

# EVALUATION OF SURFACE NET AND LOCAL HYDROPHOBICITIES DURING ACID- AND SALTS-INDUCED CONFORMATIONAL CHANGE OF CYTOCHROME C USING AQUEOUS TWO-PHASE PARTITION SYSTEMS

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**Key words:** Aqueous Two-Phase, Cytochrome c, Native, Unfolded, Molten-Globule, Conformational Change, Surface Net Hydrophobicity, Local Hydrophobicity

Quantitative correlation between the variation of surface net and local hydrophobicities and the conformational change of cytochrome c induced by added acid and salts was investigated by using aqueous two-phase partitioning systems (ATPS). Cytochrome c is unfolded at pH 2 under conditions of low ionic strength, but it is refolded to the molten-globule state by further addition of acid. By addition of salts, a conformational change from the unfolded to the molten-globule state by the anion binding to the positive charge of a protein was also observed. Surface net and local hydrophobicities observed by using the ATPS method clearly show significant differences in these three conformational states, in contrast to the conventional CD measurement. The local hydrophobicity, which can be evaluated by the increment of partition coefficient by the binding of Triton added to the ATPS, is greatest for the molten-globule state, whereas its surface net hydrophobicity is intermediate between the unfolded and native states.

## Introduction

Various surface properties of proteins and enzymes have been characterized by using the aqueous two-phase partition method, such as surface net and local hydrophobicities and net charge, since their partition coefficients in aqueous two-phase systems (ATPS) closely reflect these properties<sup>7, 8, 12</sup>. These quantitative analyses of surface properties have been successfully utilized for the rational design and development of various bioseparation processes and for novel enzyme production in the rapidly expanding field of protein engineering<sup>13</sup>.

In relation to the protein refolding and stability analysis, acid-induced denaturation of proteins such as cytochrome c and apomyoglobin has been recently studied<sup>6, 9, 12</sup>. The conformation of a protein is known to translate from the native state to the unfolded state by the addition of HCl in the absence of salts. By the further addition of HCl, however, it is translated into a third molten-globule state<sup>12</sup>. A random coil disordered secondary and tertiary structure and hydrophobic residues exposed at the protein surface are the characteristic properties of the unfolded state. The molten-globule state has a compact structure with a high content of secondary structure and with largely disordered tertiary structure in comparison with the unfolded state<sup>2, 6</sup>.

The conformational change of proteins and polypeptide caused by the binding of various surfactants as well as the interactions between proteins and surfactants have been

studied<sup>4, 10</sup>. In these, the addition of an ionic surfactant is thought to cause the conformational change of a protein from a random coil to some secondary structure such as  $\alpha$ -helix by decreasing electrostatic repulsion<sup>4</sup>.

In the present work, an attempt has been made to quantify the surface net and local hydrophobicities and the secondary structure of these three conformational states of cytochrome c using an aqueous two-phase system. In order to obtain a rather complete picture, the conformational change has been induced by various salts and a non-ionic surfactant, Triton, in addition to hydrochloric acid.

## 1. Material and Method

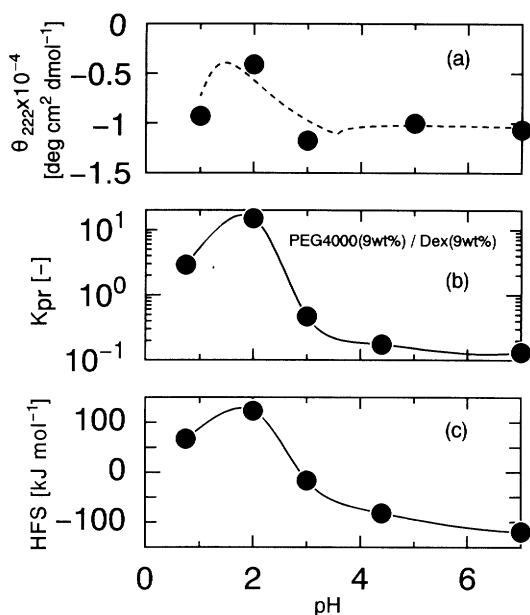
### 1.1 Materials

Dextran 100-200K and polyethylene glycol (PEG) 1540, 4000, 6000 (MW = 1500, 3000, 7500Da) were obtained from Wako Pure Chemical Ltd. Horse cytochrome c (type 3) and Octylphenylether (Triton X) series (X-100, 405) were obtained from Sigma. KCl, NaCl, NaClO<sub>4</sub>, sodium trifluoroacetate (TFANa) and other chemicals used were of analytical grade from Wako.

