BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 150–154 (1997)
ARTICLE NO. RC977313

A Stable Partly Denatured State of Trypsin Induced by High Hydrostatic Pressure

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Received July 28, 1997

The effect of hydrostatic pressure on the unfolding of trypsin was studied by fluorescence spectroscopy under pressure from 1 to 7000 bar. It was found that, at pH 3.0 or pH 7.3, a stable partly denatured state of trypsin was obtained when the applied pressure was about 6.5 kbar. This transient denatured state did not show any enzymatic activity and was different from that denatured by 8 M urea or high temperature in both intrinsic fluorescence spectrum and 8-anilino-1-naphtalene sulfonate (ANS) binding, having some obvious characteristics of 2 molten globule state of protein. It was also found that the formation of this partly denatured state of trypsin was temperature dependent. Energenic values of the process were also given.

Key Words: **trypsin; pressure; denaturation; molten globule state.**

Trypsin is a well known enzyme. Its molecular properties, structure and function have been investigated in detail. Some studies of the effect of hydrostatic pressure on trypsin have been reported (1–3). However, most of them concentrated on the effect of high pressure on the enzymatic activity (1, 2). There were few papers concerned with the effect of pressure on the trypsin conformation and structure. Recently it has been shown that under mild denaturing conditions, proteins undergo transitions toward partially unfolded states called molten globule state (MG). In this paper, we studied the pressure induced unfolding of trypsin in a range from 1 bar to 7 kbar by fluorescence spectroscopy. A stable transient denatured state of trypsin was obtained when pressure was about 6.5 kbar, with no any enzymatic activity, and different from that obtained by chemical or temperature denaturation. These results are interpreted as a pressure denatured trypsin which displays the characteristics of molten globule state of protein, an intermediate between native and unfolded states (4–6).

MATERIALS AND METHODS

Enzyme and chemicals. The bovine pancreas trypsin and Nα-benzoyl-L-arginine p-nitroaniline (L-BAPNA) were obtained from Sigma Co. SDS-PAGE was carried out by the procedure of Laemmli to determine the purity of trypsin (7). Heavily loaded samples of the trypsin employed showed a single compact band, indicating that the purity of the sample satisfied the requirement of the experiments. ANS was purchased from Molecular Probe Co. All other reagents were of A.R grade. Distilled water was further purified by a Millipore system to a resistance of 18 MΩ.

The trypsin was desolved in 0.001 M HCl (pH 3.0) as stock solution (5 mg/ml) and then stored at 0°C. All the samples of the trypsin at pH 7.3 (0.05 M Tris-HCl) or pH 3.0 (0.001 M HCl) were freshly prepared from the stock solution and used after one hour incubation at the required temperature. Tris-HCl buffer was chosen because of its almost pressure-independent pK. The enzyme activity was assayed using L-BPANA as substrate according to Erlanger et al. (8).

Fluorescence measurements. All the fluorescence measurements were carried out on AB2 fluorospectrophotometer (SLM Co.) which was modified in the INSERM laboratory to measure fluorescence in the pressure range from 1bar to 7 kbar through a thermostated pressure bomb. The fluorescence spectra of trypsin was quantified by specifying the center of spectral mass $\langle\nu\rangle$ which was defined and used by Weber and coworkers:

$$\langle\nu\rangle = \sum \nu_i * F_1/\sum F_i, \quad (1)$$

where ν_i is wavenumber and F_i is the fluorescence intensity at ν_i (9, 10). The excitation wavelength for the intrinsic fluorescence spectrum of trypsin in this study was 295 nm which only excite selectively the tryptophan residues. Absorption was measured using a spectrophotometer Cary 3E (Varian) which has also been modified to measure absorption spectra under pressure from 1 bar to 4.5 kbar (11).

RESULTS AND DISCUSSION

Intrinsic fluorescence. Figure 1 shows the effect of hydrostatic pressure from 1 bar to 6.8 kbar on the fluorescence spectrum of trypsin at pH 7.3 and 0°C. It can

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Abbreviations: ANS, 8-anilino-1-naphtalene sulfonate; MG, molten globule state; L-BAPNA, Nα-benzoyl-L-arginine p-nitroaniline.

