Pressure Dependence of Carboxypeptidase A Action

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The effect of pressure on the peptide hydrolysis by carboxypeptidase A and its inhibition by several substrate-analogs were investigated. The activation volumes for k_{cat} were -4 and -8 ml mol^{-1} for the hydrolysis of 3-(2-furyl)acryloyl-Gly-L-Phe and 3-(2-furyl)acryloyl-L-Phe-L-Phe, respectively. The reaction volumes for the binding process (ΔV_{ass}) for these dipeptide substrates and for the competitive inhibitors of the peptide hydrolysis (peptide-like inhibitors), such as Gly-L-Tyr and L-Phe, were about 30 ml mol^{-1}. In contrast, ΔV_{ass} values for 3-phenyllactate and L-mandelate, both of which are classified as the noncompetitive inhibitors for the peptide hydrolysis (ester-like inhibitors), were comparably small, 5 and 6 ml mol^{-1}, respectively. Furthermore the pressure dependence of the inhibition by D-Phe or D-mandelate was largely different from that of the corresponding L-form; namely ΔV_{ass} for D-Phe was 4 ml mol^{-1} and that for D-mandelate was 21 ml mol^{-1}. The pH dependence of the inhibition constant was classified into two groups; Gly-L-Tyr and L-Phe inhibited more strongly at higher pH while 3-phenyllactate and others did more effectively at lower pH. Taking into account the previously published result of the X-ray crystallographic studies (*e. g.*, J. A. Hartsuck and W. N. Lipscomb, "The Enzymes," 3rd ed, ed by P. D. Boyer, Academic Press, New York (1971), Vol. 3, p. 1), the present results were accounted for in terms of two different (electrostatic) interactions between the substrate or inhibitor and the active site of the enzyme.

The activation volumes or reaction volumes evaluated from the pressure dependence of chemical reactions often reflect the differences in the reaction mechanisms.[1] These parameters also provide important information on enzyme reaction,[2] as shown by the recent examinations of trypsin[3] or α-chymotrypsin catalysis.[4] By measuring the pressure dependence of enzyme reaction under appropriate experimental conditions, we can obtain the information on volumetric changes occurring in the reaction process, such as those due to changes in charge and bond states at the active site, higher-order structure of protein molecule and the hydration of the enzyme and the substrate. In the present study, we examined pressure dependence of peptide hydrolysis and the binding of several kinds of substrate analogs in bovine carboxypeptidase A (CPA). CPA is a representative zinc-containing metallo-protease, and various kinds of studies, such as primary and three dimensional structures,[5,6] metal substitution,[7,8] chemical modification,[9,10] and cryoenzymolozy,[11] have been carried out to reveal the role of the zinc together with that of other functional amino acid residues in the active site. However, due to the apparent complexities in its catalytic behavior toward peptides and esters or their analogs,[12] the detailed functional roles of these groups and the mode of catalytic functions are still ambiguous. Above all, the multiple binding of the substrate or inhibitor and a difference in the peptidase and esterase actions have prevented the straightforward interpretation of the kinetic data of this enzyme. In order to know much more details of the mechanism, especially to differentiate the mechanisms of the two kinds of enzyme activities, we measured the pressure dependence of kinetic parameters and inhibitory actions for several substrates and inhibitors.

Experimental

Materials. Bovine carboxypeptidase A (Cox) was purchased as an aqueous suspension with toluene preservative from Sigma Chemical Co., (St. Louis, Missouri, USA) (lot 119c8030). It was washed three times with cold deionized water followed by centrifugation and finally dissolved in 0.05 M Hepes buffer containing 1 M NaCl (1 M=1mol dm^{-3}). The enzyme concentration was determined spectrophotometrically at 25 °C, using ε=64200 at 280 nm.[13] 3-(2-Furyl)acryloylglycyl-L-phenylalanine (FuaGlyPhe), FuaGlyLeu and FuaPhePhe were synthesized according to the method in the literature.[14] FuaGlyPhe; mp 184—185 °C, Found; C, 62.93; H, 5.30; N, 8.12%. Calcd for $C_{18}H_{18}N_2O_5$: C, 63.15; H, 5.26; N, 8.18%. FuaGlyLeu; mp 202—204 °C, Found; C, 58.58; H, 6.56; N, 9.04%. Calcd for $C_{15}H_{20}N_2O_5$: C, 58.44; H, 6.49; N, 9.09%. FuaPhePhe; mp 203—205 °C, Found; C, 66.88; H, 5.87; N, 6.22%. Calcd for $C_{25}H_{24}O_5N_2\cdot H_2O$: C, 66.65; H, 5.82; N, 6.22%. Thinlayer chromatography on Kieselgel 60E (E. Merck, Darmstadt, West Germany) using chloroform–methanol–acetic acid (20:1:1) and/or 1-butanol–acetic acid–water–pyridine (15:10:12:3) showed a single UV quenching spot for each substrate. Inhibitors used were as follows ; Gly-L-Tyr, L-Phe, ZPhe (Protein Research Foundation, Osaka), L-2-hydroxy-3-phenylpropionic acid (3-phenyllactate), L-mandelate, D-mandelate (Aldrich, Milwaukee, Wisconsin, USA), 3-phenylpropionate, D-Phe (Wako Pure Chemicals, Osaka), L-Phe–L-Phe (Vega, Tucson, Arizona, USA). Good's buffers ; Mes, Hepes, and Taps were from Dojin Chem. Lab. (Kumamoto).