### 1.2 Methods

**1) Partition procedure** PEG 1540 / Dex (10.8/9wt%), PEG 4000 / Dex (9/9wt%) and PEG 6000 / Dex (9/9wt%) were utilized as aqueous two-phase systems (ATPS). The phase systems were prepared by mixing stock solutions of 30% Dex and 30% PEG together with protein solution. pH was adjusted by the addition of an appropriate amount of

\* Received August 31, 1994. Correspondence concerning this article should be addressed to R. Kuboi.



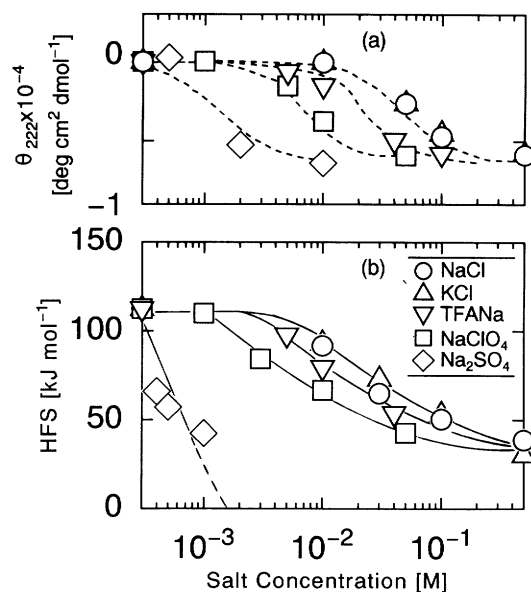
**Fig. 1** Effect of pH on (a) molecular ellipticities at 222 nm, (b) partition coefficient and (c) *HFS* of cytochrome c (15  $\mu$ M). The broken line is the result from Goto *et al.*<sup>2)</sup>.

HCl.

After mixing by gentle inversion, the systems were centrifuged for 10 min at 3,000 rpm at 25°C. Partition coefficients of cytochrome c ( $K_{pr}$ ) were determined from its concentrations in both phases of the ATPS. Protein concentrations were measured by the absorbance at 410 nm using a Shimadzu UV-visible spectrophotometer (Model UV-160A). The hydrophobic factor of the phase system (*HF*), surface net hydrophobicity (*HFS*) and local hydrophobicity ( $\Delta \ln K_{pr}$ ) of the protein surface were determined by the methods previously described<sup>8, 13)</sup> and only a brief description is made here.

The value of hydrophobic factor for the ATPS (*HF*) was determined by the partition behaviors of amino acids of which the relative hydrophobicities were defined by Nozaki and Tanford<sup>11)</sup>. By plotting the partition coefficient ( $K_{pr}$ ) of the protein to the respective *HF*, a straight line was obtained. The slope of this line was defined as surface net hydrophobicity (*HFS*) of the protein. Local hydrophobicity ( $\Delta \ln K_{pr}$ ) was determined by the increment of partition coefficient by the addition of Triton in the PEG 4000 / Dex.

**2) Circular dichroism measurements** CD measurements were carried out with a Jasco spectropolarimeter (Model J-500A) at 25°C. The results were expressed as mean residue ellipticity ( $\theta$ ), which was defined as  $\theta = 100\theta_{obsd}/lc$ , where  $\theta_{obsd}$  was the observed ellipticity in degree,  $c$  was the concentration of residue in moles per liter, and  $l$  was the length of the light path in centimeters<sup>2)</sup>. The CD spectra were measured at protein concentration of 15  $\mu$ M with a 1 mm path cell at 222 nm.