0006-291X/97 $25.00

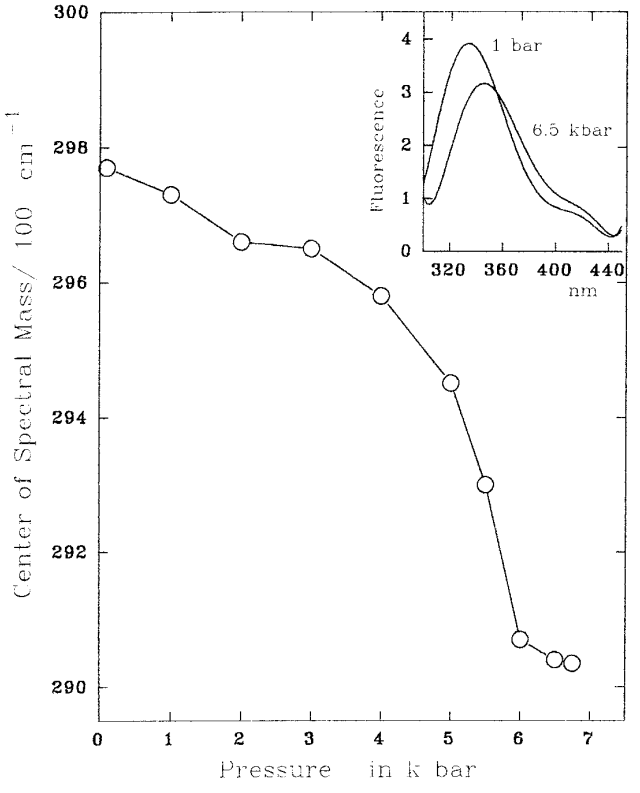

FIG. 1. Pressure dependence of the center of the spectral mass of trypsin intrinsic fluorescence at pH 7.3. (Inset) Fluorescence spectra of trypsin at 1 bar and 6.5 kbar, respectively. Concentration of trypsin, 0.2 mg/ml; temperature, 0°C. Excitation wavelength, 295 nm; slits for excitation and emission, 3 nm.

be seen in Figure 1 that the center mass of the intrinsic fluorescence spectrum of trypsin decreases as pressure increases. This indicates that the environment polarity of the tryptophan residues in trypsin becomes stronger, which is caused by the pressure-induced unfolding of trypsin molecule. From 1 bar to 3 kbar the slope of $\langle\nu\rangle$ is very gentle. However, from 3 kbar to 6 kbar the slope becomes very sharp. About 80 % of the total change in $\langle\nu\rangle$ upon the denaturation was observed in this pressure range. When the applied pressure was above 6 kbar, the change in $\langle\nu\rangle$ upon the further increase in pressure became smaller and smaller. And finally, a plateau in the center of spectral mass was obtained at about 6.75 kbar, indicating that all the trypsin was transformed into a stable pressure denatured state. At 6.75 kbar the center of the spectral mass of the pressure-denatured trypsin had a big change of about 800 cm^{-1} comparing with that of native trypsin at 1bar. The inset in Figure 1 shows the intrinsic fluorescence spectra of trypsin at 1 bar and 6.5 kbar respectively, indicating that the maximum emission wavelength of trypsin had a red shift of 12 nm upon the pressure induced denaturation and that the fluorescence intensity decreased by about 20 %. From the data shown in Figure 1 the standard volume change of trypsin upon the denaturation was determined as $\Delta V = -91 \pm 5$ ml/mol and the free energy $\Delta G = 50 \pm 5$ kJ/mol. The denaturation of trypsin caused by pressure at pH 3 was very similar with that at pH 7.3 (see Figure 2). The stable partly denatured state was obtained at 6.5 kbar, a little lower than that for trypsin at pH 7.3. The standard volume change upon the denaturation and free energy were -73 ± 5 ml/mol and 38 ± 4 kJ/mol respectively, close to that for trypsin at pH 7.3. However, at pH 3.0, the $\langle\nu\rangle$ of trypsin at 6.5 kbar was about 28850 cm^{-1} lower than that of trypsine at pH 7.3 under the same pressure, which may be caused by the effect of acidic solution on the trypsin molecule.

The enzymatic activity of trypsin under pressure between 1 bar and 4.5 kbar was assayed. It was found that the activity of trypsin began to be decreased after the pressure exceeded 1.7 kbar and was totally lost when the pressure was higher than 3.5 kbar. These experiments suggested that the pressure denatured trypsin observed above should have no enzyme activity, although the enzyme activity at pressure higher than 4.5 kbar was not assayed in this study due to the limitation of the maximum pressure (4.5 kbar) for absorption measurement in our laboratory.

