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Salt-dependent studies of NADP-dependent isocitrate dehydrogenase from the halophilic archaeon *Haloferax volcanii*

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Abstract The salt-dependent stability of recombinant dimeric isocitrate dehydrogenase [ICDH; isocitrate: NADP oxidoreductase (decarboxylating), EC 1.1.1.42] from the halophilic archaeon *Haloferax volcanii* (*Hv*) was investigated in various conditions. *Hv* ICDH dissociation/deactivation was measured to probe the respective effect of anions and cations on stability. Surprisingly, enzyme stability was found to be mainly sensitive to cations and very little (or not) sensitive to anions. Divalent cations induced a strong shift of the active/inactive transition towards low salt concentration. A high resistance of *Hv* ICDH to chemical denaturation was also found. The data were analysed and are discussed in the framework of the solvation stability model for halophilic proteins.

Keywords Stability · Isocitrate dehydrogenase · *Haloferax volcanii* · Salt dependence · Halophilic · Protein–solvent interactions

Introduction

Archaeal and bacterial extremophilic micro-organisms thrive in ecological niches in which physico-chemical conditions define the limits of life. The understanding of the evolutionary adaptation of their biochemical machinery under these highly stressful conditions is of general importance for fundamental and applied biology.

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A number of studies on proteins from extremophiles have shown that they are perfectly adapted to function under conditions where their non-extremophilic counterparts unfold and precipitate (Jaenicke and Böhm 1998; Scandurra et al. 2000). The case of thermal adaptation is well documented. The comparative analysis of psychrophilic to hyperthermophilic proteins has permitted the establishment of some of the links that exist between biochemical properties and 3-D structure (Bell et al. 2002; Mandrich et al. 2004). In particular, it has been suggested that increased thermal stability occurs through the optimisation of electrostatic interactions (Karshikoff and Ladenstein 1998).

Proteins from extreme halophiles have also received a great deal of attention (for reviews, see Madern et al. 2000; Mevarech et al. 2000). Most have been isolated from halophilic Archaea (*Halobacteriaceae*). Halophilic proteins are soluble, stable and active in high salt concentrations. The difficulties encountered during their purification have been a stringent factor that has restricted the number of very detailed studies. Crystallographic studies have also been hampered, because the solubility of halophilic proteins remains high, even in the presence of salts well known to promote crystallisation (Pieper et al. 1998). Only six 3-D structures of halophilic proteins have been published so far (Dym et al. 1995; Frolow et al. 1996; Pieper et al. 1998; Richard et al. 2000; Yamada et al. 2002; Bieger et al. 2003). In this context, it is important to extend the biochemical studies of halophilic proteins.

Halophilic proteins in general deactivate and unfold at NaCl concentrations below 1–2 M (Pundak et al. 1981). Among these, malate dehydrogenase from *Haloarcula marismortui* (*Hm* MalDH) is the most extensively studied and has been proposed as a paradigm for halophilic adaptation. Its 3-D structure has been solved by X-ray crystallography and a wide range of biochemical and biophysical experiments have been published on its behaviour in solution. Studies on the effect of salts on *Hm* MalDH demonstrated that its stability, solvation and dynamics depend on the nature

of the salt (Zaccai et al. 1989; Bonneté et al. 1994; Madern and Zaccai 1997; Ebel et al. 1999b, 2002; Tehei et al. 2001). *Hm* MalDH solubility is governed by weak repulsive protein–protein interactions (Costenaro et al. 2002). Those studies led to a model in which protein–salt–water interactions drive halophilic stabilisation mechanisms (Zaccai and Eisenberg 1990; Ebel et al. 1999a). In salting-out salts, *Hm* MalDH behaves like a non-halophilic protein with similar solvation and thermal stabilisation whereas, in KCl or NaCl, the protein associates large amounts of salt and water in its solvation shell, co-ordinated by the excess of acidic residues, which might provide specific binding sites for hydrated ions. The crystallographic structure of *Hm* MalDH has allowed the observation of: (1) an increase in complex salt-bridge networks compared with non-halophilic homologues and (2) specific ion-binding sites (Richard et al. 2000). In particular, four strong chloride-binding sites are located at the dimer–dimer interface of the tetrameric enzyme, which allows an approach to understanding the effects of different ions on *Hm* MalDH stability (Ebel et al. 1999b, 2002; Madern and Zaccai 1997).

