Effects of High-Pressure on the Activity and Spectroscopic Properties of Carboxypeptidase Y

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The effects of high pressure, up to 400 MPa, on the catalytic activity and the fluorescence and CD of carboxypeptidase Y (CPDY) were investigated.

CPDY showed a pH-dependent Suc–Ala–Ala–Pro–Phe–*p*NA hydrolysis similar to other neutral substrates. The apparent second-order rate showed a gradual decrease with increasing pressure, which was related to an increase in K_m and a decrease in k_{cat}.

The intrinsic fluorescence of CPDY showed a gradual decrease in the intensity and a red shift in the maximum wavelength with pressure. The transition curve did not follow a simple tow-state transition, but contained at least three states. The first transition occurred at around 100 MPa and the second one occurred at pressures higher than 200 MPa. After incubation at 300 MPa, both the peak intensity and the maximum wavelength did not show complete restoration; the pressure-induced change is substantially irreversible.

The latter change corresponds to the increased binding of a fluorescent hydrophobic probe molecule (8-anilino-1-naphthalenesulfonic acid) to this protein; however, the CD spectrum showed practically no evidence of irreversible changes in the protein's secondary structure.

Carboxypeptidase Y (CPDY) [E.C.3.4.16.1] was isolated from yeast and is classified as a serine-containing carboxypeptidase.[1] Its crystal structure has recently been elucidated[2] and efforts have been made to modify its enzymatic properties through genetic manipulation.[3—7] Most of the enzymes of this group show esterase and amidase activities besides the intrinsic carboxypeptidase activity[8,9] and hence have been used for peptide synthesis or transformation by the acyl transfer reaction[6,7,10—12] as well as by condensation reaction.[13]

We have made a series of physicochemical studies on the enzymes of this group[13—25] to elucidate their reaction mechanisms and also to show that peptide formation reactions can be improved by performing them under high pressure.[20,21]

During these extensions of our study, we noticed that CPDY showed some characteristic activity change under moderately high pressure as well as an irreversible inactivation at extremely high pressure. Since the mechanistic study of our previous report[14—16] utilized pressures only up to 150 MPa due to equipment limitations, here we investigated the effect of much higher pressure on the catalytic activity and the spectral change of the protein.

Materials and Methods

CPDY was obtained from the Oriental Yeast Co. (Osaka, Japan. Lots 2100361 and 21003402). The enzyme was first dissolved in deionized water. Its concentration was determined from absorbance at 280 nm using $A\%_{1cm} = 15.0$ and $M_r = 61000$ as corrected by active site titration[26] or routinely by the second-order rate of the Bz–Tyr–*p*-nitroanilide (*p*NA) hydrolysis under our standard conditions.[22] The stock solutions were stored at 4 °C. Suc–Ala–Ala–Pro–Phe–*p*NA was obtained from Sigma (St Louis, Mo., USA). Ammonium salt of 8-anilino-1-naphthalenesulfonic acid (ANS) was purchased from Wako Pure Chemicals (Osaka, Japan). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2-morpholinoethanesulfonic acid (MES) were purchased from Dojindo Laboratories (Kumamoto, Japan). Dimethyl sulfoxide (DMSO) was of analytical grade. The other reagents were commercially available.

The hydrolytic activity of the enzyme in regards to Suc–Ala–Ala–Pro–Phe–*p*NA was measured by using a Shimadzu UV-2200 spectrophotometer or Union SM-401 spectrophotometer. The incubation of protein solutions under elevated pressure was performed in a high-pressure bomb that was constructed by the Yamamoto Suiatsu Co. (Toyonaka, Japan). The spectrophotometric and fluorometric measurements under high pressure were performed by using high-pressure optical cells produced by Teramecs (Kyoto, Japan) that were connected through optical fibers to a PRAS-5000 high-sensitivity spectrophotometer (Ohtsuka Electronics, Hirakata, Japan) (absorbance) or settled inside of a Shimadzu RF-5000 spectrofluorometer (fluorescence). The measured absorbance and fluorescence intensities were corrected for the contraction of the volume under high pressure, based on the published compressibility data of water and solutions. The CD spectrum was measured on a J-720 JASCO spectropolarimeter. In all of these instruments the temperature of the measuring solutions was controlled by circulating thermostated water through optical cell jackets.