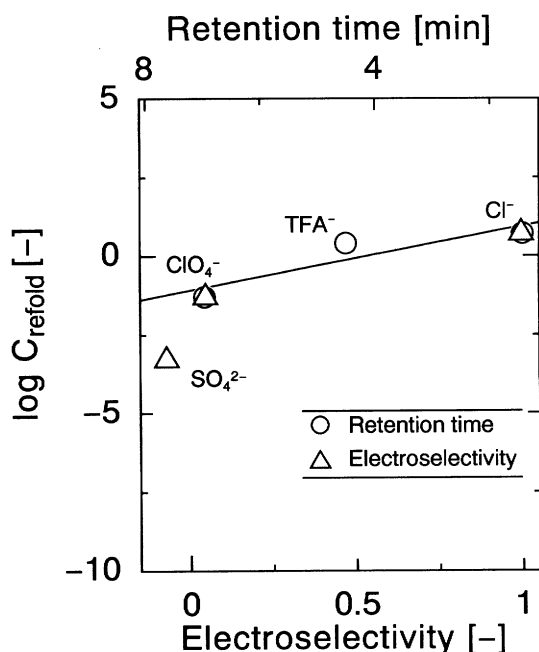
**Fig. 2** Effects of concentration of various salts on (a) molecular ellipticities at 222nm and (b) *HFS* of cytochrome c (15  $\mu$ M). Initial state: unfolded state by addition of 18mM HCl. The broken lines are the results from Goto *et al.*<sup>2)</sup>.

## 2. Results and Discussion

### 2.1 Effect of HCl on surface net hydrophobicity and conformational change of cytochrome c

The effect of HCl on molecular ellipticities at 222 nm ( $\theta_{222}$ ) is shown in **Fig. 1(a)** together with (b) partition coefficients ( $K_{pr}$ ) and (c) surface net hydrophobicities (*HFS*). The values of  $\theta_{222}$  are used for the quantitative measure of secondary structure of a protein. The line is drawn based on the experimental data. The present results of  $\theta_{222}$  correspond well with those of Goto *et al.*<sup>2)</sup> shown by the broken line. It was also reported that each conformational state was determined by pH and ionic strength<sup>2, 6)</sup>. Thus, the conformational states were evaluated based on the values of  $\theta_{222}$  as well as the conditions of pH and ionic strength employed. Addition of HCl induces a conformational change to the unfolded state, which has a disordered secondary structure due to electrostatic repulsion forces. Hydrophobic residues are, therefore, exposed at the protein surface and the protein becomes more hydrophobic. By the further addition of HCl, however, its secondary structure is reformed to the molten-globule state by neutralization of the positive charge of the protein. Some hydrophobic residues are buried again in the interior of the loosely packed structure<sup>2)</sup>, and therefore, its surface net hydrophobicity is expected to be intermediate<sup>12)</sup>.

The observed *HFS* value for the native state is -113 kJ/mol at pH 7. This low value indicates that most hydrophobic residues are buried in the interior of the protein. Therefore, the protein was preferentially partitioned to the relatively hydrophilic bottom dextran-rich phase rather than hydrophobic PEG-rich top phase and the partition coefficient ( $K_{pr}$ ) was low. In contrast with the native state, the *HFS* value for the unfolded state at pH 2

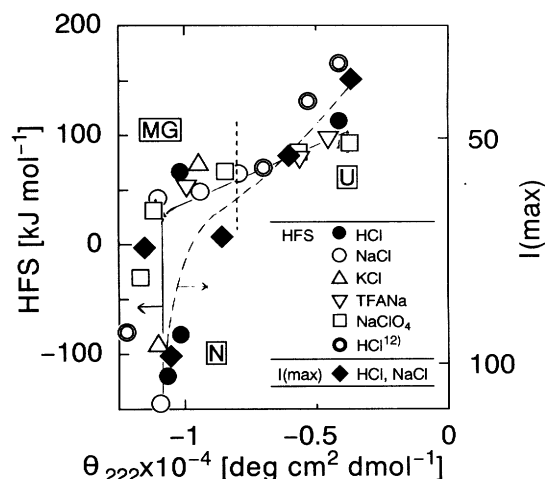


**Fig. 3** Relationship among selectivity or retention time of anion-exchange resins and the critical refolding concentration. Retention time and electroselectivity are the results from Gjerde, D.T. *et al.*<sup>1)</sup> and Gregor, H.P. *et al.*<sup>3)</sup>, respectively.

is over 100 kJ/mol, which shows that most hydrophobic residues in the interior of the protein may be exposed at the protein surface by the conformational change. Therefore, the protein was partitioned to the relatively hydrophobic top phase and  $K_{pr}$  became a high value of about 20. In the case of the molten-globule state,  $HFS$  and  $K_{pr}$  show intermediate values, about 66 kJ/mol and 2.9, respectively. These results clearly show that at least some hydrophobic residues are partly buried.

