}+[S]}, \quad (3)$$

where [E] and [S] denote the enzyme and substrate

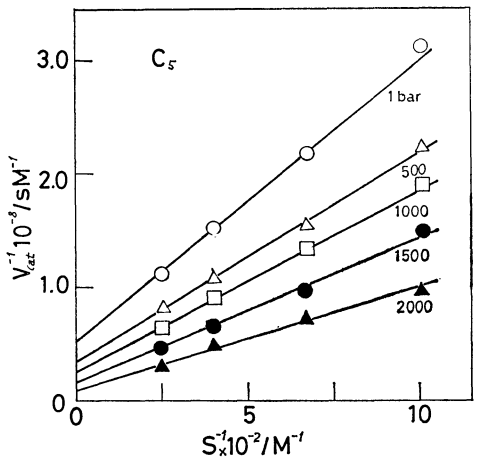

Fig. 1. Lineweaver-Burk plots of the enzyme hydrolysis of C_5 ester at pH 7.8 (0.05 M Tris–HCl) in 9% methanol–water at 30 °C. The enzyme concentration is 2×10^{-6} M.

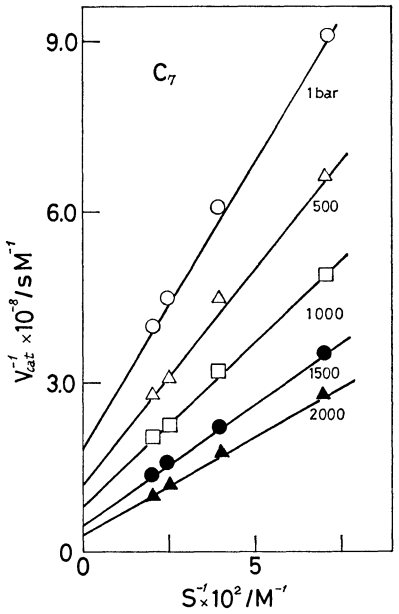

Fig. 2. Lineweaver-Burk plots of the enzyme hydrolysis of C_7 ester at pH 7.8 (0.05 M Tris–HCl) in 9% methanol–water at 30 °C. The enzyme concentration is 2×10^{-6} M.

concentrations. K_m^{app} and k_{cat} were determined by the least-squares method from the Lineweaver-Burk plots between $1/V_{cat}$ and $1/[S]$ in Eq. 4, as is shown in Figs. 1 (C_5) and 2 (C_7):

$$\frac{1}{V_{cat}} = \frac{K_m^{app}}{k_{cat}[E]}\cdot\frac{1}{[S]} + \frac{1}{k_{cat}[E]} \quad . \qquad (4)$$

These values are shown in Table 1. From the linear relationship of the logarithms of K_m^{app} and of k_{cat} *vs.* the pressure established in Figs. 3 and 4, the volume change ΔV accompanying the dissociation of the Michaelis complex and the activation volume, ΔV^*, accompanying the process of the product formation were determined to be -6.3 ± 2 and -20 ± 2 cm³/mol for

Table 1. Kinetic parameters of the hydrolysis of C_5 and C_7 esters catalyzed by α-CHT at various pressures and 30 °C[a)]

Pressure	K_m^{app}/mM		$k_{cat}\times10^3/s^{-1}$	
bar	C_5	C_7	C_5	C_7
1	5.9, 6.1[b)]	4.7, 3.0[b)]	11	37
500	6.7	5.6	16	57
1000	7.4	6.9	23	78
1500	8.4	8.3	35	122
2000	9.9	10	52	200

a) Reaction conditions: pH 7.8 in a 0.05 M Tris buffer solution, 9% methanol; the enzyme concentration, 2×10^{-6} M. b) Ref. 8.

