THE EFFECT OF HYDROCHLORIC ACID ON HYDROPHOBICITY AND PARTITION OF PROTEIN IN AQUEOUS TWO-PHASE SYSTEMS

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Key Words: Aqueous Two-Phase, Surface Hydrophobicity, Refolding, Molten Globule, Cytochrome c, Acid-Denaturation

Introduction

The partition behavior of proteins in aqueous two-phase systems is affected by surface properties such as net charge and surface hydrophobicity. Effective separation of the proteins can be achieved by exploiting the differences in their surface properties[5]. The surface properties of a proteins are dependent on its conformational structure, which can be altered in the presence of affinity ligands, salts and other ionic species[6]. Little attention has, however, been paid to the effect on partition behavior of modifications to the protein structure.

Recently, Goto *et al.*[3] have studied acid denaturation of proteins, and found that several proteins such as cytochrome c and apomyoglobin change from native to an unfolded conformation (random coil) by the addition of a small amount of HCl at low ionic strength. By further addition of HCl, these molecules refold into a compact structure with a secondary structure similar to the native state but with disordered tertiary structures (specific molten globule state). Other proteins such as lysozyme and T4lysozyme are found to show only slight acid-induced unfolding and refolding. The major factor influencing the conformational transition is considered to be electrostatic interaction between the anions in the system and the positively charged amino acid residues of the proteins. The resulting electrostatic repulsion prevails over hydrophobic interaction in an unfolded state, and this is then followed by hydrophobic refolding in a molten globule state.

The present work is carried out as an extension of the previous work[5,6]. The acid denaturation of proteins is used as a typical example of structural or conformational change caused by the binding of chemical species to proteins. The acid-induced structure conformational change of several proteins has been measured, together with the resulting surface properties and partition behavior of the system.

1. Experimental

1.1 Materials

Dextran (Dex) 60000–90000 and Dex100000–200000 were supplied by Wako Pure Chemical Ltd. (Waco). Polyethylene glycol (PEG) 1540, 4000, 6000 (Mw= 1500, 3000, 7500) were also supplied by Wako. Horse cytochrome c (Type III) and papaya papain (Type III) were supplied by Sigma. Chicken lysozyme was supplied by Wako. Apomyoglobin was prepared from horse myoglobin (Sigma) by 2-butanone extraction of heme[4]. HCl was supplied by Wako as analytically pure reagent.

1.2 Methods

Systems with the following total composition, (9%) Dex, (9–13%) PEG and (0–480 mmol/kg) HCl, were used. The phase systems were prepared by mixing stock solutions of 30% Dex and 30% PEG together with HCl and protein solutions. The protein concentration in the solution was 1 mg/ml. After mixing by gentle inversions at 4°C, the systems were centrifuged at 5000 rpm for 20 min. The protein concentrations were measured using the pyrogallol red method[2]. The colour developed was measured

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at an absorbance of 600 nm. Circular dichroism (CD) measurements were carried out at a protein concentration of 0.1 mg/ml using a Jasco spectropolarimeter (Model J-500A) at 25°C.

1.3 Measurement of the surface hydrophobicity of proteins (*HFS*)

The change in *HFS* after acid denaturation was determined by the method described in the previous work[5]. The following four systems with different hydrophobic factors were utilized: (i) PEG1540 (10.8%)/Dex10^5–2×10^5 (9%), $HF=7.03\times10^{-3}$ mol/kJ, (ii) PEG1540 (13%)/Dex6×10^4–9×10^4, $HF=9.02\times10^{-3}$, (iii) PEG4000 (9%)/Dex10^5–2×10^5 (9%), $HF=1.26\times10^{-2}$, and (iv) PEG6000 (9%)/Dex10^5–2×10^5 (9%), $HF=2.00\times10^{-2}$. The effect of PEG molecular weight on the conformational transition of the present proteins was examined by CD spectra and was found to be insensitive to PEG molecular weight in the range observed.

