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ORIGINAL PAPER

Kinetics of salt-dependent unfolding of [2Fe–2S] ferredoxin of *Halobacterium salinarum*

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Abstract The [2Fe–2S] ferredoxin from the extreme haloarchaeon *Halobacterium salinarum* is stable in high (>1.5 M) salt concentration. At low salt concentration the protein exhibits partial unfolding. The kinetics of unfolding was studied in low salt and in presence of urea in order to investigate the role of salt ions on the stability of the protein. The urea dependent unfolding, monitored by fluorescence of the tryptophan residues and circular dichroism, suggests that the native protein is stable at neutral pH, is destabilized in both acidic and alkaline environment, and involves the formation of kinetic intermediate(s). In contrast, the unfolding kinetics in low salt exhibits enhanced rate of unfolding with increase in pH value and is a two state process without the formation of intermediate. The unfolding at neutral pH is salt concentration dependent and occurs in two stages. The first stage, involves an initial fast phase (indicative of the formation of a hydrophobic collapsed state) followed by a relatively slow phase, and is dependent on the type of cation and anion. The second stage is considerably slower, proceeds with an increase in fluorescence intensity and is largely independent of the nature of salt. Our results thus show that the native form of the haloarchaeal ferredoxin (in high salt concentration) unfolds in low salt concentration through an apparently hydrophobic collapsed form, which leads to a kinetic intermediate. This intermediate then unfolds further to the low salt form of the protein.

Keywords *H. salinarum* · Ferredoxin · Fluorescence · Circular dichroism · Protein unfolding

Abbreviations

CAPSO	3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid
CD	Circular dichroism
FL	Fluorescence
HmMDH	*Halobacterium marismortui* malate dehydrogenase
HmFd	*Halobacterium marismortui* ferredoxin
HsFd	*Halobacterium salinarum* ferredoxin
MES	2-morpholinoethanesulfonic acid
MOPS	3-(*N*-morpholino)propanesulfonic acid
NATA	*N*-acetyltryptophanamide

Introduction

Understanding of the molecular mechanism by which a protein unfolds would help in exploring the contributions of the free energy of folding arising from electrostatic as well as hydrophobic interactions. The unfolding of small globular proteins follows a single kinetic phase and a single

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rate constant (Creighton 1991), except a few cases (Kuwajima et al. 1991). A single rate-limiting step is, therefore, involved in the unfolding process as all the molecules have the same probability of unfolding. This is because the molecular state of the native protein is conformationally homogenous. Under a particular unfolding condition the changes are uniform as has been shown by linear Arrhenious plots. In extreme environments such as high salt and temperature which a mesophilic counterpart may not withstand, electrostatic and hydrophobic interactions must contribute to the stability of biomolecules.

Halophilic archaea are the extremophiles that can thrive at salt concentration close to saturation (~5 M NaCl). As a part of the adaptive mechanism these organisms are known to harbor intracellularly, very high salt concentrations measured up to 4.5 M (Christian and Waltho 1962; Ginzburg et al. 1970; Lanyi and Silverman 1972; Eisenberg et al. 1992). Such high concentration of salts has deleterious effects on various biochemical processes. Several studies on proteins and enzymes from mesophilic sources have shown them to be non-functional in presence of molar concentration of salt. Other biological processes such as protein synthesis machinery and protein–nucleic acid interactions, which are essential for biological specificity, are known to be adversely affected in the presence of multimolar salt concentration (Von Hippel and Wong, 1964, 1965; Von Hippel and Schleich 1969). Since haloarchaea survive and thrive under these conditions, it would be interesting and important to study the parameters that contribute to the stability of their biomolecules like proteins and nucleic acids.

The principle mode of stabilization of halophilic proteins has been attributed to screening of negative charges at low (<0.5 M) salt concentration (Baxter 1959; Lanyi 1974) as most of these proteins have higher abundance of acidic over basic residues (Rao and Argos 1981; Madern et al. 1995). It has been pointed out that halophilic proteins behave differently from mesophilic ones. Function of many non-halophilic enzymes may involve association of several subunits and such association equilibria were shown to be affected by high concentration of salts (Nagy and Jencks 1965; Kawahara et al. 1965; Benesch et al. 1965). In contrast, it has been shown that multisubunit enzymes from the haloarchaeon *Halobacterium marismortui* require more than 2 M NaCl for stability (Eisenberg et al. 1992). Most of the prescribed non-covalent forces that are responsible in maintaining the three dimensional architecture of a protein molecule were shown to play important role in halophilic adaptation process (Eisenberg et al. 1992; Eisenberg 1995). The crystal structures of two of the halophilic proteins namely, *Hm*MDH and *Hm*Fd (Dym et al. 1995; Frolow et al. 1996) and the NMR solution structure of ferredoxin from *Halobacterium salinarum* (*Hs*Fd) has been reported by Oesterhelt et al. (Schweimer et al. 2000; Marg et al. 2005). This might help in understanding the general mechanism of adaptation of halophilic proteins in their extreme environment. Interestingly, variations in their adaptation process were observed. An increased number of salt bridges in *Hm*MDH and tightly bound water molecules in the extra stretch of the sequence of the *Hm*Fd contribute sufficiently to the halophilic adaptation process. The acidic amino acid-rich N-terminal extra domain of *Hs*Fd seems to play a significant role in its halo-adaptation (Bandyopadhyay et al. 2001; Marg et al. 2005). Furthermore, the N-terminal domain also seems to be essential for the incorporation of 2Fe–2S cluster into the protein (Hirota et al. 2005). In contrast, extensive biophysical studies on *Hm*MDH show clearly that its adaptation in high salt solution is dependent on the protein–solvent and protein–protein interactions (Bonneté et al. 1994; Tehei et al. 2001; Ebel et al. 2002; Costenaro et al. 2002; Irimia et al. 2003; Tehei and Zaccai 2005). These, in turn, modulate the oligomeric states of the protein. Specific interaction of cations and anions with this protein reveals that salt induced solvent properties are involved in the adaptation of the protein in saturated salt solution (Ebel et al. 1999). Although several enzymes from extreme haloarchaea have been investigated in the recent past (Hezayen et al. 2002; Liu et al. 2002; Camacho et al. 2002; Yoshimatsu et al. 2002; Ishibashi et al. 2003; Finn and Tabita. 2003; Bieger et al. 2003; Gadda and McAllister-Wilkins 2003), quantification of these data in terms of stability (using thermodynamics and kinetics) is clearly lacking due to the fact that (1) only few of the studies have been carried out in highly purified state due to difficulty in the purification of these biomolecules in presence of high concentration of salt and (2) in most of the cases the studied enzymes/proteins are fairly big (di- or tetra-meric), in which case equilibrium and kinetic measurements are not feasible due to irreversible denaturation (Aitken and Brown 1972; Louis and Fitt 1971; MacDonald et al. 1977; Hecht and Jaenicke 1989).