## 2.2 Effect of various salts on conformational change and surface net hydrophobicity of cytochrome c

Effects of the addition of various salts on  $\theta_{222}$  and  $HFS$  are shown in Fig. 2(a) and (b), respectively. In all experiments, 18 mM HCl was added to induce the same initial unfolded state before addition of salts. The results obtained by Goto *et al.*<sup>2)</sup> are also shown with broken lines in Fig. 2(a). All salts induced cooperative changes to the molten-globule state, whereas the concentration range of the salts required to bring about the change varied among salts. Corresponding to this conformational change, surface net hydrophobicity ( $HFS$ ) also decreased in the same concentration range. The  $HFS$  change due to the change to the molten-globule state caused by all the monovalent anions employed is 60–70 kJ/mol, which is similar to that in Fig. 1, but that for the divalent anion seems to be larger than the above value. This difference is expected from the results of Fig. 2 (b) and may be caused either by the increase in electrochemical potential between the two liquid phases of the systems or by the formation of a more compact form due to the decrease in electrostatic repulsion.

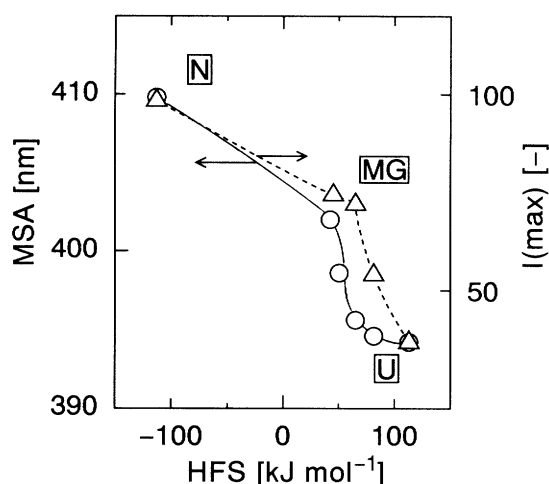


**Fig. 4** Relationship between  $HFS$ , compactness,  $I(max)$ , and molecular ellipticities at 222 nm,  $\theta_{222}$ .  $I(max)$  cited from Kataoka *et al.*<sup>6)</sup>. The experimental conditions of  $HFS$  are the same as those of Fig. 2 and Fig. 3.

The data obtained with NaCl and KCl show that  $\theta_{222}$  starts to decrease more slowly than for sodium salts such as NaClO<sub>4</sub> and TFANa. Initiation of the conformational change induced by salts is thus considered to be mainly dependent on the anion species. The net charge of cytochrome c is estimated as +24 at pH 2 by Goto *et al.*<sup>2)</sup>, and conformation is thus considered to be disordered by strong electrostatic repulsion. It has been reported that anions in the solution are directly associated with the positive charge of a protein and then refolding is initiated by neutralization of the positive charge<sup>2)</sup>.

Fig. 3 shows the relationship between both electroselectivity and retention time of anion-exchange resins and the critical refolding concentration at which  $\theta_{222}$  starts to decrease. The values of retention time were obtained from the work of Gjerde *et al.*<sup>1)</sup> in which an anion-exchange column with trimethylamino groups in the presence of 0.1 mM phthalate at pH 6.26 was used. The values of electroselectivity were cited from the work of Gregor *et al.*<sup>3)</sup> for the affinity of monovalent anions to benzyl(hydroxyethyl) dimethylammonium anion-exchange resin. In the case of monovalent anions, a good linear relationship is seen among these data. This indicates that conformational changes are induced by the anion binding to the positive charge of a protein.

The result of the divalent anion deviates from this relationship. Gjerde *et al.* have reported that the difference in retention time between the monovalent anion and the divalent anion becomes large at lower pH<sup>1)</sup>, and pH in the present experimental condition (pH 2) is lower than that employed by them (pH 6.25). The difference in actual retention time between the monovalent and the divalent anions may be larger than that of Fig. 3. Thus, it can be considered that the result for SO<sub>4</sub><sup>2-</sup> is on the linear relationship obtained with the monovalent anions if the same pH is employed. The conformational change induced by divalent anions may possibly be similar to that induced by



**Fig. 5** Correlation between the *MSA*, the maximum intensity of the SAXS in the Kratky plot, *I(max)* and *HFS*. *MSA* cited from Goto *et al.*<sup>2)</sup>, and *I(max)* from Kataoka *et al.*<sup>6)</sup>. The experimental conditions of *HFS* are the same as those of Fig. 2.

the monovalent anion.