Methods. The rate of substrate hydrolysis was measured by following the absorbance change at around 340 nm due to the scission of the *C*-terminal peptide bond of Fua substituted amino acid, analogous to the case of thermolysin.[15,16] The difference extinction coefficient at 340 nm was determined as 870 (M^{-1} cm^{-1}) for FuaPhePhe and 830 (M^{-1} cm^{-1}) for FuaGlyPhe. An ultraviolet-visible spectrophotometer with a Drickamer type high pressure optical cell was used as previously described.[3] When the kinetics seem to follow a simple Michaelis-Menten scheme including only a competitive product inhibition, the steady-state rate expression is given by

$$v = -\mathrm{d}[S]/\mathrm{d}t = k_{cat}[E][S]/(K_m(1+([S]_0-[S])/K_p)+[S]), \quad (1)$$

where t is time, $[S]_0$ is the initial substrate concentration, [S] is the substrate concentration at time t, K_p is the product inhibitory constant and k_{cat} and K_m have their usual meanings.

In order to measure the second order rate constant (k_{cat}/

K_m), the substrate concentration is set sufficiently low with respect to both K_m and K_p, and Eq.1 is reduced to

$$v = -d[S]/dt = k_{cat}[E][S]/K_m. \quad (2)$$

In our experimental conditions, 0.03—0.05 mM FuaGlyPhe, Eq.2 is always satisfied (see results). The observed k_{cat}/K_m from the pseudo-first-order conditions (Eq. 2) is about 98% (pH 5—8), 95% (pH8—9), and 93% (pH above 9) confidence level. K_i values were measured under the condition of $[S]_0 \ll K_m$, K_p, where we cannot distinguish competitive and noncompetitive modes and therefore the inhibition constant was obtained from Eq. 3.

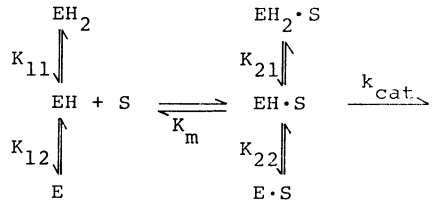
$$1 + [I]/K_i = (k_{cat}/K_m)_{[I]=0}/(k_{cat}/K_m)_{app}. \quad (3)$$

In the study of pH dependence, 0.05 M Hepes, 0.05 M Taps or 0.02 M Mes containing 0.33 M NaCl was used. At such a concentration the buffer dependence of the reaction was not significant.

The pressure dependence of pH of these buffers was examined according to the method of Neuman *et al.*[17] Hepes, Taps and Mes were rather pressure independent as was the case with Tris–HCl. The pH value given in this paper is the corrected one for the pressure dependence. Pressure dependence of K_i was measured in Hepes buffer at 270, 500, 750, and 1000 atm against the hydrolysis of FuaGlyPhe. In the case of L-Phe, FuaGlyLeu was also used as the substrate to avoid the influence of the product inhibition by L-Phe produced from FuaGlyPhe, since the K_p of L-Phe becomes markedly low at pH above 8, as shown in the results section. The change in the concentration of the solute in the solution under high pressure was corrected as previously reported.[3] The nonlinear regressions were carried out following the principle given in the literature.[18]