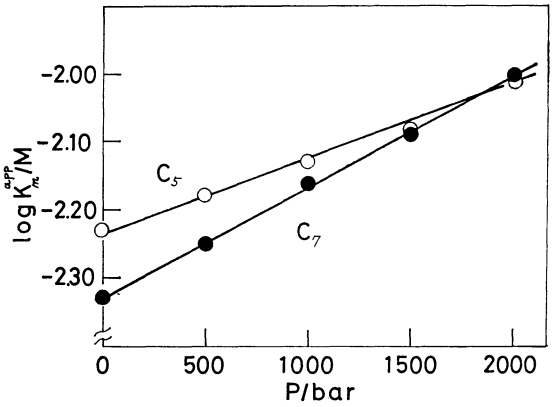

Fig. 3. Pressure *vs.* log K_m^{app} at 30 °C.

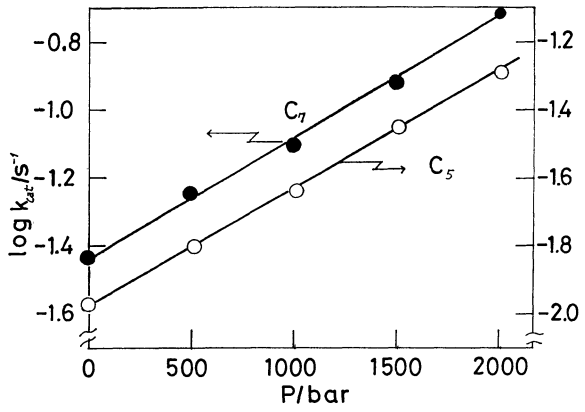

Fig. 4. Pressure *vs.* log k_{cat} at 30 °C.

the C_5 ester -9.5 ± 2 and -21 ± 2 cm³/mol for the C_7 ester respectively.

Hydrolysis of PNPA. PNPA is also hydrolyzed spontaneously in the absence of the enzyme. The rate of the spontaneous hydrolysis is proportional to the PNPA concentration. Therefore, the enzyme rate, V_{cat}, was obtained by means of Eq. 1. When $[E]\ll[S]$ at PNPA concentrations higher than $[S]=1.6\times10^{-5}$ M,[6)] the reaction rate can be considered to be substrate-independent and to be proportional to the enzyme concentration. At each pressure, the rate constants, k_{cat}, were obtained by means of Eq. 5:

$$k_{cat} = V_{cat}/[E]. \qquad (5)$$

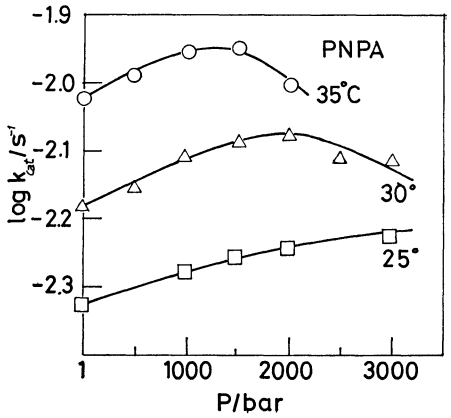

Fig. 5. Pressure effects of log k_{cat} for enzyme hydrolysis of PNPA at pH 7.8 (0.05 M Tris-HCl) in 1.6% acetonitrile–water at 25 °C, 30 °C, and 35 °C, respectively. The enzyme concentration is 2×10^{-6} M, and PNPA concetration is 9.56×10^{-5} M.

The relationship between the logarithm of k_{cat} and the pressure at 30 °C and 35 °C in Fig. 5 shows the maxima at a certain pressure, while at 25 °C it simply increases up to 3000 bar. Lockyer *et al.*[3] reported that the value of log k_{cat} for PNPA increases linearly up to 2000 bar at 20 °C and that the activation volume is −6 cm³/mol. Therefore, phenomena in which the k_{cat} values decrease above a certain pressure (2000 bar at 30 °C and 1500 bar at 35 °C) indicate the loss of the activity of α-CHT under pressure. This interpretation is also directly supported by a recent study of the pressure denaturation based on the measurement of the fluorescence of α-CHT up to 5000 bar.[7] The activation volumes at 1 bar are −3 cm³/mol at 25 °C and −4 cm³/mol at 30 °C and 35 °C respectively.

