Pressure Dependence of L-Leucine-*p*-nitroanilide Hydrolysis by Leucine Aminopeptidase

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Synopsis. The effect of pressure on the hydrolysis of L-leucine-*p*-nitroanilide catalyzed by leucine aminopeptidase from pig kidney was investigated. The activation volumes for k_{cat} process were $-8\,cm^3/mol$ and $-9\,cm^3/mol$ for the native enzyme and the modulated one by Mg^{2+}, respectively. The volume changes for the K_m process were $\simeq 0\,cm^3/mol$ for both enzymes.

Aminopeptidase is most widely distributed in the living organisms,[1] but least studied among the several types of proteinases and peptidases. Leucine aminopeptidase (LAP) from pig kidney has a molecular weight of 320000 composed of six identical subunits[2] and contains one Zn^{2+} per subunit at the active site.[3] LAP from various sources requires divalent metal ions for their activities.[4] Recently, Van Wart and Lin purified pig kidney enzyme by affinity chromatography[2] and found that one subunit has an additional metal binding site for activating metal ion such as Mg^{2+} or Mn^{2+} (regulatory site) beside the one occupied by Zn^{2+} (catalytic site).

We have investigated the pressure dependence of catalytic reactions of several proteases[5] and obtained important informations on their mechanisms. In the present study, we applied this technique to the hydrolysis of L-leucine-*p*-nitroanilide (Leu-*p*NA) catalyzed by LAP to investigate its reaction mechanism.

Experimental

LAP from porcine kidney (type III-CP, Lot 111F-8015) was purchased from Sigma Chemical Co. (St. Louis, USA) and purified by affinity chromatography over L-leucylglycyl-AH-Sepharose according to the literature.[2] For native enzyme with Zn^{2+} (represented by [(LAP)Zn_6-]) and Mg-incubated enzyme ([(LAP)Zn_6Mg_6]), metal analysis was carried out by atomic absorption spectroscopy with a Jarrel ash AA 780 instrument. The metal content was 5.6—5.9 mol per mol enzyme for Zn^{2+} and Mg^{2+} on the basis of protein absorbance ($\varepsilon_{280nm}=400000\ M^{-1}cm^{-1}$ (1 M=1 mol dm^{-3})).[2] Leu-*p*NA was obtained from the Protein Research Foundation (Osaka). An ultraviolet-visible spectrophotometer with a high pressure optical cell was used as previously described.[5] The concentration of Leu-*p*NA was determined spectrophotometrically by using $\varepsilon_{320nm}=13900\ (M^{-1}cm^{-1})$ and the initial velocities were calculated from the slope of the absorbance change by using $\varepsilon_{405nm}=9900\ (M^{-1}cm^{-1})$. The extinction coefficient under high pressure was corrected as previously described.[5] The k_{cat} and K_m values were evaluated from Eadie plot. Tris(hydroxymethyl)aminomethane (Tris) buffer was used[1,2,6] and the pressure dependence of its pK_a was corrected.[5a]

Results and Discussion

Figure 1 shows the pH dependence of k_{cat}/K_m for [(LAP)Zn_6Mg_6] at 0.1 MPa ($\simeq$1 atm) and 100 MPa ($\simeq$1000 atm). Under 100 MPa the activity increased

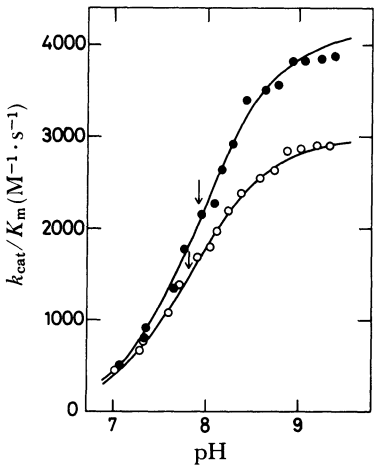

Fig. 1. The pH dependence of k_{cat}/K_m for the hydrolysis of Leu-*p*NA catalyzed by [(LAP)Zn_6Mg_6] at (○) 0.1 MPa and (●) at 100 MPa
$[S]_0<0.05$ mM, $[E]_0=0.018\,\mu M$, 25 °C. 0.02 M Tris-HCl, containing 5 mM $MgCl_2$, 0.5 M KCl and 0.8% (v/v) dimethyl sulfoxide. Arrows indicate the position of pK_a.