Halophilic organisms exhibit flexibility for salt requirement for biomolecules present in their cellular interior. This criterion is often used in classifying halophilic proteins as extremely halophilic, moderately halophilic and non-halophilic categories (Lanyi 1974). *Hm*Fd and *Hs*Fd have 88% sequence homologies. Both these proteins are small and single subunit having 128 amino acids, two tryptophan side chains with no disulphide bonds and a [2Fe–2S] chromophore center. Therefore, it is reasonable to assume that they might have similar structural features to help them to remain stable in multi molar salt concentration inside the cell. However, biophysical studies have shown that *Hm*Fd is moderately halophilic in nature as it requires only 40 mM salt to maintain its structural

stability over an extended period of time (>6 months) at pH 6.0–8.0 and its stability decrease with increasing the pH (Werber and Mevarech 1978). This experimental observation has been further supported by theoretical calculations (Elcock and McCammon 1998) based on the crystal structure of *Hm*Fd (Frolow et al 1996). *Hs*Fd, on the other hand, is extremely halophilic and is stable only in salt concentrations ≥1.5 M (Bandyopadhyay and Sonawat 2000). We had reported earlier that *Hs*Fd unfolds as salt concentration is lowered. This unfolding is partial. The partially unfolded protein was characterized and compared with the completely unfolded form of the protein (Bandyopadhyay et al. 2001). To gain further insight into the stability of *Hs*Fd we have investigated the kinetics of unfolding of the protein. The results of these experiments on *Hs*Fd are presented here. Our experiments conclusively show that the kinetic product in very low salt is a partially unfolded form and its mechanism involves repulsive columbic interactions. At intermediate salt concentrations the major mechanism of destabilization originate from solvent perturbation effects mediated by salt. These experiments demonstrate clearly that the adaptation of this protein in saturated saline environment involves not only electrostatic stabilization but also hydrophobic interactions.

Materials and methods

Purification and characterization of ferredoxin from *Halobacterium salinarum* (M1) were performed by the method reported earlier (Bandyopadhyay and Sonawat 2000). The concentration of ferredoxin were determined spectrophotometrically using a molar extinction coefficient, ε = 10,000 M^{-1} cm^{-1}, at 420 nm. Typically 4.0–25 μM protein concentration was used unless otherwise specified. CAPSO, MES, MOPS, and Tris were purchased from Sigma Chemical Company, USA. All other reagents were analytical grade and were used as supplied.

The solutions used in the study were double filtered. All spectroscopic measurements were carried out at ambient temperature in 10 mM sodium phosphate buffer (buffer-A), pH 7.3 unless mentioned otherwise. Temperature and pH of the different experiments are indicated in the corresponding figure legends. For fluorescence experiments the intensity at 360 nm was monitored while for CD measurement the ellipticity at 217 nm was used for assessing the rate of unfolding. In kinetic experiments of less than 10 min duration, the salt-jumps were achieved by injecting the incubated sample into the cuvette containing buffer of required salt concentration, and mixing manually. Efficient mixing was usually achieved in less than 10 s and measurements could be started immediately afterwards. Thus in these experiments the dead time was ~10 s. The data were fitted to a first order process by the following equation:

$$y(t) = a + b(1 - e^{-kt}) \quad (1)$$

where y is the observable CD or fluorescence parameter, a the initial value and b the amplitude.

Some experiments involved more than one phase. For fitting the data from such experiments the following equation was used:

$$y(t) = y(\infty) + \sum_i \Delta\alpha_i e^{-k_i t} \quad (2)$$

with modification, as necessary, to obtain the best fit of kinetic parameters. Millisecond kinetic experiments were performed in an SF-61MX multi-mixing stopped-flow spectrometer (Hi-Tech Scientific Co.) using fluorescence detection mode with filters cut off below 320 nm. The kinetic traces from these experiments were also fitted to Eq. 2.