Clearly, however, it is important to characterise a larger variety of proteins from extreme halophiles. The present publication describes results on the salt-dependent behaviour of isocitrate dehydrogenase from *Haloferax volcanii* (*Hv* ICDH). The approaches used are similar to those used to study the effect of ions on *Hm* MalDH (Madern and Zaccai 1997; Ebel et al. 1999b). The *Hv* ICDH-encoding gene was previously cloned and overexpressed in *Escherichia coli* (Camacho et al. 2002), leading to sufficient amounts of enzyme for physicochemical, biochemical and structural studies. The recombinant halophilic protein has similar characteristics to the wild type (Camacho et al. 1995).

First, the effects of high and low NaCl concentration on the oligomeric state, secondary structure content, solvent exposure of tryptophan residues and deactivation of *Hv* ICDH were recorded. Second, we examined the effect of various cations and anions on protein stability. As could be predicted from *Hm* MalDH behaviour, divalent cations strongly increased *Hv* ICDH stability against low salt deactivation. However, in contrast to our expectations, *Hv* ICDH was found to be very little (or not) sensitive to anion type. The data were analysed in the context of results for *Hm* MalDH and other halophilic proteins. They provided evidence that the physical basis of the salt requirement may be different from that seen in the other systems, in which discrete ion-binding sites are clearly implicated.

Materials and methods

Effect of salts on the stability of *Hv* ICDH

All experiments were performed with *Hv* ICDH stock solutions (in 4.0 M NaCl) diluted to the desired salt and buffer conditions. Consequently, a residual NaCl concentration of 0.2 M was present in all conditions tested. Samples were incubated in various salt concentrations for 24 h at 20°C. After this treatment, various parameters (residual activity and/or spectral properties) were monitored, as described below.

Residual activity measurements

Residual activity measurements were done in the standard enzymatic assay for *Hv* ICDH, as described by Camacho et al. (1995). They were expressed as the percentage of enzyme activity maintained with respect to incubation in 4.0 M NaCl.

Spectroscopic measurements

A Jobin Yvon CD6 circular dichroism (CD) spectropolarimeter with thermostated sample holder was used. Data were recorded between 190 nm and 260 nm at 25°C with an interval of 1 nm and an integration time of 2 s, using cells with a path length of 0.1 cm. The protein concentration was 0.5 mg/ml in solutions buffered with Tris–HCl at pH 8. Each spectrum was corrected for buffer. The dependence of molar ellipticity values at 222 nm was expressed as a percentage of the value found for the enzyme maintained in 4.0 M NaCl before incubation (100%). Data recorded after 24 h of incubation at 0 M NaCl was taken as the zero value, rather than the one recorded after incubation in guanidine hydrochloride (GdnHCl).

Fluorescence measurements were carried out in a thermostated Aminco Bowman series 2 luminescence spectrometer. The spectra were recorded between 300 nm and 450 nm at 20°C in a 1-cm thermostated cuvette with an excitation wavelength of 295 nm and an integration time of 1 min. The dependence of fluorescence values at 335 nm was expressed as a percentage of the value found for the enzyme maintained in 4.0 M NaCl before incubation and corrected for the value found after 24 h of incubation at 0 M NaCl.

For each method, the spectra of control samples (not containing protein) were recorded and subtracted from the experimental samples, to correct for background interference.

Sedimentation velocity

Detailed calculations of the sedimentation coefficient ($S_{20,w}$) for halophilic proteins were as described by Franzetti et al. (2001) and Solovyova et al. (2001). Experiments were performed on a Beckman XLA analytical ultracentrifuge equipped with a UV scanning system, using a four-hole AN-60 Ti rotor with double centre-pieces and a path length of 1.2 cm. The sedimentation velocity profiles were monitored at 280 nm every 5 min, at 20°C and 42,000 rpm, in various NaCl

concentrations and analysed by dcdt$^+$ software (Philo 2000). The calculated (via amino acid composition) partial specific volume value was $\bar{v} = 0.7316$ ml/g for *Hv* ICDH. Data were corrected for density and viscosity. The mathematical function $g(s)$ represents the distribution of s values in the solution (i.e. the weight fraction of material with the sedimentation coefficient value s; Stafford 1992). It is calculated from an analysis of the time derivative using experimental sedimentation profiles. With this method, the baseline contribution is completely eliminated, allowing a proper analysis of the distribution of sedimenting material. The term $g(s^*)$ corresponds to the distribution of apparent sedimentation coefficients (s^*), uncorrected for the effects of diffusion. The values of sedimentation coefficient returned by the fit represent the values for the individual species. The time-derivative analysis was shown to be relevant for characterising the induced switch in protein oligomerisation state (Akoev et al. 2004).

