EVALUATION OF SURFACE HYDROPHOBICITIES DURING REFOLDING PROCESS OF CARBONIC ANHYDRASE USING AQUEOUS TWO-PHASE PARTITIONING SYSTEMS

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Key Words: Biochemical Engineering, Carbonic Anhydrase, Partition, Aqueous Two Phase, Protein Refolding, Hydrophobic Interaction, Molten Globule, Nonionic Detergent, Triton

Changes in conformational and surface properties in refolding processes were quantitatively investigated in aqueous two-phase systems, using bovine carbonic anhydrase (CAB) as a model protein. Surface net hydrophobicity (*HFS*) of a native CAB was determined as -84kJ·mol^{-1}, showing a moderately hydrophilic surface. By addition of 1.5-2M guanidine hydrochloride (GuHCl), CAB was denatured and HFS increased drastically up to about 300kJ·mol^{-1}, accompanied by some increment of local hydrophobicity. The fully unfolded state was achieved in 5M GuHCl. The unsteady change of local hydrophobicity during CAB refolding was also quantified using phase separation of Triton solution. Yield of CAB reactivation was enhanced by the addition of Triton X-405, due to hydrophobic interaction with hydrophobic residues exposed during the refolding process.

Introduction

Genetic modifications of microorganisms to produce eucaryotic proteins often cause the formation of inactive aggregates called inclusion bodies[1, 10]. Active target protein can be recovered by solubilizing the inactive aggregates in a strong denaturant, followed by refolding the protein to its native state. This protein refolding can be initiated by removal or dilution of the denaturants. Protein refolding processes have been investigated by several methods such as esterase activity[2], ANS fluorescence[13], quasi-elastic light scattering[1] and circular dichroism[4, 6]. During refolding, a protein undergoes a series of conformational changes including molten globule state to the native state, but in many cases these result in biologically inactive aggregates[2, 13]. It is, therefore, very important to understand surface properties, especially surface hydrophobicity and conformational changes in the intermediate states, in order to control time-consuming protein refolding processes.

In the present work, an attempt was made to quantify the surface net and local hydrophobicities together with secondary and tertiary structures and the enzyme activity of bovine carbonic anhydrase in its refolding state. The effect of addition of polymer and nonionic detergent on the refolding process was examined to improve refolding yields and process time.

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1. Experimental

1.1 Reagents

Bovine carbonic anhydrase (CAB, EC 4.2.1.1, MW = 30KDa, pI = 5.9) was purchased from Sigma Chemical Co. (Sigma). Guanidine hydrochloride (GuHCl) used for the denaturation of CAB was supplied by Wako Pure Chemical Industries, Ltd. (Wako). The substrate, *p*-nitrophenyl acetate (*p*NPA), used for the measurement of CAB activity, was supplied by Wako. Dextran (Dex) 100K-200K and polyethylene glycol (PEG) 1540, 4000, 6000 (MW = 1500, 3000, 7500) were supplied by Wako. Triton X series were supplied by Sigma. Other chemicals were of analytical grade.

1.2 Procedures

Three aqueous two-phase systems (ATPS) were utilized. The first ATPS used was PEG/Dex system of various molecular weights, such as PEG 1540 (10.8%), 4000 (9%), and 6000 (9%)/Dex 100K-200K (9%). The phase systems were prepared by mixing stock solutions of 30% Dex and 30% PEG together with protein and buffer solutions. CAB solutions were prepared in 10 mM Tris-HCl, pH = 7.4. After mixing by gentle inversions, the systems were centrifuged for 15 minutes at 5000 rpm at 25°C. The hydrophobic factor of phase system (*HF*) and that of protein surface (*HFS*) were determined by the previously used method[7, 8]. *HF* was determined by the partition behaviors of amino acids of which the relative hydrophobicities were defined by Nozaki and Tanford[11]. By plotting the natural logarithm of the partition coefficient of protein ($\ln K_{P,r}$) to respective *HF*, a straight line is observed. The slope of this line is *HFS*.

