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Behaviour in different denaturant agents and structural characterization by fluorescence techniques of *Haloferax mediterranei* D-2-hydroxyacid dehydrogenase

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ABSTRACT

The halophilic enzyme D-2-hydroxyacid dehydrogenase (D2-HDH) from *Haloferax mediterranei* is found to be an oligomeric enzyme composed of two identical subunits. Fluorescence spectra of native and denatured protein and effect of denaturants such as urea and guanidine hydrochloride on enzyme activity of halophilic D-2-hydroxyacid dehydrogenase have been analysed. Native D2-HDH shows the maximum emission at 340 nm. The denaturation process caused an exposure to the solvent of the tryptophan residues, as manifested by the red shift of the emission maximum from 340 to 350 nm. When urea was used as denaturant agent the enzyme required long incubation times, higher to 24 h, to unfold. Fluorescence quenching by KI and acrylamide was also carried out; showing that the tryptophan residues are mainly located near the enzyme surface.

1. Introduction

A new D-2-hydroxyacid dehydrogenase (EC 1.1.1.) with dual coenzyme specificity from the halophilic Archaeon *Haloferax mediterranei* has been heterologous overexpressed in *Escherichia coli*, purified and characterized in our laboratory [1]. It has a native M_r of 111 ± 9 kDa determinated by gel filtration and it catalyses the stereospecific and reversible NAD(P)H-dependent reduction of α-ketocarboxylic acids into the corresponding α-hydroxy carboxylic acids, as depicted in equation:

$$RCOCOOH + NAD(P)H + H^+ \leftrightarrow RCH(OH)COOH + NAD(P)^+$$

The family of D-2-ketoacid dehydrogenases includes enzymes such as D-2-hydroxyisocaproate dehydrogenase (HicDH), formate dehydrogenase (FDH), D-glycerate dehydrogenase (GDH), vancomycin-resistant protein H (VanH), D3-phosphoglycerate dehydrogenase (D-PGDH) and D-lactate dehydrogenase (D-LDH).

The three-dimensional structures of D-lactate dehydrogenase from *E. coli* [2], D-3-phosphoglycerate dehydrogenase from *Mycobacterium tuberculosis* [3] and D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei* [4] have been determined, providing considerable insights for structure–function relationships studies of this class of enzymes. The study of halophilic proteins has a particular interest, since subunit interactions may be additionally involved in protein stabilization.

As a common theme of the enzymes from some halophilic organisms, in the D-2-hydroxyacid dehydrogenase from *H. mediterranei* we found the cumulative content of acidic residues to be much higher than the cumulative content of basic residues [1]. This strong acidic composition makes the structures of these enzymes strongly salt dependent [5,6].

Conformational and dynamics changes of proteins can be studied by characterizing the fluorescence emission from intrinsic fluorophores such as tryptophan, tyrosine and phenylalanine. In proteins containing all three aromatic amino acids, fluorescence is usually dominated by the contribution of the tryptophan residues as their absorbance at the wavelength of excitation and their quantum yield of emission are considerably greater than the respective values for tyrosine and phenylalanine. Tryptophan fluorescence is highly sensitive to environment; therefore, these residues are useful structural probes. Quenching studies provide valuable information concerning the exposure of tryptophan residues and the dynamics of the protein matrix surrounding such residues [7,8].

In the present paper we report fluorescence and quenching studies of the native and denatured protein as well as the effect of denaturants on activity of D-2-hydroxyacid dehydrogenase from

Abbreviations: D2-HDH, D-2-hydroxyacid dehydrogenase; GdmCl, guanidine hydrochloride.

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H. mediterranei in order to allow a better understanding of some aspects of its structure.

2. Experimentals

2.1. Biological material

D-2-hydroxyacid dehydrogenase from the extremely halophilic Archaea *H. mediterranei* was overexpressed and purified as previously described [1] and used as biological material for all experiments.

2.2. Chemicals

α-Ketoisocaproate was obtained from Sigma–Aldrich and the coenzyme NADH was purchased from Roche. All other chemicals were purchased from Sigma–Aldrich. All salts were of analytical grade and acrylamide, guanidine hydrochloride and urea were of molecular biology grade. Ultrapure water type Milli Q 18 mΩ was used for all experiments. All buffers and reagents were filtered before fluorescence measurements.

