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EFFECTS OF NEUTRAL SALTS ON THERMAL STABILITY OF SPINACH FERREDOXIN

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Summary: The effects of various neutral salts on the thermal stability of spinach ferredoxin were studied by means of differential scanning calorimetry and circular dichroism. Upon addition of 1 M NaCl, the apparent denaturation temperature increased by about 13°, and the enthalpy and activation energy for the denaturation also increased by 27% and 44%, respectively. The salt effect was ascribed essentially to the ionic strength of the environment. The circular dichroism measurements showed that the interaction between the iron-sulfur cluster and the protein moiety was modulated by the salt, resulting in stabilization of the protein. It is therefore highly probable that the native ferredoxin structure is maintained through the interaction with the iron-sulfur chromophore.

It is known that iron-sulfur proteins are markedly stabilized by the addition of neutral salts (1-4). Petering et al. (1) have reported that NaCl protects spinach ferredoxin from the bleaching of the visible absorption, which is usually taken as an index of denaturation of the protein. It has also been reported that the optical properties of ferredoxin treated with denaturants (2) or acetic anhydride (3) could be reversed almost completely to those of the native ferredoxin by the addition of neutral salts. Hasumi and Nakamura (4) have shown that the reactivity of p-chloromercuribenzoate with ferredoxin decreases with the increase of NaCl concentration. These results suggest that the addition of neutral salts may modulate the iron-sulfur cluster and/or the conformation of the protein moiety. However, the addition of neutral salts caused no detectable change in the absorption spectrum, which indicated that there occurred no alteration in the integrity of the native protein structure (5).

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In order to get further information as to the effect of neutral salts on the stability and structure of ferredoxin, DSC[1] and CD measurements were made in the presence and absence of neutral salts. The details will be described.

Materials and Methods

Ferredoxin was prepared from spinach leaves by the method similar to that described by Tagawa and Arnon (6). The concentration of this protein was determined spectrophotometrically by the use of the molar extinction coefficient of 9.68 $mM^{-1}cm^{-1}$ at 420 nm (7). Apoferredoxin was prepared by incubating a ferredoxin sample at room temperature for more than 24 hr. and the sample with the absorbance ratio (A_{465}/A_{276}) of less than 0.028 was used for DSC and CD measurements.

DSC measurements were made with a Rigaku Denki DSC-meter. A DSC heating rate of 5°/min was adopted in the present study. A sample pan was filled with 25 µl of sample solution and a reference pan with the same volume of buffer solution. The thermal denaturation parameters were determined essentially after Donovan and his coworkers (8,9). CD spectra were measured at room temperature with a Union Giken Dichrograph III-J, which has been interfaced to a computer, Union Giken System-77. The spectra were collected and stored in digital form, and generated after computer averaging. The difference CD spectrum was produced by subtracting one spectrum from another on computer. A sample cell of 5-mm path length was used throughout the experiments.

Results

Ferredoxin was incubated at room temperature under low ionic strength conditions and the absorption spectra were recorded at intervals (Fig. 1). The absorption band in the visible wavelength region due to the iron-sulfur chromophore was substantially bleached in 24 hr. On the other hand, the absorption around 280 nm remained without significant change. However, the CD spectrum in the far-UV region showed that there remained no significant amount of ordered structures in the protein moiety after the incubation. This is indicative that the iron-sulfur cluster plays an important role in maintaining the steric structure of the protein moiety. As depicted in the inset of the figure, the rate of the denaturation was remarkably lowered by the addition of 1 M NaCl.

The stabilizing effect of NaCl was quantitatively investigated by the DSC method. Fig. 2 shows the thermograms of ferredoxin in the absence (a) and presence (b) of 1 M NaCl. The thermograms indicate that thermal denaturation of the protein is endothermic. The stabilization by NaCl is evident from this

1) Abbreviations used: DSC, differential scanning calorimetry; CD, circular dichroism.

