Salt Dependent Stability and Unfolding of [Fe2-S2] Ferredoxin of *Halobacterium salinarum*: Spectroscopic Investigations

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ABSTRACT Ferredoxin from the haloarchaeon *Halobacterium salinarum* is a 14.6-kDa protein with a [Fe2-S2] center and is involved in the oxidative decarboxylation of 2-oxoacids. It possesses a high molar excess of acidic amino acid residues and is stable at high salt concentration. We have purified the protein from this extreme haloarchaeon and investigated its salt-dependent stability by circular dichroism, fluorescence, and absorption techniques. The predominantly β-sheeted protein is stable in salt concentrations of $\geq$1.5 M NaCl. At lower concentrations a time-dependent increase in fluorescence intensity ratio (I_{360}:I_{330}), a decrease in the absorption at 420 nm, and a decrease in ellipticity values are observed. The rate of fluorescence intensity change at any low salt concentration is the highest, followed by absorption and ellipticity. This suggests that at low salt the unfolding of ferredoxin starts with the loss of tertiary structure, which leads to the disruption of the [Fe2-S2] center, resulting in the loss of secondary structural elements.

INTRODUCTION

Archaea are a group of ancient organisms that are found in adverse environmental conditions like high salinity, high temperature, and high or low pH conditions; the haloarchaea grow in nearly NaCl-saturated concentration (Ginzburg et al., 1970; Kushner, 1978). This group of organisms is known as "extreme halophiles." The intracellular salt concentration is found to be very high in extreme halophiles (Ginzburg et al., 1970). As a consequence, universally conserved molecules like proteins and nucleic acids may have also adapted to function at such a high salt concentration (Eisenberg et al., 1992). Indeed, several of the functional proteins in haloarchaea have been reported to be halo-adapted in that they are structurally intact and functionally active at high salt concentrations only (Eisenberg et al., 1992; Lanyi, 1974; Hecht et al., 1990; Krishnan and Altekar, 1993; Madan and Sonawat, 1996). For biochemical characterization of the effect of salt on these proteins and enzymes, it is necessary to purify them to homogeneity. In the recent past halophilic proteins have been amenable to purification by ammonium sulfate-mediated chromatography (Mevarech et al., 1976; Von der Haar, 1976; Leicht et al., 1978; Werber and Mevarech, 1978; Danson et al., 1984; Bonete et al., 1986; Guinet et al., 1988).

Ferredoxin (Fd) is a ubiquitous protein found in almost all living organisms, with variations in molecular weight, composition of amino acids, and primary sequence. Because of its structural diversity and presence in a wide range of phylogenetically unrelated organisms, from primitive anaerobic prokarya to all eukarya, this protein has served as a useful molecular probe for understanding biological evolution. The nonheme, iron-sulfur center in ferredoxins acts as an electron carrier in a variety of biochemical processes like carbon metabolism, nitrogen fixation, photosynthesis, and steroid hydroxylation (Hall and Evans, 1969; Buchanan and Arnon, 1970). Ferredoxin from *Halobacterium salinarum*, like that from spinach, contains a [Fe2-S2] center, unlike in the case of *Escherichia coli*, and its function as an electron carrier in the decarboxylation of α-ketoacids has been demonstrated (Kerscher and Oesterhelt, 1977). The crystal structure of ferredoxin of *Haloarcula marismortui* has been reported (Frolow et al., 1996).

Because the halophilic proteins, in general, need high salt concentrations to maintain their biological activity, it is pertinent to study the possible role of salt in the stability of its structure. Very few haloarchaeal proteins, such as glutamate and malate dehydrogenases from *Haloarcula marismortui* (Leicht et al., 1978; Mevarech et al., 1977; Madern and Zaccai, 1997; Ebel et al., 1999) and *Haloferax mediterranei* (Ferrer et al., 1998), glucose dehydrogenase from *H. salinarum* (Madan and Sonawat, 1996) and *Haloferax mediterranei* (Bonete et al., 1996), and dihydrolipoamide dehydrogenase (Jolley et al., 1997) and dihydrofolate reductase from *H. volcanii* (Zusman et al., 1989; Pieper et al., 1998), have been examined in detail for the role of salt in the stabilization of protein structure. The paucity of data from such studies may stem from the fact that halophilic proteins need special approaches for their purification and characterization. We report here a procedure for purifying the ferredoxin from *H. salinarum* (HsFd) that not only gives a higher yield but also a preparation with the maximum A_{420}/A_{275} ratio that characterizes the integrity of the protein. We provide evidence that this protein requires a high salt concentration to remain intact over extended periods of time, and withdrawal of salt results in the gradual loss of its tertiary and secondary structures, ultimately leading to its denaturation. Our data conclusively show that *H. salinarum*

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ferredoxin is indeed adapted to the intracellular high-salt environment.

MATERIALS AND METHODS

Chemicals

Sepharose-4B, diethyl aminoethyl-cellulose (DEAE-cellulose), acrylamide, and 3-(*N*-morpholino)propane-sulfonic acid (MOPS) were procured from Sigma (St. Louis, MO). Bacteriological peptone (L37) was from Oxoid (Hampshire, UK). All other chemicals were of analytical grade and were used as supplied. Double glass-distilled water was used for purification and for all experiments.

Bacterial strain and growth medium

The *H. salinarum* (strain M1) cells were grown in a complex medium containing 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, 3 g sodium citrate, and 10 g bacteriological peptone per liter. The inoculum was built up by at least three successive transfers in the fresh medium. The autoclaved medium was inoculated with 5% (v/v) of this inoculum and incubated in a rotary shaker at 37°C and 150 rpm. For frequent use the culture was maintained by transfer to fresh medium at 4-day intervals. When not required for prolonged periods it was maintained by subculturing, once every 20 days, on slants prepared by solidifying the above medium with 1.8% (w/v) agar.