The second system was PEG 4000 (9%)/Dex

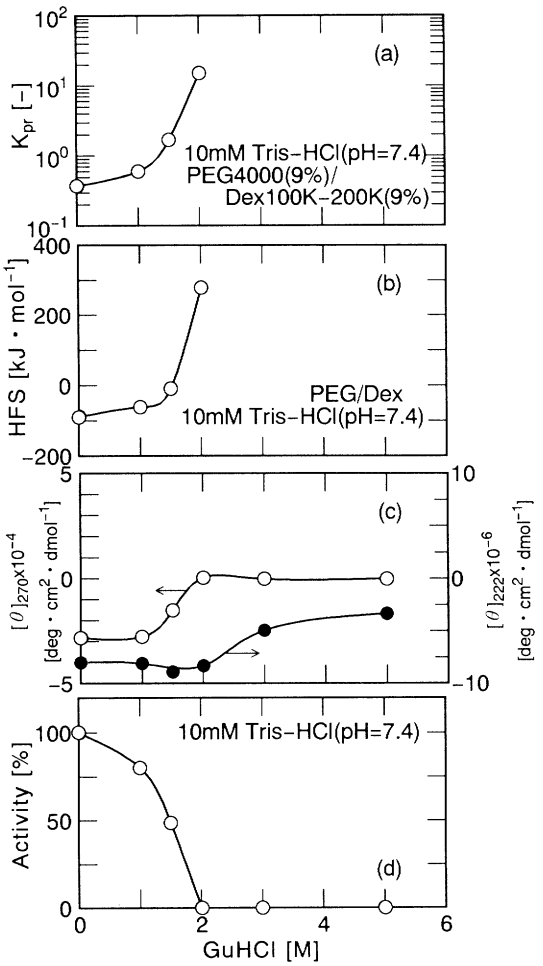

Fig. 1 Effects of concentration of GuHCl on (a) partition coefficient, (b) *HFS*, (c) molecular ellipticities at 270nm and 222nm and (d) activity of CAB

100K-200K (9%) with added nonionic detergent Triton X-405 (0-5 mM). The preparation method of this system was the same as the first ATPS except for the addition of Triton.

The third system was Triton X-114(1%)/salt (150 mM potassium phosphate, KPi) without PEG. Phase separation of the solution at increased temperature and salt concentration[9, 14] was utilized. CAB samples were prepared in 5 ml of 10 mM Tris-HCl, pH = 7.4, 150 mM KPi and 1% Triton X-114 at room temperature. The solutions were cooled to 0°C for 30 seconds and then raised to 30°C for 2 minutes. Clouding of the solution occurred. The tube was centrifuged for 2minutes at 3000 rpm at 30°C.

Partition coefficients (K_{Pr}) of CAB for the preceding two ATPSs, PEG/Dex with and without Triton X-405 systems, were determined by the concentrations in both phases of ATPS. Partition coefficients (K) for the third ATPS, Triton/KPi system, were determined by the ratio of the concentration in the bottom (Triton-rich) phase calculated from mass balance to the measured concentration in the top phase. Protein concentration was determined by the Pyrogallol red method[3]. Activity of CAB was determined by the esterase activity method

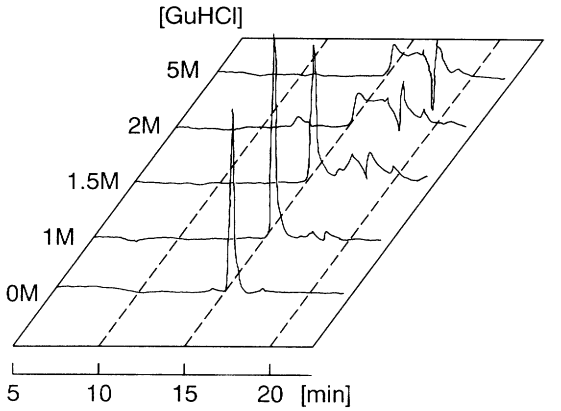

Fig. 2 Elution profiles of CAB equilibrated in 0.01 M Tris-HCl (pH=7.4), and with addition of various concentrations of GuHCl

using *p*-nitrophenyl acetate (*p*NPA) as a substrate following the previous method and a definition[1, 12]. The change of absorbance at 348 nm was measured by a UV spectrophotometer at room temperature. Recovery of activity was determined using the ester hydrolysis rate constant of the native protein at the same concentration. CD spectra were measured by using a Jasco spectropolarimeter. Molecular analyses were conducted by a size-exclusion HPLC equipped with a Shimazu Shim-pack Diol-150 column, and a UV-265FW as a detector followed at 280 nm.