Results

(1) Effect on Activity: Besides carboxypeptides, CPDY

can hydrolyze amino acid alkyl esters, amino acid amides, and other *endo*-protease substrates[8,9,15,17] such as *p*-nitrophenyl esters, *p*-nitroanilides and *N*-(4-methyl-2-oxo-2*H*-1-benzopyran-7-yl)amides. Figure 1 shows the apparent second-order rate constant (measured at a fixed low concentration of the substrate: $k_{cat}/K_{m(app)}$) for the CPDY-catalyzed hydrolysis of Suc–Ala–Ala–Pro–Phe–*p*NA (25 °C) at various pH levels (a) and pressures (b). Apparently the catalytic activity decreased with an increase in the pressure irrespective of the pH value. The pH profile was similar to the hydrolysis of neutral substrate such as Fua–PheOEt, Fua–PheOMe, and Fua–PheNH$_2$ (where Fua denotes 3-(2-furyl)acryloyl).[14] For these substrates, the apparent activation volumes ($\Delta V^{\ddagger}_{(app)}$) for $k_{cat}/K_{m(app)}$ were reported as 4.5, 2.5, and 8.2 ml mol^{-1}. respectively. The decrease in the $k_{cat}/K_{m(app)}$ in Fig. 1-b gave a roughly estimated value of $\Delta V^{\ddagger}_{(app)}$ as around 5 to 10 ml mol^{-1} for the present substrate.

The hydrolytic rate was measured with changes in the concentration of the substrate at 0.1, 100, 200, and 300 MPa. The kinetic parameters were obtained from Eadie plot analysis as shown in Table 1. The pressure dependence of the k_{cat}/K_m values calculated from these parameters are compared with the pressure-dependence of the $k_{cat}/K_{m(app)}$ in Fig. 1-b. It is clear that the apparent decrease in k_{cat}/K_m was rather related to the increased K_m value and k_{cat} showed only a moderate decrease even at an elevated pressure.

To know the reversibility of these activity changes, we measured the hydrolytic activity after the prolonged incubation of enzyme solutions under elevated pressure. Figure 2 shows the results. Up to 5 h of incubation and 200 MPa, the residual activity of the enzyme solution is almost the same as the intact or control, although the data at 100 MPa showed some characteristic increase in the remaining activity. The relatively low K_m value that was observed in Table 1 for this pressure value may be related to this kind of peculiar apparent pressure-activation, although the reason for this is not yet clear. Incubation 300 MPa resulted in a gradual (irreversible) inactivation; furthermore, incubation overnight at 200 MPa caused the loss of a substantial part of the initial activity. All the kinetic measurements shown in Fig. 1 and Table 1 were completed in several minutes. Thus the depressed in situ activity at lower than 200 MPa has a reversible nature and is recovered after the liberation of the extraneous pressure.

Table 1. Kinetic Parameters of Suc–Ala–Ala–Pro–Phe–*p*NA Hydrolysis Catalyzed by Carboxypeptidase Y at Various Pressures[a)]

Pressure / MPa	K_m / mM	k_{cat} / s^{-1}
0.1	1.14±0.02	1.03±0.05
50	1.52±0.03	1.00±0.09
100	1.17±0.06	0.68±0.12
150	1.71±0.09	0.66±0.09
200	1.26±0.07	0.40±0.06
250	3.92±0.07	0.58±0.08
300	9.23±0.73	0.68±0.09

a) 25 °C, pH 7.5, and 2% (v/v) DMSO.