Results and discussion

*Hs*Fd unfolding: salt affects the influence of pH

Unfolding of *Hs*Fd has been monitored at two extreme of salt concentrations. The native protein (in 4.5 M NaCl) was either transferred to medium with low (0.05 M) NaCl or 8 M urea was added to it. The resultant unfolding was studied as a function of pH using fluorescence and CD spectroscopic techniques. Fluorescence measurements were made by monitoring the ratio of intensities at 360 nm (tryptophans in polar environment) and 330 nm (tryptophans in non-polar environment). The use of this parameter has two main advantages. Firstly, it is independent of the protein concentration. Secondly, upon unfolding, the fluorescence intensity at 360 nm increases with a concomitant decrease of intensity at 330 nm, making this quotient a more sensitive parameter than the absolute values of either of the two intensities. Figure 1 shows the kinetics of unfolding observed when the native ferredoxin in high salt was exposed to (a) 8 M urea or (b) low salt concentration (without urea) at various pH. The urea-induced unfolding in high salt starts from a F_{360}:F_{330} ratio which is essentially same as that of the native protein. This kinetics is very slow. The time taken to reach an apparent steady state is pH-dependent. The end point of this kinetics is same at all pH values (pH 5.5, 7.4 and 9.6). In contrast, unfolding of *Hs*Fd in low salt proceeds in a manner that has several distinctive features when compared with that of the unfolding of native protein in presence of urea (Fig. 1).

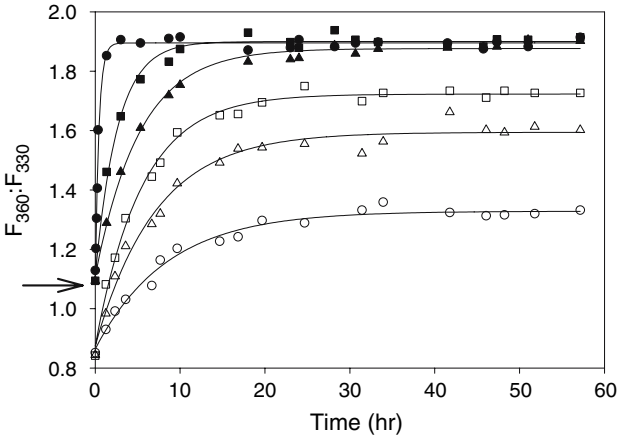

Fig. 1 Kinetics of unfolding of ferredoxin from *Halobacterium salinarum* in low salt (*open symbols*) and in high salt with 8 M urea (*closed symbols*) monitored by fluorescence. Ferredoxin in MES pH 5.5 (*open circle*, *filled circle*), Tris pH 7.4 (*open triangle*, *filled triangle*) and CAPSO pH 9.6 (*open square*, *filled square*) buffer with 0.05 M NaCl for the low salt. For high salt experiments the above three buffers were supplemented with 4.5 M NaCI and 8 M urea. Excitation wavelength was 295 nm. F_{360}/F_{330} ratio was computed from the emission spectra. *Symbols* are the experimental points and the *solid line* is the fitted curve assuming first order kinetics. The *arrow* points to the position of the native protein (4.5 M) when diluted in the same buffer

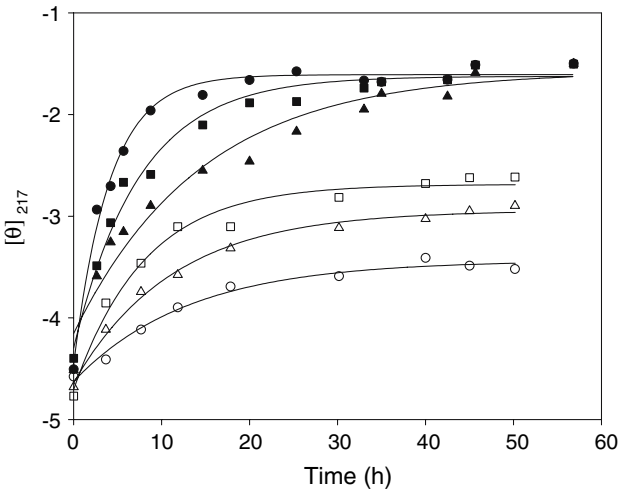

Fig. 2 Kinetics of unfolding of ferredoxin from *H. salinarum* monitored by CD. Ferredoxin in MES pH 5.5 (*open circle*, *filled circle*), Tris pH 7.4 (*open triangle*, *filled triangle*) and CAPSO pH 9.6 (*open square*, *filled square*) buffer with 0.05 M NaCl for the low salt (*open symbols*). For high salt experiments (*closed symbols*) the above three buffers were supplemented with 4.5 M NaCI and 8 M urea. CD ellipticity at 217 nm is plotted. Spectra were baseline corrected using the corresponding buffer

Firstly, the initial fluorescence ratio is far lower than the native value. Secondly, the apparent end point of unfolding is different at different pH values in that pH 5.5 proceed slowest, which is followed by the pH 7.4 and 9.6.

We have also followed the kinetics of unfolding of ferredoxin by CD spectroscopy. Unlike fluorescence, which sometime gives information about the immediate environment of tryptophans, CD-spectroscopy directly assesses the changes that take place in the secondary structure elements themselves. Thus the CD measurement is expected to provide a more global information. The results are presented in the Fig. 2. A comparison of the kinetics of unfolding of ferredoxin measured by CD with that of the fluorescence (Figs. 1, 2) shows that the initial difference between the native and the low salt unfolding seen in the fluorescence measurements is not apparent in CD measurements. The unfolding is faster when measured by fluorescence in comparison with CD spectroscopy. It is clear from the foregoing data that ferredoxin unfolds in low salt in a pH dependent manner. The lower the pH, less the unfolding. This agrees well with our earlier observation of the protein being relatively more stable at low pH values (Bandyopadhyay et al. 2001). However, such orderly pH dependence is not exhibited by the native protein when subjected to urea-induced unfolding. In this case the protein at neutral pH shows slower unfolding than either in acidic or alkaline conditions. In addition, the end point of unfolding is similar at all the pH values studied. The rate constants for unfolding, calculated assuming a simple first order process, are presented in Table 1. The unfolding of *Hs*Fd is pH dependent both in high salt (with urea) and low salt as shown by CD and fluorescence measurements. This observation implies that the unfolding is an electrostatically driven process. The mechanism by which *Hs*Fd unfolds at different pH values in low salt condition (0.05 M NaCl) might involve nonspecific classical electrostatic