2. Results and Discussion

2.1 Effect of GuHCl on surface net hydrophobicity and conformation of CAB

The effect of GuHCl concentration on the partition coefficients K_{Pr} is shown in **Fig.1**(a) together with (b) surface net hydrophobicities (*HFS*), (c) molecular ellipticities at 270 nm and 222 nm (θ_{270} and θ_{222}) and (d) esterase activities. The values of θ_{270} and θ_{222} were used for reflecting tertiary and secondary structure of protein respectively[2]. *HFS* value of CAB at its native state is -84 kJ·mol^{-1}. It is a hydrophilic protein like α-chymotrypsin. An increase in GuHCl concentration from 1.5M to 2M induces a drastic increase in K_{Pr} and *HFS*. This closely corresponds to the increase in θ_{270}, which shows loss of tertiary structure. A sharp decrease in esterase activity was also found.

Secondary structure, however, still remains in this region and the fully unfolded state is achieved in 5M GuHCl. In similar experiments, the complete denaturation of CAB has been observed in this condition[1].

The results were compared with those obtained with molecular exclusion chromatography. Elution profiles of CAB equilibrated in Tris buffer (pH=7.4) and CAB added with various concentrations of GuHCl are shown in **Fig.2**. The peak of native state appears after about 16 minutes. With increase in concentration of GuHCl this peak becomes smaller and new broadened peaks appear between 17 and 20 minutes. Three or more peaks appear in the elution profiles for denatured states.

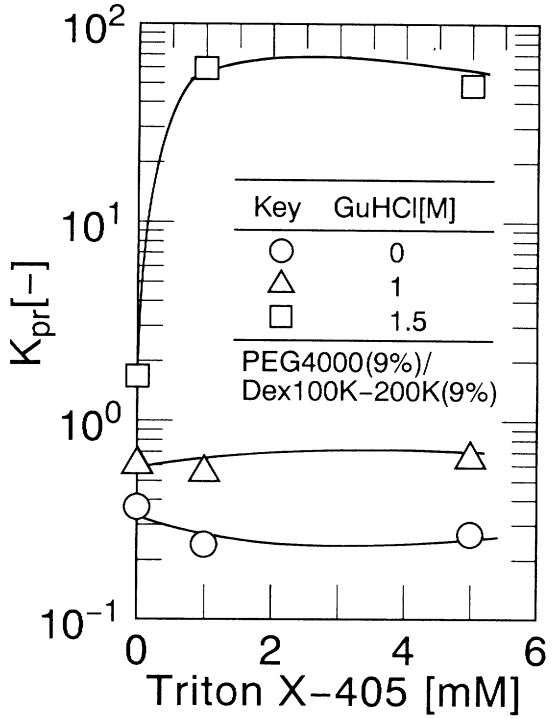

Fig. 3 Effect of addition of Triton X-405 on partition coefficient of CAB in native and denatured states

They are likely to correspond to peaks in native, intermediate and unfolded states of CAB respectively. This suggests that the denatured state is a mixture of these states. In the range of GuHCl concentrations less than 1.5M the first peak remains, indicating that the tertiary structure of native state is preserved to some extent. The elution profiles, however, have been reflected as a single peak in respective states in another experiment using gel exclusion chromatography[13]. A detailed comparison with other results is needed.

2.2 Variation of local hydrophobicities

The local hydrophobicities of CAB were estimated by the increment in partition coefficient, K_{Pr}, caused by the addition of Triton in the ATPS, that is, by the second ATPS. The effect of Triton addition on the partition behavior of CAB in the several states is shown in **Fig.3**. In the range of GuHCl concentrations up to 1M the effect was not so clear, though small peaks for denatured states are observed in Fig.2, while the addition of 1.5M GuHCl seems to cause a drastic increase in partition coefficients in the presence of 1 to 5mM Triton. The coefficients are 40 times those in the absence of Triton.

