Pressure-Assisted Cold-Denaturation of Carboxypeptidase Y

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The pressure and temperature dependence of the hydrolytic rate of carboxypeptidase Y was studied in the range of 5—40 °C and 0.1—400 MPa. The iso-activity contours have more or less positive slopes. The pressure apparently disactivates the enzyme at a constant temperature, and the apparent activation volume ($\Delta V^{\ddagger}$) became smaller with increasing temperature; $\Delta V^{\ddagger}_{app} = -46$ ml mol^{-1} at 10 °C and -11 ml mol^{-1} at 40 °C. At a constant pressure, the activity increased with increasing temperature, and the evaluated activation enthalpy ($\Delta H^{\ddagger}$) increased with an increase in pressure from 13 kcal mol^{-1} at 0.1 MPa to 25 kcal mol^{-1} at 400 MPa.

The intrinsic fluorescence spectrum changed with temperature and pressure and the highest intensity was recorded at ambient pressure and the lowest temperature, while the intensity decreased with increasing temperature or pressure. The intensity also decreased with decreasing temperature under elevated pressure, while the peak wavelength shifted to the red side according to the increase in pressure and temperature.

The disactivation of the catalysis and the decrease in the fluorescence peak intensity at a higher pressure and lower temperature were considered to be the so-called pressure-assisted cold-denaturation of the enzyme protein. Pressure can be a perturbation agent to "increase" the transition temperature above 0 °C.

Carboxypeptidase Y (CPDY) [E.C.3.4.16.1] is a serine-containing carboxypeptidase from yeast.[1] Its crystal structure has been elucidated[2] and several amino acid-substitution studies have been made to modify its enzymatic properties.[3—8] This enzyme has both esterase and amidase activities, besides the intrinsic carboxypeptidase activity,[9,10] which has been utilized to synthesize peptide bonds.[5,6,11—14] During our physicochemical studies on the enzymes of this group,[15—26] in order to elucidate their reaction mechanisms and to find an application to peptide formation reactions, we have found that peptide-formation reactions can be improved by performing the catalytic reaction under high pressure.[22,23]

In previous report we showed that CPDY exhibits some characteristic activity change under moderately high pressure as well as that the enzyme is irreversibly inactivated at extremely high pressure, showing some molten-globule like properties.[27] Recently, Dumoulin et al. showed that these conformational stabilities were affected by elimination of the carbohydrate moiety from the protein.[28] Such a pressure-induced inactivation or conformational change is rigorously influenced by temperature, and it was reported that this enzyme also showed an irreversible inactivation after a freeze-thawing process at sub-zero temperature and high pressure.[29]

Considering this background, we studied the in situ activity and conformational change, as studied by fluorescence, of CPDY over a wide range of pressure-temperature planes, and found that this enzyme shows a kind of pressure-assisted cold-denaturation, as was demonstrated for ribonuclease A by Jonas' group.[30]

Materials and Methods

CPDY was obtained from the Oriental Yeast Co. (Osaka, Japan. Lot 21003801 and 21003805). The enzyme concentration was determined from the absorbance at 280 nm using $A^{1\%}_{1cm} = 15.0$ and $Mr = 61000$ as corrected by active site titration[31] or routinely by the second-order rate of the hydrolysis of Bz–Tyr–*p*-nitroanilide (*p*NA) or succinyl (Suc)–Ala–Ala–Pro–Phe–*p*NA under our standard conditions.[24] These *p*NA substrates were obtained from Sigma (St Louis, Mo., USA) and Suc–Ala–Ala–Pro–Phe–4-methylcoumarin-7-amine (MCA) was from the Peptide Institute (Minoo, Japan). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Dojindo Laboratories (Kumamoto, Japan). Dimethyl sulfoxide (Me_2SO) was of analytical grade. Other reagents were commercially available.