Table 1 Rate of unfolding of ferredoxin from *Halobacterium salinarum* at pH 5.5, 7.4 and 9.6 and in low (0.05 M NaCl) and high (4.5 M NaCl) salt in presence of 8 M urea

Transitions	pH	Rate constant k_i (h^{-1})	
		Fluorescence	CD
N > U	5.5	7.20 ± 0.009	0.220 ± 0.015
	7.4	0.19 ± 0.010	0.100 ± 0.010
	9.6	0.34 ± 0.010	0.170 ± 0.010
N′ > L	5.5	0.11 ± 0.020	0.070 ± 0.010
	7.4	0.14 ± 0.006	0.085 ± 0.010
	9.6	0.18 ± 0.006	0.120 ± 0.020

pH 5.5, 7.4 and 9.6 were achieved with MES, Tris and CAPSO buffers, respectively.

N represents the native protein in 4.5 M NaCI, *U* is the unfolded protein at the steady state in 8 M urea, *N*′ the state of the protein in the initial stage of unfolding when the native protein is transferred into low salt concentration, *L* is low salt form at steady state of unfolding of the protein at different pH

repulsion, which may originate from the high charge density on the surface of the protein. The structure of *Hm*Fd and *Hs*Fd exhibits cluster of these amino acids on the surface (Frolow et al. 1996; Marg et al. 2005). It is seen that the rate of unfolding of *Hs*Fd follows the order $k_L^{FL/CD,\ 5.5} < k_L^{FL/CD,\ 7.4} < k_L^{FL/CD,\ 9.6}$ (Table 1). At acidic pH surface negative charges are partially neutralized, thus reducing the repulsive electrostatic interaction, which may affect the rate of unfolding. At high pH the ionized carboxyl groups of the acidic amino acid residues get hydrated, more so than at low pH (Kuntz 1971). Excess hydration of these groups in alkaline condition cause lowering of activation free energy 'barrier' (Kertesz et al. 1977), which may contribute to the enhanced rate of unfolding of *Hs*Fd at alkaline pH.

It is interesting to note that the order of urea-induced unfolding in high salt, which is $k_u^{FL/CD,\ 5.5} > k_u^{FL/CD,\ 9.6} > k_u^{FL/CD,\ 7.4}$, differs from that in low salt. This may not be due to classical electrostatic effects (Dill 1990) as side chains of aspartate and glutamate are expected to remain neutral in presence of high salt. As a possible mechanism the origin of this rate-enhancement seems to be guided by protein group transfer-hydration mechanism (Low and Somero 1975). Specific ion-pair(s) may get disrupted either at acidic or alkaline pH (at pH 5.5 or 9.6) and eventually its exergonic transfer to the protein–water interface leads to a lowering of the activation free energy. This might accelerate the rate of unfolding (Kertesz et al. 1977; Somero and Low 1976).

The rate of unfolding in presence of high concentration of urea is higher than low salt unfolding rate (i.e. $k_u^{FL/CD} > k_L^{FL/CD}$). Although velocity of reaction either in presence of urea or in low salt is slow, the rate observed by fluorescence are higher than that by CD measurement. This observation could imply that the loss of tertiary structure is followed by that of loss of secondary structure of the protein. Slow unfolding in low salt has been reported in other halophilic proteins as well (Pundak et al. 1981). Salts bind to the protein molecule at specific sites and help in stabilizing non-covalent interactions such as electrostatic, hydrophobic and hydrogen bonding (Fridovich 1963; Bello et al. 1966; Damodaran and Kinsella 1981). This intrinsic binding constant that affects protein stability, varies widely as a function of the conformational states of the binding site of the protein molecule.

To check the nature of the pH dependence, logarithm of the rate of unfolding has been plotted as function of pH (Fig. 3). A linear relationship is observed for the unfolding in low salt by both fluorescence and CD spectroscopic measurements. This could be an indication of a typical monophasic process (Dobson et al. 1994). Non-specific electrostatic repulsion of the exposed carboxylates (Dill 1990; Lanyi and Stevenson 1970) and charge dependent hydration (Kuntz 1971) would be the major operators for the above observable. In contrast, for the unfolding in urea, log of the rates of unfolding shows nonlinear pH dependence. The native protein would have different conformational states that determine activation energy barrier. In the crystal structure of *Hm*Fd (Frolow et al. 1996) and the NMR solution structure of *Hs*Fd reported by the group of Oesterhelt (Marg et al. 2005) buried salt-bridges are observed. Exergonic transfer of titrated cationic or anionic group to protein–water interface might cause the rate enhancement (Low and Somero 1975). This suggests the accumulation of intermediate(s) at different pH values, as deviation of log of rate of unfolding from linearity is an indication of accumulation of intermediate(s) (Jackson and Fersht 1991a, b).