Surface net hydrophobicity (*HFS*) was increased with the addition of 1.5M GuHCl, as shown in Fig.1(b). Significant increase in local hydrophobicity clearly indicates that hydrophobic residues, which bind strongly with Triton, have been exposed. The hydrophobicity in intermediate state or molten globule state has been observed to be very high[5, 13, 15]. The transition from the native state to intermediate (molten globule) state is likely to occur in the concentration range from 1.5M to 2M GuHCl.

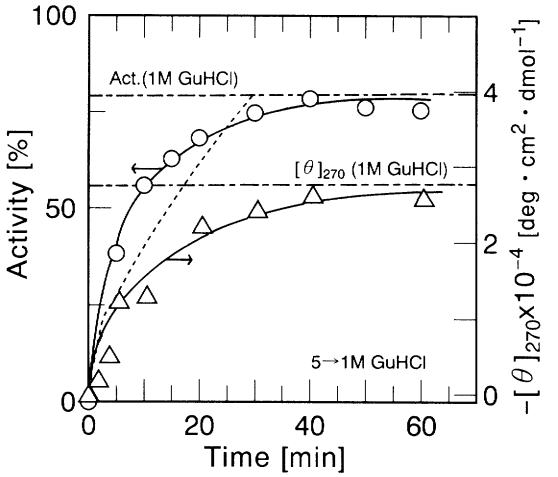

Fig. 4 Time dependences of activity and molecular ellipticity at 270 nm during CAB refolding at final concentrations of 0.1mg·ml^{-1} CAB and 1M GuHCl. The dashed line represents reactivation of CAB at final concentrations of 0.5 mg·ml^{-1} CAB and 1M GuHCl[1].

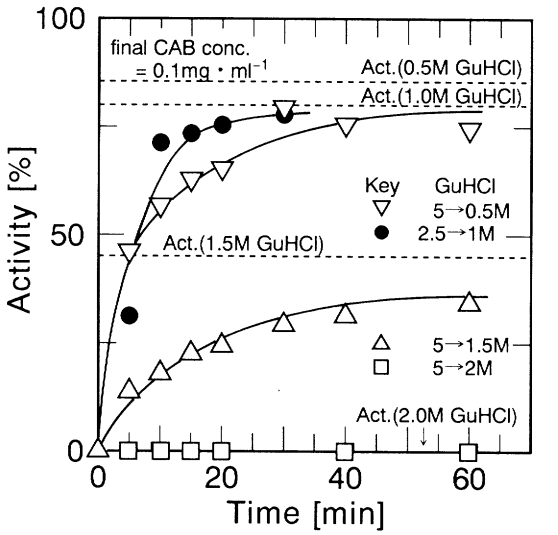

Fig. 5 Reactivation of CAB when diluted from 5M GuHCl to various concentrations

2.3 Variations of activity and conformation of CAB during refolding process

Transient behavior of the CAB refolding process was investigated using the time variation of the recovery of esterase activity initiated by dilution of the GuHCl concentration from 5M to 0.5-2M. Variations in activity and θ_{270} with time in CAB refolding are shown in **Fig.4**. The protein was diluted to a final concentration of 0.1 mg·ml^{-1}. Both values increased with time, finally approaching asymptotes that corresponded to the data obtained otherwise with 1M GuHCl. The dashed line in Fig.4 represents reactivation of CAB reported by others[1]. The condition is different only in the final concentration of CAB (0.5 mg·ml^{-1}) from the present experiment (0.1 mg·ml^{-1}). Although the rate is slightly different in the initial stage of refolding, the yield after 30 minutes agrees very well, indicating that the activity of CAB is closely related to tertiary structure.