Discussion

In the hydrolysis catalyzed by α-CHT, the pathway containing acylation and deacylation is shown by Eq. 6:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{acyl}} \mathrm{Acyl\text{-}E} + \mathrm{P_1} \qquad \mathrm{Acyl\text{-}E} \xrightarrow{k_{deacyl}} \mathrm{E} + \mathrm{P_2}. \tag{6}$$

The kinetic parameters can, then, be expressed by:

$$K_m^{app} = K_m \cdot k_{deacyl}/(k_{acyl}+k_{deacyl}), \tag{7}$$

$$k_{cat} = k_{acyl} \cdot k_{deacyl}/(k_{acyl}+k_{deacyl}), \tag{8}$$

where:

$$K_m = (k_{-1}+k_{acyl})/k_1. \tag{9}$$

Then,

$$K_m^{app} = K_m \cdot k_{cat}/k_{acyl}. \tag{10}$$

From the pressure dependences of K_m^{app} and k_{cat}, ΔV and ΔV^* are given by the following equations:

$$\Delta V = \Delta V_m + \Delta V^* - \Delta V^*_{acyl}, \tag{11}$$

$$\Delta V^* = \Delta V^*_{acyl} + \Delta V^*_{deacyl} + RT\partial \ln (k_{acyl}+k_{deacyl})/\partial P. \tag{12}$$

From the study of the pH effect on the hydrolysis of 2-acyloxybenzoic acids catalyzed by α-CHT, Hofstee[8] suggested that the rate-determining step for the overall reaction of the rate constant, k_{cat}, is the acylation. The k_{acyl} value is nearly equal to the k_{deacyl} for 2-acyloxybenzoic acids catalyzed by α-CHT because the k_{cat} values for C_5 and C_7 esters, as shown in Table 1, are approximately half of the k_{deacyl} values: 16.8×10^{-3} s^{-1} for the C_5 ester and 69×10^{-3} s^{-1} for the C_7 ester,[9] respectively. The ΔV^* values of −20 cm³/mol for the C_5 ester and −21 cm³/mol for the C_7 ester at 30 °C agree with those of −20 cm³/mol, accompanied by the hydrolysis of 3-nitro-4-butyryloxybenzoic acid (NBBA) and 3-nitro-4-valeryloxybenzoic acid (NPeBA) catalyzed by the copolymer of 1-vinyl-2-methylimidazole with 1-vinylpyrrolidone (MI-VP),[1a] in which the deacylation process is not the rate-determining step, at least. In the hydrolysis of the PNPA ester, in which the deacylation process is the rate-determinig step,[6] the ΔV^* values are −3 cm³/mol at 25 °C and −4 cm³/mol at 30 °C and 35 °C. The difference in the sizes of the activation volume between the two process, acylation and deacylation, is of interest.

The detailed mechanism of the charge-relay system accompanying the acylation and the deacylation of α-CHT was proposed by Blow *et al.*[10] Following the model of the acylation step in Fig. 6, the formation of one hydrogen bond between the NH group of the imidazole ring of His-57 and the carbonyl oxygen atom of the substrate, and one covalent bond between the alkoide anion of Ser-195 and the carbonyl carbon atom of the substrate is expected to be established on the transition state, accompanied by the formation of the tetrahedral intermediate between the substrate and α-CHT. The volume changes accompanying the formation of such bonds are established to be about from −10 to −15 cm³/mol for one covalent bond[11,12] and from −5 to −7 cm³/mol for one hydrogen bond.[13,14] Therefore, the value of −20 cm³/mol accompanying the acylation process, which is independent of the length of the alkyl chain of the substrate, is reasonable.