Buffer solutions

The following buffers were used during various stages of purification: 0.025 M MOPS + 4.5 M NaCl, pH 7.5 (buffer A); 40% $(NH_4)_2SO_4$ + 0.025 M MOPS, pH 7.5 (buffer B); 60% $(NH_4)_2SO_4$ + 0.025 M MOPS, pH 7.5 (buffer C); 30% $(NH_4)_2SO_4$ + 0.025 M MOPS, pH 7.5 (buffer D). Circular dichroism (CD), fluorescence, and optical absorption studies were performed in 10 mM sodium phosphate buffer (pH 7.3) supplemented with various concentrations of NaCl as noted for specific experiments.

Purification of ferredoxin

H. salinarum cells were grown to stationary growth phase ($A_{660} \approx 1.5$) in the complex medium. The cells were harvested by centrifugation at 2000 × *g* for 30 min and resuspended in 2 ml of buffer A/g of cells. The resuspended cells were sonicated in ice for 5 × 1 min. The sonicate was frozen and thawed once and then subjected again to sonication for a second round (5 × 1 min). The sonicate was then centrifuged (Beckman L8–60M) at 50,000 × *g* for 2 h at 4°C. The supernatant was collected and diluted with half the volume of buffer A. This was dialyzed overnight against buffer B to exchange the NaCl with 40% $(NH_4)_2SO_4$. The precipitate was removed by centrifugation (50,000 × *g*, 2 h), and the new supernatant was dialyzed again against buffer C. The precipitate appearing at this stage was removed by centrifugation (75,000 × *g*, 1 h). The clear supernatant obtained was loaded onto a Sepharose-4B column (2 cm × 35 cm) preequilibrated with buffer C. Under these conditions HsFd binds to the column. The column was washed with the same buffer until the A_{280} reached <0.1, then elution was carried out with a linear reverse gradient of $(NH_4)_2SO_4$ (60% to 0%) in 25 mM MOPS buffer pH 7.5 (1 ml/min), and absorbance of the eluent was monitored at 420 nm. The first peak fractions eluting at ~26% $(NH_4)_2SO_4$ containing ferredoxin were pooled and were readjusted to 60% with neutralized saturated solution of $(NH_4)_2SO_4$. Some precipitate appearing at this stage was removed by filtration. The clear filtrate was loaded onto a DEAE-cellulose column (1 cm × 20 cm) preequilibrated with buffer C and was washed with 5 bed volumes of the same buffer. Ferredoxin, which bound as a very dark band at the top of the column, was eluted with buffer D. The condensed dark brown band spread over the column at this stage and was eluted over several fractions. The fractions with A_{420}:A_{275} >0.2 were pooled, and an equal volume of saturated, neutralized $(NH_4)_2SO_4$ was added and rechromatographed on a DEAE-cellulose column. This chromatographic step was repeated until a A_{420}:A_{275} ratio of 0.33 was finally obtained in buffer C. The protein was then loaded on a small DEAE-cellulose column, eluted with buffer A and dialyzed against it to remove traces of $(NH_4)_2SO_4$. The protein thus obtained was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and it was found that the major protein was ferredoxin, along with a small amount of impurities. This protein was further purified on a Sephadex-G50 column (1 cm × 60 cm) preequilibrated with buffer A. The pooled fractions, exhibiting an A_{420}:A_{275} ratio of 0.35, were then concentrated by ultrafiltration (Amicon), using YM10 membrane. Ferredoxin prepared by this method was essentially homogeneous by SDS-PAGE analysis.

Optical measurements

Absorbance spectra were recorded on Shimadzu and Spectronic-1201 spectrophotometers, using cuvettes of 1 cm path length.

CD spectra

CD spectra were recorded on a Jasco-600 Spectropolarimeter. CD data are reported as mean residue ellipticities with a mean residue molar mass of 110. The path lengths of the cuvettes were 1.0, 0.1, or 0.05 cm, depending on the protein concentration and wavelength region. For each spectrum 5–10 scans were co-added, buffers along with the corresponding salt concentration were subtracted, and the spectra were smoothed using mild smoothing function.

Fluorescence measurements

Fluorescence intensities and emission spectra were measured on a Spex spectrofluorimeter with a cuvette of 1 cm path length. All measurements were made with excitation and emission bandwidths of 3.77 nm and 7.54 nm, respectively, using a single photomultiplier. The excitation wavelength was 295 nm. Emission spectra were recorded between 310 and 400 nm. They were baseline corrected for the corresponding buffer. A measurement of the intensity at wavelengths 360 and 330 nm was made for the calculation of the ratio of I_{360}:I_{330}. This ratio was used for the assessment of the overall protein structure (see text for details).

Protein estimation

Protein was estimated by the method of Lowry et al. (1951), as modified by Peterson (1983). Bovine serum albumin was used as the standard. For some experiments the A_{420} value was also used.

RESULTS

Purification and characterization of ferredoxin

H. salinarum ferredoxin was purified by a typical halophilic approach (Werber and Mevarech, 1978) as detailed in Materials and Methods. Its purity was checked by SDS-PAGE, which showed a single band corresponding to ferredoxin. An A_{420}:A_{275} ratio of 0.35 is routinely obtained. The purified protein yield was estimated by Lowry's modified method (Lowry et al., 1951; Peterson, 1983) as well as by its

absorption coefficient at 420 nm. These two methods gave similar estimates, and typically 3 mg purified HsFd per liter of culture was obtained. The protein was highly stable when stored at 25°C in 4.5 M NaCl at pH 7.3 and did not show changes in its optical spectra or A_{420}:A_{275} ratio over extended periods of time (>120 h).