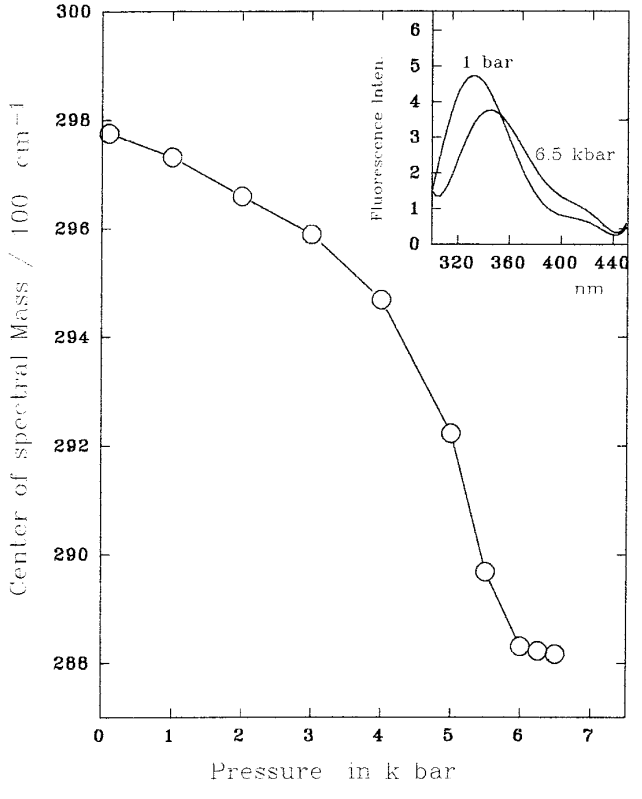

FIG. 2. Pressure dependence of the center of the spectral mass of trypsin intrinsic fluorescence at pH 3.0. (Inset) Fluorescence spectra of trypsin at 1 bar and 6.5 kbar. Other experimental conditions were the same as in Fig. 1.

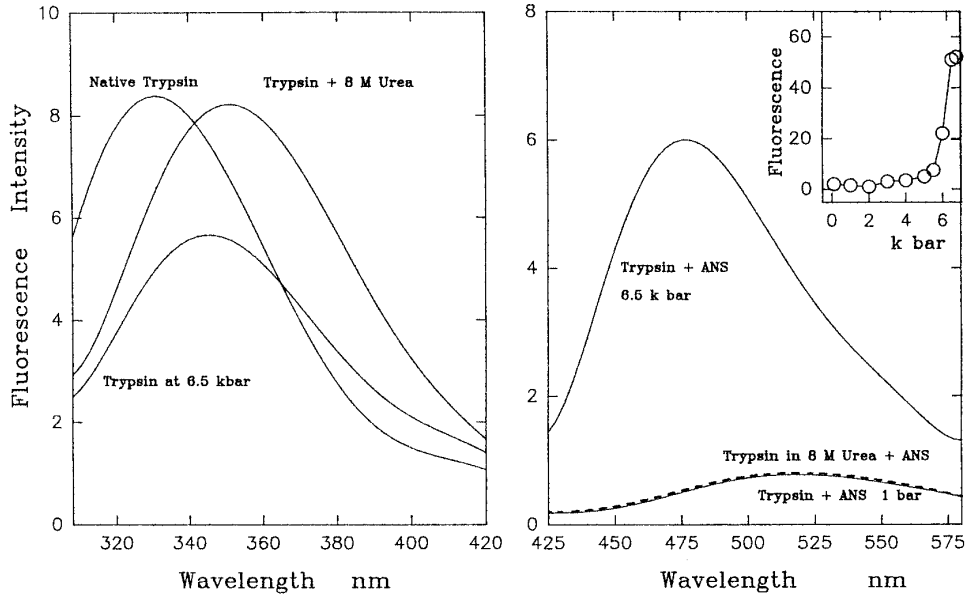

FIG. 3. Difference in intrinsic fluorescence spectrum and ANS binding between pressure-denatured and 8 M urea denatured trypsin. (Left) Intrinsic fluorescence spectra of native trypsin and denatured trypsin by pressure or 8 M urea. The spectrum of 8 M urea denatured trypsin was normalized to that of native trypsin. Experimental conditions were the same as in Fig. 1. (Right) Fluorescence spectra of ANS-trypsin at different conditions. Concentrations: trypsin, 0.2 mg/ml; ANS, 0.018 mg/ml. (Inset) Fluorescence intensity of ANS at 480 nm in the presence of trypsin as a function of pressure. Excitation wavelength, 350 nm; slits for excitation and emission, 3 nm. Temperature, 0°C.

