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Hydrophobic core of molten-globule state of bovine carbonic anhydrase B

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The interaction which stabilizes the intermediate state of the protein folding and/or unfolding is important for understanding the structure formation mechanism of proteins. The partitioning of a hydrophobic fluorescence probe, pyrene, into the core of a 'molten globule' structure of bovine carbonic anhydrase B was measured, revealing a partition coefficient of about 10^4. The result leads to the conclusion that the compact structure of the molten-globule state is formed by the hydrophobic interaction, as detergent micelles are formed by the same interaction.

1. Introduction

The study of intermediate conformations of proteins is important for understanding the mechanism of protein folding. In general, two kinds of information are required for elucidating the formation mechanism of intermediate states: one is the structure of a state and the other is the interaction stabilizing the state. Recently, a novel intermediate state, a molten-globule state of a soluble protein which has properties intermediate between the native and the completely unfolded states, has been described [1–3]. The structural features of the molten-globule state have been elucidated: the compactness and the secondary structure were maintained, while the tertiary structure was destroyed. As for the interaction stabilizing the intermediate, the importance of the hydrophobic interaction have been suggested [4]. However, quantitative measurements of the interaction have not been carried out. Therefore, the question in this work is whether the hydrophobic interaction is strong enough to form the globular structure of the intermediate state, as micellar aggregates of detergents are formed by the hydrophobic interaction.

Carbonic anhydrase B is suitable for such studies, because it is a globular protein of a single polypeptide with no S–S bonds and shows the intermediate structure in equilibrium [5,6]. Therefore, the denaturation behavior of carbonic anhydrase B has been studied extensively by means of circular dichroism, fluorescence, X-ray scattering, NMR, viscometry, kinetics and activity measurements [2,5–7,4,8–11], from which it has been established that the intermediate state is characterized by the compactness and the high mobility of side chains. Furthermore, the molten-globule state of carbonic anhydrase B was observed not only in equilibrium but also kinetically [2].

The hydrophobicity of a molecule or a residue is usually estimated by measuring the partition

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coefficient between a reference nonpolar solvent and water, from which the transfer free energy is calculated [12]. On the other hand, the hydrophobicity of a medium, i.e. organic solvents or microenvironments such as the inside of micelles and intermediate structure of proteins, may be evaluated from the partition coefficient of a reference probe molecule between the medium and water. A hydrophobic fluorescence probe, pyrene, is suitable for this purpose, because the fine structure of the fluorescence spectrum changes according to the polarity of the medium [13,14]. Therefore, the hydrophobicity of the core portion of the intermediate structure may be estimated quantitatively using the fluorescence spectra of pyrene irrespective of possible fluorescence intensity changes due to the turbidity increase or the bleaching of chromophore. The present work describes the importance of the hydrophobic interaction for the stability of the molten-globule state.

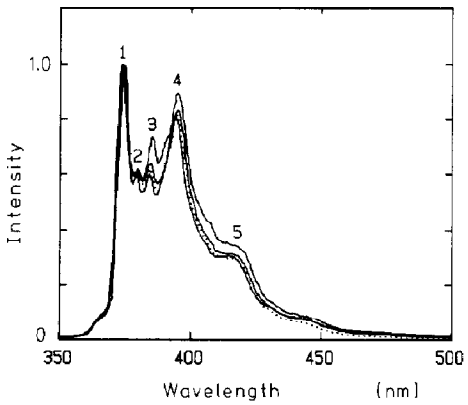


Fig. 1. The three solid curves are the pyrene fluorescence spectra of carbonic anhydrase solutions at 30 °C containing guanidinium chloride of 0 *M*, 1.6 *M* and 3.8 *M*. The concentration of carbonic anhydrase B was 51 μM and pyrene of 0.3 μM was used for the fluorescence measurement. The broken line represents the spectrum at 1.6 *M* of guanidinium chloride in the absence of protein. The intensity ratio of peak 3 to peak 1 is significantly larger at the denaturant concentration of 1.6 *M* than at 0 *M* and 3.8 *M*. This reveals that the environment of pyrene is hydrophobic at the intermediate concentration of guanidinium chloride.

2. Materials and methods

Bovine carbonic anhydrase B and pyrene were obtained from Sigma Chem. Co., and guanidinium chloride was purchased from Wako Chem. Co.

Fluorescence spectra were measured by Hitachi F3000. The excitation wavelength was 334 nm for the pyrene fluorescence measurements and 280 nm for the intrinsic fluorescence measurements. Circular dichroism measurements were carried out in a JASCO J-40AS. The secondary structure of protein was monitored at 222 nm, whereas the molar ellipticity at 274 nm was used for the study of the tertiary structure.