Estimate of secondary structure contents by CD spectra

The far-UV CD spectra of HsFd in high and low NaCl are presented in Fig. 1. In high NaCl, a double minimum was observed near 210 and 217 nm. However, a completely different spectrum was seen for the protein in low NaCl. Intermediate NaCl concentrations showed a gradual decrease in the ellipticity. Ellipticity at 217 nm has been plotted as a function of time at different NaCl concentrations (Fig. 2). In concentrations above 1.0 M NaCl, ellipticity remains more or less unchanged with the progress of time, whereas at lower concentrations the protein shows time-dependent reduction in ellipticity values, indicating the loss of secondary structure. This loss of secondary structure became prominent at concentrations below 0.5 M NaCl. The CD spectra for several such NaCl concentrations were analyzed for secondary structural elements by the method of Andrade et al. (1993). The results for the high- and low-salt concentrations are presented in Table 1. It is seen that in high salt the protein has a predominantly β-sheet content. As the NaCl concentration is lowered, the protein loses a substantial amount of secondary structure, in which the loss of β-sheet is more pronounced than the loss of helix. The rate constants for the decrease in mean molar ellipticity at 217 nm calculated from these spectra are shown in Table 2.

Fig. 3 shows the near-UV CD spectra of HsFd in high and low NaCl. In the presence of high salt, negative peaks were observed at 267, 286, and 293 nm. These minima are indicative of specific tertiary connectivities with the aromatic side chains (Krishnan and Altekar, 1993). At low NaCl concentration, however, these ellipticity values were lower, indicating the loss of tertiary structure.

In the visible region, the CD spectra (Fig. 3) of ferredoxin in high NaCl showed positive peaks at 362 and 429 nm and minima at 328, 339, 397, 510, and 553 nm. In low NaCl an overall decrease in the ellipticity was again observed.

Secondary structure in the vicinity of the [Fe2-S2] center changes in low salt

In Fig. 4, the absorbance spectra of HsFd for high and low NaCl concentrations are presented. Ferredoxin shows prominent absorption peaks at 277, 329, 421, and 467 nm in high salt. These are characteristic of halophilic ferredoxin (Werber and Mevarech, 1978). At lower salt concentration specific enhancement at 277 nm was observed, whereas other major peaks became relatively less intense. We have monitored the characteristic ratio A_{420}:A_{275} for various salt concentrations as a function of time (Fig. 5). The data show that the ratio does not change for a long time if the salt concentrations are in the range of 2.0–4.5 M NaCl, but at lower salt concentrations a reduction in this ratio is clearly seen. We conclude from these data that the [Fe2-S2] center is destabilized upon exposure of HsFd to low salt.

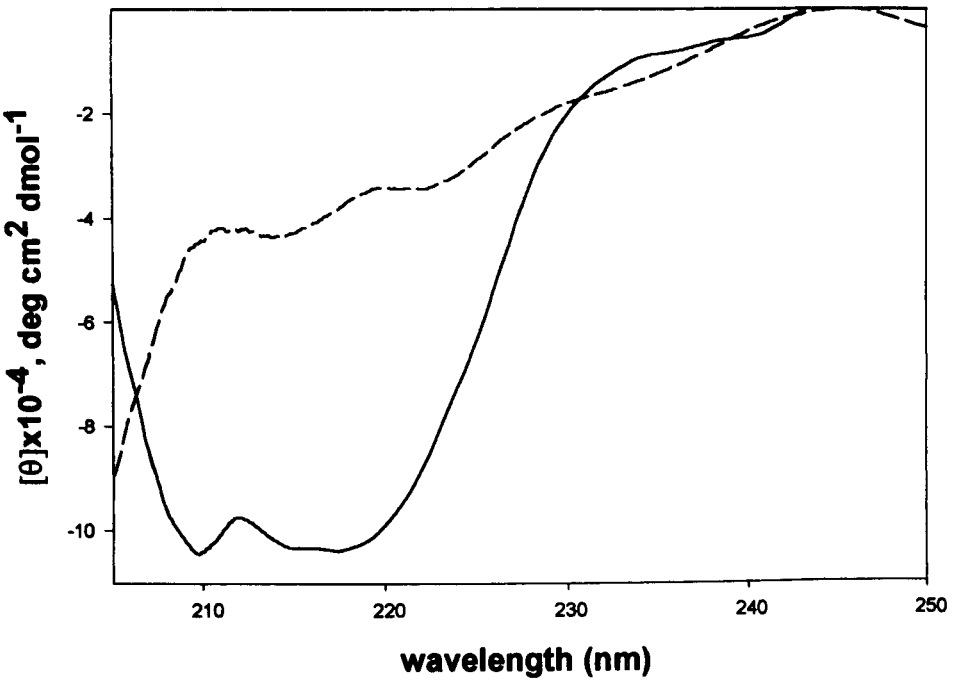

FIGURE 1 Far-UV CD spectra of ferredoxin of *Halobacterium salinarum.* ——, Salt concentration of 4.5 M NaCl; – – –, after incubation in 0.1 M NaCl for 60 h.

