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Effects of Salts on the Stability of Maleylated Tropomyosin

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Tropomyosin, a typical fibrous protein which has almost 100% α-helix, takes a random coil conformation when lysyl residues are maleylated. This conformational change is due to the increase in charge repulsions between negative charges produced by maleylation. When KF is added to MTM solutions, the recovery of α-helix can be observed by the decrease of ellipticities at 222 nm and 208 nm of circular dichroic spectrum (CD). Therefore helix-coil transitions induced by heat on the salt-induced α-helix were measured by CD measurements. Thermodynamical quantities, transition temperature, T_m, enthalpy and entropy of the transition were obtained by the analysis of the transition curves using a curve-fitting technique at various salt concentrations (C_s). The results show that T_m increases with the increase in C_s with a linear relation between T_m and ln C_s. It is inferred from this result that MTM behaves like a polyelectrolyte, and the major origin of the salt effect is the shielding of charges by small ions to reduce the electrostatic free energy.

INTRODUCTION

Tropomyosin, one of the muscle proteins, is a typical fibrous protein constituted of two subunits, which is a rod-like molecule of 40 nm in length and a coiled-coil of α-helices. The characteristic property of tropomyosin[1] may be the polymerizability to form a long fiber at a low salt concentration, which dissociates with the increase in the salt concentration probably due to electrostatic interaction between the molecules. The dependence of the polymerization and the stability of α-helices on temperature[2] has been investigated by viscosity[3] and circular dichroic measurements and the results showed that the molecules are stable at neutral pH up to *ca.* 50°C where the thermal transition to random coil occurs in parallel with the depolymerization.

On the other hand, maleylated tropomyosin (MTM), in which positive charges of lysyl residues in the intact molecule are converted to negative charges, has no more polymerizability[4]. In addition, the molecule dissociates into subunits owing to the charge repulsion against the attraction which associates the subunits together. Since the electrostatic energy may be modified in the presence of salts, the effects of salts on the conformation and the stability of MTM are expected to be significant. The present study is to elucidate the effects of salts on MTM as a function of temperature.

EXPERIMENTAL PROCEDURE

Tropomyosin was prepared from rabbit skeletal muscle and purified according to the procedure of Ebashi *et al.*,[5] and the purity was examined by the ultraviolet absorption

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spectrum to confirm the absence of tryptophan residues and by SDS-gel electrophoresis to detect any other contaminations.

Maleylation of tropomyosin was performed by the addition of maleic anhydride to the purified tropomyosin[6] and the extent of maleylation was measured by the ultraviolet absorption spectrum and the ninhydrin reaction; all the lysyl residues were found to be modified.

Circular dichroic measurements were carried out with a JASCO J-20 spectropolarimeter using a cell of 0.1 mm path length equipped with a thermostated jacket to control temperature.

Potassium fluoride was selected as a salt in the present measurements because of a low extinction at a low wavelength of ultraviolet light. (Except for the low wavelength region less than 210 nm, the use of NaCl gave the similar results to KF).

Phosphate buffer of pH 6.9 was used to maintain the pH of the solutions. A protein concentration around 1 mg/ml was employed throughout the experiments, since no appreciable difference was found at one-tenth of mg/ml.

The curve fitting of the thermal transitions was performed by FACOM 230-48 at the Computing Center of the Institute for Chemical Research, Kyoto University.

RESULTS AND DISCUSSIONS

Figure 1 shows circular dichroic spectra of MTM; in the presence of guanidine HCl, in the absence of KF at 23°C, in 1.2 M KF at 80°C, and in 1.5 M KF at 30°C. In the absence of excess salts, CD spectrum is not α-helical shape but approximately the same as in guanidine HCl, where MTM is assumed to be random coil. With the increase in the salt concentration, the spectrum changes to a typical curve of α-helix of the intact molecule, having minima at 222 nm and 208 nm. The result illustrates that the recovery of α-helix of MTM is almost complete at a high salt concentration. Apparently neutralization of charges on the molecule may be achieved by the addition of salt, thus giving rise to the recovery of α-helix. Upon heating, the salt-induced α-helix is transformed to random coil as usually observed.

The stability against temperature of the α-helix induced by the salt is shown in Fig. 2. At a given salt concentration, the curve of $[\theta]_{222}$ *versus* temperature exhibits a transition from α-helix to random coil. It is obvious from this result that the salt has an effect of stabilizing α-helix by raising the transition temperature. The effect may be shown by the increase in α-helical content with the increase in the salt concentration at a certain temperature.