The above observations and discussion lead to the following schemes of unfolding:

$$N > I > U \quad \text{(for high salt)} \tag{3}$$

$$N > N' > L^{9.6} > L^{7.4} > L^{5.5} \quad \text{(for low salt)} \tag{4}$$

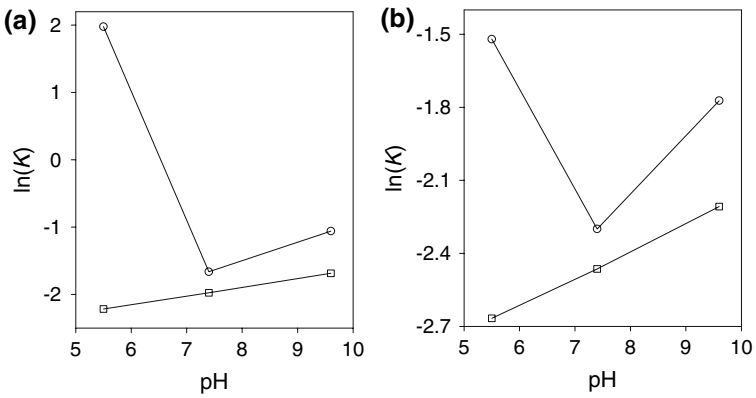

Fig. 3 pH dependence of unfolding rates of ferredoxin of *H. salinarum* measured by **a** fluorescence and **b** CD spectroscopy. The experimental conditions are as mentioned in the text. The *symbols* are unfolding in low salt (*open sqaure*) and in high salt with 8 M urea (*open circle*)

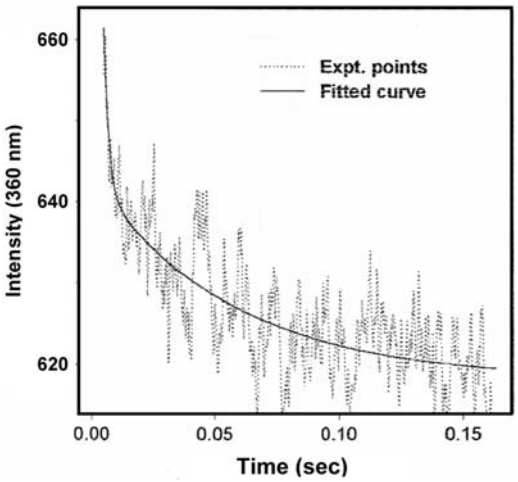

Fig. 4 Unfolding kinetics of ferredoxin of *H. salinarum* monitored by stopped flow. The protein is in 10 mM sodium phosphate buffer pH 7.3. Salt concentration was initially 3.0 M. The final salt concentration is 0.43 M and protein concentration is 10 μM. Detection was done by fluorescence. *Continuous line* corresponds to the best fit to a double exponential function

where N is the native *Hs*Fd, N′ is a form immediately after dilution to low salt concentration, L is the low salt form, U is urea unfolded protein, and I is an intermediate in urea-induced unfolding.

Kinetics of *Hs*Fd unfolding

To understand the nature of the fast phase involved in the low salt transition (N > N′) stopped flow kinetics were carried out by fluorescence. A typical kinetic trace is presented in Fig. 4. Kinetic profile shows a double exponential decay, which may be contrasting with the observation in our slow kinetic measurements presented above (Figs. 1, 2), where an increase in either F_{360}:F_{330} or CD signal was seen. Although we used fluorescence ratio for slow kinetics assessment, which is a more sensitive and protein concentration independent parameter, the kinetics of F_{360} follow similar pattern (data not shown). Therefore, the decrease in intensity at pH 7.4 (using NATA as reference) (Fig. 4) is due to a mechanism which is distinct compared with that of slow kinetics (Figs. 1, 2). The electrostatic shielding effect is completed at about 0.25 M NaCl (Lanyi 1974). However, since the final concentration of salt in this reaction mixture is 0.45 M, we believe that the dominant effect of salt in our stopped flow experiments, like any other additive, is due to its effect on the solvent properties of the solution (Ebel et al. 1999).

The nature of the fast phase component(s) was investigated further. Figure 5 shows the typical kinetic traces obtained using different salt jumps. As expected the native protein does not show any time dependent decay of the F_{360} (Fig. 5a). A jump from 4.3 M → 0.53 M NaCl (Fig. 5b) shows time-dependent decay of fluorescence intensity. When the salt concentration was jumped from 4.3 M → 0.02 M NaCl, an enhancement of fluorescence intensity was observed (Fig. 5d). Interestingly, a jump to a salt concentration intermediate (4.3 M → 0.22 M) of these two extremes resulted in both the decay and the rising components (Fig. 5c). Rate constants calculated from various salt jump experiments starting from the native are tabulated in Table 2.

Broadly two distinct components of fluorescence intensity change are seen from these experiments. One is decreasing and the other increasing. The following features may be noted from these experiments: (a) A salt concentration change of the native protein (in 4.3 M NaCl) to about 0.5 M NaCl shows only the decreasing component.

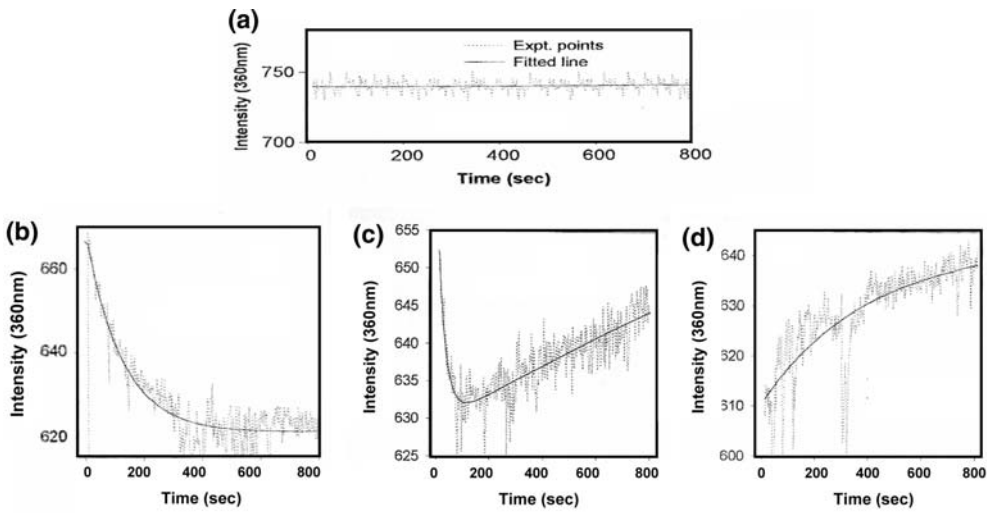

Fig. 5 Unfolding kinetics of ferredoxin from *H. salinarum*. The native protein in 4.3 M NaCl was transferred to **a** 4.3 M, **b** 0.52 M, **c** 0.22 M and **d** 0.02 M NaCl in 25 mM sodium phosphate buffer, pH 7.5. The fluorescence emission was monitored at 360 nm. Excitation was at 295 nm. The experimental data point is presented in *dashed line* and the *solid line* is the fitted regression line. Mixing was achieved manually and the dead time was approximately 12 s