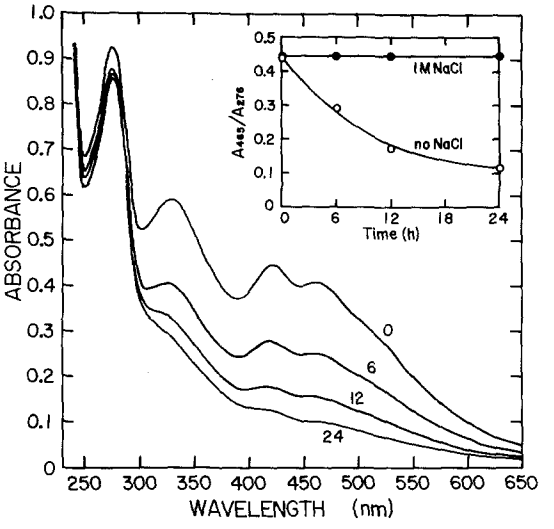

Fig. 1 Absorption spectra of ferredoxin after incubation of 0, 6, 12 and 24 hr. at room temperature in 10 mM Tris-HCl buffer, pH 7.4. The inset shows the time course of denaturation in the absence and presence of 1 M NaCl. The ratio of A_{465} to A_{276} was taken as the index.
Ferredoxin concentration: 46 μM.

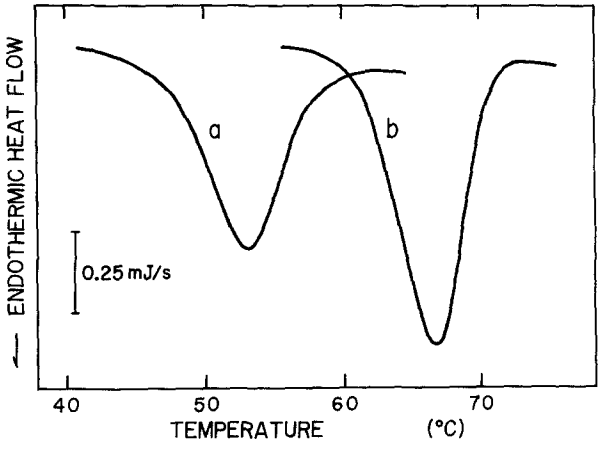

Fig. 2 DSC thermograms of ferredoxin in the absence (a) and presence (b) of 1 M NaCl. The sample pans contained 25 μl of 8.89 mM ferredoxin. The DSC heating rate was 5°/min.

figure, i. e., 1) the apparent denaturation temperature, T_d, shifted from 53.3° to 66.6°, 2) the denaturation enthalpy, ΔH_d, increased by about 27%, and 3) the activation energy for the denaturation reaction, E_a, also increased by

Table I. Thermal Denaturation Parameters of Ferredoxin

Addition	T_d (°C)	ΔH_d (kJ/mol)	E_a (kJ/mol)
None	53.3	233	327
1.0 M NaCl	66.6	296	470
1.0 M KCl	65.8	276	451
0.33 M Na_2SO_4	66.0	294	465
1.0 M KNO_3	61.8	239	427
0.33 M $MgCl_2$	59.6	260	368
1.0 M NaSCN	52.1	228	306

about 44% (see Table I). No detectable heat flow was observed by DSC when the apoferredoxin was applied. This is in agreement with the result of CD measurement that no ordered structures are present in the chromophore-free protein.

The thermograms in the presence of various inorganic salts with the same ionic strength were also investigated, and the results are shown in Fig. 3 in the forms of the Arrhenius plots. As seen in this figure, the effects of KCl and Na_2SO_4 were practically identical with that of NaCl, indicating that the stabilization is essentially due to the effect of ionic strength. However at the same time, some ion-specific interactions are also noticeable. The stabi-

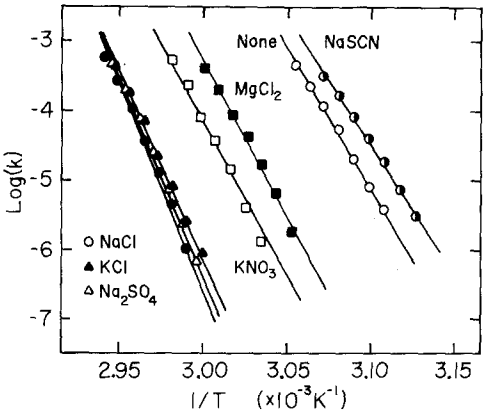

Fig. 3 Arrhenius plots for the denaturation of ferredoxin with various salts of the same ionic strength, 1.0. For each DSC experiment, 25 µl of 7.26 mM ferredoxin was used. Other conditions were the same as in Fig. 2.