2.3. Enzyme assay

D2-HDH was routinely assayed spectrophotometrically (Ultrospec 2000 spectrophotometer from Pharmacia-Biotech) at 40 °C in Tris–HCl buffer, pH 8.0, containing 4 M NaCl, 0.3 mM NADH and 25 mM α-ketoisocaproate. One unit of enzymatic activity was defined as the amount of enzyme which catalyzes the consumption of 1 μmol of NADH min^{-1} under assay conditions.

2.4. Fluorescence spectroscopy

The ultraviolet fluorescence spectra were determined in a FP-6500 Jasco spectrofluorimeter. Measurements were carried out using 1 cm path-length cuvettes. The intrinsic fluorescence emission spectrum was scanned from 310 to 400 nm after excitation at 295 nm. All emission spectra were recorded using the 500 nm min^{-1} scanning speed of the fluorimeter. The slit width on the excitation and emission monochromators was 3 and 10 nm, respectively. The buffer used was 20 mM Tris–HCl, pH 8.0, containing 2 M NaCl and 3 mM EDTA for all experiments. Fluorescence spectra of control samples, without protein, were recorded and subtracted from the experimental samples to correct the background interference in all experiments. Samples for all spectroscopic measurements were previously filtered through 0.20 μm pore size membrane filters (Millipore 0.2 μm).

2.5. Denaturation studies

The enzyme denaturation by urea and guanidine hydrochloride (GdnCl) was performed by incubation in 20 mM Tris–HCl buffer, pH 8.0, containing 3 mM EDTA and 2 M NaCl. The enzyme concentration used was 2.5 μg ml^{-1}. Urea or guanidine hydrochloride was added to the denaturation mix. The stock solution used for Urea was 9 M Urea in buffer 20 mM Tris–HCl pH 8.0, 3 mM EDTA and 2 M NaCl and for GdnCl was 6 M GdnCl in the same buffer.

The emission spectrum was recorded at 25 °C. The incubation time was 5 min. The peak fluorescence of the native protein was taken as 100% and the fluorescence peak values under the given experimental conditions were calculated relative to this value.

The renaturation process was carried out using Tris–HCl 20 mM, pH 8.0 buffer containing 4 M NaCl, 3 mM EDTA by fast dilution of the denaturated enzyme.

2.6. Fluorescence quenching studies

Quenching of protein intrinsic fluorescence by KI or acrylamide was carried out at two different temperatures (25 and 40 °C) in order to determine whether the quenchers were dynamic or static. 0.1 M sodium thiosulfate was added to the KI solution to prevent free iodine formation [9]. The ionic strength of the solutions was kept constant by increasing the unquenching salt concentration and adding Tris–HCl 20 mM, pH 8.0 buffer without salt.

The initial fluorescence (F_0) of the solution was measured. The enzyme fluorescence from the tryptophan residues was then quenched by the progressive addition of small aliquots of the stock solution of the quenchers to the fluorimetric cuvette, and the fluorescence intensity (F) was measured again. The data on quenching were analyzed according to the Stern and Volmer equation,

$$\frac{F_0}{F} = 1 + K_q[Q] \quad (1)$$

where K_q is the rate constant characteristic for the quenching process and [Q] indicates the concentration of the quencher. Eq. (1) was modified by Lehrer Eq. (2) [9],

$$\frac{F_0}{\Delta F} = \frac{1}{[Q]} f_a K_q + \frac{1}{f_a} \quad (2)$$

where F and F_0 are the quenched and unquenched fluorescence quantum yields or intensities, respectively, and f_a is the quenchable fraction of fluorophores. Eq. (2) shows that the values of f_a and K_q can be determined from the slope and intercept of the linear fit to the plot of $F_0/\Delta F$ versus $[Q]^{-1}$. $(f_a)_{eff}$ is the value of the 'effective' fractional quenchable fluorophores from 1/intercept, and $(K_q)_{eff}$ is the 'effective' quenching constant obtained from the intercept/slope.

Fluorescence quenching studies were carried out in denaturing and non denaturing conditions after incubation of the enzyme (2.5 μg ml^{-1}) for 5 min in 20 mM Tris–HCl buffer, pH 8.0, 3 mM EDTA, 2 M NaCl, with and without, respectively, of 3 M GdnCl. The emission spectra were registered at 25 °C.