Results

Kinetic Parameters. First we examined the effect of pressure on the acid-base equilibria of the enzyme active site (Fig. 1) by using pseudo-first-order rate measurement with a substrate of poor K_m values (FuaGlyPhe). The pH dependence of k_{cat}/K_m at 25 °C, 1 atm showed a bell-shaped profile, as was previously reported for the hydrolysis of tripeptide substrate.[19] Under 1000 atm, though the activity was lower by a factor of three than that at 1 atm, a similar simple bell-shaped pH profile was obtained. By increasing pressure, the pK_{a1} shifted toward lower pH (from 6.04 to 5.72) and pK_{a2} changed in the same direction to a less extent (from 9.10 to 8.98). Though, in general, there can be a number of factors which may complicate the pH dependence of the enzyme and several mechanisms which can fit the result obtained, the simplest mechanism to explain these pH dependences is as follows:



Scheme 1. K_{ij} means the proton dissociation constant for each species.

Then the observed two pK_a's are corresponding to the acid-base equilibria of the free enzyme (K_{11}, K_{12}), which shifted to the deprotonated state with an increase in pressure. We can estimate ΔV values for these deprotonation processes from the pK_a shifts; 18±5 ml mol^{-1} for pK_{a1} and 6±3 ml mol^{-1} for pK_{a2}. The following measurement under high pressure, therefore, was carried out at pH around 7 to minimize the influence from these pK_a shifts. FuaGlyPhe showed a substrate activation at high substrate concentration as shown in Fig. 2-a, similar to some other dipeptide

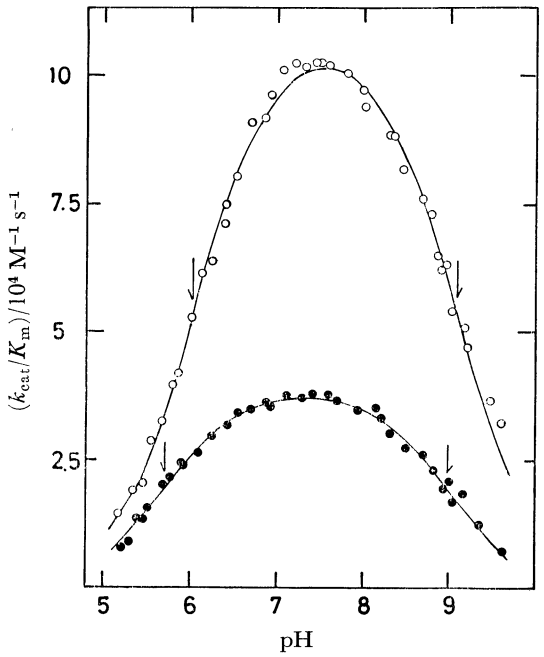


Fig. 1. The pH dependence of k_{cat}/K_m for FuaGlyPhe hydrolysis, (○) 1 atm, (●) 1000 atm. [FuaGlyPhe] = 0.03—0.05 mM, [E] = (1—3) × 10^{-7} M, 25 °C. 0.02 M Mes (pH 5.2—7.2), 0.05 M Hepes (pH 6.5—8.2), 0.05 M Taps (pH 7.5—9.5), NaCl 0.5 M. Arrows indicate the position of pK_a.

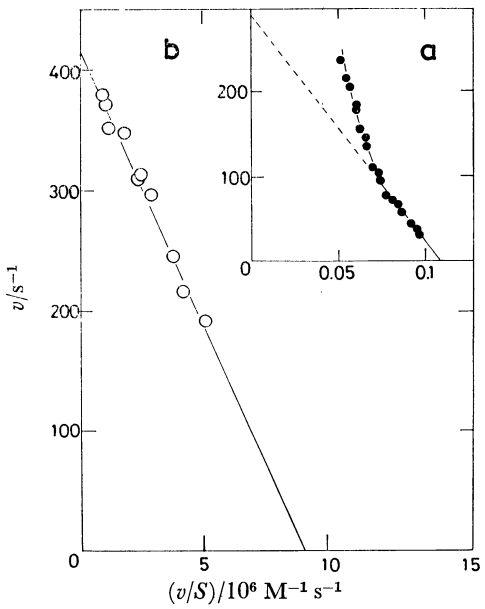


Fig. 2. Eadie plots for hydrolysis of FuaGlyPhe (a), and FuaPhePhe (b). 0.05 M Hepes, 0.33 M NaCl, pH 7.35, 25 °C.

substrates reported in the literature.[20] On the contrary, FuaPhePhe did exhibit neither substrate activation nor substrate inhibition within the concentration range studied here and its reactivity was considerably high (Fig. 2-b).