The pressure denatured state of trypsin is different from that denatured by 8 M urea or by high temperature (65 ° C). Figure 3 shows the fluorescence spectrum of trypsin at pH 7.3 denatured by 8 M urea. The spectrum of 8 M urea denatured trypsin is almost the same with that of free tryptophan in the same buffer, its maximum wavelength red-shifted by 18 nm to about 350 nm, compared with the native trypsin fluorescence spectrum. This indicated that the trypsin in 8 M urea was completely denatured. However the spectrum of the pressure denatured trypsin at 6.5 kbar had only about 12 nm red shift compared with that of native trypsin, implying that the tryptophan residues in trypsin at 6.5 kbar were not totally exposed to the solvent. This means that the pressure-denatured trypsin obtained above is only partially unfolded and thus differs from the 8 M urea denatured trypsin. The fluorescence spectrum of thermally-denatured trypsin is very similar to that of 8 M urea denatured trypsin (see Figure 4). Its maximum wavelength is red-shifted by about 18 nm compared with that of native trypsin at 0 ° C, and the efficiency of fluorescence is decreased by about 50 %, which also indicates that the pressure-denatured trypsin is not the same as the thermal denatured form (see the comparison between Figures 3 and 4). The plateau of the center mass of the spectrum shown in Figure 1 and Figure 2 indicates that no more unfolding takes place when the pressure is further increased to about 6.5 kbar, which means that this denatured state of trypsin is a stable equilibrium state and should be an intermediate between native and completely denatured trypsin. This phenomenon also indicates that the pressure denaturation of trypsin should be a multistep process, so the energy needed for the denaturation of tryp-

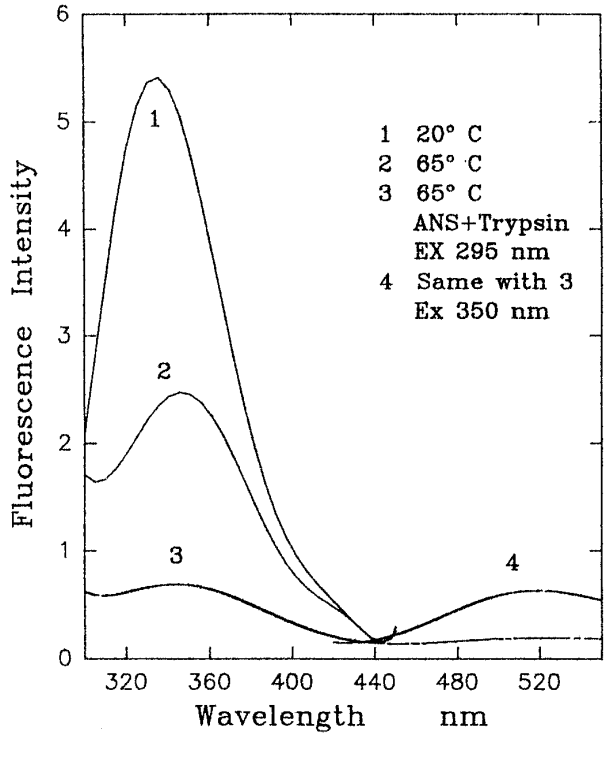

FIG. 4. Fluorescence spectra of thermal-denatured trypsin at 65°C. Other experimental conditions were the same as in Fig. 1.

sin is not continuous. Further unfolding of trypsin needs higher pressure (higher than 7 kbar), to result in complete denaturation. We must note that it is not possible to observe the denaturation of trypsin at a pressure higher than 7 kbar because of the technical limitation of our instrument. It is interesting that the pressure denaturation of chymotrypsinogen which is very similar with trypsin in molecular structure is different from its denaturation behavior. Chymotrypsinogen was completely denatured by pressure of about 9 kbar and there was no any stable intermediate of partly denatured form to be observed (12).