The hydrolytic activity of the enzyme in regards to Suc–Ala–Ala–Pro–Phe–MCA was measured by using a high-pressure optical cell produced by Teramecs (Kyoto, Japan) that was settled inside of a Shimadzu RF-5000 spectrofluorometer; the temperature of the measuring solutions was controlled by circulating thermostat-regulated water through the cell block. The inside temperature was detected by a Cu-constantan thermocouple. The extraneous pressure was applied by a high-pressure hand pump equipped with an intensifier (ratio 8.5 : 1) (Teramecs), and the pressure medium was deionized water. The pressure was measured by a Bourdon tube-type pressure gauge. For calculating of the apparent second-order rate of the hydrolysis, the pressure and temperature dependence of the fluorescence from the liberated 4-methylcoumarin-7-amine was corrected.

The differential scanning calorimetry (DSC) was measured by a high-sensitive DSC meter Nano-DSC Model 5100 (Calorimetry

Science Co., UT, USA). One mg protein per 1 ml buffer solution was introduced to the sample tube of the apparatus and the temperature scanning rate was 1 °C min^{-1}. The reference solution was the equilibrium solution of the dialysis of the sample for overnight.

Results and Discussion

First, the pressure and temperature dependence of the hydrolytic rate (apparent second-order rate constant) was studied in the range of 5—40 °C and 0.1—400 MPa at a sufficiently low substrate concentration (3 μM). The obtained dependence is presented in Fig. 1 as iso-activity contours in a *P–T* plane. The logarithmic scale of the *z*-axis (as described in M^{-1} s^{-1}) is to be noted in this figure (M = mol dm^{-3}). The iso-activity contours have more or less positive slopes. Thus, the highest activity was observed at the highest temperature and the lowest pressure in the measured range, and the lowest activity was at the highest pressure and the lowest temperature. At 350 or 400 MPa and 5 or 10 °C, practically no activity was observed in the measured timescale. At a constant temperature, the pressure apparently disactivated the enzyme, as we have reported before for certain temperatures and for other substrates.[16,27]

Generally, the pressure-dependent events, rate constants, or equilibrium constants, at constant temperatures are analyzed by an equation comprising the first and second derivatives of the free energy with respect to pressure and, in the case of the rate constant (k) as in the present results, the first derivative is interpreted as the volume of activation ($\Delta V^{\ddagger}$) and the second derivative as the (absolute) compressibility of activation ($\Delta\beta^{\ddagger}$) (Eq. 1, where the subscript o means the standard condition and here at 0.1 MPa. Please remember that this (absolute) compressibility takes a negative value when the volume decreases with pressure, while the conventional relative compressibility takes a positive value with decreasing volume).

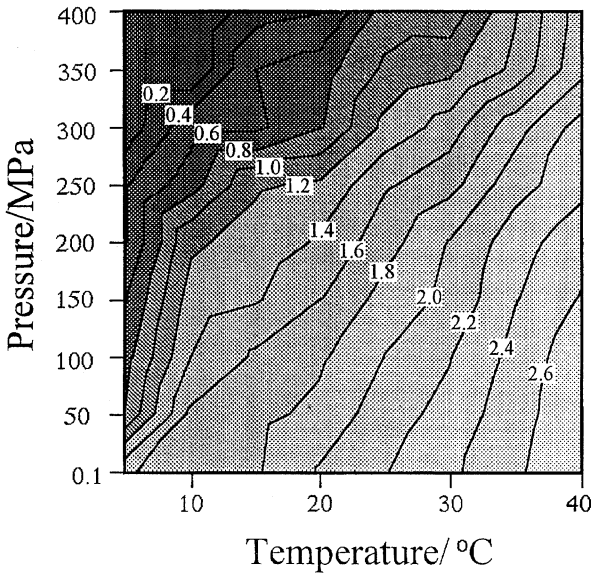

Fig. 1. The temperature and pressure dependence of the apparent second-order rate constant of the CPDY-catalyzed hydrolysis of Suc–Ala–Ala–Pro–Phe–MCA at pH 7 (0.1 M Hepes/NaOH containing 2% Me_2SO). [S] = 3 μM, [E] = 87—440 nM (depending on the activity). Numbers on the contours denote the log ($k_{cat}/K_{m_{app}}$) in M^{-1} s^{-1}.