The solvent was buffered by a phosphate buffer of pH 7.0. The pyrene concentration was kept constant to 0.3 μM throughout the denaturation measurements.

3. Results and discussion

There are five major peaks in a pyrene fluorescence spectrum. The intensity ratio of peak 3 to peak 1 is dependent on the solvent polarity. Figure 1 shows the pyrene fluorescence of carbonic anhydrase B solution at 30 °C in the presence of guanidinium chloride. The spectrum is normalized by the intensity of peak 1, so that the change in the intensity ratio is visualized. The fluorescence intensity ratio appeared larger at a guanidinium chloride concentration of 1.6 *M* than at 0 *M* and 3.8 *M*. Figure 2 exhibits the intensity ratio of carbonic anhydrase B solution as a function of the guanidinium chloride concentration at 30 °C. The intensity ratio of the protein solution showed a maximum around 1.5 *M*, while only a gradual increase was observed in the absence of protein. The anomalous part of the intensity ratio indicates that a hydrophobic microenvironment appears in carbonic anhydrase B at the intermediate concentration range of guanidinium chloride.

By plotting the range, in which the difference of the intensity ratio between a protein solution and the solvent was larger than 0.1, a temperature vs. denaturant concentration phase diagram was obtained, as shown in Fig. 3. The phase space was divided into three areas, native (N), intermediate (I) and random coil (R) states. The intensity ratio was substantially the same as that of pure aqueous pyrene solution in the native and the random coil

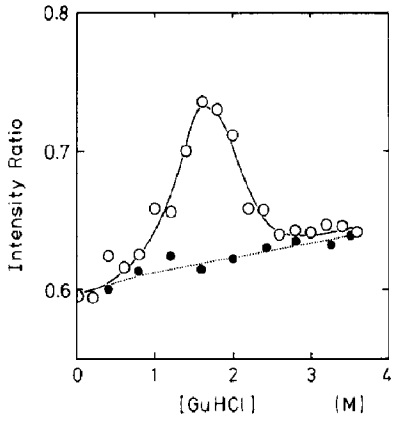


Fig. 2. The intensity ratio of pyrene in 51 μM carbonic anhydrase B solution (○) and in solvent (●) at 30 °C is plotted as a function of the guanidinium chloride. The anomalously large intensity ratio in the intermediate concentration range indicates the presence of a hydrophobic core in the molten-globule structure. On the other hand, the low intensity ratio shows that pyrene molecules are excluded from the hydrophobic core of the native structure and that there is no hydrophobic environment in the random coil structure.

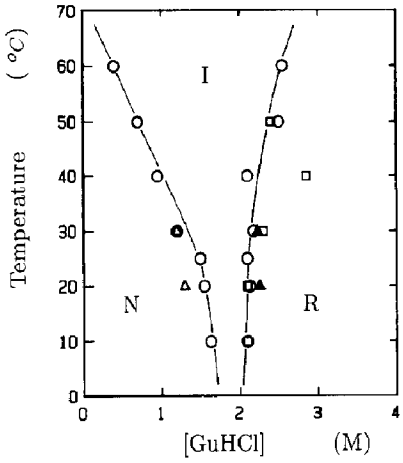
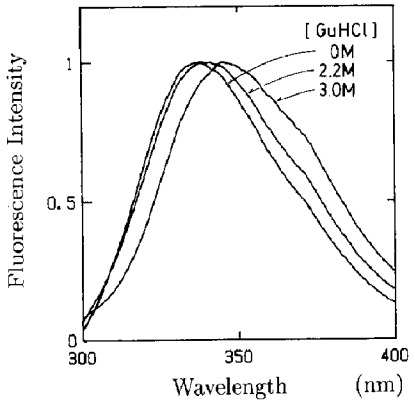


Fig. 3. The area of the intermediate state as measured by the pyrene fluorescence spectra is plotted by open circles (○) in a phase space of temperature vs. guanidinium chloride concentration. The denaturation points by the intrinsic fluorescence spectra (□), the molar ellipticity at 222 nm (▲) and at 274 nm (△) are also shown. There are three areas: the native (N), the random coil (R) and the intermediate (I) state, respectively. The intermediate state, which is characterized by the absence of the tertiary structure with the secondary structure maintained, rendering it stabler at higher temperature.