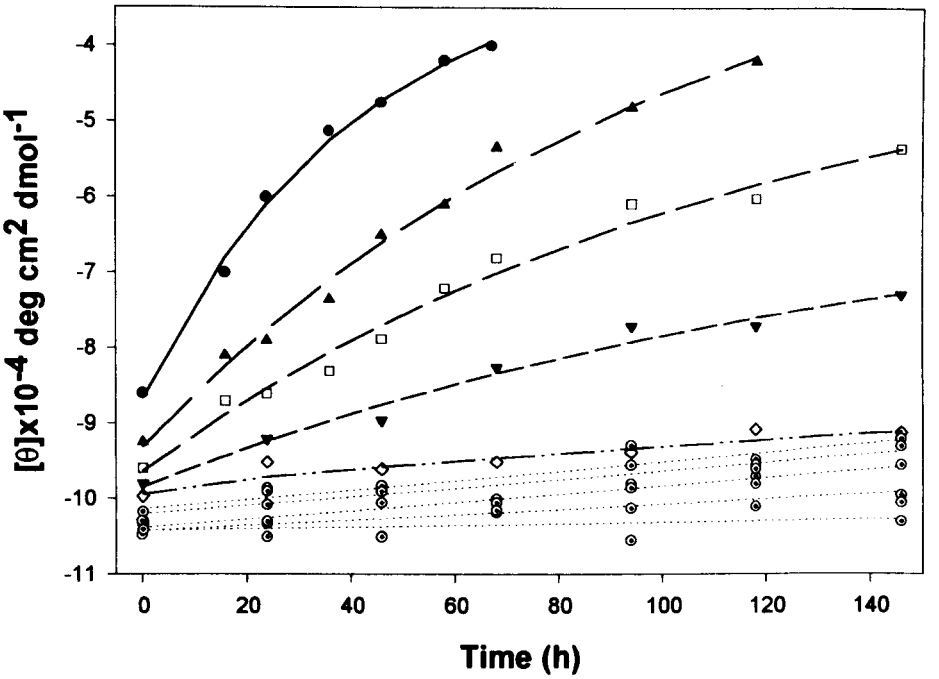

FIGURE 2 Kinetics of unfolding of ferredoxin of *H. salinarum*. CD ellipticity at 217 nm with protein in 0.1 M (●), 0.25 M (▲), 0.5 M (□), 1.0 M ([itrif), 1.5 M (◇), and 2.0–5.0 M (○) NaCl. The symbols represent experimental points and the lines correspond to two-state exponential fits.

Transfer of HsFd to low salt changes fluorescence characteristics of tryptophan residues

Fig. 6 shows the fluorescence spectra of ferredoxin at high and low NaCl. An emission maximum at 335 nm was observed for ferredoxin at high salt, whereas at low salt concentration the emission maxima shifted to 355 nm. All of the intermediate NaCl concentrations showed values intermediate between these two extremes and therefore were clearly salt concentration dependent. We have monitored the I_{360}:I_{330} ratio of the protein at different NaCl concentrations over a period of several days (Fig. 7). In high concentrations of NaCl the ratio remains more or less invariant, whereas at concentrations below 1.0 M a time-dependent increase was observed. The redshift of the λ_{em} and an increase in the I_{360}:I_{330} ratio indicate exposure of the tryptophan residues to an aqueous environment. Unfolding of the protein at low salt concentration is clearly apparent. An estimate of the rate constants of this unfolding process at the various salt concentrations is presented in Table 2. The data show that the rate of unfolding is dependent on the salt concentration. The HsFd unfolding rate is enhanced when it is transferred from high to low salt.

TABLE 1 Secondary structure elements of *H. salinarum* ferredoxin calculated from CD spectra in 10 mM phosphate buffer (pH 7.3) with the indicated salt concentrations

NaCl concentration (M)	α-Helix	β-Sheet	Others
4.5	23.0 ± 3	43.0 ± 2	32.0 ± 3
0.1	10.3 ± 4	15.5 ± 2	74.2 ± 4
% change	55.0	64.0	131

The fractions were determined at ambient temperature by the method of Andrade *et al* (1993).

DISCUSSION

H. salinarum is a facultative anaerobe that belongs to the archaeal domain. It grows in high salt environments. This microbe has adapted the mechanism of osmoprotection by accumulating a high concentration of salt inside the cell (Ginzburg et al., 1970). As a result of this, proteins from haloarchaea, in general, require high salt to maintain their structural and functional integrity. The structures of a few of the enzymes like malate dehydrogenase, glutamate dehydrogenase, dihydrofolate reductase, lactate dehydrogenase, and glucose dehydrogenase have been studied in great detail

TABLE 2 Rate constants (*k*) for salt-dependent unfolding of ferredoxin from *Halobacterium salinarum* measured by CD, fluorescence, and optical absorbance

Salt concentration (M)	$10^{-3} \times k$ (h^{-1})		
	CD	Fluorescence	Absorbance
0.10	24.0 ± 0.003	62.0 ± 0.003	23.0 ± 0.002
0.25	9.6 ± 0.002	20.0 ± 0.002	12.0 ± 0.003
0.50	8.6 ± 0.002	15.0 ± 0.005	10.0 ± 0.003
1.00	7.0 ± 0.003	10.0 ± 0.004	8.6 ± 0.004
1.50	4.8 ± 0.010	5.9 ± 0.070	5.5 ± 0.051

The rate constants for CD experiments were determined from ellipticity measurements at 217 nm, for fluorescence from the I_{360}:I_{330} ratio, and for absorbance from the A_{420}:A_{275} ratio. The protein was in 10 mM phosphate buffer (pH 7.3), and measurements were performed at ambient temperature.