$$\ln k = \ln k_o - ((\Delta V^{\ddagger}/RT)\cdot P + (\Delta\beta^{\ddagger}/2RT)\cdot P^2). \quad (1)$$

The values of $\Delta V^{\ddagger}$ and $\Delta\beta^{\ddagger}$ were calculated from the data in Fig. 1 by curve fitting to Eq. 1. The apparent activation volume ($\Delta V^{\ddagger}$) became smaller with increasing temperature; namely $\Delta V^{\ddagger}_{app} = -46$ ml mol^{-1} at 10 °C and -11 ml mol^{-1} at 40 °C. The absolute compressibility of activation ($\Delta\beta^{\ddagger}$) didn't show a clear temperature dependence, and it was about -0.1 ml mol^{-1} MPa^{-1} throughout the measured range. At a constant pressure, the activity increased as the temperature increased, and the evaluated activation enthalpy ($\Delta H^{\ddagger}$) increased with pressure from 13 kcal mol^{-1} at 0.1 MPa to 25 kcal mol^{-1} at 400 MPa.

Figure 2 shows the changes in the intrinsic fluorescence spectrum with temperature and pressure, as illustrated by the relative fluorescence peak intensity (a) and peak wavelength (b). In (a) the peak intensity at 20 °C and 0.1 MPa was arbitrarily chosen as the control. The highest intensity was recorded at ambient pressure and the lowest temperature in the measured range, while the intensity decreased with increasing temperature or pressure. However, the intensity also decreased with decreasing temperature under elevated pressure. The peak wavelength (b) shifted to the red side as the pressure and temperature increased, and the longest wavelength was recorded at 40 °C and 400 MPa in this plane. The changes to a higher pressure and lower temperature, as was observed in the panel (a) and also in the catalytic activity (Fig. 1), were not clearly seen in this case, but it can be estimated that the contours seem round-shaped in this region and that the peaks show further red-shift in the lower temperature range at much higher pressures than that can be applied in the present system.

The catalytic disactivation and the decrease in the fluorescence peak intensity at a high pressure and lower temperature range will lead us to consider the so-called pressure-assisted cold-denaturation of the enzyme protein. The cold-denaturation of proteins is a thermodynamically universal phenomenon where the denaturation is described in a simple two-state model.[32—34] In practice, however, this type of denaturation can be observed only when the transition temperature has an observable value: above 0 °C when any special technique is applied. Very few examples are known to clear this limitation. In most cases, some perturbation agents, such as acids or other chemical denaturants, were introduced in the system in order to make the proteins less stable and to raise the (lower) transition temperature.

According to Hawley's equation (Eq. 2) and its approximated form supposing $T \simeq T_o$ [i.e., $(T-T_o)/T_o \ll 1$] (Eq. 3),[35,36]

$$\begin{aligned}\Delta G = {} & \Delta\beta\cdot(P-P_o)^2/2 + \Delta\alpha\cdot(P-P_o)\cdot(T-T_o) \\ & -\Delta C_p\cdot[T\cdot\ln(T/T_o) - 1) + T_o] \\ & +\Delta V_o\cdot(P-P_o) - \Delta S_o\cdot(T-T_o) + \Delta G_o, \end{aligned} \quad (2)$$