ANS binding fluorescence. The fluorescence of ANS is enhanced when bound to solvent-exposed hydrophobic areas of proteins (13). It was observed that ANS can bind to the transient pressure denatured trypsin but not to the trypsin denaturated by 8 M urea or by high temperature. In the Figure 4, spectrum 4, and in the right plot in Figure 3 both the fluorescence spectra of the native trypsin, of the 8 M urea and of the thermal denatured enzymes exposed to ANS were shown. The maximum wavelength at 530 nm is very similar with that of free ANS in the same buffer, indicating that ANS cannot bind or only has very low affinity to both native and completely denatured trypsin, in a good agreement with Li & Weber and Goto & Fink (12,14). The fact that no fluorescence of ANS was observed when the trypsin was excited at 295 nm also indicated no ANS binds to trypsin molecule (see spectrum 3 in Figure 4). However, when trypsin was compressed, especially when the applied pressure was above 5 kbar the fluorescence intensity of ANS was greatly enhanced (see the inset in Figure 3). At 6.5 kbar, the fluorescence intensity of ANS in the presence of trypsin was enhanced about 50 fold and its emission maximum wavelength shifted from 530 nm to 480 nm, indicating that ANS binds to the trypsin molecule at this pressure. This means that the pressure denatured trypsin has some specific conformation which is different from both native and completely denatured trypsin. This conclusion is in a good agreement with the difference observed in the intrinsic fluorescence spectra of the pressure denatured trypsin and the 8 M urea or thermal denatured trypsin described above. It has been shown that there exists an intermediate, termed a molten globule state (MG), between the native and denatured forms of protein upon the protein denaturation (15–17). One characteristic of MG is that the hydrophobic core of the protein in this form is more accessible to ANS than in native state. Our results suggested that the pressure denatured trypsin might be a molten globule state, an observation already reported for other proteins (13, 18–21). Hydrostatic pressure is a mild denaturant and the unfolding of polypeptide chain occurs usually at pressure higher than 3 kbar (22, 23). According to this view it is not surprising to get the MG state of protein at

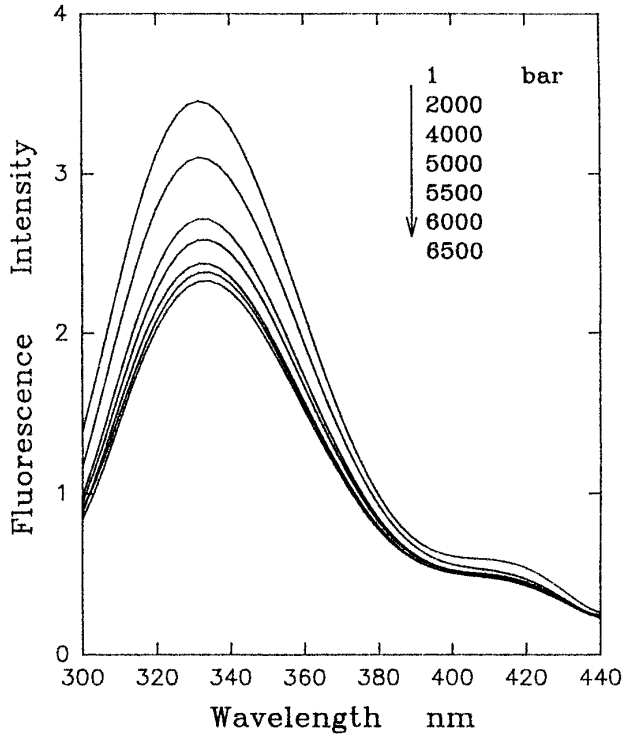

FIG. 5. Effect of high pressure on trypsin at 35°C. Other experimental conditions were the same as in Fig. 1.

about 6.5 kbar. Notably, previously reported MG states were obtained at lower pressure, such as those of cholinesterase and Arc repressor obtained at pressures lower than 3 kbar (13, 18) or ribonuclease A obtained at about 4 kbar (17). As reported here, the stable partly denatured state of trypsin was obtained at much higher pressure of 6.5 kbar, which is in a good agreement with the view that the structure of MG has probably a wide distribution from highly ordered MG (close to the native state) to pre-molten globule (close to the unfolded state) (24, 25), indicating that the energy needed to get MG should be different.

It was noticed that the stable partly denatured state of trypsin under pressure was obtained only at low temperature (i.e. closed to 0 ° C). At high temperature, 20 or 35 ° C, this transient denatured state could not be obtained in the pressure range from 1 bar to 7 kbar. At 20 ° C, the center of the spectral mass still had obvious decrease upon the further pressure increase from 6.5 kbar to 6.75 kbar, and the plateau observed in Figures 1 or 2 could not be obtained. More interesting is that at 35 ° C, there was almost no red shift in the trypsin intrinsic fluorescence spectrum to be observed, even if the applied pressure was as high as 5 kbar (see Figure 5). This means that the trypsin is more stable at high temperature under pressure, suggesting that the higher temperature could protect trypsin from pressure-denaturation, in a good agreement with the phase diagram proposed by Hawley (26).

ACKNOWLEDGMENTS

The authors thank Mr. J.-L. Saldana for technical assistance and Dr. J. Connelly for critical reading of the manuscript. This work was supported by a grant from INSERM/Academia China (K.R. and C.B.) and in part by La Direction de la Recherche et de la Technologie, Grant 94/05.

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