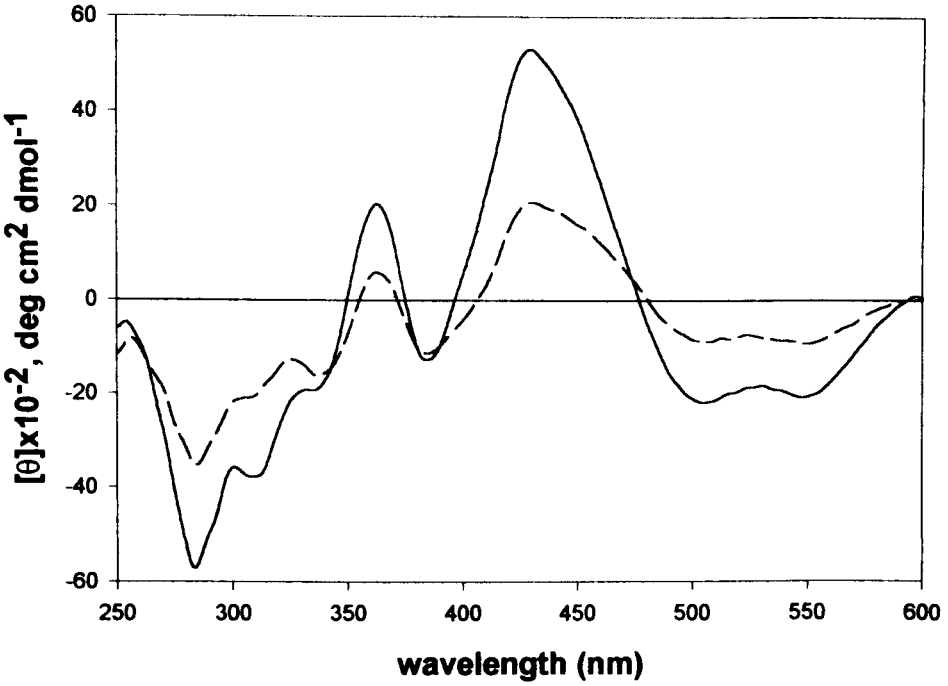

FIGURE 3 UV-visible CD spectra of ferredoxin of *H. salinarum* in 4.5 M NaCl (——) and after incubation in 0.1 M NaCl for 60 h (– – –).

as a function of salt concentration (Eisenberg et al., 1992; Hecht et al., 1990; Madan and Sonawat, 1996), and it has been shown that at low salt these enzymes lose their activities. The effect of salt on the structure of some halophilic proteins has been explored in great detail (Zaccai et al., 1989; Cendrin et al., 1993; Bonnete et al., 1994; Madern and Zaccai, 1997; Ebel et al., 1999). Haloarchaeal ferredoxin acts as a cofactor in decarboxylation reaction catalyzed by oxidoreductase. Therefore, the study of the effect of salt on its structure as well as its function may provide useful information about the structural biology of this protein.

Ferredoxin was purified earlier by a procedure that involved its exposure to low salt (Kerscher and Oesterhelt,

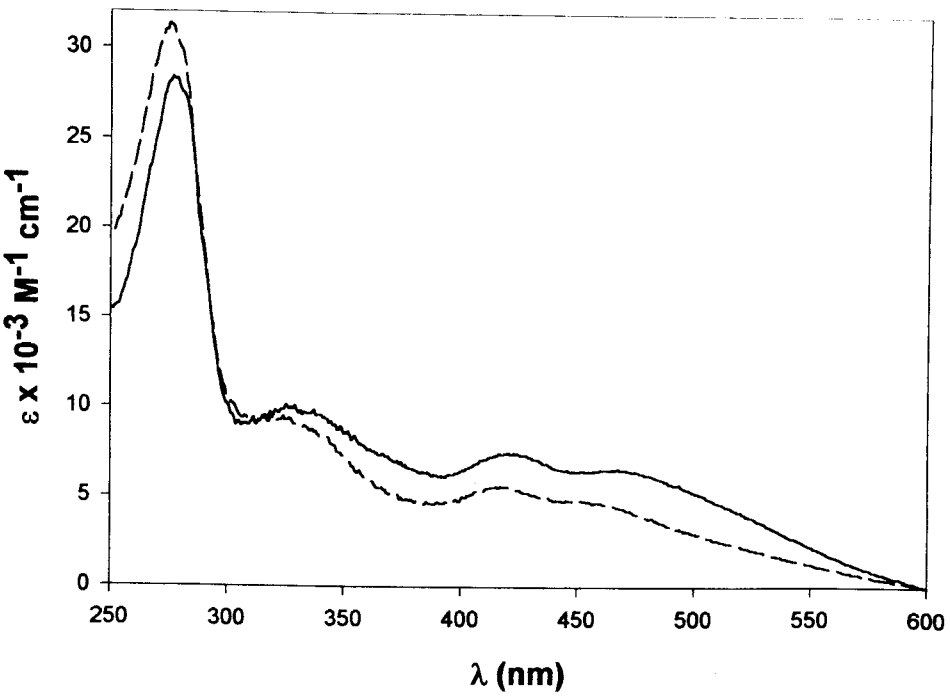

FIGURE 4 Absorption spectra of *H. salinarum* ferredoxin in 4.5 M NaCl (——) and after incubation in 0.1 M NaCl for 60 h (– – –).