states. Figure 3 represents also the denaturation points as measured by the intrinsic fluorescence spectra, the molar ellipticity at 222 nm as well as 274 nm. The intrinsic fluorescence spectra in Fig. 4 indicate the red shift at the same guanidinium chloride concentration of the sharp decrease of pyrene fluorescence intensity ratio, that is, the branch of higher denaturant concentration in the phase diagram. The denaturation points by the circular dichroism measurements at 222 nm agrees to the results of fluorescence measurements. Therefore, the decreasing of the intensity ratio of



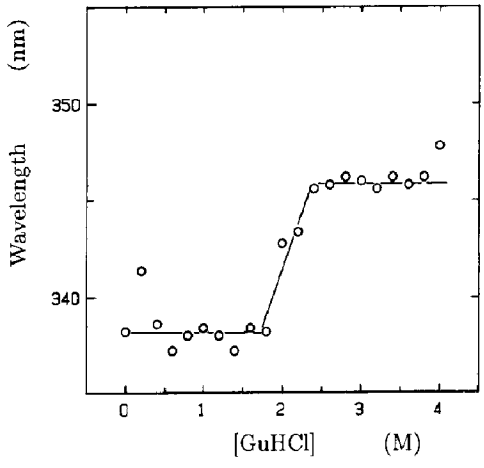


Fig. 4. The intrinsic fluorescence spectra of carbonic anhydrase B solutions are shown for guanidinium concentrations [GuHCl] of 0, 2.2 and 3.0 M. (a) The maximum wavelength of the intrinsic fluorescence at 20 °C is plotted as a function of guanidinium chloride concentration, (b) indicating stepwise red shift around 2 M.

pyrene fluorescence has good correlation with the disappearance of the secondary structure. On the other hand, the destruction of the tertiary structure monitored by the circular dichroism at 274 nm correlates well with the increasing phase of pyrene intensity ratio. The results of the circular dichroism measurements show good agreement with previous reports, in that the denaturation point at 222 nm differs with that at 274 nm [11]. This leads to the conclusion that the guanidinium concentration range of the anomalous intensity ratio corresponds to the molten-globule state.

The pyrene fluorescence behavior seems to clarify the formation mechanism of the molten-globule state. The reason for the low intensity ratio in the native state, as shown in Fig. 2, is the rigid protein structure of carbonic anhydrase B. Pyrene could not, therefore, penetrate into the hydrophobic core of the native structure. On increasing the concentration of guanidinium chloride from the native to the intermediate state, the rigid tertiary structure was destroyed gradually, providing room for pyrene molecules in the hydrophobic core. Further increase of the guanidinium chloride caused complete denaturation and the polypeptide chains were exposed to water. Therefore, the hydrophobic probes also became exposed to water. This is why the hydrophobic microenvironment gradually decreased and the intensity ratio of pyrene became low. It should be reasonable to assume that the hydrophobic microenvironment is provided by the molten core of the intermediate structure. In this area, the secondary structure was maintained with tertiary structure destroyed. Therefore, the intermediate structure, the molten-globule state, is similar to detergent micelles, in that it has a hydrophobic core and internal freedom of motion.

The degree of hydrophobicity in the molten-globule state may be estimated from the partition coefficient of pyrene between the hydrophobic core and the bulk water. A pyrene molecule binds to the hydrophobic core of the protein when this core is accessible to the pyrene molecule. The binding may be expressed formally by a first-order reaction (in protein concentration) of the binding constant K, when the fluorophore concentration is much lower than that of protein:

$$\mathrm{P} + \mathrm{F} \rightleftharpoons \mathrm{PF}, \tag{1}$$

$$K = \frac{[\mathrm{PF}]}{[\mathrm{P}][\mathrm{F}]}, \tag{2}$$

in which P, F and PF represent protein, fluorophore and their complex. Assuming that the fluorescence intensity ratio depends linearly on the fraction of bound fluorophore, the following equation is obtained,

$$\frac{R - R_0}{R_{\max} - R_0} = \frac{[\mathrm{PF}]}{[\mathrm{PF}]_{\max}} = \frac{[\mathrm{PF}]}{[\mathrm{F}]_{\mathrm{total}}} = \frac{K[\mathrm{P}]}{1 + K[\mathrm{P}]}. \tag{3}$$

Here, $[\mathrm{F}]_{\mathrm{total}}$ denotes the total concentration of the fluorophore, pyrene. In the case in which $[\mathrm{F}]_{\mathrm{total}}$ is much smaller than $[\mathrm{P}]_{\mathrm{total}}$, the protein concentration [P] is approximately equal to $[\mathrm{P}]_{\mathrm{total}}$. Figure 5 shows the anomalous part of the intensity ratio, $R - R_0$, as a function of the protein concentration, R_0 being the intensity ratio of pure pyrene solution of about 0.6. The result was in good agreement with eq. (3), with a binding constant of $1 \times 10^5\ M^{-1}$ in the intermediate state, whereas the binding constant of pyrene was very small in the native and the random coil states.