2. Results and Discussion

2.1 Partition behavior and conformation of proteins

The effect of various amounts of HCl on the partition behavior of several proteins is shown in **Fig. 1**(a). The acid, added in the amount of 30 mM, has a dramatic effect in enhancing the partition of the two hydrophilic proteins cytochrome c and apomyoglobin. The partition coefficients then decrease exponentially with increasing HCl concentration, but a further increase from 300 to 500 mM has a slight effect on the partition. The partition of papain is also affected by adding 60 and 120 mM HCl. The data for papain are, however, very limited, since it precipitates upon the addition of 480 mM HCl. For the partition of lysozyme, HCl has less effect, and the partition coefficients increase slightly upon the addition of 60 mM HCl.

The effect of HCl on the ellipticity at 222 nm, (θ_{222}), of the protein in the solution with the same composition as the top phase of the system is shown in Fig. 1(b). HCl, added up to 60 mM, has a significant effect on the ellipticity of cytochrome c and apomyoglobin as shown in Fig. 1(a) for the partition behavior of the two proteins. The ellipticity then decreases with increasing HCl concentration to respective asymptotic values. The ellipticity of lysozyme, on the other hand, is rather insensitive to HCl. These results suggest that the conformation of cytochrome c and apomyoglobin changes from the native to the unfolded state by addition of a small amount of HCl, and then changes to a compact conformation containing substantial secondary structure at higher HCl concentrations. The constant ellipticity observed for lysozyme suggests that no significant unfolding occurs. The results of the ellipticities and interpretation for cytochrome c and

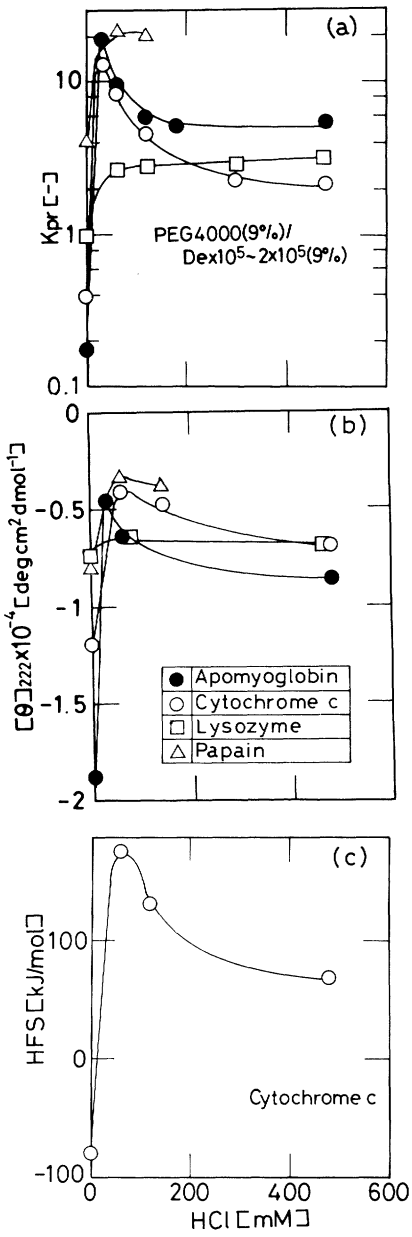

Fig. 1. Effect of HCl on partition coefficient, ellipticity and surface hydrophobicity

apomyoglobin are in line with those presented by Goto *et al.*[3].

2.2 Surface hydrophobicity of proteins

The value of the surface hydrophobicity of the protein (*HFS*) has been correlated with the hydrophobic factor of the system *HF* and the partition coefficient of the protein K_{pr} in the system by the following equation[5]:

$$\ln K_{pr} = HF \times HFS \qquad (1)$$

The values of *HFS* of the native and acid-denatured proteins were determined by partitioning the proteins in the four systems with $HF=7.03\times10^{-3}$, 9.02×10^{-3}, 1.26×10^{-2} and 2.00×10^{-2} mol/kJ. The values of *HFS* thus obtained are plotted against HCl

concentration in Fig. 1(c) for cytochrome c. The variation of *HFS* is seen to correspond closely with that of the partition coefficient K_{pr} and the ellipticity θ_{222} show in Figs. 1(a) and (b). A linear relationship between K_{pr} and θ_{222} and between *HFS* and θ_{222} can be seen in **Fig. 2**(a) and (b). This indicates that the partition coefficient, i.e. the surface hydrophobicity of the protein, directly reflects the conformational transitions in the partition systems employed.