Results

Effect of NaCl on *Hv* ICDH oligomeric state

Most of the archaeal ICDHs have been found to be dimeric (Steen et al. 2001). Analytical ultracentrifugation (AUC) is a particularly powerful method for determining the oligomeric state of a halophilic protein, because it accounts for their specific solvation, which could be significantly different from that of non-halophilic proteins. In order to probe the effect of salt concentration on the *Hv* ICDH structure, sedimentation velocity boundary profiles were recorded in various NaCl concentrations, as described in the Materials and methods. A first set of experiments was performed in 4.0 M NaCl, using three protein concentrations (0.15, 0.5, 2.0 mg/ml). The apparent differential distribution of the sedimentation coefficient [$g(s^*)$] calculated for each protein concentration was centred at the same position (data not shown), demonstrating that there was no dissociation at lower protein concentrations. The effect of NaCl concentration was tested at 0.5 mg/ml of *Hv* ICDH. The elapsed time from the sample preparation to the beginning of the AUC run was approximately 1 h. The data reflect, therefore, an initial snapshot of *Hv* ICDH behaviour when incubated at low salt concentration. The $g(s^*)$ profiles calculated for 4.0, 2.0, 0.4 and 0.3 M NaCl are presented in Fig. 1 (black lines). At high NaCl concentration, the profiles reflect a single species. The calculated $S_{20,W} = 5.4$ Svedberg units (S) for *Hv* ICDH (in 4.0 M NaCl) is in agreement with values (4.9–5.4 S) found for other dimeric archaeal ICDH (Pieper et al. 1998; Steen et al. 2001). The calculated molecular mass (91 kDa) of *Hv* ICDH was found to be in very close agreement with the theoretical value (91,678 Da) of a dimer calculated from its amino acid composition. When recorded at concentrations below 0.5 M NaCl, two species with different s values (pale grey lines) were taken into account to fit the $g(s^*)$ profiles of *Hv* ICDH. When the NaCl concentration decreases (compare profiles at 0.4 M and 0.3 M NaCl), the concentration of dimeric species ($S_{20,W} = 5.4$ S) decreases, whereas the