1.36 fold for [(LAP)Zn_6Mg_6] and 1.40 fold [(LAP)Zn_6-] at pH around 9. The logarithm of k_{cat}/K_m *vs.* pressure showed a linear correlation for both enzyme forms. The pressure effect was fully reversible in the present pressure range (0.1 MPa—100 MPa) and the observed time scale (less than one hour). These results indicate that there occurs no significant pressure-induced denaturation or dissociation of the hexamer under our experimental conditions,[7] though several oligomeric enzymes were reported to be denaturated or inactivated by medium pressure.[8] The apparent pK_a value was determined for [(LAP)Zn_6Mg_6] by nonlinear regression[5a] and it shifted toward higher pH (pK_a=7.91) under 100 MPa from atmospheric value (7.82). The moderate increase in enzyme activity and the slight pK_a shift to a higher value by pressure increase apparently resemble those of a neutral protease from *Bacillus subtilis*, another type of zinc protease.[5c]

Table 1 shows $\Delta V^{\neq}$ of k_{cat} and ΔV of K_m for [(LAP)-Zn_6Mg_6] and [(LAP)Zn_6-]. Mg^{2+} enhanced the enzyme activity more than 10 fold, which is related not to K_m but k_{cat}.[2] For both forms of the enzyme, k_{cat} increased with increasing pressure, while K_m values were rather pressure independent. Interestingly $\Delta V^{\neq}$ values of the two forms of the enzyme are practically equal. Activation by Mg^{2+} doesn't accompany drastic structural or hydrational change. The reaction mechanisms of LAP was proposed more than twenty years ago[9] and no significant interpretation has been made since then. $\Delta V^{\neq}$ values similar to the present ones were obtained in other zinc protease such as carboxypeptidase

TABLE 1. ACTIVATION VOLUMES AND VOLUME CHANGES FOR Leu-*p*NA HYDROLYSIS AT 0.1 MPa[a)]

Enzyme	k_{cat}	$\Delta V^{\neq}$ for k_{cat}	K_m	ΔV for K_m	k_{cat}/K_m	$\Delta V^{\neq}$ for k_{cat}/K_m
	s^{-1}	cm^3/mol	mM	cm^3/mol	$M^{-1}s^{-1}$	cm^3/mol
[(LAP)Zn_6Mg_6]	1.73	−8±1	0.60	−1±1	2890	−7.6±0.7
[(LAP)Zn_6^-]	0.13	−9±3	0.61	0±3	210	−8.9±1.0

a) Assays are carried out in 0.02 M Tris-HCl buffer containing 0.5 M KCl and 5.6%(v/v) dimethyl sulfoxide, pH 9.0 at 25 °C, except for the presence of 5 mM $MgCl_2$ for [(LAP)Zn_6Mg_6]. [Leu-*p*NA]=0.03—0.57 mM.

A (CPA)[5b)] where the ligating group of the substrate on the active site zinc ion is considered as carbonyl oxygen of the scissile bond. These $\Delta V^{\neq}$'s are to be interpreted by a common mechanism for these metalloproteases.[10)]

The nearly zero ΔV values for K_m show that the substrate binding process does not primarily contain the electrostatic interaction, provided that the apparent K_m value mainly reflects the properties of the substrate binding process, which is consistent with the lack of the evidence of acyl-enzyme intermediate.[11)] This result is also consistent with the facts that LAP favors more hydrophobic side chain in both the amino-terminal and the next residues and that the hydrolysis requirs free amino group.[1,6)] The volume change upon the formation of hydrophobic interaction is considered to be very small.[12)] In this context LAP reaction is in contrast with the reaction of CPA,[5b)] which also favors hydrophobic side chain but requires an ionized (carboxylate) terminal to give a large ΔV value (20—35 cm^3/mol) of K_m^{-1}, reflecting the electrostatic aspect of the substrate recognition. With respect to the pressure dependence of the catalytic process, LAP resembles CPA[5b)] but as for the substrate binding it resembles the mesophilic neutral protease,[5c)] where mostly the hydrophobic nature of the enzyme-substrate interaction was observed.

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7) This statement does not exclude the possibility that a reversible dissociation of the hexamer enzyme into protomers or oligomers without changing any catalytic activity of each subunit can occur upon the pressure application, which is not detectable by the present principle of the measurement.
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