For the quantitative estimate of the effect, the transition curves shown in Fig. 2 were analyzed by a curve-fitting technique. When we assume the two-state transition (*i.e.*, helix to coil transition) for the present curves, the enthalpy for the transition may be obtained by a slope of the linear relation of ln K *versus* 1/T (T is the absolute temperature), where K=A/(1-A), A being a helical content. Therefore, the plots of ln K against 1/T should be linear as far as we assume the two-state model for the transition. Since a full transition curve could not be realizable at a low salt concentration, such a treatment is inevitable for the present system. As shown in Fig. 3, the linear relations of ln K *versus* 1/T were obtained by varying the helical contents at the fully helical region, which could not be measured. From this analysis, the transition

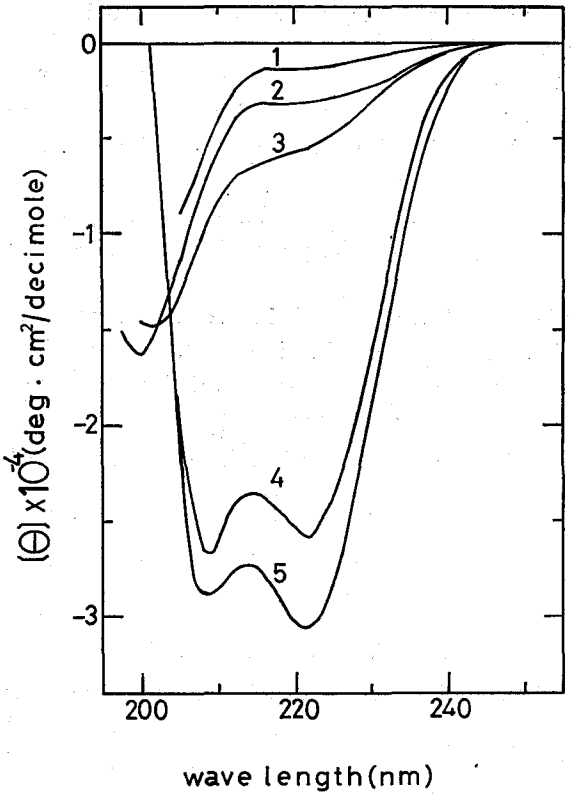

Fig. 1. Circular Dichroic Spectra of Maleylated tropomyosin at various conditions.
(1) MTM in 6 M GuHCl at 30°C, (2) MTM in 0 M KF at 23°C,
(3) MTM in 1.2 M KF at 80°C, (4) MTM in 1.5 M KF at 30°C,
(5) Intact tropomyosin in 0.5 M KF at 20°C, pH 6.9

temperature (T_m), the enthalpy (ΔH_0) and the entropy (ΔS_0) of the helix-coil transition as a function of the salt concentration were estimated as listed in Table I. T_m increases, as expected, and both ΔH_0 and ΔS_0 decrease with the salt concentration. The calculated curves of $[\Theta]_{222}$ *versus* temperature were shown in Fig. 2. The positive enthalpy arises from the disruption of interaction energies which hold the α-helical conformation, and the positive entropy is due to the increase in chain freedom accompanying the transition from a regular conformation to random coil. Furthermore, solvent water would play a role in the stabilization of the conformation presumably through hydration about the charged groups, since extents of both ΔH_0 and ΔS_0 decrease with the salt concentration as shown in Table I.

The transition temperatures obtained above are plotted against natural logarithm of the salt concentration (C_s) in Fig. 4. Interestingly, the plots lie on a line, indicating the presence of a linear relation between T_m and ln C_s. Such a linear relation between T_m and ln C_s was reported for the denaturation of DNA helix, and the quantitative analyses were performed by Kotin,[7] by Oosawa,[8] and by Record.[9] On the other hand, the effects of salts on the stability of protein conformations have been studied extensively;[10,11] *e. g.*, the transition temperature of RNase A varies depending on a kind of salts and their concentrations,[12] and T_m of collagen decreases linearly with the molarity

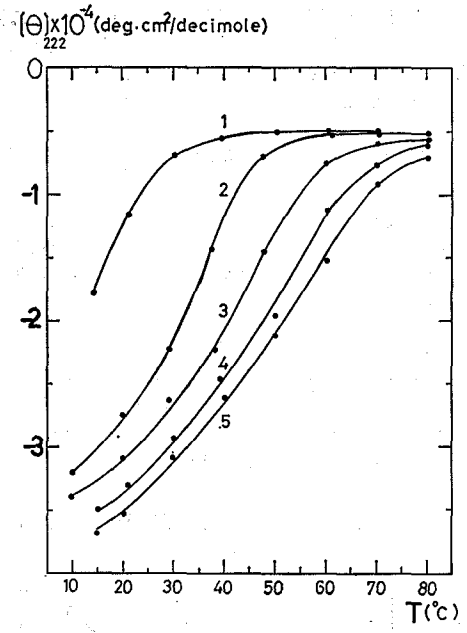

Fig. 2. Thermal transitions measured by ellipticity at 222 nm, $[\Theta]_{222}$, which is a measure of the content of α-helix, for various KF concentrations. pH 6.9
(1) 0.4 M KF, (2) 1.0 M KF, (3) 1.5 M. KF, (4) 2.0 M KF,
(5) 2.4 M KF.
The solid lines represent the computed curve after the curve-fitting shown in Fig. 3.