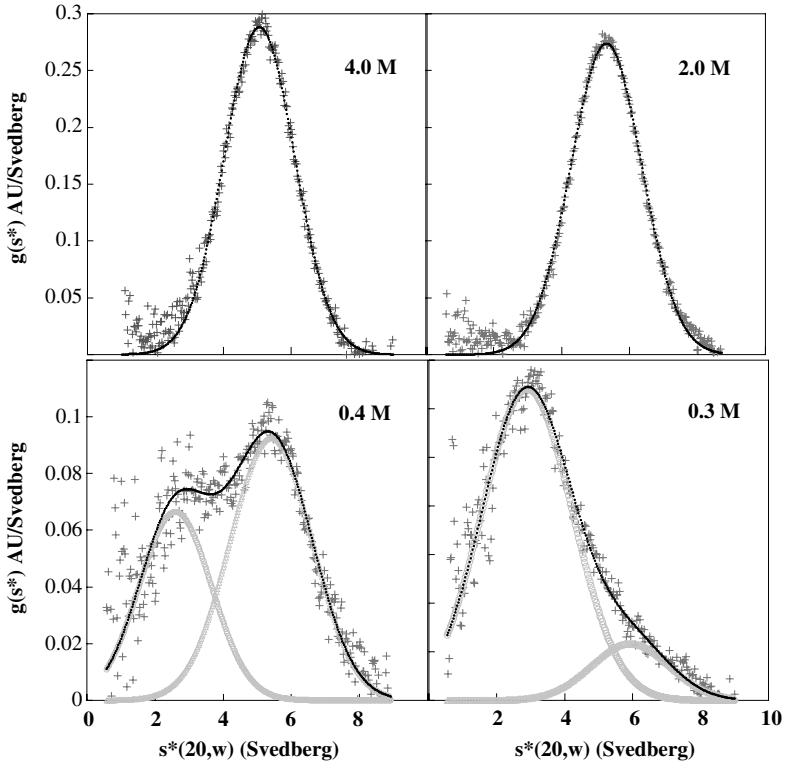

Fig. 1 Sedimentation velocity of *Hv* ICDH. Sedimentation velocity data (+) for *Hv* ICDH in various NaCl concentrations analysed by the time-derivative method (see Materials and methods). The *black lines* show apparent distribution functions $g(s^*)$ vs the sedimentation coefficient $s^*_{20,W}$ in Svedberg units (S). In high NaCl concentration (4.0 M, 2.0 M), the fitted $g(s^*)$ profiles (*black lines*) correspond to a single species. At low NaCl concentration (0.4 M, 0.3 M), the fitted $g(s^*)$ profiles (*black lines*) reflect the relative amount of two species (*pale grey lines*)

concentration of lower species ($S_{20,W}$≈2.5 S) increases. It should be pointed out that there is a discrepancy between the theoretical values ($S_{20,W}$≈3.6 S) of a compact folded monomer of *Hv* ICDH and the experimental values. Values of the sedimentation coefficient depend on the frictional coefficient (f/f_0), a parameter which reflects the protein shape. The calculation (data not shown) using the experimental $S_{20,W}$ values of *Hv* ICDH gives f/f_0≈1.9 for the monomer. Compared with the f/f_0 values of ≈1.25 generally observed for globular compact proteins, the higher value found for the monomer strongly suggests an extended conformation. The conformational change or partial unfolding explains why the calculated molecular mass (39 kDa) of the slowly sedimenting species was found slightly lower than that of a monomer (45.5 kDa) determined from the *Hv* ICDH sequence.

The AUC data demonstrated that a drastic change (dissociation) of dimeric *Hv* ICDH occurs when the protein is incubated at a low salt concentration.

Effect of NaCl on *Hv* ICDH stability

The AUC data showed that *Hv* ICDH starts dissociating when exposed to low NaCl concentrations. In order to refine the understanding of *Hv* ICDH behaviour at low salt concentrations, different parameters (secondary structure content, solvent exposure of tryptophan residues, residual activity) were recorded after 24 h of incubation at 20°C as a function of NaCl concentration. The data are presented in Fig. 2. In 4.0 M NaCl, the protein has a CD spectrum (left panel, black triangles) with a negative peak at 222 nm, specific for a folded protein with a high α-helical content, and a fluorescence spectrum with a maximum emission centred at 335 nm (central panel, black triangles). A CD spectrum indicating complete loss of secondary structure was obtained after *Hv* ICDH incubation at 4.0 M GdnHCl for 24 h (left panel, crosses). The fluorescence spectrum, recorded in the same condition, displayed a shift of its maximum emission toward 350 nm (central panel, crosses). The AUC data showed *Hv* ICDH exists as an extended or unfolded monomer at low NaCl concentration. Samples incubated at 0 M (left panel, open triangles), have a CD spectrum indicating a partially folded structure with a lower amount of helical structures, whereas the fluorescence spectrum (central panel, open triangles) is similar to the one obtained in 4.0 M GdnHCl. The residual activity of these three *Hv* ICDH samples was tested, using the standard assay as described in Materials and methods. Both samples incubated at 0 M NaCl and 4.0 M GdnHCl were inactive, in contrast to the sample incubated at 4.0 M NaCl, which was fully active after 24 h.

It was checked whether or not the low salt deactivation is a reversible process. Samples of *Hv* ICDH were incubated in 0.2, 0.6 and 2.0 M NaCl at 20°C for 24 h, followed by an additional 24 h of incubation at 3.3 M NaCl. The highest concentration was obtained by the addition of 5.0 M NaCl to the samples. Both samples incubated at 0.6 M and 2.0 M displayed the same high level of residual activity (100%), whereas those incubated at 0.2 M NaCl had only 5% of residual activity. This indicates that, below a cut-off NaCl concentration, *Hv* ICDH undergoes an irreversible deactivation. In some cases, a small precipitate was observed at low salt concentrations, suggesting an evolution of the dissociated *Hv* ICDH toward aggregates.