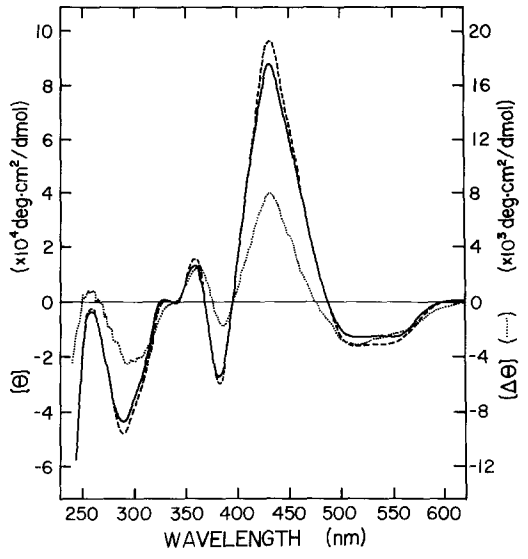

Fig. 4 CD spectra of ferredoxin in the absence (solid line) and presence (broken line) and the difference CD spectrum (dotted line) between them. The difference spectrum was obtained by subtracting the spectrum in the absence of NaCl from that in the presence of the salt by the use of the computer. Measurements were made in 10 mM Tris-HCl buffer, pH 7.4, at room temperature by using a 5-mm cell. The spectra are of the average of 8 scannings. Ferredoxin concentration: 140 μM.

lizing effects of $MgCl_2$ and KNO_3 were not so remarkable as those of NaCl, KCl and Na_2SO_4. Furthermore, the addition of NaSCN even lowered the stability of ferredoxin. The thermal denaturation parameters are summarized in Table I. It is noteworthy that the increase or decrease in the denaturation temperature is well paralleled by those of the denaturation enthalpy and the activation energy.

Since it was shown that the chromophore of ferredoxin is closely related to the maintaining of the steric protein structure and the addition of NaCl stabilizes the protein, the effect of the salt on the chromophore was investigated by means of CD measurements. Fig. 4 shows the CD spectra of ferredoxin in the absence and presence of 1 M NaCl. The difference CD spectrum ascribable to the addition of the salt is also shown (dotted line). The results indicate that the difference spectrum can essentially be attributed to the perturbation of the iron-sulfur chromophore. Since the CD spectrum in the visible wavelength

region has been ascribed to an asymmetric interaction between the chromophore and the protein moiety (10), the distinct difference CD spectrum implies a stronger interaction between them as compared with that in the absence of the salt. This is probably the reason for the increase of the stability of the protein.

Discussion

As has been observed, high ionic strength environment generally protects ferredoxin from denaturation during the incubation at room teperature. The bleaching of the visible color, or the destruction of the iron-sulfur cluster, is concomitantly accompanied by the destruction of the secondary structure of the protein moiety. This was evidenced by the observations that the apoferredoxin did not show any detectable heat flow on the DSC measurement, and that the CD spectrum of the apoferredoxin in the far-UV region was of an unordered conformation. The addition of neutral salts, therefore, is considered to stabilize the protein conformation against denaturing treatments. This was actually the case as already seen in Fig. 2, i. e., ferredoxin was greatly stabilized against thermal treatment in the presence of 1 M NaCl. The same line of evidence has been provided by Petering et al. (1) that the stability against urea treatment also increased as the increase of NaCl concentration.

Although the mechanism of stabilization of ferredoxin by neutral salts is not precisely known, it appears likely that the high ionic strength environment results in a stronger interaction between the iron-sulfur cluster and the polypeptide chain as already mentioned (Fig. 4). Since many proteins are known to increase thermal stability when bound to added compounds, such as metal ions, cofactors, inhibitors or second proteins (8,9, 11, 12), the stabilization is reasonably interpreted as being due to the modulation of the interaction between the chromophore and the protein moiety.

The effect of $MgCl_2$ may need some comments. According to Matsubara et al. (3), $MgCl_2$ is more effective than NaCl in restoring the visible absorption spectrum of acetylated ferredoxin. However as described above, the effect of

$MgCl_2$ on the thermal stability was not so effective as that of NaCl. It is well known that Na^+, K^+ and SO_4^{2-} ions are generally more effective in stabilizing native protein structures than Mg^{2+}, NO_3^- and SCN^- ions (13). This may lead to a supposition that the salt effect of restoration of the absorption spectrum would be distinct from that of stabilization of the protein structure.

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