2.7. Calorimetric studies

High sensitivity DSC measurements were carried out using an ultra-sensitive differential scanning calorimeter (VP-DSC, MicroCal). It is especially designed for studies on dilute aqueous solutions of biological macromolecules. Protein concentrations used in the DSC studies varied between 0.7 and 0.8 mg mL^{-1}. The buffer was Tris–HCl 20 mM, pH 8.0, 3 mM EDTA, 4 M NaCl. All samples and buffers were degassed by stirring under vacuum before loading into the sample and reference cells. The heat flow required to keep the sample cell and reference cell thermally balanced was recorded from 25 to 100 °C, using scan rate of 1.0 °C/min. The buffer background was subtracted from each sample-reference scan during the data analysis process. T_m is the midpoint transition temperature at which folded and unfolded molecules are equally populated. The calorimetric enthalpy ΔH was determined by integrating the area under the transition peak. Data manipulation was performed using Origin® software provided with the instrument.

3. Results

3.1. Effect of denaturing agents on the fluorescence of D2-HDH

The fluorescence spectra of native D2-HDH showed a maximum at 340 nm. No changes in maximum excitation and emission wavelengths were found in fluorescence spectra at 25 and 40 °C. Incubation of D2-HDH at increasing concentrations of GdnCl resulted in a progressive change of intrinsic fluorescence emission

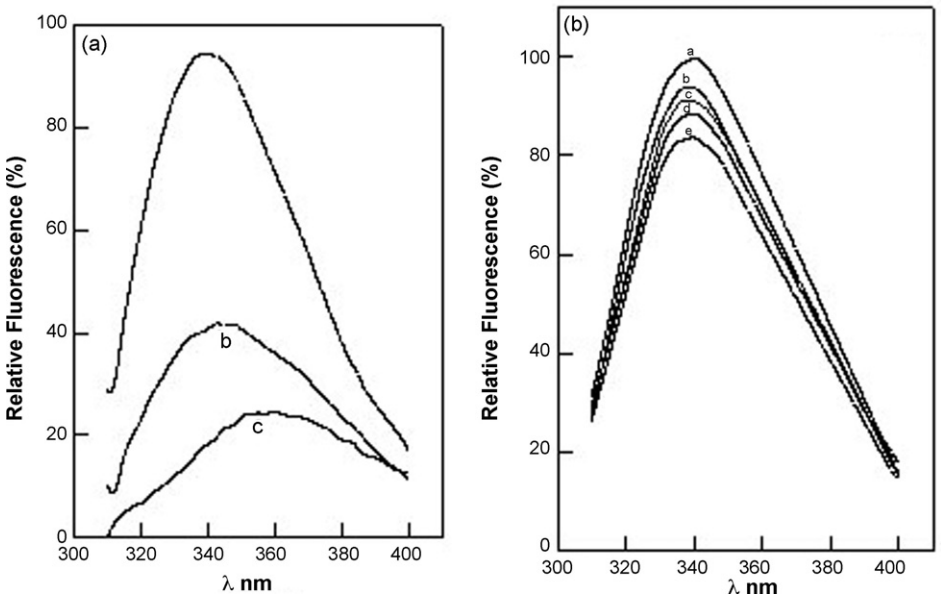

Fig. 1. Fluorescence spectra of D-2-hydroxyacid dehydrogenase from *H. mediterranei*. (a) With guanidine hydrochloride: (a) 0 M, (b) 2 M and (c) 5 M. (b) With urea: (a) 0 M, (b) 2 M, (c) 4 M, (d) 5 M and (e) 8 M. All spectra were obtained after 5 min of incubation in 20 mM Tris–HCl, pH 8.0, 4 M NaCl, 3 mM EDTA plus the denaturant agent.

to a longer wavelength, 355 nm, showing that tryptophan residues had been exposed to the aqueous solvent as a result of the enzyme denaturation by GdnCl (Fig. 1(a) and Fig. 2(a)). The D2-HDH fluorescence was quenched upon unfolding with GdnCl, decreasing 53% and 64% of the original value after incubation with 2 and 3 M GdnCl, respectively. However, addition of urea did not cause a shift of emission wavelength in the same period of time and, throughout the concentration range tested going from 0 to 8 M, urea exerted a quenching effect much lower than GdnCl (Fig. 1(b) and Fig. 2(a)). This quenching effect exerted by the denaturant agents has also been described in other halophilic proteins such as halophilic class I aldolase and glyceraldehyde-3-phosphate dehydrogenase from *Haloarcula vallismortis* [10]. Therefore, as increasing concentrations of urea do not cause unfolding of the protein, we checked the effects of incubation in 8 M urea during long periods of time (Fig. 2(b)).