(2) Effect on Spectroscopic Properties: The intrinsic fluorescence of CPDY was measured with excitation at 280 nm (at pH 7.5). A gradual decrease in the peak intensity

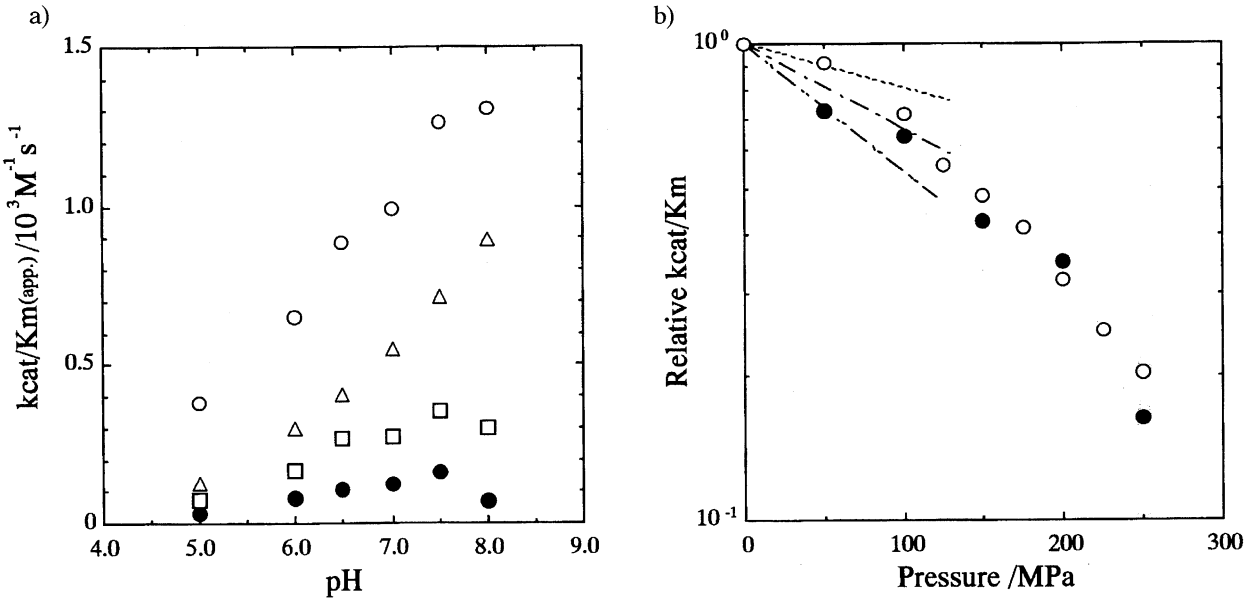

Fig. 1. The apparent second-order rate constant of the CPDY-catalyzed hydrolysis of Suc–Ala–Ala–Pro–Phe–*p*NA at various pH and pressure. 25 °C, 2% (v/v) DMSO. (a) pH dependence. ○, 0.1 MPa; △, 100 MPa; □, 200 MPa; ●, 300 MPa. 50 mM MES–NaOH. [S] = 0.05—0.20 mM, [E] = 18—660 nM (depending on the activity). (b) Pressure dependence at pH 7.5 (○), shown in the relative value to that at 0.1 MPa. [S] = 0.04—22 mM, [E] = 90—100 nM (depending on the activity). The k_{cat}/K_m values calculated from the data in Table 1 are shown in closed symbols for comparison. The lines indicate the predicted decrease in k_{cat}/K_m with $\Delta V^{\ddagger} = 5$ ml mol (····), 10 ml mol (– · –), and 15 ml mol (– · · –), near atmospheric pressure.