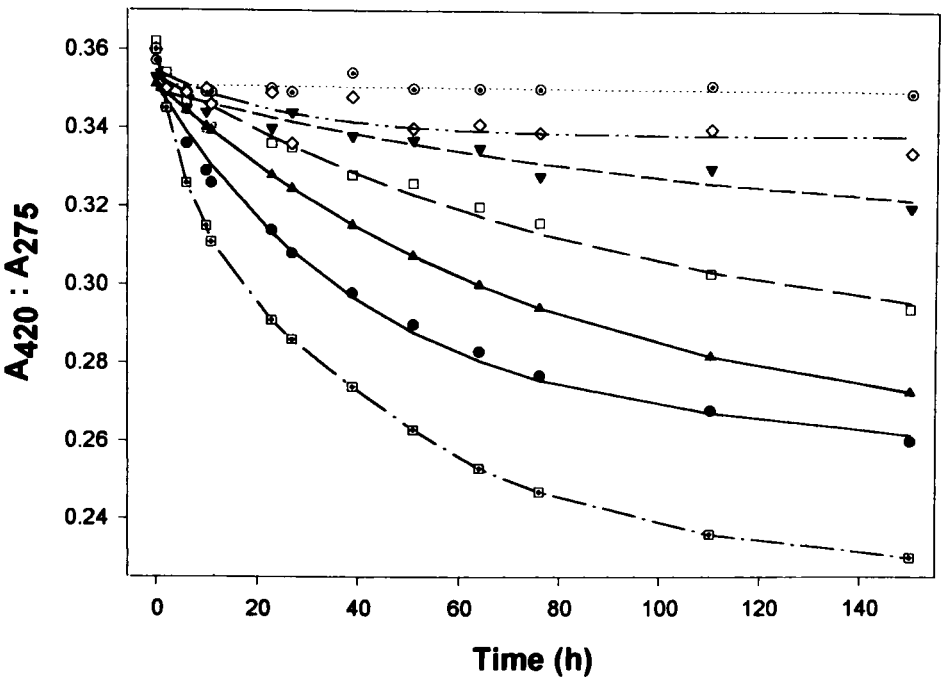

FIGURE 5 Unfolding kinetics of *H. salinarum* ferredoxin. The absorption ratio A_{420}:A_{275} corresponds to the unfolding in 0.05 M NaCl (⊠) and 4.5 M NaCl (·····). Other symbols are as in Fig. 2. The symbols represent experimental points, and the lines correspond to two-state exponential fits.

1976). This was improved by Werber and Mevarech (1978), who employed a method that maintains a high salt concentration throughout the purification procedure. In view of the instability of the *H. salinarum* ferredoxin at low salt, we used a procedure retaining several features of the latter method. In our procedure, a yield of 3 mg/liter cell culture with a high A_{420}:A_{275} ratio of 0.35 has been reproducibly obtained over several batches of purification. This may be contrasted with the nonhalophilic procedure, which has a lower yield (0.4 mg/liter cell culture) and an A_{420}:A_{275} ratio of 0.25 (Kerscher et al., 1976), and the modified procedure (Werber and Mevarech, 1978). The introduction of a Sepharose-4B column results in additional purification. This has also been reported for ferredoxin from *H. marismortui* (Werber and Mevarech, 1978). The most significant point in the procedure reported here is that the salt concentration has

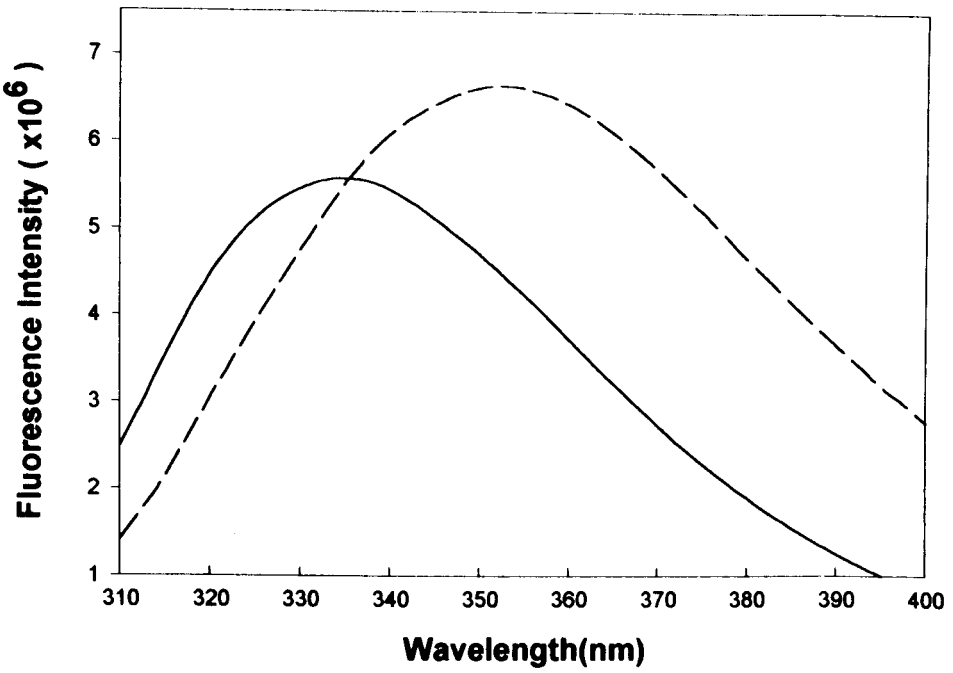

FIGURE 6 Fluorescence emission spectra of *H. salinarum* ferredoxin in 10 mM phosphate buffer containing 4.5 M NaCl (——) and 60 h after incubation in the same buffer with 0.1 M NaCl (– – –).