Table 2 Kinetic parameters for the unfolding of ferredoxin at different salt jump experiments at pH 7.5

Salt jump (M)	Rate constant k_1 (h^{-1}) for the first component	Rate constant k_2 (h^{-1}) for the second component
4.3 > 1.02	0.00015 ± 0.00004	–
4.3 > 0.52	0.00810 ± 0.0004	–
4.3 > 0.22	0.03800 ± 0.0004	0.0002 ± 0.00010
4.3 > 0.12	0.10450 ± 0.0040	0.0026 ± 0.00040
4.3 > 0.07	–	0.0087 ± 0.00023
4.3 > 0.02	–	0.0248 ± 0.00036

The fit of the intrinsic fluorescence kinetic data at various salt concentrations. The initial part (12 min) which cause sharp rise due to instrumental set up has been deducted. The residual data has been fitted to first order increasing or decreasing exponential. When both the components are present the data were fitted to a double exponential

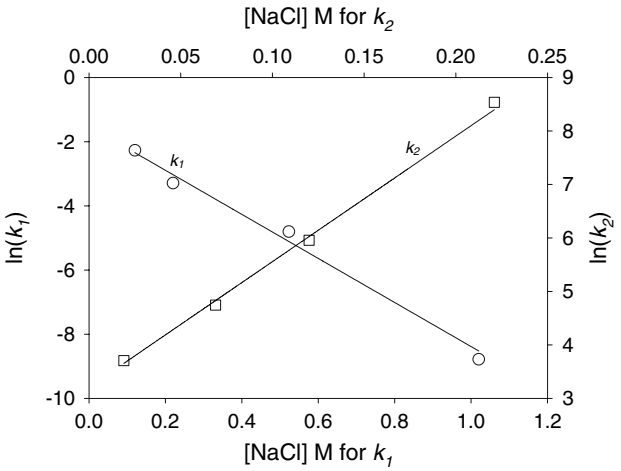

Fig. 6 Dependence of the unfolding rate constants of ferredoxin from *H. salinarum* on salt concentration. The k_1 (*decreasing*) and the k_2 (*increasing*) components are plotted separately. Experimental conditions as mentioned in the text. Note that the rate constant k_1 has a *negative sign* just to indicate the decreasing fluorescence intensity

(b) The native protein, again in 4.3 M NaCl, when transferred to salt concentration lower than 0.07 M NaCl leads to only the rising component. (c) Interestingly, lower the final salt concentration, greater is the rate of change of fluorescence intensity.

In Fig. 6 the logarithm of the rates obtained for decay and rising components for unfolding of the protein different salt jump experiments are presented. The functional relation of logarithm of rates for these two components against salt concentration is largely linear. Such a relation has been reported in cases where intermediates are not populated (Jackson and Fersht 1991a, b; Khorasanizadeh et al. 1993). Several questions can be raised for these two phases of kinetics. Firstly, are these two phases of kinetics related to unfolding of the protein? Secondly, if an increase in the fluorescence emission is indicative of unfolding of the protein then its decrease should not indicate the unfolding of the protein.

Characterization of the two phases of *Hs*Fd unfolding

To elucidate the nature of these two kinetic phases, different cation and anion were used for lowering the salt concentration. Figure 7(a–c) shows a family of curves obtained from these experiments. It is clear from Fig. 7a and b that the velocity of the decreasing component depends on the nature of cations and anions, whereas in the case of rising component (at low salt) the velocity is largely independent of the type of salt (Fig. 7c). It is known that different salt species, particularly at high concentration, affect protein stability to different extent. This follows the 'Hofmeister' series (Von Hippel and Schleich 1969; Arakawa and Timasheff 1984). This effect is taken as an indirect evidence for hydrophobic interactions (Von Hippel and Schleich 1969; Collins and Washabaugh 1985).

The rate constants computed from the experiments are plotted in Fig. 8. It may be seen from the kinetic traces (Fig. 7a–c) as well as from the plot of the rate constant against salt-type (Fig. 8) that the velocity is largely independent of the type of cation and anion in the low salt range (4.0 M > 0.05 M). Interestingly, at moderately high concentration of salt jump (4.0 M > 0.5 M) such rates are cation and anion specific. Furthermore, the anion effect is much more prominent than the cation (compare Fig. 7a, b). This is an indirect evidence of the involvement of solvent perturbation effect mediated by specific cations and anions. At a salt concentration of ~0.5 M electrostatic charge shielding is mostly satisfied (Lanyi 1974), thus the effect of salt-type on the reaction rate is most likely due to hydrophobic interactions. It is also known that sulfate acts as stabilizer or structure former (Von Hippel and Schleich 1969). Therefore the downward trend (and its enhancement in the presence of sulfate) may be considered to be the stabilized state of the protein mediated and facilitated by hydrophobic interactions.