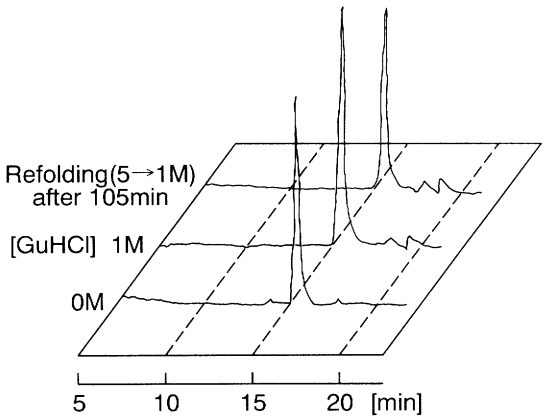

Fig. 6 Elution profiles of native CAB, CAB in 1M GuHCl and refolded CAB by dilution from unfolded state in 5M GuHCl to 1M

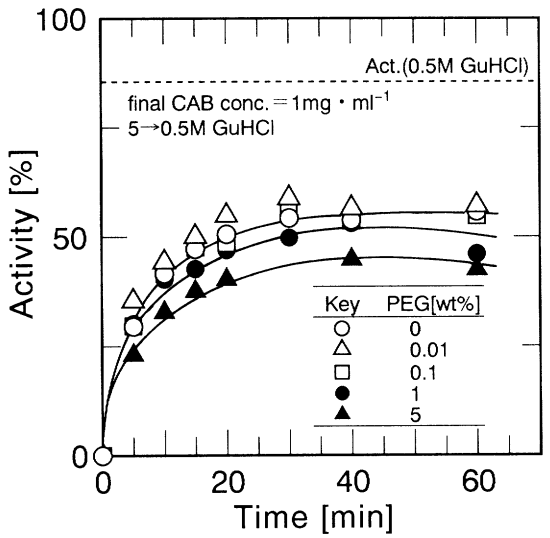

Fig. 7 Effect of addition of PEG4000 on reactivation of CAB

Other data of reactivation of CAB by dilution obtained under different conditions are shown in **Fig.5**. The activities approach the final values obtained otherwise with respective concentrations of GuHCl. The refolding of protein has been considered to be conformational change towards native state from denatured state[1, 2, 13]. The present results suggest that the conformational change is reversible and dependent on the final concentration of GuHCl in the CAB refolding process.

Elution profiles of molecular exclusion chromatography are shown in **Fig.6**, for native CAB, partly unfolded CAB in 1M GuHCl and refolded CAB by dilution from 5M to 1M GuHCl. The elution profile after 105 minutes refolding is more similar to that of the partly unfolded CAB than to that of the native state. This result is in line with those obtained with the variations of activity and conformation of CAB.

2.4 Effect of addition of polymer or nonionic detergent in the refolding process

Since CAB at the intermediate state has exposed hydrophobic binding sites, the addition of polymer or Triton may have some effects on the refolding yield and

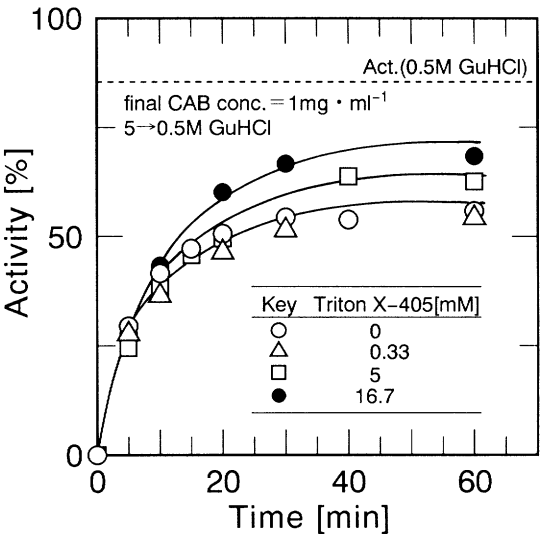

Fig. 8 Effect of addition of Triton X-405 on reactivation of CAB

process time. The effects of PEG4000 and Triton X-405 are shown in **Figs.7** and **8** respectively. In these cases, refolding is performed at 0.5M GuHCl and 1 mg·ml^{-1} protein. It is reported that hydrophobic aggregation occurs in this condition[1]. In the absence of additives, the yield of CAB reactivation is at most 50% because of hydrophobic aggregation. Reactivation of CAB is not improved by a small amount of PEG such as 0.01 to 0.1 wt%. Further increase in PEG concentration seems to inhibit the reactivation. This may be caused by a strong stabilizing effect of PEG on hydrophobic aggregation or salting-out effect. Reactivation of CAB is, however, enhanced by the addition of Triton. The transient weak binding of Triton with hydrophobic residues of CAB may inhibit hydrophobic aggregation of CAB, and thus enhance the refolding process.