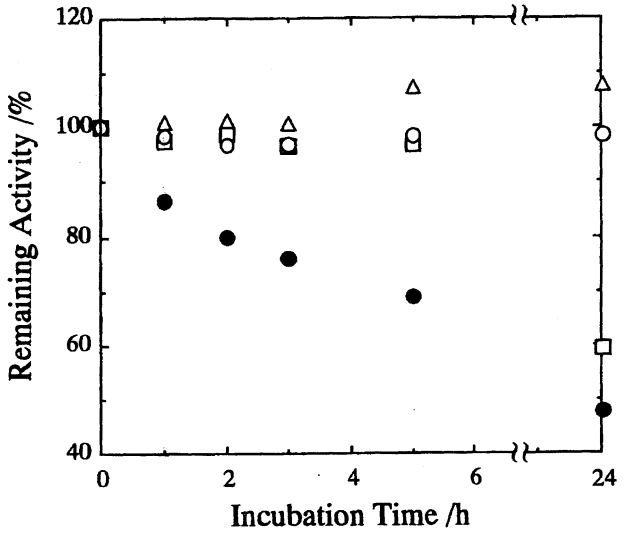

Fig. 2. Residual activity of CPDY after incubation under various pressures. Activity was assayed against Suc–Ala–Ala–Pro–Phe–*p*NA. ○, 0.1 MPa; △, 100 MPa; □, 200 MPa; ●, 300 MPa. Activity was measured after incubation at 25 °C for indicated periods at pH 7.5 and [E] =4 μM. Assay conditions; [S] = 0.2 mM, [E] =0.13 μM, 2%(v/v) DMSO, and pH 7.5.

was observed with increasing pressure (Fig. 3-a). The peak wavelength showed a gradual red shift upon the application of pressure (Fig. 3-b). The transition curve did not follow a simple two-state transition, but the process seemed to contain at least three states (two-step change). This is evident from the information in the peak wavelength shift. The first transition occurred at around 100 MPa and the second one occurred at pressure higher than 200 MPa. After the release of the highest pressure (300 MPa) application, both the peak intensity and the maximum wavelength did not show complete restoration; the change induced by high pressure is substantially irreversible.

The change in the intrinsic fluorescence at higher pressure corresponded to the increased binding of a fluorescent hydrophobic probe molecule (ANS) to this protein (Fig. 4). At pressure values lower than 200 MPa, practically no increase in the ANS fluorescence was observed. ANS can bind to CPDY even under atmospheric pressure (although incompletely). The large shift of the peak wavelength at lower pressure strongly reflects the contribution of the fluo-

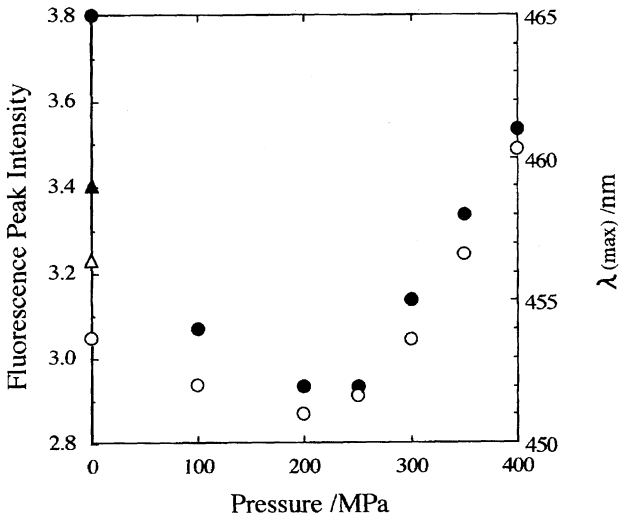

Fig. 4. Pressure dependence of the fluorescence peak intensity (○,△) and maximum wavelength (●,▲) of the solution containing CPDY and ANS. [E] =6 μM, [ANS] =6 μM, 25 °C, and pH 7.5. Excitation at 350 nm. ○ and ● were measured under the indicated pressure. △ and ▲ were after pressure release (from 400 MPa).