($\Delta\beta$, $\Delta\alpha$, and ΔC_p are the changes of (absolute) compressibility [= $(\partial\Delta V/\partial P)_T$], (absolute) expansivity [= $(\partial\Delta V/\partial T)_P = -(\partial\Delta S/\partial P)_T$], and the heat capacity at constant pressure [= $T(\partial\Delta S/\partial T)_P$], respectively; the standard state was taken at 0 °C and 0.1 MPa)

$$\Delta G = \Delta\beta\cdot(P-P_o)^2/2 + \Delta\alpha\cdot(P-P_o)\cdot(T-T_o) - \Delta C_p\cdot(T-T_o)^2/2T_o + \Delta V_o\cdot(P-P_o) - \Delta S_o\cdot(T-T_o) + \Delta G_o, \quad (3)$$

the denaturation produces an ellipse when $-\Delta\beta\cdot\Delta C_p/T_o - \Delta\alpha^2 > 0$.

It is clear that pressure can be a perturbation agent to "increase" the transition temperature above 0 °C, when the center of the ellipse is at the negative (non-real) pressure region; for this $\Delta V_o\cdot\Delta C_p/T_o - \Delta S_o\cdot\Delta\alpha$ should be < 0 (Fig. 3-a), or, due to the positive $\Delta V/\Delta S$ of the ice-water transition under high pressure, one can make the observation at sub-zero temperatures and cold-denaturation can be observed even when $\Delta V_o\cdot\Delta C_p/T_o - \Delta S_o\cdot\Delta\alpha > 0$ (Fig. 3-b). A clear-cut example of the former case was exhibited by Jonas' group for ribonuclease A.[30] The present results can be another example of cold-denaturation that was made observable by the help of a high-pressure application (pressure-assisted cold-denaturation).