### 2.3 Surface net hydrophobicity and compactness of proteins

**Fig. 4** shows the relationship between the surface net hydrophobicity (*HFS*) and molecular ellipticities at 222 nm ( $\theta_{222}$ ). The solutions were adjusted to give each conformational state as follows:

Native state: pH 7.0

Unfolded state: pH 2.0 and NaCl less than 0.08 M

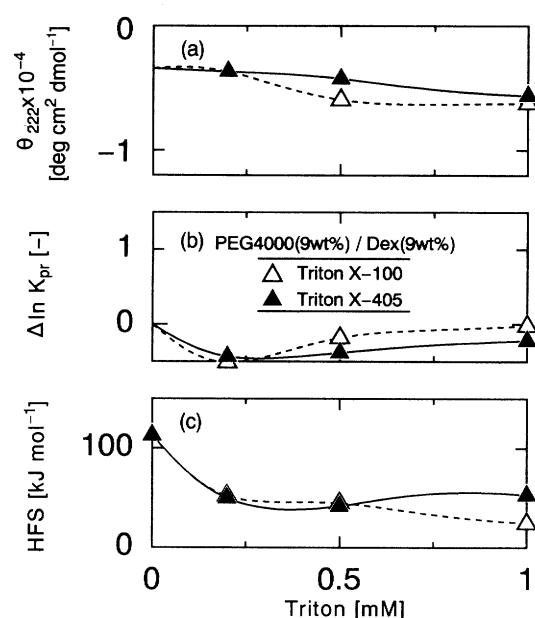
Molten-globule state: pH 2.0 and NaCl greater than 0.08 M, or below pH 1.0

The dotted line dividing the unfolded state and the molten-globule state was decided based on pH and ionic strength as well as the value of  $\theta_{222}$ . The data shown with double circle keys are cited from previous works<sup>12)</sup>. The *HFS* change from the unfolded state to the molten-globule state is on the same line for all salts used. Salt-induced change can be considered as an identical process, whereas the concentration range of the salt needed for the change is different. There are significant differences between the *HFS* values of the native and the molten-globule states, while the ellipticities at 222 nm are almost the same.

The size, compactness and shape of a protein can be measured by the SAXS (small angle X-ray scattering) method. The data of compactness, *I(max)*, reported by Kataoka *et al.*<sup>6)</sup> are also shown in Fig. 4. The *I(max)* values correspond to the present *HFS*. The fully expanded form of the unfolded state is continuously approaching a compact form of the native state. The unfolded state has a small amount of secondary structure,  $\alpha$ -helix, and the form may be an expanded and large random coil. Thus, hydrophobic residues are exposed at the surface of the protein and the surface hydrophobicity is greatest.

### 2.4 Surface net hydrophobicity and other conformational properties of proteins

The relation between *HFS* and *MSA* (maximum Soret

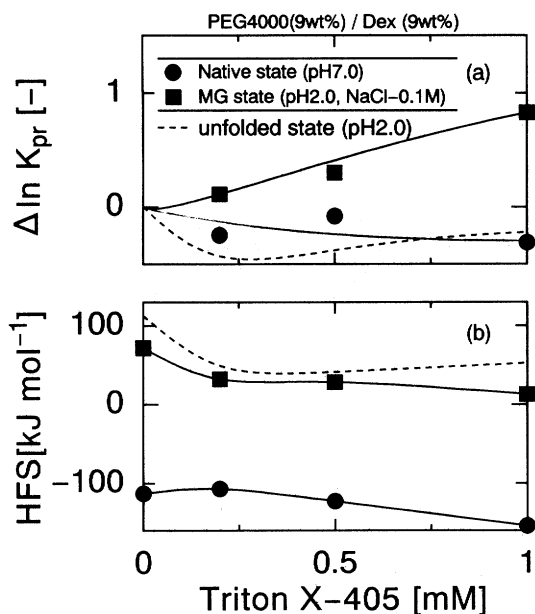


**Fig. 6** Effects of concentration of Triton in the unfolded state (pH 2.0) on (a) molecular ellipticities at 222 nm, (b) partition coefficient and (c) *HFS* of the unfolded state of cytochrome c (15  $\mu$ M)

adsorption) and that between *HFS* and *I(max)* measured by SAXS are shown in **Fig. 5**. The data of *MSA* and *I(max)* for the three states are cited from Goto *et al.*<sup>2)</sup> and Kataoka *et al.*<sup>6)</sup>, respectively. The *MSA* and *HFS* are seen to be directly and closely related to the results of the SAXS method, and all are effective in detecting conformational change of proteins. The *MSA* reflects the spin state of iron and, as a result, depends on the conformational state of the protein. The native state is low spin with both Met-80 and His-18 coordinated to the iron and the maximum adsorption of the native state is observed at 410 nm. On the contrary, the unfolded state, which has an extended form, is a high spin with neither Met-80 nor His-18 coordinated and it translates to 394 nm, whereas that of the molten-globule state has an intermediate value of 397 nm<sup>2)</sup>.