Taken together, our data demonstrate that deactivation due to incubation of *Hv* ICDH at low NaCl concentrations is related to irreversible dissociation of the active dimeric species towards a monomer, which can be described as an extended inactive monomeric species with a residual amount of secondary structure. Solvent exposure of tryptophan residues occurs during dissociation, suggesting they are located in the vicinity of the monomer–monomer interface making the dimer.

Residual activity, CD and fluorescence data as a function of NaCl incubation concentration are plotted

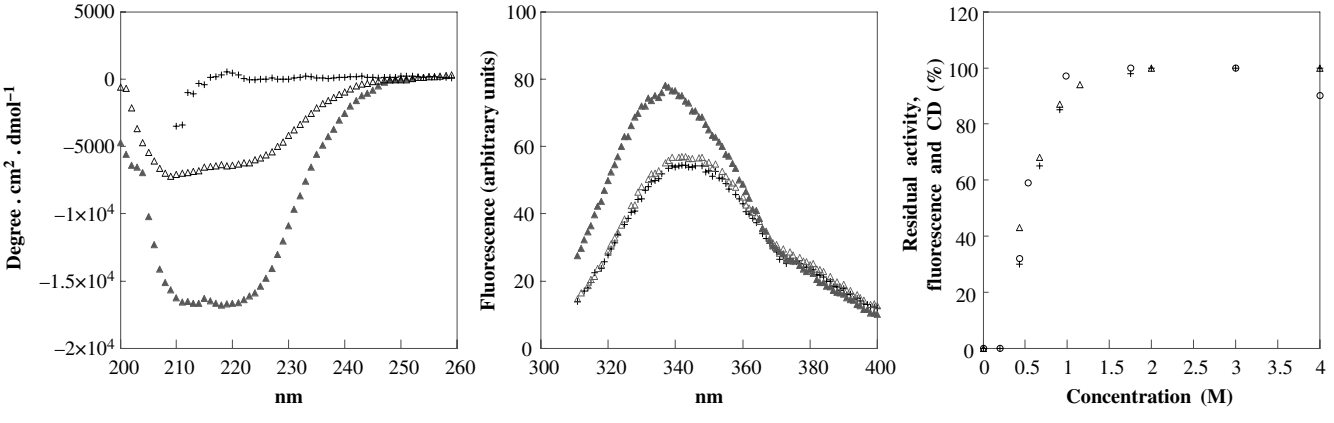

Fig. 2 Effect of NaCl on *Hv* ICDH stability. *Left panel* CD spectra recorded after 24 h of incubation at 20°C in 4.0 M NaCl (▲), 0 M NaCl (△) and 4.0 M GdnHCl (+). *Central panel* Fluorescence spectra recorded in similar conditions. *Right panel* Percentage of residual activity (+), fluorescence (○) and CD (△) recorded in various NaCl concentrations