The hydrolytic rate of Fua–Phe was found to be very slow and its contribution was safely neglected in the interpretation of the observed rate process for FuaPhePhe. Table 1 shows k_{cat}, K_m, and k_{cat}/K_m at different pressures. The logarithms of these parameters were in linear relations with pressure and the activation and reaction volumes given in this paper were calculated at 1 atm.

The temperature dependence of k_{cat} and K_m was also studied for FuaPhePhe between 10 to 35 °C (in 0.05 M Hepes, 0.33 M NaCl pH 7.3). The thermodynamic parameters calculated are; $\Delta H^* = 8.0$ kcal mol^{-1} (1 cal=4.184 J) and $\Delta S^* = -20$ cal deg^{-1} mol^{-1} for k_{cat} and $\Delta H = 1.7$ kcal mol^{-1} and $\Delta S = 25$ cal deg^{-1} mol^{-1} for K_m^{-1}. The ΔH_{a1} value for pK_{a1} obtained from the temperature dependence of the pH profile of k_{cat}/K_m was about 4 kcal mol^{-1} for FuaGlyPhe hydrolysis.

Inhibition Constants. Figure 3 shows the pH dependence of the inhibition constants for various inhibitors. In order to prevent the complex multiple binding of these inhibitors on CPA,[21] the concentration of inhibitors were set below 1.5 fold of K_i over the whole range of pH examined. Under this condition, the [I] *vs.* [I]/K_i plot gave straight lines. As shown in Figs. 3-a and 3-b, K_i of GlyTyr, L-Phe and D-Phe increased as the pH became lower, and for GlyTyr, K_i showed a minimum at pH 8.5. On the contrary, the K_i values of 3-phenyllactate, L-madelate (Fig. 3-c), D-mandelate, 3-phenylpropionate and ZPhe (3-d) increased with pH increase. The profile of the last three were quite similar but the first two showed

TABLE 1. KINETIC PARAMETERS FOR PEPTIDE HYDROLYSIS BY CPA

Pressure atm	For FuaGlyPhe[a]			For FuaPhePhe[b]		
	k_{cat} / s^{-1}	K_m / 10^{-5} M	k_{cat}/K_m / 10^4 M^{-1} s^{-1}	k_{cat} / s^{-1}	K_m / 10^{-5} M	k_{cat}/K_m / 10_4 M^{-1} s^{-1}
1	85±2	52±3	16±1.3	410±10	4.6±0.2	980±60
500	91±4	98±8	9.3±0.8	480±20	8.3±0.5	580±40
1000	100±10	180±20	5.5±0.4	570±35	15±2	390±30
ΔV or $\Delta V^{\neq}$	−4±2	31±2		−8±2	30±3	

a) In 0.1 M Hepes, 0.1 M NaCl, pH 7.35, 25 °C. b) In 0.05 M Hepes, 0.33 M NaCl, pH 7.35, 25 °C.