The overall kinetic model for the decreasing and increasing components can be the following:

$$N \overset{k_1^{fast}}{\leftrightarrows} D1 \overset{k_1^{slow}}{\leftrightarrows} N' \quad \text{for the decreasing component}$$

$$N' \overset{k_2}{\leftrightarrows} L \quad \text{for the increasing component}$$

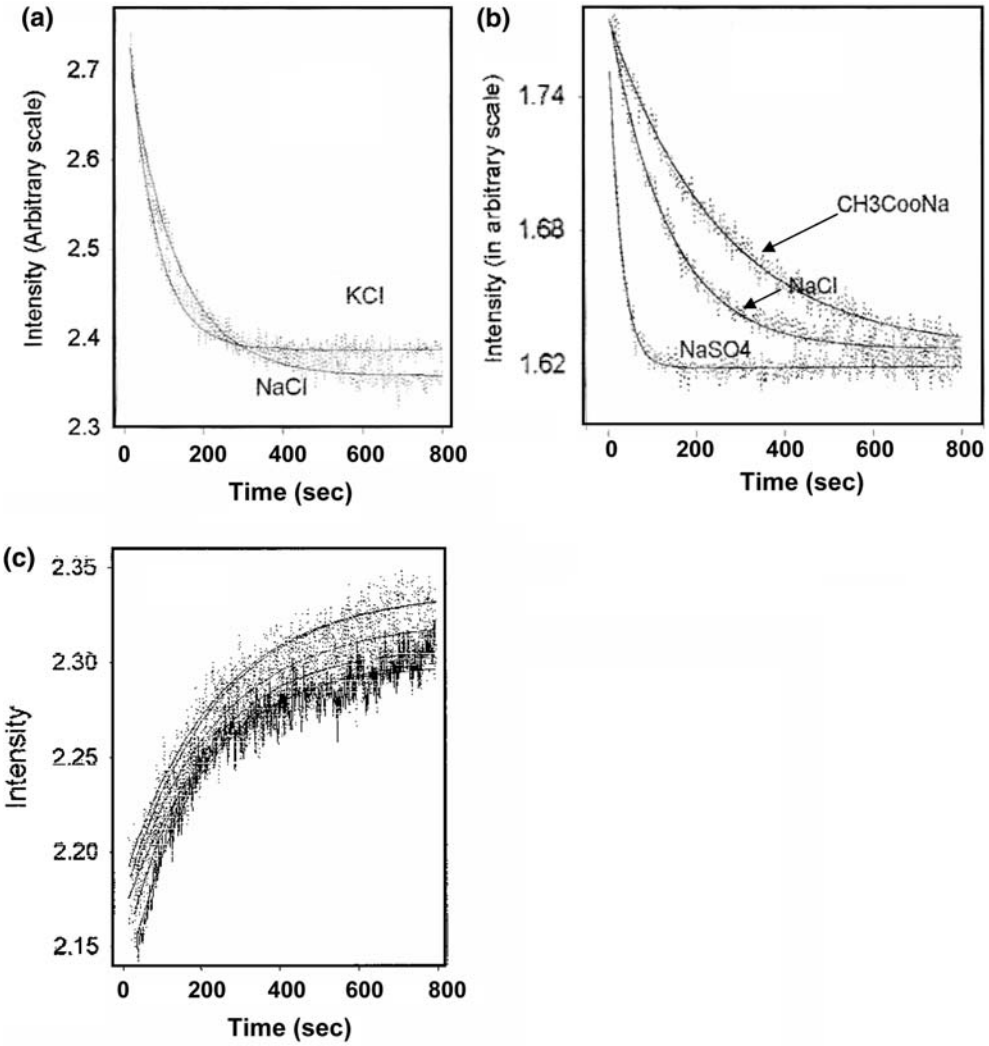

Fig. 7 Unfolding kinetics of ferredoxin from *H. salinarum* in presence of various cations (**a**), anions at high (**b**) and low (**c**) salt concentration. The anions used in **c** are SO_4, Cl, CH_3COO, NO_3 and Br. Experiments were performed as described in Fig. 5. Experimental conditions are similar to that of Fig. 5

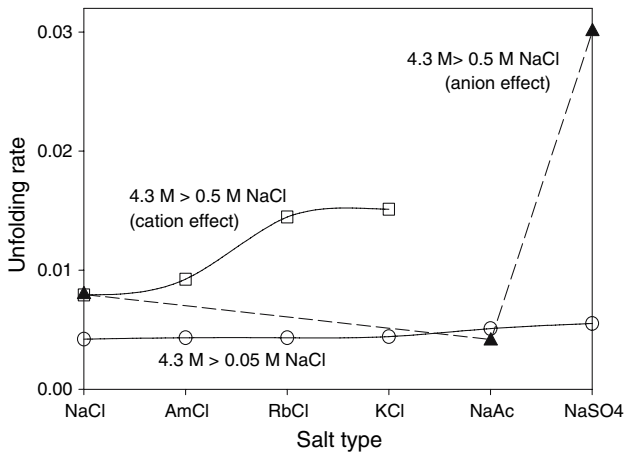

Fig. 8 Plot of rate constants as a function of salt-type

where D1 may be considered as a hydrophobic collapsed state which then unfolds due to increased charge repulsion and lead to the kinetic product N′.