In the deacylation step shown in Fig. 7, at first there occur the formation of one covalent bond between the oxygen atom of the H_2O molecule and the carbon atom of the carbonyl group, and the rupture of one covalent bond, accompanied by the release of the acyl group from acyl-α-CHT. Secondly, there occur a breaking of the weak interaction (ionic bonds, hydrogen bonds, and hydrophobic interactions) between the acyl group and the side-chain groups of amino acids around the active center of α-CHT. As the volume change, accompanied by the appearance and the disappearance of covalent bonds, can be expected to cancel each other out, the observed activation volumes for each substrate, −13 cm³/mol for L-tyrosyl-,[2] −7 cm³/mol for 3-(3-indolyl)-acryloyl-,[15] −6 to −4 cm³/mol for acetyl[3] −3 cm³/mol for isobutyryl,[3] and −2 cm³/mol for pivaloyl-α-CHT,[3] would come from the latter term. The differences in these volume changes can be discussed from the point of view of the detailed structure of the active center of acyl-α-CHT, as determined in the X-ray studies.

From the structure of 3-(3-indolyl)acryloyl-α-CHT

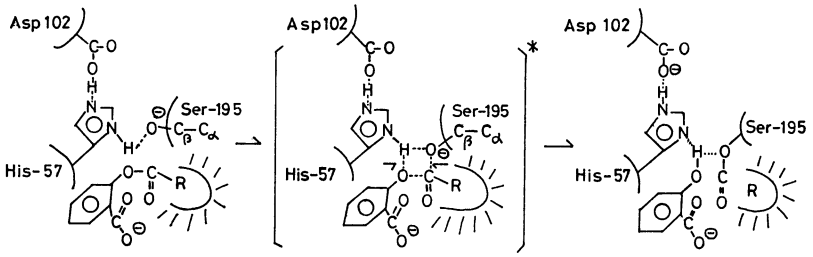

Fig. 6. Modified mechanism for the acylation step of α-CHT proposed by Blow *et al.*, (1969). R is the alkyl chain.

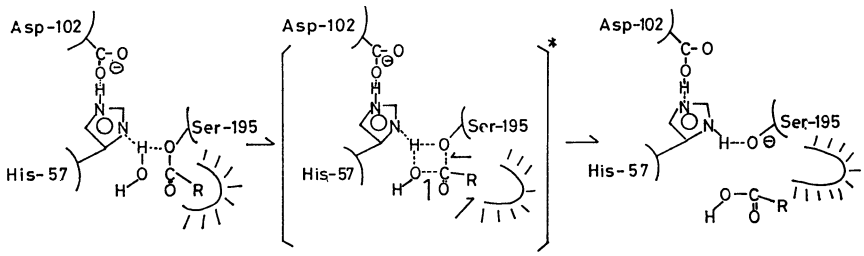

Fig. 7. Modified mechanism for the deacylation step of acyl-α-CHT proposed by Blow *et al.*, (1969). R is the alkyl chain.

determined by Henderson,[16] it is clear that one H_2O molecule acts as a hydrogen bond bridge between the acyl oxygen atom and the NH group of the imidazole ring of His-57 and that the indole ring exists in the hydrophobic cavity. Simultaneously with rupture of the hydrophobic interactions in the deacylation process, the NH group of the indole ring is freed from hydrophobic interaction and can form a hydrogen bond with a H_2O molecule. If a hydrogen bond connected to the carbonyl oxygen atom does not change, the value of -7 cm^3/mol for the 3-(3-indolyl)acryloyl group corresponds to the formation of a hydrogen bond between the NH group of indole and a H_2O molecule, because the rupture of such hydrophobic interaction does not contribute to the volume change; *i.e.*, the volume change accompanied by the formation of hydrophobic interaction containing the benzene ring is nearly zero.[17]

In the deacylation of L-tyrosinyl-α-CHT, the OH group of tyrosine ($pK_a=10$) ionizes when the tyrosine residue is transferred from the hydrophobic cavity of α-CHT to the pH 7.8 buffer solution. The volume change accompanied by the ionization of the tyrosine residue was estimated to be -11.3 cm^3/mol from the study of the pressure effect on the ionization of *p*-nitrophenol.[18] Therefore, the value of -13 cm^3/mol can be explained as being due to the ionization of the tyrosine residue.