### 2.5 Effect of Triton and local hydrophobicity of cytochrome c

Triton, non-ionic surfactant, is known to be less effective than ionic surfactants as a protein denaturant but is bound to the exposed hydrophobic sites of proteins. The partition coefficient is increased by the binding of Triton and the increment ( $\Delta \ln K_{pr}$ ) is considered to represent the local hydrophobicity of a protein<sup>8, 13)</sup>. The effects of Triton on  $\theta_{222}$  together with the increment of partition coefficient by the addition of Triton ( $\Delta \ln K_{pr}$ ) and *HFS* at pH 2 are shown in **Fig. 6** (a), (b) and (c), respectively. pH was adjusted to pH 2 by the addition of HCl and thus, the initial conformational state was the unfolded state. The values of  $\theta_{222}$  only slightly decreased with the addition of Triton.  $\Delta \ln K_{pr}$  and *HFS* also changed in the same manner. A slight conformational change is considered to have occurred not by the decrease in electrostatic repulsion but by the increase in hydrophobic effect due to the binding of Triton.



**Fig. 7** Effect of the concentration of Triton X- 405 on (a)  $\Delta \ln K_{pr}$  and (b)  $HFS$  for the three states

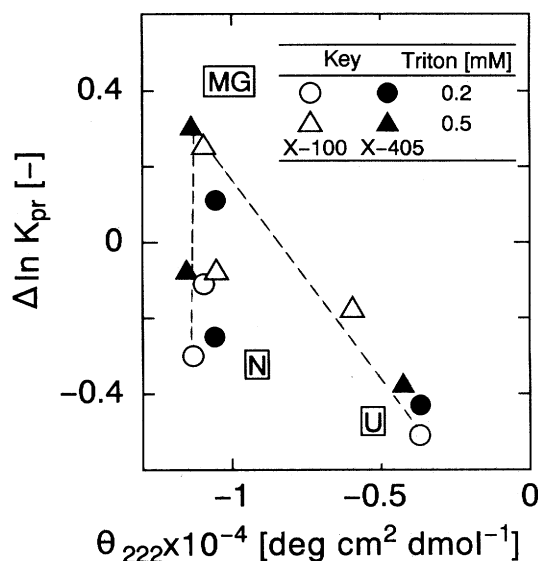
Triton is likely to be associated with the protein as a monomer at low Triton concentration, because the critical micelle concentrations (cmc) estimated from the surface tension measurements of Triton X-100 and Triton X-405 in the present ATPS are about 0.24 mM and 0.36 mM, respectively<sup>5)</sup>.

The effects of addition of Triton X-405 on the hydrophobic characters of the protein for the three states are compared in **Fig. 7**. For the native and molten-globule states, the conformation and  $HFS$  values are not significantly influenced by the addition of Triton. Therefore, the change in  $\Delta \ln K_{pr}$  is not brought about by the conformational change but by the Triton binding to the local hydrophobic binding site.  $\Delta \ln K_{pr}$  values of the native and the unfolded states vary only slightly with the addition of Triton. However, the  $\Delta \ln K_{pr}$  value of the molten-globule state shows a distinct increase, which strongly suggests the existence of some strong local hydrophobic sites in the molten-globule state of the protein.

The relationship between  $\Delta \ln K_{pr}$  and  $\theta_{222}$  is shown in **Fig. 8**. The increase in  $\Delta \ln K_{pr}$  from the unfolded to the molten-globule state may occur by localization of the hydrophobic residues due to the conformational change, whereas surface net hydrophobicity was decreased as shown in Fig. 4. Furthermore, the hydrophobic residues are more tightly buried in the interior of a protein by the formation of a tertiary structure during the change from the molten-globule to the native states. This brings about a decrease in both  $\Delta \ln K_{pr}$  and  $HFS$ .