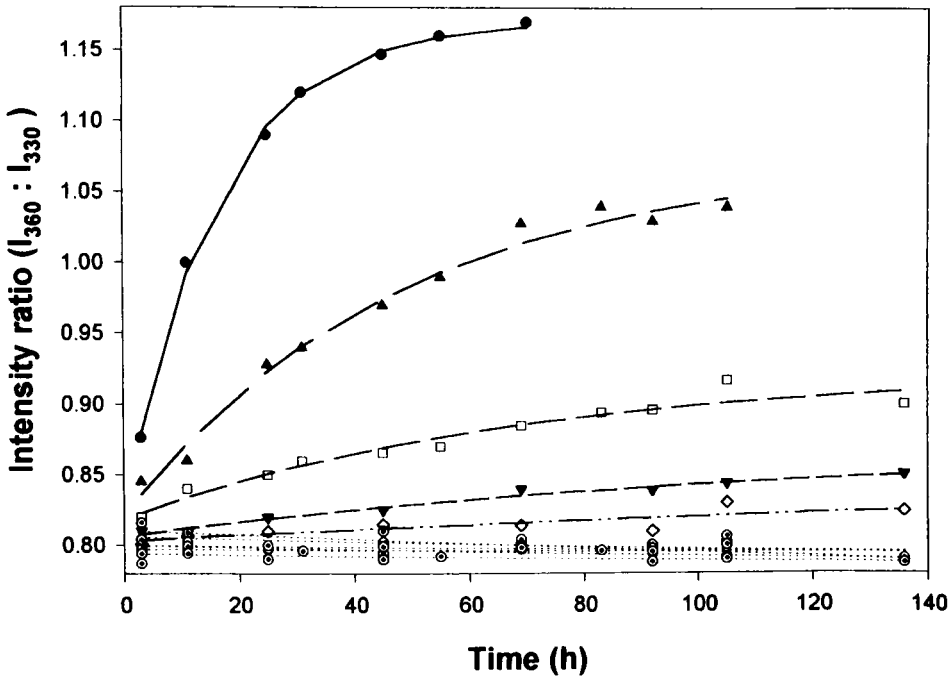

FIGURE 7 Unfolding kinetics of *H. salinarum* ferredoxin from the fluorescence intensity ratio I_{360}:I_{330}. The protein is in 10 mM phosphate buffer (pH 7.3) containing the indicated salt concentrations. Symbols are as in Fig. 2. The symbols are experimental points and the lines are the best fits, assuming a two-state process.

not been reduced below 1 M in any of the steps of purification. All of the purification steps were carried out in buffer containing ammonium sulfate, which is known to stabilize halophilic proteins (Eisenberg et al., 1992). Thus, during the purification procedure, ferredoxin could be kept intact in the absence of sodium chloride. Exposure to low salt has deleterious effects on halophilic proteins, which might be the prime factor for the observation of a lower absorption ratio in the earlier procedures. As we show later, exposure of purified HsFd to low salt may reduce the A_{420}:A_{275} ratio to <0.25 in $\sim$48 h. Prolonged exposure to low salt leads to aggregation and eventual protein precipitation. These features explain why the purified HsFd had a lower A_{420}:A_{275} ratio in apparently electrophoretically homogeneous preparations obtained in earlier procedures. Although, ammonium sulfate-mediated chromatography has advantages over the nonhalophilic procedure, it has to be ensured that the protein is not exposed to ammonium sulfate for extended periods, because in such cases protein precipitation occurs and may result in a low yield.

HsFd has two tryptophan residues that are localized in two distinct regions of the protein, namely, W16 at the N-terminal end and W59 in the vicinity of the conserved [Fe2-S2] center. Our observation of a fluorescence emission maximum of 335 nm implies that these tryptophans are in a hydrophobic environment. At low salt, an increase in quantum yield and a red shift as high as 20 nm in its emission maximum suggest that local environments of tryptophans are affected, leading to their transfer to more aqueous environment. This may reflect the loss of secondary and tertiary structure (Chen et al., 1967; Altekar, 1977a,b). This unfolding process is kinetically slow and continues for several hours, even days, to reach an apparent end point by spectroscopic criteria. The fluorescence intensity increase upon exposure to low salt has also been seen in other cases (Irace et al., 1981; Hargrove et al., 1994). The increased intensity may result when the tryptophans are relieved from an internal quenching in the folded state. Overall, the red-shift, along with the change in the spectral behavior, could be ascribed to loss of tertiary and/or secondary structure of ferredoxin at low salt (Staniforth et al., 1998).

The CD of salt-stabilized HsFd indicates 1) predominant β-sheet structure ($\sim$43%), 2) low α-helical content (23%), and 3) an intact [Fe2-S2] center. Our observation of a low α-helical content in the case of HsFd is in agreement with earlier reports (Werber and Mevarech, 1978; Frolow et al., 1996). The crystal structure of *Haloarcula marismortui* ferredoxin has been reported to have a β-sheet content of 25% (Frolow et al., 1996). Exposure to low salt induces unfolding, as indicated by an incremental random structure and a decrease in the secondary structure elements, in which the loss of β-sheet is greater than the loss of α-helix. In addition, low salt also affected the tertiary connectivities of the aromatic residues and the [Fe2-S2] center.

That the HsFd structure is destabilized in low salt is again revealed by optical spectra. The absorption spectrum for the haloarchaeal ferredoxin in its salt-stabilized state exhibits maxima at 275 and 420 nm. The A_{420}:A_{275} ratios for various ferredoxins have been determined and are found to have a characteristic value for any given species. This ratio can therefore be taken as a signature of the integrity of the [Fe2-S2] center and that of functionally intact protein.

Lower values of this ratio, if they occur because of external perturbation, may therefore be ascribed to unfolding (Keresztes-Nagy and Margoliash, 1966; Werber and Mevarech, 1978). Our observation that this ratio gradually decreases upon exposure to low salt in a time-dependent manner clearly suggests that the integrity of the [Fe2-S2] center is affected by protein unfolding.

The time-dependent destabilization of tertiary and secondary structure and the integrity of the [Fe2-S2] center were followed at salt concentrations in the range 0.1–5 M NaCl. A comparison of the rate constants (Table 2) reveals that for each of the salt concentrations the change is at maximum as determined by fluorescence and at minimum by CD spectroscopy. However, given that these disparate techniques monitor different aspects of overall protein structure and these three independent methods show a similar trend in the manner in which HsFd unfolding occurs, the half-lives determined by these methods are in general agreement with one another (Fig. 8). At the lowest salt concentration studied, ferredoxin tends to aggregate and precipitate after ~70 h.