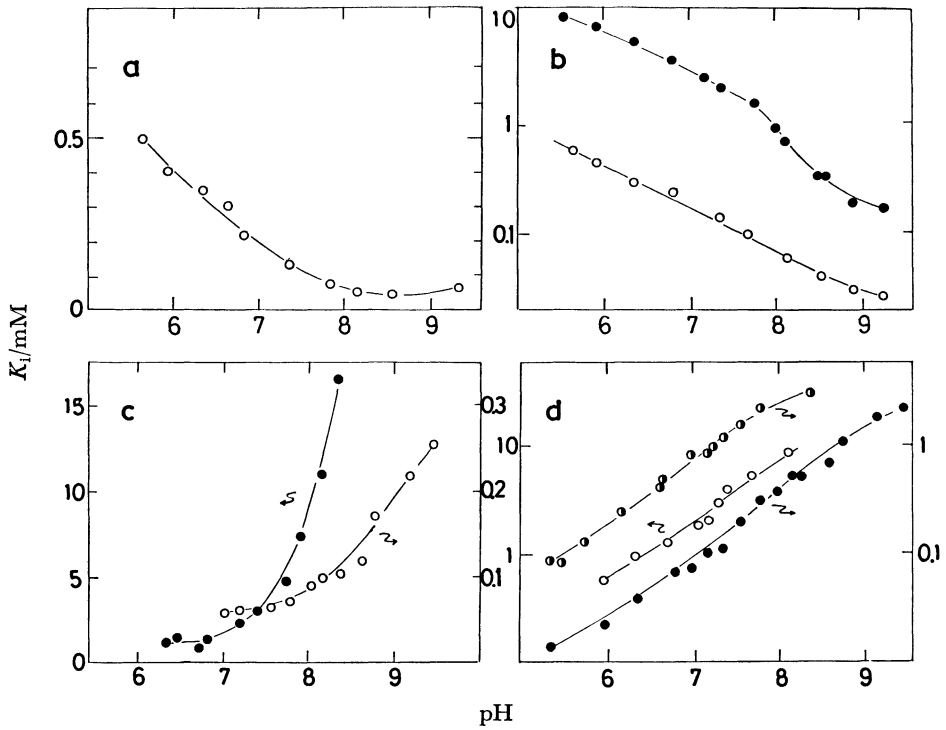


Fig. 3. pH dependence of K_i at 25 °C.
(a) GlyTyr (b) L-Phe (●), D-Phe (○) (c) 3-phenyllactate (○), L-mandelate (●) (d) ZPhe (◑), 3-phenylpropionate (●), D-mandelate (○), 0.02 M Mes, 0.05 M Hepes, or 0.05 M Taps, 0.33 M NaCl.

some characteristic features.

We note that the inhibitors of zwitter-ion type showed K_i decreasing with increasing pH while those having only carboxylate as the ionic residue showed K_i increasing with increasing pH. This classification is similar to that observed in the inhibitor interaction with thermolysin.[16] The fundamental feature of the pH dependence of the K_i of these zinc proteases, therefore, seems to be determined by the relation of the net charges of the inhibitor and the active site of the enzyme in both instances.

The pressure dependence of K_i was measured at pH around 7 (Table 2). Though, for some inhibitors which do not show maxima or minima around this pH, the measured pressure dependences involve influences from the pK_a shifts of the residues which control the binding process of these inhibitors, the logarithm of the observed K_i values were in good linear relations to pressure as shown in Fig. 4. Inhibitors of CPA have been grouped into two; one such as GlyTyr acts competitive towards peptidase activity[20] and the other such as 3-phenylpropionate acts noncompetitive towards peptidase but competitive towards esterase.[7,20] By considering the structure of the inhibitors presently used and the inhibition studies on the related substances,[21,22] we understand that PhePhe, ZPhe and L-Phe fall in the former category (peptide-like inhibitors) while 3-phenyllactate and L-mandelate do in the latter (ester-like inhibitors). For the apparent reaction volumes of the formation process of the complex (ΔV_{ass}) we notice that the former group gave considerably large values, similar to that for the binding of the peptide substrates, FuaGlyPhe and FuaPhePhe, while the latter gave smaller values. We also notice that D-isomers of Phe and mandelate gave ΔV_{ass} values quite in contrast to the corresponding L-isomers.

Table 2. Reaction volumes and inhibition constants of several substrate analogs[a]

Inhibitor	ΔV_{ass}/ml mol^{-1} [b]	$K_i/10^{-4}$ M
GlyTyr	33±2	1.3±0.1
PhePhe	34±5[c]	6.3±0.8
ZPhe	27±1[d]	11±1
L-Phe	23±1	23±2
D-Phe	4.0±0.5	1.4±0.1
3-Phenyllactate	5.1±1.0	0.64±0.03
3-Phenylpropionate	12±2	1.3±0.2
L-Mandelate	5.8±1.5[e]	33±2
D-Mandelate	21±3	13±2

a) Pressure dependence measured at pH 7.3, 0.05 M Hepes, 0.33 M NaCl, 25 °C unless otherwise mentioned. K_i's at pH 7.35, 0.05 M Hepes, 0.33 M NaCl, 25 °C, 1 atm. FuaGlyPhe was used as the substrate except for the case with L-Phe (see experimental). b) Volume change according to the complex formation. c) At pH 7.80. d) At pH 6.60. e) At pH 6.70.

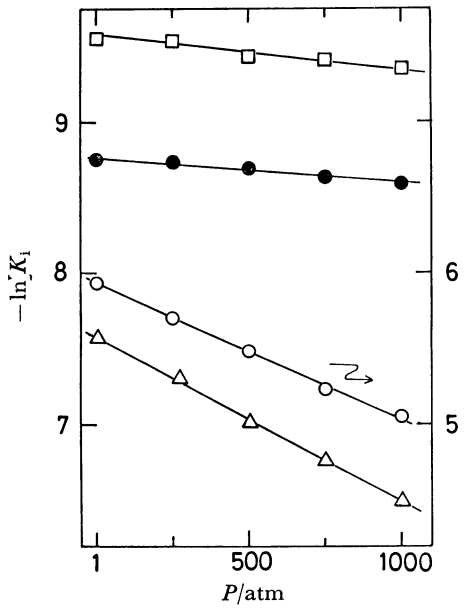


Fig. 4. Pressure dependence of K_i. Hepes 0.05 M, NaCl 0.33 M, pH 7.35 except for ZPhe (pH 6.60), 25 °C. (□) 3-phenyllactate, (●) D-Phe, (○) L-Phe, (△) ZPhe.