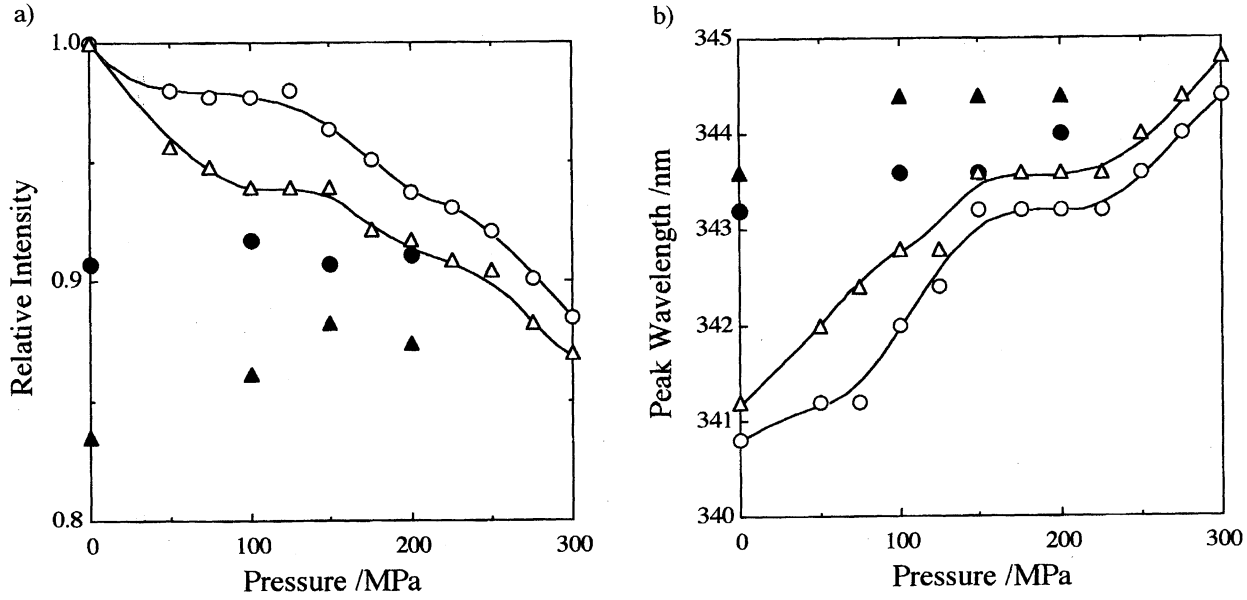

Fig. 3. Pressure dependence of the peak intensity (a) and maximum wavelength (b) of the intrinsic fluorescence of CPDY. ○, 25 °C; △, 40 °C. Open symbols were measured under the indicated pressure. Closed ones are for after pressure release (from 300 MPa). [E] =5.5 μM HEPES (pH 7.5). Excitation at 280 nm.

rescence of the free ANS molecules, whose peak wavelength was 510 nm. After the release of high pressure, a substantial part of the increased fluorescence intensity and the peak shift remained irreversibly.

Both of these fluorescence results show that at pressure values higher than 250 MPa, the protein surface develops a more hydrophobic character, or the protein hydrophobic core becomes more accessible, in order to increase the number of bound ANS molecules; the tryptophan residues becomes more exposed to the solvent (water).

Figure 5 shows the far UV region CD spectra after the incubation of the CPDY solution for 2 h as measured under atmospheric pressure. There was practically no indication of any irreversible changes in the secondary structure of the CPDY protein, even after incubations at 300 or 400 MPa where the catalytic activity was substantially lost.

Discussion

At least two steps of the pressure-induced change in CPDY were observed in both kinetic activity and spectroscopic properties: one occurs at 100—200 MPa and the other at near 300 MPa. The latter one is accompanied by a large increase in K_m values, as well as by the irreversibility of the activity change. As for the first step of the change, we measured the pressure dependence (up to 100 MPa) of the kinetic parameters for simple amino acid ester substrates.[14] They showed an almost linear dependence and gave the following parameters: $\Delta V_{(Km)}$ and $\Delta V^{\ddagger}_{(kcat)}$ were -6.0 and -1.5 ml mol^{-1}, respectively, for Fua–PheOEt and -6.2 and -3.7 ml mol^{-1}, respectively, for Fua–PheOMe. However, peptide substrates such as Fua–Gly–Phe and Fua–Phe–Phe showed positive $\Delta V^{\ddagger}_{(kcat)}$ values (27 and 10 ml mol^{-1}, respectively), though $\Delta V_{(Km)}$'s are also negative. The present tetrapeptide amide substrate somehow gave more complicated behavior even in the lower pressure range. This might be explained by the activated character of its scissile bond and/or by extended subsite interactions of this substrate in the enzyme binding site.