Three distinct phases of structural destabilization are observed in our experiments. The low-salt range (0.1–0.25 M) corresponds to electrostatic charge repulsion. This originates largely from the large excess of negatively charged residues, aspartates and glutamates. HsFd has a total of 21 each of Asx and Glx (Kerscher et al., 1976), as compared to 10 Asx and 14 Glx of *E. coli* (Knoell and Knappe, 1974). Negatively charged residues cover the entire surface, barring the [Fe2-S2]center of *H. marismortui* ferredoxin (Frolow et al., 1996), which shows 88% homology to HsFd. These residues exist preferentially on the solvent-exposed side of α-helices and in loops that connect the various secondary structure elements. The crystal structure of this protein also suggests that the negative charges are shielded from each other by extensive solvation and are thus noninteracting (Frolow et al., 1996). The stability of HsFd in this range of salt concentration is, therefore, due to screening of these charges and the consequent stabilization of the α-helices and the loops. This is even more evidently the case for the amphipathic helix in the N-terminal 22-residue extra segment reported in *H. marismortui* ferredoxin. Because of the high homology, this helix is also expected in the HsFd, where the N-terminal segment contains nine negatively charged residues and no positive charges. This hyperacidic region of the protein, it is reasonable to assume, contributes largely to the stability of HsFd in the low-salt concentration range. Enhanced stability at lower pH values, where the side-chain carboxyl groups of these residues are undissociated (protonated), and in the presence of lower concentration of di- and multivalent cations supports the electrostatic charge screening (Baxter, 1959; Brown, 1963, 1964a,b, 1965; Lanyi, 1974). In addition, the negatively charged residues have an excellent solvation capacity, and the binding of water and/or salt to halophilic proteins is ~10 times more than that for nonhalophilic proteins (Ebel et al., 1992, 1995; Bonnete et al., 1993). Indeed, the surface water in *H. marismortui* ferredoxin has been shown to have 40% more

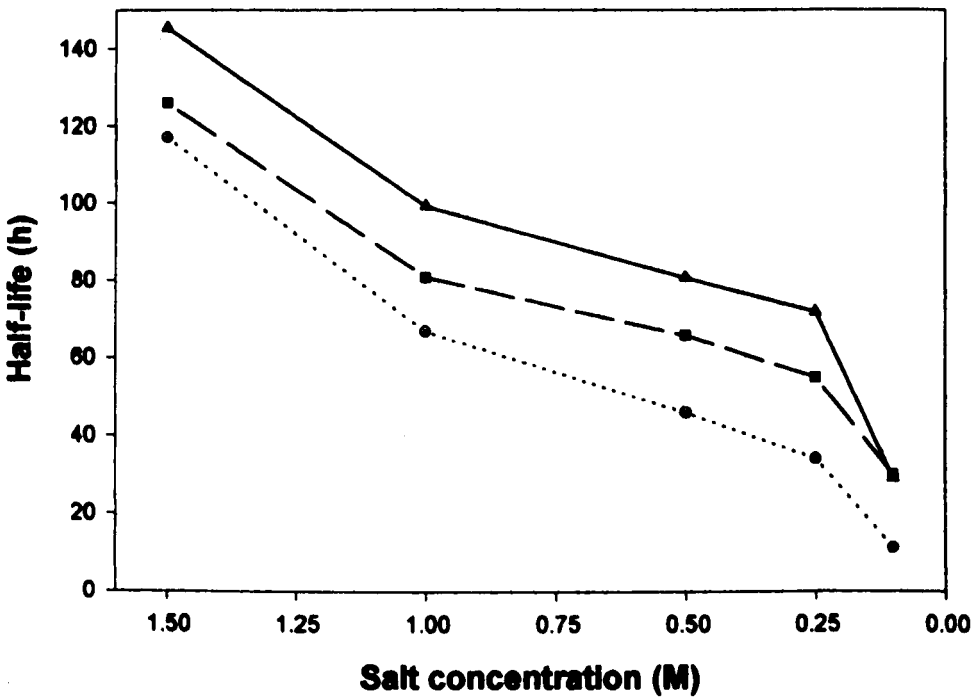

FIGURE 8 Half-life of *H. salinarum* ferredoxin determined by fluorescence (○), absorption (□), and CD (△) as a function of NaCl concentration.

hydrogen bonds (Frolow et al., 1996). The stabilization of the hyperacidic segment in HsFd is probably due to excessive solvation. The charge screening seems to continue in the intermediate salt concentration range as well, although its contribution to the overall stability is much less. In the high-salt concentration range the hydrophobic effects play a dominant role in the stabilization of halophilic proteins (Lanyi, 1974). The electrostatic interactions are affected to a smaller extent in this range (Elcock and McCammon, 1998). HsFd also possesses a large excess of valine, glycine, alanine, and tyrosine (Kerscher et al., 1976) in comparison to the nonhalophilic counterpart (Knoell and Knappe, 1974). The contacts among these residues are expected to be affected by a change in the salt concentration in this range. A comparison with the crystal structure of the homologous *H. marismortui* ferredoxin reveals that a significant fraction of these residues occur in the β-sheets. Our observations (Table 1) therefore suggest that the β-sheets are preferentially destabilized by a reduction of salt and that their stabilization contributes significantly to the stability of the protein in this concentration range. The intermediate salt range effects could be due to contributions from hydrophobic and specific residue interaction along with electrostatic charge screening. Further investigations with various cations and organic solvents with varying degrees of hydrophobicity might be helpful in evaluating the relative contributions of negative charges and hydrophobic residues to the stability of ferredoxin from this haloarchaeon.

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