2.5 Variation of local hydrophobicity during CAB refolding

Transient variation of local hydrophobicity during CAB refolding was evaluated by the phase separation of Triton solution, that is, by the method using the third ATPS[9]. This method is suitable for measurement of unsteady-state phenomena, since quick operation is possible. In this system, hydrophobic proteins partition in Triton phase due to hydrophobic binding with Triton. The K value is inverse to the general $K_{P,r}$. Both values correspond in that the concentrations of protein in a more hydrophobic phase like PEG or Triton phase are used as numerator when calculated. Variations of activity and partition coefficient (K) with time after dilution obtained by phase separation of Triton solution in the CAB refolding are shown in **Fig.9**. In the initial stage of the refolding, the partition coefficient of CAB is high and CAB preferably partitions to the Triton phase. The local hydrophobicity decreases with CAB refolding.

Ptitsyn *et al.*[13] studied the refolding process of CAB by using a hydrophobic probe ANS. The hydrophobicity change is measured by a sharp increase of

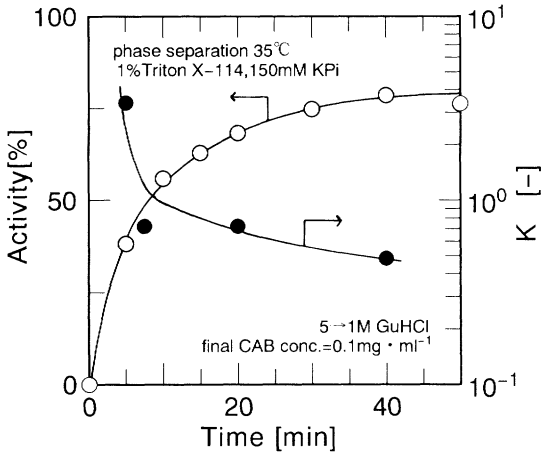

Fig. 9 Time dependences of activity and partition coefficient measured by phase separation of Triton solution during CAB refolding

ANS fluorescence upon its binding to a protein molecule. They confirmed that an intermediate (molten globule) state exists during the refolding in various proteins and shows a very hydrophobic surface. Their results support the present data, though the principles of the two experiments are different. Binding of Triton with CAB is found to be rather reversible, and the present method is effective in quantifing variations in conformational state and surface properties.

Conclusion

The variation of hydrophobicity with conformational change in the refolding process of CAB was evaluated, using aqueous two-phase systems (ATPS), with the following results.

(1) Surface net hydrophobicity (*HFS*) and local hydrophobicity originating from the hydrophobic binding sites on the surface of CAB were quantified in the native state and in various conformational (denatured) states.

(2) The change of conformational states depends on the final concentration of GuHCl in the course of CAB refolding. It is a reversible transformation towards the equilibrated state under respective conditions.

(3) The local hydrophobicity change during CAB refolding was quantified, using the phase separation method with Triton solution. The protein refolding proceeds with variation of hydrophobicity, and a strongly hydrophobic intermediate state is induced in the initial stage of refolding.

(4) In the refolding process, yield of CAB reactivation was enhanced by the addition of Triton X-405. In the case of PEG4000, reactivation was not affected significantly compared with no additive, and inhibit ion was observed at a high concentration of PEG. This is induced by hydrophobic interaction between the additive and hydrophobic residues exposed during the refolding process. The transient binding of Triton plays a role in protection of the aggregation between hydrophobic residues, and the addition of PEG is likely to induce precipitation of CAB.

Nomenclature

HF	=	hydrophobic factor of two-phase system	[mol·kJ^{-1}]
HFS	=	surface net hydrophobicity of protein	[kJ·mol^{-1}]
K	=	partition coefficient of CAB measured for phase separation of Triton solution	[-]
K_{Pr}	=	partition coefficient of CAB measured for PEG/Dex systems	[-]
θ_{270}, θ_{222}	=	molecular ellipticities at 270 and 222 nm	[deg·cm^2·dmol^{-1}]

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