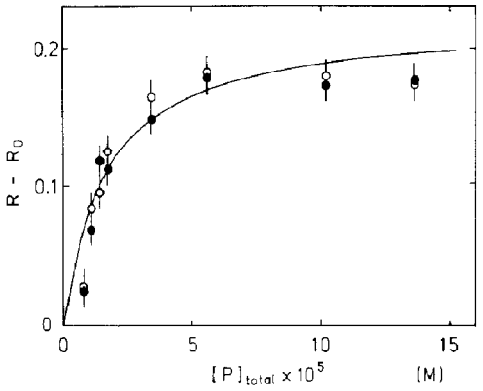


Fig. 5. The intensity ratio at guanidinium concentrations of 0 M (△), 1.0 M (○), 1.2 M (●) and 3.0 M (□) plotted as function of protein concentration at 40 °C. The experimental result could be analyzed well by a first-order reaction in [P] with a binding constant K of 1×10^5 l/mol in the intermediate state. Whereas, the binding in the native and the random coil states was rather low. Assuming the partition process of pyrene between the hydrophobic core and water as the binding mechanism, a partition constant of about 1×10^4 l/mol is obtained.

The binding of pyrene with carbonic anhydrase B has to be by some nonspecific mechanism, because the tertiary structure is destroyed and different hydrophobic probes bind to the molten globule state [4]. Because the maximum value of the anomalous intensity ratio was about 0.2 as shown in Fig. 5, the fluorescence intensity ratio of pyrene in the molten-globule state of carbonic anhydrase B has to be larger than 0.8. This value was in the same range as that in bulk alcohols [13], and mere adhesion of pyrene to the hydrophobic surface may not account for the large intensity ratio. Therefore, pyrene has to bind to the intermediate state through partitioning into the hydrophobic core of the molten globule structure. The partition coefficient κ is the ratio of pyrene concentration in the hydrophobic microenvironment to that in water:

$$\kappa = \frac{[PF]}{\phi[F]}, \tag{4}$$

in which ϕ is the volume fraction of the hydrophobic microenvironment in solution. Because the volume fraction ϕ is proportional to the protein concentration, the following proportionality between the partition coefficient and the apparent binding constant is obtained:

$$\kappa = \frac{1000\rho K}{M f_1 f_2}, \tag{5}$$

M and ρ being the molecular weight and the density of the protein, respectively. In this expression, two factors f_1 and f_2 are introduced. When the guanidinium chloride concentration is small, the native and the molten-globule states coexist and the fraction f_1 of opening of hydrophobic core has to be small. When the molten-globule structure transforms to the random coil state, as the guanidinium chloride concentration increases, the fraction f_2 of hydrophobic core to the total volume of protein decreases. The product $f_1 f_2$ has maximum at the guanidinium concentration of molten-globule state. The magnitude of $f_1 f_2$ has to be smaller than 1, because globular protein has hydrophilic skin regions and rigid secondary structures that will exclude probe molecules. The partition coefficient κ was estimated from the apparent binding constant as 2400 for $f_1 f_2 = 1$ and 12000 for $f_1 f_2 = 0.2$, respectively. This result leads us to the conclusion that the partition coefficient should be as large as 10^4 in order of magnitude. This value of the partition coefficient almost equals that of bulk liquid alcohols and (the inside of) detergent micelles, strongly suggesting that the globular form of the intermediate state is stabilized primarily by the hydrophobic interaction [15]. It is pointed out that the acid denaturation state of various globular proteins is similar to the molten-globule state of bovine carbonic anhydrase B described here [16,17]. It is suggested that the role of the hydrophobic interaction in the folding and the unfolding processes is different from those of the polar interactions.

According to the folding and the unfolding mechanism of globular proteins proposed by Saitô [18], α-helices and β-sheets are first formed and then assembled by hydrophobic interaction between hydrophopbic residues successively from the nearer ones to form the tertiary structure. On denaturation a reverse process is observed, keeping secondary structures intact with the hydrophobic core partially opened which is represented by a fraction f_1 introduced above. This is also consistent with the picture of the molten globule state proposed by Kuwajima which is composed of partly rigid and partly loose parts [3]. Thus the present experiment supports the essential role of the hydrophobic interaction in the folding and the unfolding of globular proteins as proposed by Saitô.

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