The low *HFS* value for the native state indicates that the hydrophobic residues are buried in the interior of the protein. The partition coefficient of native proteins is therefore low, and the protein is preferentially partitioned to the more hydrophilic bottom phase. When HCl is added, proteins change to the unfolded state because of electrostatic repulsion forces. The hydrophobic residues are thus exposed at the protein surface and the protein becomes most hydrophobic. With further addition of HCl, however, the protein changes to a compact conformation (refolding) as a result of the neutralization of positive charges by anions in the system and thus the partition coefficient decreases. A different behavior is seen for lysozyme. It maintains its native-like secondary structure in the presence of the acid, as shown in Fig. 1(b). A small increase in the partition coefficient in Fig. 1(a) and Fig. 2(a) is likely to be caused by a small change in its tertiary structure.

2.3 Hydrophobicity difference caused by folding

Assuming that the fully unfolded state of cytochrome c, apomyoglobin and papain may be attained in the extreme case of their ellipticities being zero, an approximate estimation of the partition coefficient and accordingly the surface hydrophobicity in this state can be made from the linear relationship shown in Fig. 2. The partition coefficient, *K*, the surface hydrophobicity in the native state, *HFS* and the difference in surface hydrophobicity (ΔHFS) between the native state and the fully unfolded state are shown in **Table 1** for cytochrome c, apomyoglobin and papain. Eisenberg and McLachlan[2] showed that the solvation free energy of folding of haemerythrin and that of (V_L) domain which has 113 side chains are −472.34 kJ/mol and −443.08 kJ/mol, respectively. Cytochrome c, apomyoglobin and papain have 103, 152 and 211 side chains, respectively. The have, thus, estimated *HFS* values of the same order as the solvation free energy of folding shown in Table 1.

Conclusion

The effect of hydrochloric acid-induced conformational change of several proteins on surface hydrophobicity and thus partition behavior have been studied in the PEG and dextran aqueous two-phase system. The partition coefficient, the surface hydrophobicity and the conformational change of proteins

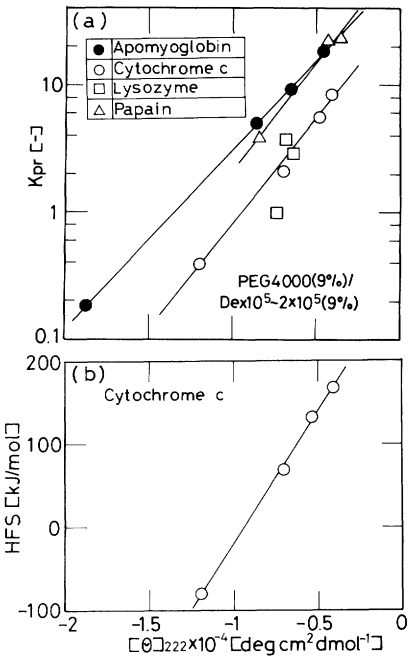

Fig. 2. Relationship between partition coefficient and ellipticity, and between surface hydrophobicity and the ellipticity

Table 1. The partition coefficient in unfolded state, the surface hydrophobicity in the native state and the difference in surface hydrophobicity between native state and unfolded state ΔHFS ($= HFS_{native} - HFS_{fully\ unfolded}$).

	K [—]	*HFS* [kJ/mol]	ΔHFS [kJ/mol]
Cytochrome c	31.5	−98.4	−365
Apomyoglobin	86.5	−110	−491
Papain	97.5	43.7	−253

are found to be directly related, and the structural changes resulting from binding various ligands to the proteins will therefore be very effective in separating proteins by phase partitioning. Partitioning in an aqueous two-phase system can be used as a highly sensitive and versatile method for analysis of the properties of the protein surface.

Acknowledgments

The authors gratefully acknowledge Dr. Y. Goto, Dept. of Biology, Osaka University, for his valuable suggestion to initiate this work.

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