Further characterization of the N′ was done by changing the salt concentration from 0.8 (N′) to 0.25 or 0.05 M (L) and from 4.3 (N) to 0.25 or 0.05 M NaCl in separate experiments. Interestingly the two sets of experiments resulted in different kinetic fate (Figs. 9, 10). The rates (0.0039 ± 0.0001 and 0.0052 ±0.0001) of former transitions are faster than those (0.0002 ± 0.0001 and 0.0034 ± 0.00023) of the latter. This could mean that in 0.8 M NaCl the protein is in a state (N′ state) quite distinct from native one. The comparison of the salt jump results presented in the Fig. 9 with that of Fig. 5 confirm the observation. In either 0.8 M → 0.05 M or 0.8 M → 0.25 M salt the first component is absent, which is seen in the 4.3 M → 0.25 M salt jump. Another interesting observation is that the rates in 0.8 M → 0.05 M and 0.8 M → 0.25 M salt jumps are higher than the 4.3 M → 0.05 M and 4.3 M → 0.25 M salt jumps, respectively. This is in agreement with our earlier equilibrium studies where we show the haloarchaeal protein to be in a sort of intermediate state in 0.8 M NaCl both during

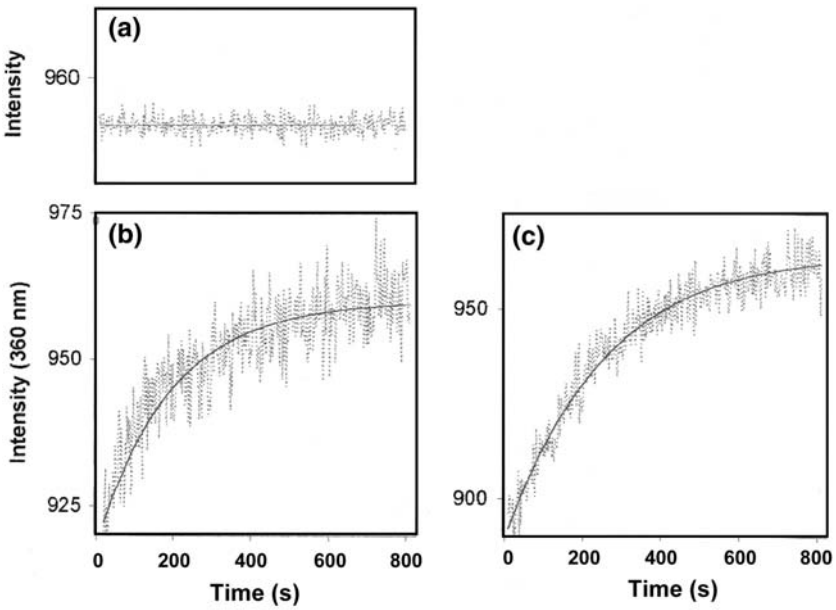

Fig. 9 Unfolding kinetics of ferredoxin from *H. salinarum*. **a** The protein, equilibrated in 0.8 M NaCl, was transferred to **b** 0.05 M and **c** 0.25 M NaCl in 25 mM sodium phosphate buffer pH 7.5. Other experimental details as mentioned in Fig. 5

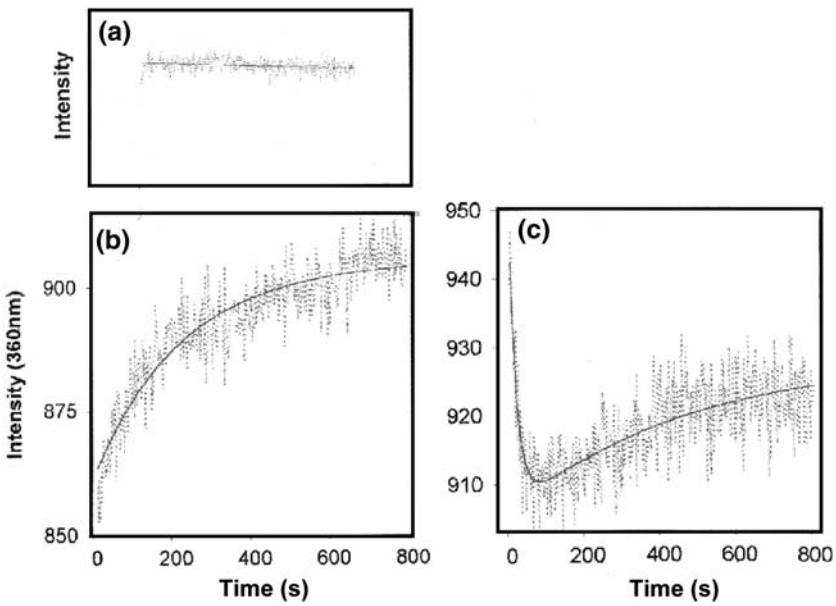

Fig. 10 Unfolding kinetics of ferredoxin from *H. salinarum*. **a** The native protein, in 4.3 M NaCl, was transferred to **b** 0.05 M and **c** 0.25 M NaCl in 25 mM sodium phosphate buffer pH 7.5. Other experimental details as in Figs. 5 and 9

unfolding in low salt and refolding when the salt concentration is increased (Bandyopadhyay et al. 2001).

Conclusions

Our results suggest that the unfolding of *Hs*Fd in low salt and urea induced unfolding of the native *Hs*Fd are influenced by pH. While the former does not involve kinetic intermediates and the rates of unfolding increase with increasing pH values, the latter indicates formation of kinetic intermediate and that the protein is most stable at neutral pH, the unfolding rates being higher at either acidic or alkaline pH values. This implies that although the native form is stable and is not perturbed by change of pH over a wide range (2), a pH change in presence of urea does destabilize the protein leading to its completely unfolded state. This is indicated by the similar unfolding end points (Figs. 1, 2). However, we had earlier shown that high salt concentration does confer stability on the native protein against denaturation by urea. The unfolding in low salt seems to be a two phase process. The first—fast phase—exhibits opposite effect to that of the

later—slow phase (Fig. 5). This might suggest that the protein undergoes transient compaction during the unfolding process when exposed to low salt. The rates of both the phases show an inverse linear dependence on salt concentration. Furthermore, our data clearly demonstrates that the initial unfolding phase, and therefore the stabilization of the protein, is dependent on the type of anion(s) and cation(s), whereas the latter phase is independent of the nature of the salt type.

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