Discussion

Assignment of Catalytic Group. From the pH dependences of the kinetic parameters of CPA, there have been arguments on the ionic state of the active site amino acid residues.[22-25] Based on the X-ray crystallographic studies of this enzyme which binds GlyTyr, three interpretations were given so far for the origin of the pK_{a1}: The ionization of the water molecules coordinated on Zn; the dissociation of a Zn–His bond followed by pH effects associated with His 69 or His 196; the interactions of the charged residue(s) (probably Glu 270) in the active site.[6] The measured ΔH_{a1} for the pK_{a1} process (4 kcal mol^{-1}) is comparable to the corresponding values (≈3 kcal mol^{-1}) reported for the hydrolysis of ZGlyGlyVal and ZGlyGlyLeu.[25] These values are intermediate between those reported for a free carboxyl (±1 kcal mol^{-1}) and a free imidazole or a water coordinated on zinc (6—7 kcal mol^{-1})[26-28] The presently obtained ΔV_{a1} value is apparently comparable to the ΔV value reported for a free carboxyl group (≈15 ml mol^{-1}).[27] Thus the ΔH_{a1} and ΔV_{a1} might give different interpretations. In general, ΔV has strong correlation with ΔS for various reactions in solution[2] and hence the problem is equivalent to telling which of the enthalpic and entropic factors plays a dominant part to determine the unusual (perturbed) pK_a values in the active site of the enzyme. The accumulated data on the thermodynamic measurement of the pK_a's of various acids in mixed solvents[28] tell us that, by changing the solvent composition, the entropic term varies significantly and the enthalpic one does partly compensate this change. This allows us to say that, at least in the case of pK_a, the enthalpic parameter is less influenced by the perturbation of the environments and it is better to assume the nature of the dissociative residues on the basis of this term. Then the value of ΔH_{a1} (3—4 kcal mol^{-1}) is considered to reflect the contributions from both carboxyl group and the H_2O on the zinc. In the hydrolysis by ther-

molysin, which is a microbial zinc protease similar to CPA with respect to the amino acid components of the active site and showed ΔH_{a1} of $\simeq 8$ kcal,[16] there were two possible functional groups which dominated the pK_{a1}; the imidazol of His231 and the proton-sharing combination of the carboxylate of Glu and the zinc-coordinated water. Taking the absence of histidine residue in the active site of CPA into consideration, we can consider the latter is the case for the group controlling the pK_{a1} in the present enzyme.

Two Binding Modes of Inhibitors and Substrates. The X-ray crystallographic studies suggested that Arg-145, Glu270, Tyr248, and zinc ion at the active site are important in the CPA catalysis.[12] There have been many studies concerning the roles of these residues in the peptidase and esterase activities. A mechanistic difference between these two types of catalyses was inferred in most of the studies; *e.g.* different influence of metal substitution or metal extraction on the binding and/or catalytic processes of two types of the substrates[30] and a different influence of the chemical modification of the Arg residue.[9] One interpretation of these results was that the carboxyl group of the ester substrates interacts with zinc ion in the binding process while the carbonyl group of the peptide substrate does so during the hydrolytic process.[8] That is, in the binding process of peptide substrate, the Arg145 residue is considered to interact electrostatically with the terminal carboxylate. Such a purely electrostatic interaction is similar to the one observed in the substrate binding process of trypsin and its homologous proteases. Judging from the result on the pressure dependence of trypsin catalysis[3] and of other low molecular weight ionic associations[29] a fairly large and positive volume change is expected for such an association. In fact the pressure dependence of K_m^{-1} gave a large and positive ΔV_{ass} ($+30$ ml mol^{-1}) and the ΔV_{ass}'s for the peptide-like inhibitors such as GlyTyr, PhePhe, L-Phe, and ZPhe were also positive and large. In contrast, fairly small ΔV_{ass} values were observed for the binding of ester-like inhibitors such as 3-phenyllactate and L-mandelate. These smaller ΔV_{ass} values, then, would be related to the interaction of terminal carboxylate directly with the zinc ion instead of the guanidinium cation of Arg145. (Though these ester-like inhibitors behave noncompetitive to the peptidase activity, their competitive actions on ester one[22,23] and other physicochemical studies[6,30,31] comfirmed direct interaction of these inhibitors with the active site of enzyme.) The interaction of divalent metal ion with various carboxylic acids in aqueous solution has shown relatively small ΔV values (<10 ml mol^{-1}) as known in the literature.[29] Thus the present result gives strong support for the interpretation of Auld and Holmquist.[7] However it cannot exclude the possibility that such a direct coordination of terminal carboxylate to the zinc ion is limited to the case of ester-like *inhibitor* interacting with the active site where the peptide *substrate* is already located on the Arg residue and that the ester *substrate* might be bound in a similar manner to the peptide substrate.[32,33]