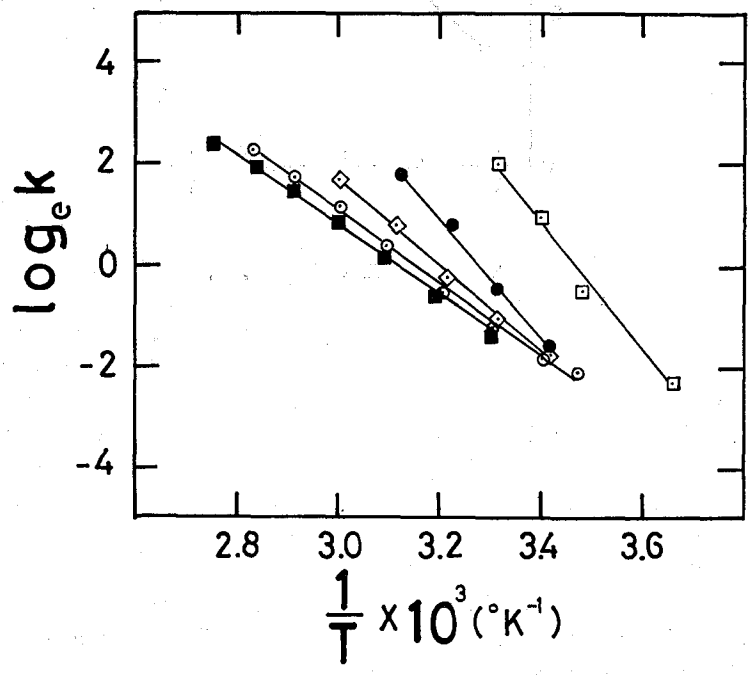

Fig. 3. van't Hoff plots of thermal transitions of MTM shown in Fig. 2 obtained by adjusting the values of 100% and 0% of helical content.
⊡: 0.4 M KF, ●: 1.0 M KF, ◇: 1.5 M KF, ⊙: 2.0 M KF, ■: 2.4 MKF

Table I Thermodynamical quantities, transition temperature (T_m), enthalpy (ΔH_0) and entropy (ΔS_0) of helix-coil transition obtained by the analysis of the curve fitting technique are shown at various salt concentrations (C_s)

C_s (M)	T_m K° (°C)	ΔH_0 Kcal/mol	ΔS_0 eu/mol	$n(\gamma_{p,c}-\gamma_{p,h})$*
0.4	288.4 (15.4)	24.9	86.3	2.69
1.0	305.0 (23.5)	23.5	77.1	2.28
1.5	312.2 (39.2)	17.0	54.4	1.57
2.0	317.4 (44.4)	14.2	44.7	1.27
2.4	320.5 (47.5)	13.0	40.6	1.14
2.7**	323.0 (50.0)	12.0	37.1	1.03

* From the slope of the line shown in Fig. 4 (18°), the difference of activity coefficients of the protein at helix and coil states are calculated according to Equation (2).

** Extrapolated values to the saturation of the salt effect.

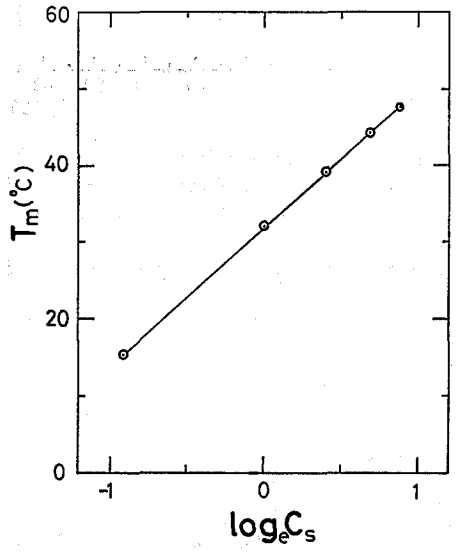

Fig. 4. Plots of T_m against $\log_e C_s$.

of $CaCl_2$.[13] Apart from such effects on proteins, the present result shows that the effect of salts on T_m is just the same as that for DNA. Therefore, it is inferred that MTM behaves like a polyelectrolyte, charges on which are neutralized by counter ions of added salts. The only difference is the range of the salt concentration observed for the linear relation; 0.4 to 2.4 M for MTM, in contrast to 0.002 to 0.1 M for DNA.