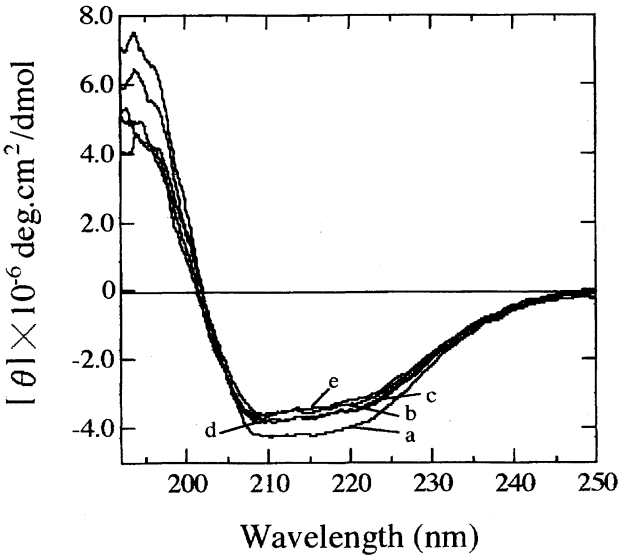

Fig. 5. Effect of incubation under high-pressure on CD spectrum of CPDY solutions. Curves a, b, c, d, and e correspond to 0.1, 100, 200, 300, and 400 MPa, respectively. CD spectrum was measured after incubation (2 h) at each pressure. pH 7.5, 25 °C, and [E] = 15 μM.

The hydrolytic reaction of CPDY is believed to proceed via serine-acylated enzyme intermediate, but it is somehow different from the typical serine proteases such as chymotrypsin and trypsin; the rates for the acylation and deacylation steps are not much different even with ester substrates. Therefore, a clear observation of their presteady state process could be done only with a substrate with an acyl-enzyme-stabilizing acyl group such as pivalate,[4,5,26] although the ester substrates has a acylation-limited character and Fua–PheOEt showed a very small trace of pre-steady state process.[27] The substrate in this study is an activated amide and has more ester substrate character. In regards to these, we can postulate that the change at the first step (under moderate pressure) is accompanied with a change in the rate-limiting process; the acylation process is rate-limiting at low pressure but the deacylation process becomes rate-limiting at higher pressure. This might be alternatively explained by some difference in the subsite interactions. If the first (mostly reversible) change affects only the extended subsite interaction sites, an extended substrate such as the present one would be affected to a larger extent. However, the smaller substrates would suffer from this level of pressure-induced change to a limited extent and show pressure responses mostly due to the catalytic reaction.

A further increase in pressure would cause a collapse in the protein structure, known as the irreversible change in the intrinsic fluorescence and the (fairly irreversible) increase in binding of ANS molecules, which is coupled with an irreversible inactivation of the enzyme. However, the secondary structure of the protein seemed to be maintained almost intact even under (and after) extreme pressure conditions where substantial parts of the activity were lost. These two characteristic of CPDY after high pressure incubation, an increased ANS binding and a mostly intact secondary structure, fit two of the three criteria for molten globule structure.[28,29] The third one, a compactness in structure, was not examined here. However, we can image the structure of the pressure-denatured CPDY molecule as a molten globule-like one. Similar characteristics of pressure-denatured proteins have been indicated also for other proteins.[30—32]

The irreversibility of the structural change in CPDY is not simply explained by the aggregation of the proteins, as judged from the observation of the solutions after pressure treatment and the gel electrophoretic patterns (unpublished result). This might be related with the necessity of a pro-region of the pre-matured protein for the correct (re)folding of the matured CPDY,[33,34] which is common to the case of subtilisin.[35]

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