The volume changes accompanying the deacylation of acyl (acetyl, isobutyryl, pivaloyl, valeryl, heptanoyl)-α-CHT come mainly from the rupture of the hydrophobic interaction between the alkyl chain of acyl-α-CHT and the hydrophobic cavity of α-CHT. This view is supported by the two facts of a negative volume change accompanied by the rupture of the hydrophobic interaction[19] and of the observed negative values of the experimental activation volume for the deacylation of acyl-α-CHT. Moreover, the observed binding constants of the esters and α-CHT increase with an increase in the length of the alkyl chain of the acyl group up to the size of C_7, which is the optimum hydrocarbon chain.[8] Therefore, it is to be expected that the longer the hydrocarbon chain, the more negative the value of the volume change will be on the rupture of hydrophobic interaction. The expected volume change is -6.3 cm^3/mol for the C_5 ester and -9.5 cm^3/mol for the C_7 ester, judging from a study of the pressure effect on K_m^{app} to be described later. As the value of -20—-21 cm^3/mol accompanying the process of the product formation is too small to explain the rupture of the hydrophobic interaction between the acyl groups of the esters and the hydrophobic pocket of α-CHT, the rate-determining step in this experimental system seems not to be the deacylation step. On the other hand, the amount of the volume change expected from the rupture of hydrophobic interaction between the acetyl, isobutyryl, or pivaloyl group and the hydrophobic pocket of α-CHT is opposite to the experimental results (-6 to -4 cm^3/mol for acetyl, -3 cm^3/mol for isobutyryl, and -2 cm^3/mol for pivaloyl)[3]; *i.e.*, the larger the hydrophobicity of acyl groups, the less negative the value of the activation volume. This fact can be explained in terms of the relationship between the molecule size of acyl groups and the size of the hydrophobic pocket of α-CHT. The X-ray study of Steitz *et al.*[20] determined the size of the pocket to be approximately 1.0—1.2 nm × 0.55—0.65 nm × 0.33—0.45 nm. The van der Waal's radius of the acetyl group is 0.40 nm, while those of isobutyryl and pivaloyl are over 0.5 nm. Therefore, the acetyl group can fit into the pocket, but the bulky groups of isobutyryl and pivaloyl are too large to do so. The ΔV^* values, which are -4 to -6 cm^3/mol for acetyl, -3 cm^3/mol for isobutyryl, and -2

cm^3/mol for pivaloyl groups, are explained by the ratio of the hydrophobic contact of the acyl groups of the esters to the hydrophobic pocket of α-CHT.

When the acylation step is the rate-determining one, the value of K_m^{app} is found by Eq. 7 to be equal to the value of K_m. If k_{-1} were larger than k_{acyl} is Eq. 9, the value of K_m would be the true dissociation constant of the Michaelis complex. The values of -6.3 cm^3/mol for the C_5 ester and -9.5 cm^3/mol for the C_7 ester correspond to the volume change accompanying the dissociation of the Michaelis complex. The amino acids surrounding the hydrophobic pocket in contact with the acyl group through the hydrophobic interaction are Met-195, Val-213, and Try-215. As the contribution of such aromatic compounds as Try-215 to the volume change accompanied by the formation of the hydrophobic interaction is negrigibly small (nearly zero),[17] the average number of the alkyl chain of the side chains of Met-195 and Val-213 is three. Therefore, the estimated volume change for the interaction between the hydrocarbon part of C_3 and the valeryl or heptanoyl groups are in the range of -5 to -10 cm^3/mol, as established by the study of the model compounds.[19] Thus, the driving force of the association of the substrate to the enzyme, α-CHT is shown to be primarily the hydrophobic interaction, as has already been described.

It is concluded that the negative volume change accompanying the dissociation of the Michaelis complex is due primarily to the hydrophobic interaction between the substrates and α-CHT. The value of ΔV^*_{acyl} is explained by the formation of the tetrahedral intermediate in the transition state of the acylation process. These values of ΔV^*_{deacyl} are derived by breaking weak interactions between the acyl groups and side-chain groups of amino acids around the active center of α-CHT, as deduced from the X-ray study of the enzyme-substrate complex.

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