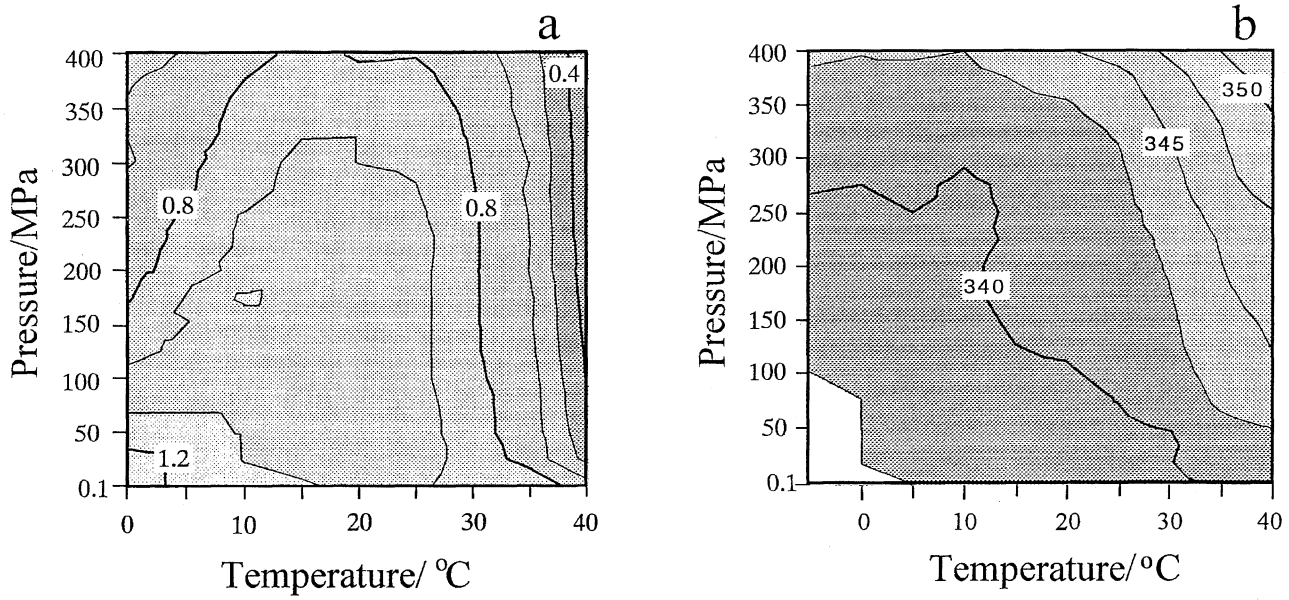

Fig. 2. The pressure and temperature dependence of the intrinsic fluorescence of CPDY at pH 7.5 (0.1 M Hepes/NaOH). [E] = 1—4 μM, λ_{ex} = 295 nm. (a) Relative fluorescence peak intensity. That at 20 °C and 0.1 MPa was arbitrarily taken as the control, and the numbers on the contours denote the relative values. (b) Fluorescence peak shift. The numbers on the contours denote the excitation peak wavelengths in nm.

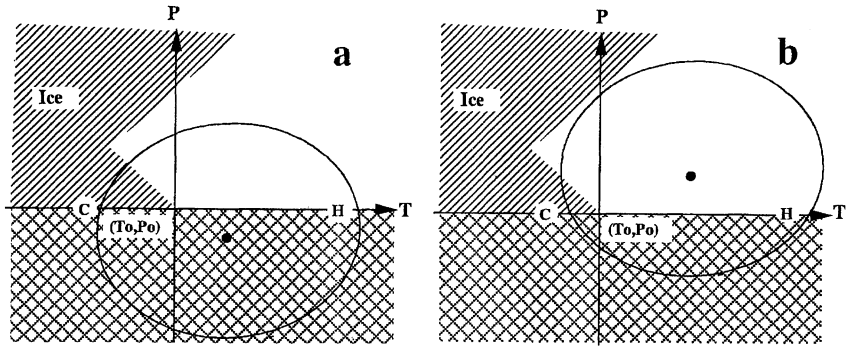

Fig. 3. Schematic presentation to explain the two possible origins of the observable pressure-assisted cold-denaturation. The ellipse shows the transition point (T, P), where the free energy difference of the two-state transition becomes zero (Eq. 2). "C" and "H" indicate the cold-denaturation and heat-denaturation temperatures under atmospheric pressure, respectively. (T_o, P_o) indicates the reference state in Eqs. 2 and 3. The////area is the ice phase of water, and the hatched area is the "negative" (not realistic) pressure zone. (a) When the center of the ellipse (●) is below the T-axis (in the "negative" pressure range), the cold-denaturation temperature increases with an increase in pressure, up to the liquid phase of the water and becomes observable. (b) Even when the center of the ellipse is above the T-axis (in the positive pressure range), the liquid phase of water is realized under certain range of high pressure, and then the cold-denaturation can be observed; in this case the cold-denaturation temperature in this pressure range might be lower than T_c.

Calorimetric measurements by the DSC technique revealed that the ΔH_m and ΔC_p of the thermal transition of CPDY are 408 kJ mol^{-1} and 10.2 kJ mol^{-1} K^{-1}, respectively (thermogram is not shown). These values and the peak temperature of the endothermic thermogram (T_p; 324.5 K or 51.5 °C) predict that the cold-denaturation of this protein occurs at around $T_c = 260$ K or -13 °C from Eq. 4,[32,33] with the approximation that T_p is practically equivalent to the transition temperature (T_m).

$$T_c = T_m^2 \cdot [2 \cdot (\Delta H_m / \Delta C_p) + T_m]^{-1}. \quad (4)$$

When we compare the results of the activity (Fig. 1) and the fluorescence (Fig. 2), it can be seen that these changes are not exactly coincidental. The higher activity of the enzyme at higher temperatures, even with deformed or denaturing protein, can be brought about by the high value of activation enthalpy of the catalytic reaction. The decreasing rate with an almost intact protein at a medium temperature and pressure can be explained by the positive activation volume of the catalytic reaction. At the low-temperature and high-pressure region, both the catalytic activity and fluorescence intensity became low, but the peak didn't show a large shift. In the case of cold-denaturation of this protein, the deformation of the protein structure could be different from that expected for a region of high pressure and temperature.

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