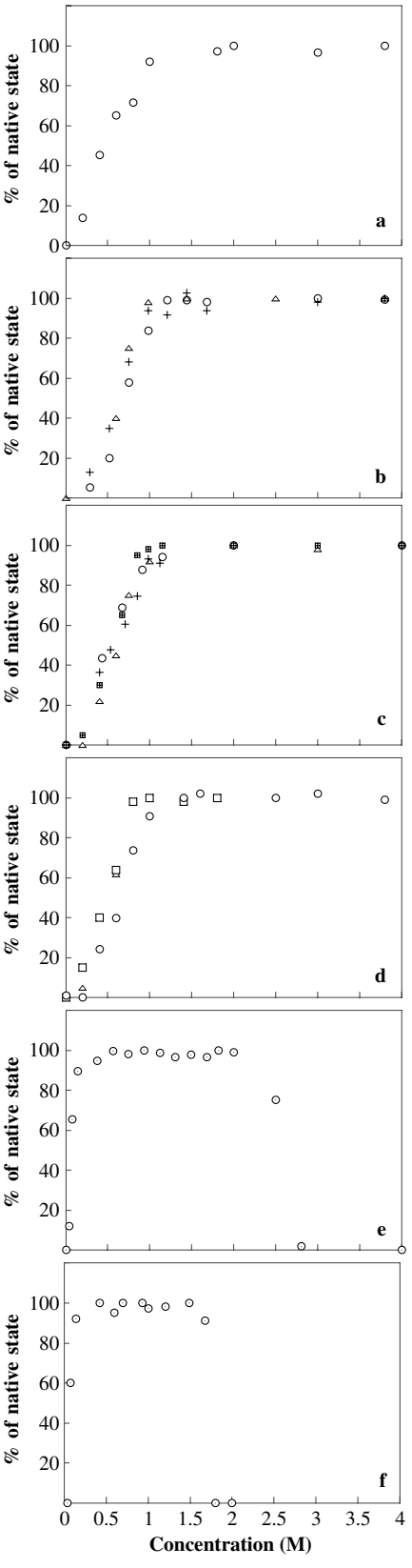

Fig. 3a–f Salt concentration dependency of *Hv* ICDH stability. Residual activity or CD was recorded after 24 h of incubation in various salts, as explained in Materials and methods. As analysed in the text, data are expressed as percentages of the native dimeric state. **a** CsCl (○), **b** KCl (○), KCH_3COO (△) and KF (+), **c** NaCl (○), NaF (+), $NaCH_3COO$ (△) and NaBr (⊠) **d** $(NH_4)_2SO_4$ (□), NH_4CH_3COO (△) and NH_4Cl (○), **e** $MgCl_2$ (○), **f** $CaCl_2$ (○). The data can be used to analyse the effects of cations, with chloride as co-ions, by comparing the *circles* in **a–f**. The effect of anions can be analysed using either K^+, Na^+ or NH_4^+ as co-ions in **b**, **c** and **d**, respectively. In all salts conditions tested, there is a residual NaCl concentration of 0.2 M

in Fig. 2, right panel. Experimental details are explained in the Materials and methods. In the case of CD, the data were expressed as a percentage relative to the values recorded between 4.0 M (dimeric state) and 0 M (partially folded monomeric state) and not relative to the unfolded state. The three curves are superimposable, indicating that the dissociation, unfolding and deactivation processes of *Hv* ICDH are concomitant.

Effect of ions on *Hv* ICDH

The deactivation of halophilic proteins incubated at low salt concentration is a kinetic process (Pundak et al. 1981; Bonneté et al. 1994; Jaenicke 2000). It was checked that *Hv* ICDH deactivation follows first-order kinetics in four different salt conditions (data not shown). As already observed with various halophilic proteins (Pundak et al. 1981; Bonneté et al. 1994; Madern et al. 1995; Bonete et al. 2003), the deactivation rate constant of *Hv* ICDH decreases when the salt concentration increases. The results are presented using similar graphic representations to those for *Hm* MalDH, i.e. in terms of a percentage of the folded fraction (Ebel et al. 1999b).

To our knowledge, the respective effects of anions and cations on the stability of a halophilic protein have been studied only in *Hm* MalDH, for which it was shown that cations and anions induced drastically different effects during low salt deactivation (Madern and Zaccai 1997; Ebel et al. 1999b). It was examined whether or not cations and anions act in a similar fashion on *Hv* ICDH stability, by recording either the CD spectrum or residual activity in various salts and salt concentrations. The effect of CsCl, KCH_3COO, KF, KCl, $NaCH_3COO$, NaF, NaCl, NaBr, $(NH_4)_2SO_4$, NH_4CH_3COO, NH_4Cl, $MgCl_2$ and $CaCl_2$ on *Hv* ICDH at 20°C are shown in Fig. 3. The data are expressed as percentages of the native state and reflect the effect of salt on the dissociation/unfolding pathway measured either by CD or by activity. It should be kept in mind that these measurements were made by dilution of a *Hv* ICDH stock solution containing NaCl, leading to a residual 0.2 M NaCl concentration in the assays. As shown by the data recorded in NaCl, this concentration is not sufficient to stabilise *Hv* ICDH.

Effect of cations

Dimeric *Hv* ICDH is stable (active and folded) in the range from 4.0 M to 1.0 M in all monovalent cations

(Cs^+, K^+, Na^+, NH_4^+) tested (Fig. 3a–d). Incubation of *Hv* ICDH at low salt concentration results in inactivation, which is related to dissociation of the dimer into inactive monomer. This phenomenon occurred when the salt concentration of the solution dropped below 1.0 M, with no noticeable discriminating effect according the nature of cation. The low salt concentration at which 50% (LSC_{50}) of *Hv* ICDH is inactive and dissociated was almost identical (0.6 ± 0.15 M) in all the salts tested. These data are in contrast to those recorded using *Hm* MalDH, for which was found a variation of wider amplitude ($\approx$1.0 M) of LSC_{50} (Ebel et al. 1999b).