D-Phe showed a considerably different ΔV_{ass} value from L-form and this can be interpreted by the scheme

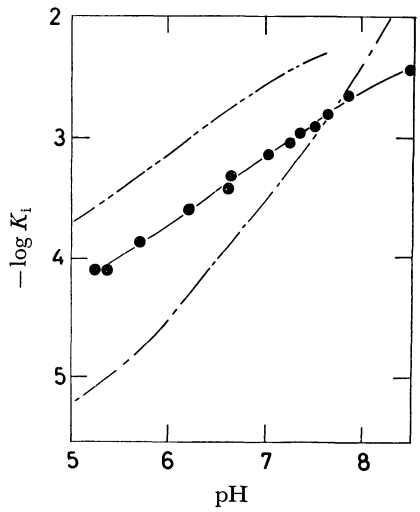


Fig. 5. Comparison of pH dependences of inhibitions by ZPhe for thermolysin and CPA.
●: Inhibition of CPA by ZPhe (taken from Fig. 3-d), ——: inhibition of thermolysin by ZPhe (taken from Ref. 16), – – – –: inhibition of thermolysin by ZLeuGly (taken from Ref. 16).

given by Lipscomb[34] that the terminal carboxylate of D-Phe does not interact with Arg but directly to zinc ion, just like the ester-type inhibitor. In fact D-Phe gave a ΔV_{ass} value which can be classfied into the latter. On the contrary D-form of mandelate showed a larger ΔV_{ass} value than L-form and in this case the D-isomer would interact with Arg residue just like peptide-like inhibitors.

pH-Dependent Alternation of Binding Modes. In the inhibition of thermolysin, ZPhe showed a very strong pH dependence and it was related to the direct interaction of terminal carboxylate of this inhibitor with zinc ion at a relatively low pH.[16,35] The inhibition of CPA by this inhibitor is less pH dependent as compared in Fig. 5 but it resembles the binding of the dipeptidyl inhibitor (ZLeuGly)[16] or tripeptidyl substrate (Fua-GlyLeuAla) (Fukuda *et al.*, unpublished result) on thermolysin. In the latter instances the pH profile can be interpreted as that the inhibitor or substrate interacts with the active site in a productive (normal) manner at neutral pH, while at lower pH it interacts in a nonproductive (reversed) manner just like ZPhe interaction with carboxylate coordinating on zinc. The situation seems analogous in the case of ZPhe interaction with CPA. At pH 7, where the pressure dependence was measured, the interaction of this inhibitor is normal and similar to the peptide substrate but at lower pH it would undergo a direct interaction with zinc ion and would have a character of ester-like inhibitor. D-Mandelate and 3-phenylpropionate showed fundamentally similar pH dependences to ZPhe and therefore a similar explanation can be valid for these inhibitors. Especially in the case of 3-phenylpropionate, we notice a ΔV_{ass} value of an intermediate between two extremes. Though this inhibitor behaved as a noncompetitive one for the peptidase,[7] a stopped-flow study revealed that an analogous inhibitor, phenylacetate, interacted with CPA in a mixed manner.[36]

Thus we can expect that the present intermediate ΔV_{ass} value of 3-phenylpropionate comes from two types of interactions. When the alternation of two binding modes occurs at relatively higher pH, as in these two instances, intermediate values of the parameters are observed even in ordinary measuring conditions. To substantiate this explanation further, we are measuring the pH dependence of ΔV_{ass} for these inhibitors.