## Conclusion

The acid- and salt-induced conformational changes of cytochrome c have been studied in aqueous two-phase



**Fig. 8** Relationship between the increment of partition coefficient and molecular ellipticities at 222nm in the presence of Triton. The experimental conditions are the same as those of Fig. 7.

systems, in relation to its surface hydrophobicities and interaction with the non-ionic surfactant Triton. The following results are obtained.

1) Conformational change from the native state, to the unfolded state, and then to the molten-globule state, is induced by the addition of hydrochloric acid at low ionic strength. The change from the unfolded to the molten-globule state is also induced by the addition of various salts. The relation between surface net hydrophobicity ( $HFS$ ) and the molecular ellipticity at 222 nm ( $\theta_{222}$ ) is expressed with a single line over the three states, induced by the addition of acid and all salts examined.

2) The surface net hydrophobicity ( $HFS$ ) of the protein varies depending on the three conformational states, and three conformational states can easily be distinguished by  $HFS$ . The local hydrophobicity, expressed by  $\Delta \ln K_{pr}$ , of the molten-globule state, which has an intermediate  $HFS$ , is the greatest.

3) The present results of  $HFS$  and  $\theta_{222}$  for the three conformational states correspond well with published results of maximum Soret adsorption and compactness measured by the SAXS method. Reasonable interpretations can, therefore, be obtained for the compactness of the protein, surface net hydrophobicity, existence of hydrophobic binding sites on the surface and structure of the protein or content of  $\alpha$ -helix in each state. The native state of cytochrome c has the most compact and hydrophilic surface (the smallest  $HFS$ ) and does not interact with Triton. The molten-globule state has the largest local hydrophobicity, whereas  $HFS$  is intermediate among the three states.

## Acknowledgments

We gratefully thank Dr. Y. Goto, Dept. of Biology, Faculty of Sci., Osaka University, for his valuable suggestions.

## Nomenclature

$C_{refold}$	=	the critical refolding concentration	[M]
$HF$	=	hydrophobic factor of two-phase system	[mol·kJ <sup>-1</sup> ]
$HFS$	=	surface net hydrophobicity of protein	[kJ·mol <sup>-1</sup> ]
$I(max)$	=	integral intensity of the Kratky plot	[-]
$K_{pr}$	=	partition coefficient of cytochrome c measured for PEG/Dex system	[-]
$MSA$	=	maximum Soret adsorption	[nm]
$\theta_{222}$	=	molecular ellipticity at 222nm	[deg cm <sup>2</sup> dmol <sup>-1</sup> ]
$\Delta \ln K_{pr}$	=	increment of partition coefficient by the addition of Triton	[-]

## Literature Cited

- 1) Gjerde, D.T., G.Schmuckler and J.S F ruitz: *J. Chromatogr.*, **187**, 35-45 (1980)
- 2) Goto, Y., N. Takahashi and A.L. Fink: *Biochemistry*, **29**, 3480-3488 (1990)
- 3) Gregor, H.P., J. Belle and R.A. Marcus: *J. Am. Chem. Soc.*, **77**, 2713-2719 (1955)
- 4) Hayakawa, K., H. Murata and I. Satake: *Colloid Polym. Sci.*, **268**, 1044-1051 (1990)
- 5) Kakinuki, T.: *MSc. Thesis*, Osaka Univ. (1995)
- 6) Kataoka, M., Y. Hagihara, K. Mihara and Y. Goto: *J. Mol. Biol.*, **229**, 591-596 (1993)
- 7) Kuboi, R., K. Yano, H. Tanaka and I. Komasa: *J. Chem. Eng. Japan*, **26**, 286-290 (1993)
- 8) Kuboi, R., K. Yano and I. Komasa: *Solv. Extr. Res. Dev. Japan*, **1**, 42-52 (1994)
- 9) M.F. Jeng and S.W. Englander: *J. Mol. Biol.*, **221**, 1045-1061 (1991)
- 10) Nishiyama, H., H. Maeda: *Biophys. Chem.*, **44**, 199-208 (1992)
- 11) Nozaki, Y. and C. Tanfold: *J. Biol. Chem.*, **246**, 2211-2217 (1971)
- 12) Tanaka, H., R. Kuboi and I. Komasa: *J. Chem. Eng. Japan*, **24**, 661-664 (1991)
- 13) Yano, K., A. Wakayama, R. Kuboi and I. Komasa: *Bunseki Kagaku*, **42**, 673-679 (1993)