In contrast to the curves observed with monovalent cations (Fig. 3e,f), the $MgCl_2$ and $CaCl_2$ stability curves of *Hv* ICDH have a bell-shape, showing that the enzyme deactivates also when the salt concentration increases. This phenomenon was observed first with *Hm* MalDH (Zaccai et al. 1989; Madern and Zaccai 1997; Ebel et al. 1999b). *Hv* ICDH is stable in the concentration ranges 0.15–1.5 M $CaCl_2$ and 0.3–2.0 M $MgCl_2$. We recall that *Hm* MalDH was found to be stable in a narrower concentration range of these salts: 0.35–0.5 M $CaCl_2$ and 0.45–0.65 M $MgCl_2$. The LSC_{50} of *Hv* ICDH (Fig. 3) was $\approx$0.04 M in both $MgCl_2$ and $CaCl_2$. Compared with the one found with a monovalent cation (0.6 M), we conclude that divalent cations induce a drastic stability shift for *Hv* ICDH towards very low salt concentrations.

Effect of anions

The effect of anions on a halophilic protein was tested using *Hm* MalDH, for which it was found that anions of high charge density are the most efficient to stabilise the native state against low salt deactivation (Ebel et al. 1999b). In particular, there is a clear stabilising effect of SO_4^{2-}, F^- ($LSC_{50} \approx 0.40$ M), compared with Cl^- ($LSC_{50} = 1.0$ M) and Br^- (unstable). This observation is valid whatever the co-cation.

In the case of *Hv* ICDH (Fig. 3b–d), when comparing transitions in KCH_3COO and KF versus KCl (panel b), $NaCH_3COO$ and NaF versus NaCl (panel c) and $(NH_4)_2SO_4$ and NH_4CH_3COO versus NH_4Cl (panel d), there is no clear evidence of a strong LSC_{50} modification. Identical LSC_{50} values ($\approx 0.6 \pm 0.2$ M) were recorded. More strikingly, *Hv* ICDH is completely stable in NaBr (panel c), whereas this was not reported to be the case with *Hm* MalDH.

Our data show that *Hv* ICDH low salt deactivation is very likely not to be sensitive to anion effects.

Discussion

The present work shows that ICDH from *Hv* is a dimer at high salt concentration, as was found with other archaeal ICDHs (Camacho et al. 1995; Steen et al. 2001). When incubated at low salt concentrations, *Hv* ICDH undergoes irreversible deactivation, due to dissociation of the dimer towards an inactive, partially folded monomeric species. The efficiency of different ions in the stabilisation of the native state of *Hv* ICDH against low salt deactivation was compared with similar data for *Hm* MalDH (Madern and Zaccai 1997; Ebel et al. 1999b; Irimia et al. 2003). The salt concentration at which 50% of *Hv* ICDH is still folded (LSC_{50}, from Fig. 3) was used to classify the order of cation and anion stabilisation efficiency, with Cl^- and Na^+ as the counter-ions, respectively:

Ca^{2+}, Mg^{2+}	$\gg NH_4^+, Na^+, K^+, Cs^+$		*Hv* ICDH
0.02 M	0.6 – 0.8 M		
Ca^{2+}, Mg^{2+}	$\gg NH_4^+, Na^+ > K^+$	$> Cs^+$	*Hm* MalDH
0.05 M	0.6 – 0.8 M	1.6 M	
	$SO_4^{2-}, CH_3COO^-, F^-, Cl^-, Br^-$		*Hv* ICDH
	0.6 – 0.8 M		
$SO_4^{2-}, CH_3COO^-, F^-$	$\gg Cl^-$	$> Br^-$	*Hm* MalDH
0.1 M	0.6 – 0.8 M	2.5 M	

In contrast to *Hm* MalDH, *Hv* ICDH transition curves in all salts tested with a monovalent cation occurred in the same narrow range of concentration (± 0.2 M), preventing the possibility to easily discriminate the relative order of their efficiency. A very similar order of cation efficiency was found with various halophilic proteins (Taupin et al. 1997; Kobayashi et al. 1994).