According to the theory of polyelectrolytes, charged groups of a macromolecule are neutralized by small salt ions due to a strong electric field formed around the charges, and the effective net charges diminish significantly by the ion binding. The linear relation between T_m and ln C_s has been formulated by several authors, which was developed to elucidate the experimental results of the DNA denaturation as mentioned above. Among them, the equation derived by Oosawa[8] seems to be adequate for the present purpose, since MTM is not regarded as a polyelectrolyte of equivalent ionizable units like DNA.

On the basis of the theory, the shift of the transition temperature, ΔT_m, may be expressed as follows,

$$\Delta T_m = RT_m^2(n/\Delta H_0)(\gamma_{pc}-\gamma_{ph})/\gamma_s \; 1n(1+\gamma_s C_s/\gamma_p C_p), \quad (1)$$

where R is the gas constant, n is the number of charged groups of the polymer, ΔH_0 is the enthalpy change of helix-coil transition, γ_{pc} and γ_{ph} are the activity coefficients of the polymer in the coil and helix state respectively, γ_s is the activity coefficient of small ions, and C_s and C_p are the concentrations of the small ions and polymer respectively. Since $\gamma_s \approx 1$ and $\gamma_s C_s \gg \gamma_p C_p$ at a high salt concentration, Eq. (1) may be written in the following form,

$$dT_m/d \; 1nC_s = RT_m^2 n(\gamma_{pc}-\gamma_{ph})/\Delta H_0 \quad (2)$$

By the use of Eq. (2), we can estimate apparent values of $n\ (\gamma_{pc}-\gamma_{ph})$ as listed in Table I.

The number of major ionizable groups in tropomyosin may be deduced from the amino acid sequence of one of the subunits, α-tropomyosin, reported recently,[14] as follows; aspartic acid=24, glutamic acid=56, lysine=39, and arginine=14. The net negative charges are converted from 27 to 105 on the maleylation, so that the change in the number is significant. The difference of the activity coefficients between the helix and coil may be estimated as 0.01 by putting n=100. This value is about one-twentieth of that for DNA denaturation, 0.25, indicating that the electrostatic free energy of α-helix due to ion binding is not so different from that of random coil of a polypeptide. This might be the reason why the linear relation between T_m and ln C_s has not been observed so far.

Since we have thermodynamical quantities of helix-coil transition as a function of the salt concentration, it may be possible to deduce the electrostatic contribution to the transition free energy. By extrapolation to a higher salt concentration where salt ions shield completely the charges on the protein, the following values are deduced for the quantities at the saturation of the salt effects; T_m=323 °K, ΔH_0=12.0 kcal/mol, ΔS_0=37.1 eu/mol and $n(\gamma_{pc}-\gamma_{ph})=1.03$. The difference from these values may be regarded as the electrostatic effects. In Table II, the calculated values are listed. The electrostatic free energy at the corresponding transition temperature (ΔG_{ele}) listed also in the table favors the coil conformation, and the extent is greater with the decrease in the salt concentration. It may be worth mentioning that ΔG_{ele} in Table II is linearly dependent on ln C_s although the reason is not clear.

The salt-induced conformational change of a polypeptide chain may be investigated

Table II Electrostatic Contributions of the Enthalpy, Entropy, and Free Energy to the Transition at Various Salt Concentrations

C_s (M)	ΔH_{ele} (Kcal/mol)	ΔS_{ele} (eu/mol)	ΔG_{ele} at T_m (Kcal/mol)
0.4	12.9	49.2	1.3
1.0	11.5	40.0	0.7
1.5	5.0	17.3	0.4
2.0	2.2	7.6	0.21
2.4	1.0	3.5	0.12

by techniques to detect the secondary structures as *CD*, and the change in the conformation is attributed to binding of small ions to reduce the strong electrostatic energy originated from ionizable groups of the polypeptide. Usually the binding capacity of ions to the charges on the chain is dependent on a kind of salts, *e. g.*, classification by Hofmeister and ranking of ions according to helix-making or-breaking capacity. In the present experiment, *KF*, which may be located in the middle of the rank, was selected as the salt. The salt-induced α-helix found in this study, however, implies that the major origin of the gain of the stability would be non-specific shielding of the charged groups in view of the success of the analysis. Actually, the use of NaCl gave the similar results although the values were slightly different. It is probable that the addition of salts induces the increase in the stability of a conformation of some protein, which may be observed experimentally.

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