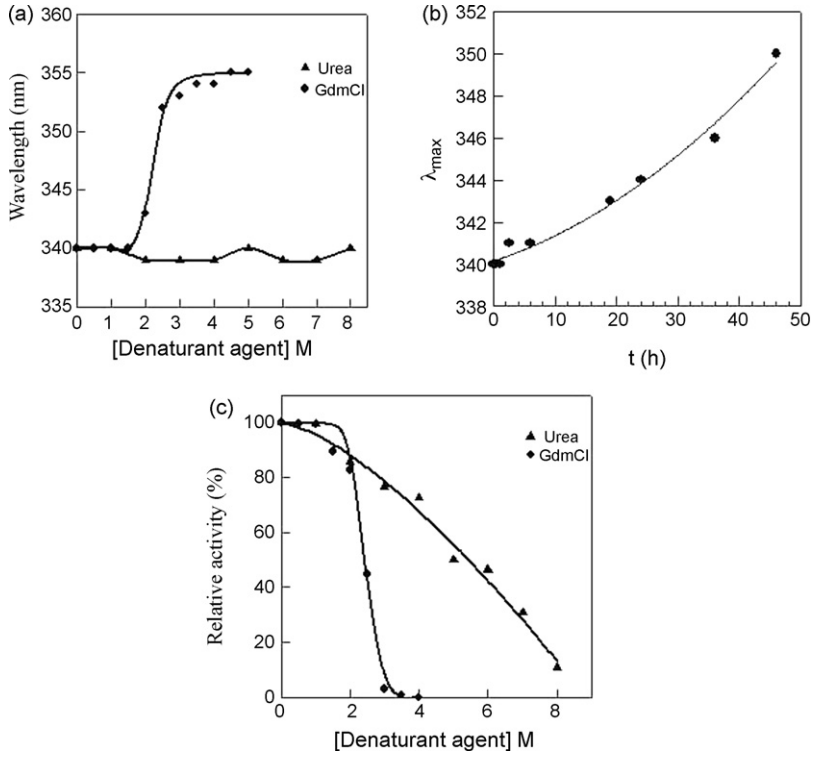

Fig. 2. Shift of the emission maximum wavelength of D-2-hydroxyacid dehydrogenase by (a) rising concentrations of denaturant agents after 5 min incubation: (▲) urea, (●) GdnCl; (b) long time incubation in 8 M urea; (c) effects of rising concentrations of denaturant agents on the activity of D-2-hydroxyacid dehydrogenase in 20 mM Tris–HCl, pH 8.5 buffer, 4 M NaCl plus 25 mM α-ketoisocaproate and 0.3 mM NADH: (▲) urea, (●) GdnCl.

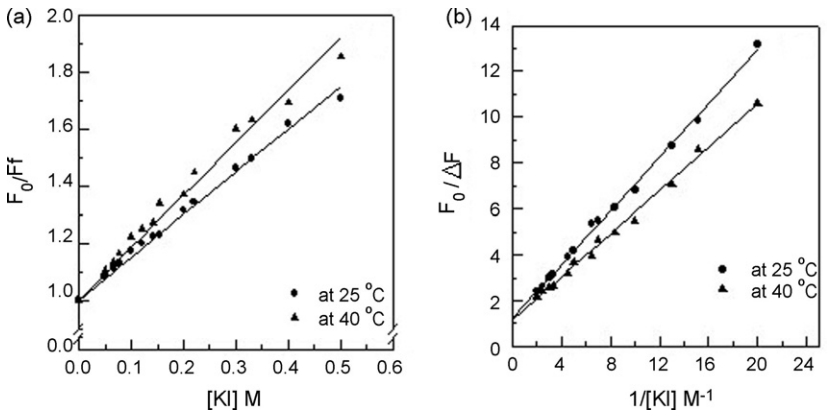

Fig. 3. Stern–Volmer plots (a) and modified Stern–Volmer plots (b) of the quenching of D-2-hydroxyacid dehydrogenase (2.5 μg ml^{-1}) by rising concentrations of KI.