Thermodynamic studies on protein hydration and solvation, in general, have shown that a hydration–solvation shell of a different composition from the bulk solvent may lead to instability and protein unfolding or precipitation (Timasheff 1992; von Hippel and Schleich 1969). Cations have been proposed to act on halophilic protein stability by a mechanism that implies the weak binding of a solvation shell (a network) by the acidic protein surface and/or by binding through weak cation-binding sites (Madern et al. 2000; Mevarech et al. 2000). Results from the present work suggest that *Hv* ICDH salt-dependent behaviour is controlled mainly by cations. There is also a strong stabilising effect against low salt deactivation and dissociation of *Hv* ICDH, which is induced by divalent cations, an observation in agreement with data for *Hm* MalDH (Madern and Zaccai 1997; Ebel et al. 1999b). Analysis of the *Hv* ICDH composition showed its acidic nature (Camacho et al. 2002). Surface acidic residues may well play a role in the organisation of a specific solvation shell. It was shown for *Hm* MalDH that such a shell (of a composition very close to the bulk solvent composition) contributes to the solubility and stability of the native structure (Ebel et al. 2002; Costenaro et al. 2002). Divalent cations are strong water-structure makers due to their high charge density. In particular, the Mg^{2+}/K^+ charge density ratio is $\approx$7 (Collins 1997). The data on *Hv* ICDH agree well with the idea that the divalent cation effect on stability is due to a more highly efficient binding of the halophilic protein to surface-exposed carboxylates (Zaccai et al. 1989; Ebel et al. 2002; Costenaro et al. 2002).

The destabilising effect of high concentrations of divalent cations on *Hv* ICDH fits well with previous observations using *Hm* MalDH (Zaccai et al. 1989; Madern and Zaccai 1997; Ebel et al. 1999a). This phenomenon is governed by the general "salting-in" effect on proteins (von Hippel and Schleich 1969; Schellman 1987; Timasheff 1992), which is proposed to be valid also for any halophilic proteins (Zaccai et al. 1989).

More strikingly, when compared with *Hm* MalDH, it was observed that *Hv* ICDH is not very sensitive to the effect of anions. AUC experiments on *Hm* MalDH, using Cl^- or F^- with K^+ as the counter-ion, showed that fluoride strongly stabilises the enzyme against dissociation, an observation that highlighted the critical role of intersubunit anion-binding sites in *Hm* MalDH (Irimia et al. 2003). The crystallographic structures of three oligomeric halophilic proteins [*Hm* malate dehydrogenase, *Hm* catalase-peroxidase (Cp), *Hm* dodecameric flavin-binding protein (DFBP); Richard et al. 2000; Yamada et al. 2002; Bieger et al. 2003] show that intersubunit chloride-binding sites might be seen as one of the main structural adaptative features selected by nature to maintain the native state of halophilic proteins.

In the case of *Hm* Cp and *Hm* DFBP, it is not possible to establish a structural link with their salt-dependent behaviour (relative to the effect of cations and anions) because it has not been documented. No anion-binding sites were described in either ferredoxin or dihydrofolate reductase (DHFR; Frolow et al. 1996; Pieper et al. 1998). The effect of salts on the stability of *Hv* DHFR was studied by Wright et al. (2002). In contrast to *Hm* MalDH, the halophilic DHFR is stabilised in NaCl or KCl by preferential hydration, in a similar way to a non-halophilic protein.

In the case of *Hv* ICDH, it is reasonable to suggest two hypotheses concerning the lack of anion effects: either (1) there are no anion-binding sites in dimeric *Hv* ICDH, or (2) there are very few weak anion-binding sites, whose role in stabilisation is masked by the high intrinsic stability of the protein. Three-dimensional protein architecture is maintained by the sum of weak forces due to electrostatic, Van der Waals, H-bonds and hydrophobic effects, both within the polypeptide chain and between the protein and the solvent. Numerous studies have shown that protein–ion interactions strongly contribute to the stability of halophilic proteins (Dill 1990; Madern et al. 2000; Mevarech et al. 2000; Minton 2001; Costenaro et al. 2002).

Without any structural information concerning *Hv* ICDH, the present study strongly suggests that *Hv* ICDH might be seen as a new type of halophilic protein never described before: an oligomeric halophilic protein devoid of intersubunit anion-binding sites. This finding shows that subtle differences in halophilic adaptation can be described at the protein level, as was recently observed with the discovery of a completely non-halophilic enzyme from an extreme halophilic micro-organism (Madern and Zaccai 2004).

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