References

1) T. Asano, Abstracts of the 23rd High Pressure Conference of Japan, p. 358 (1982).
2) K. J. Laidler and P. S. Bunting, "The Chemical Kinetics of Enzyme Reaction," 2nd ed, Clarendon Press, Oxford (1973), Chap.7.
3) S. Kunugi, M. Fukuda, and N. Ise, *Biochim. Biophys. Acta*, **704**, 107 (1982).
4) a) Y. Taniguchi and K. Suzuki, *Bull. Chem. Soc. Jpn.*, **53**, 1709 (1980); b) S. Makimoto, Y. Taniguchi, and K. Suzuki, Abstract of the 23rd High Pressure Conference of Japan, p. 328 (1982).
5) P. H. Petra and H. Neurath, *Biochemistry*, **10,** 3171 (1971).
6) W. N. Lipscomb, J. A. Hurtsuck, G-N. Reeke, F. A. Quiocho, P. H Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead, and J. C. Coppola, *Brookhaven Symp. Biol.*, **21**, 24 (1968).
7) D. S. Auld and B. Holmquist, *Biochemistry*, **13**, 4355 (1974).
8) H. E. Van Wart and B. L. Valle, *Biochemistry*, **17**, 3385 (1978).
9) J. F. Riordan, *Biochemistry*, **12**, 3915 (1973).
10) J. Suh and E. T. Kaiser, *J. Am. Chem. Soc.*, **98**, 1940 (1976).
11) M. W. Makinen, K. Yamamura, and E. T. Kaiser, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3882 (1976).
12) F. A. Quiocho and W. N. Lipscomb, *Adv. Protein Chem.*, **25**, 1 (1971).
13) R. T. Simpson, J. F. Riordan, and B. L. Vallee, *Biochemistry*, **2**, 616 (1963).
14) S. Blumberg and B. L. Vallee, *Biochemistry*, **14**, 2410 (1975).
15) J. Feder, L. R. Bougham, and B. S. Wildi, *Biochemistry*, **13**, 1186 (1974).
16) S. Kunugi, H. Hirohara, and N. Ise, *Eur. J. Biochem.*, **124**, 157 (1982).
17) R. C. Neuman, Jr., W. Kauzman, and A, Zipp, *J. Phys. Chem.*, **77**, 2687 (1973).
18) D. W. Marquardt, *J. Sos. Ind. Appl. Math.*, **11**, 431 (1963).
19) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 4352 (1970).
20) a) R. C. Davies, J. F. Riordan, D. S. Auld, and B. L. Vallee, *Biochemistry*, **7**, 1090 (1968); b) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 602 (1970).
21) B. L. Vallee, J. F. Riordan, J. L. Coombs, D. S. Auld, and M. Sokolvsky, *Biochemistry*, **7**, 3574 (1968).
22) P. L. Hall, B. L. Kaiser, and E. T. Kaiser, *J. Am. Chem. Soc.*, **91**, 485 (1969).
23) F. W. Carson and E. T. Kaiser, *J. Am. Chem. Soc.*, **88**, 1212, (1966).
24) E. T. Kaiser and B. L. Kaiser, *Acc. Chem. Res.*, **5**, 219 (1972).
25) D. S. Auld and B. L. Vallee, *Biochemistry*, **10**, 2892 (1971).
26) J. T. Edsall and J. C. Wyman, "Biophysical Chemistry," Academic Press, New York (1958), Vol. 5, p. 456.
27) P. Woolley, *Nature (London)*, **258**, 677 (1975).
28) R. M. Izatt and J. J. Christensen, "Handbook of Biochemistry and Molecular Biology," 3rd ed, ed by G. D. Fasman, CRC Press, Cleaveland (1976), Phys. Chem. Data, Vol.1, p.151.
29) T. Asano and W. J. le Nobel, *Chem. Rev.*, **78**, 407 (1978).
30) J. E. Coleman and B. L. Vallee, *Biochemistry*, **3**, 1874 (1964).
31) G. Navon, R. G. Shulman, B. J. Wyluda, and T. Yamane, *J. Mol. Biol.*, **51**, 15 (1970).
32) W. W. Cleland, *Adv. Enzymol. Relat, Areas Mol. Biol.*, **45**, 273 (1977).
33) D. C. Ress and W. N. Lipscomb, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 5455 (1981).
34) W. N. Lipscomb, *Tetrahedron*, **30**, 1725 (1974).
35) W. R. Kester and B. W. Matthews, *Biochemistry*, **16**, 1506 (1977).
36) D. S. Auld, A. S. Latt, and B. L. Vallee, *Biochemistry*, **11**, 4994 (1972).