Table 1
Quenching constants and $(f_a)_{eff}$ values of *Haloferax mediterranei* D2-HDH.

Quencher	Temperature (°C)	K_q (M^{-1})	$(f_a)_{eff}$	$(K_q)_{eff}$ (M^{-1})
KI	25	1.50 ± 0.02	0.81 ± 0.06	2.1 ± 0.2
	40	1.84 ± 0.04	0.87 ± 0.05	2.4 ± 0.3
Acrylamide	25	[a]	1	6.8 ± 0.8
	40	[a]	1	8.6 ± 0.5

[a] No determined value corresponding to the nonlinear Stern–Volmer relationships.

3.2. Effect of denaturants on enzyme activity

The enzyme activity decreased upon continuous increment of the urea concentration reaching a value under 20% of the initial activity value at 8 M urea. The enzyme activity was lost by incubation in solutions containing GdnCl at a concentration of 2.5 M or higher.

The enzyme renaturation was achieved, with both denaturants, in a few seconds after adding the renaturation buffer, recovering about 50% of the native activity and showing a shift of maximum emission wavelength from 355 to 340 nm.

3.3. KI quenching of D2-HDH fluorescence

The effects of I^- ions (0.1–0.5 M) on the emission of the enzyme were tested in native conditions at 2 M salt concentration. KI was selected as the ionic quencher to quench selectively the emission of exposed accessible tryptophan residues. Iodide quenching efficiency is influenced by the charge in the vicinity of the fluorophore and the collision frequency [7]. The Stern–Volmer relationships shown in Fig. 3(a) at 25 and 40 °C were linear. The K_q values under these conditions were determined as 1.50 ± 0.02 and 1.84 ± 0.04 M^{-1}, respectively. The modified Stern–Volmer plot (Fig. 3(b)) gave values of 81% at 25 °C and 87% at 40 °C for the tryptophan residues accessible to the quencher (Table 1).

3.4. Acrylamide quenching of D2-HDH fluorescence

The fluorescence of D2-HDH was also quenched in the presence of acrylamide. Acrylamide was chosen as a non-ionic quencher to quench both the exposed and buried fluorophores [7]. We found the Stern–Volmer relationships at 25 and 40 °C for D2-HDH to be nonlinear (Fig. 4(a)). The modified Stern–Volmer plot gave values of 100% for the tryptophan residues accessible to the quencher at the two different temperatures tested (Fig. 4(b)). The quenched fluorescence was 80% for the native enzyme and 86% in denatured conditions.

3.5. Calorimetry denaturation

The heat denaturation of D2-HDH was studied by high sensitivity DSC. The thermogram obtained (Fig. 5) was best fitted by a single two-state transition with subunit dissociation model which could follow the next process:

$$N_n \leftrightarrow nD \tag{3}$$

$$K = \frac{[A]^n}{[A_n]}$$

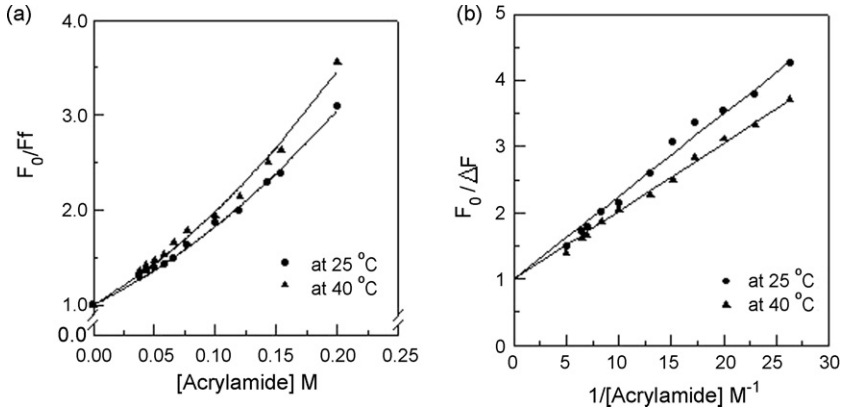

Fig. 4. Stern–Volmer plots (a) and modified Stern–Volmer plots (b) of acrylamide quenching of D-2-hydroxyacid dehydrogenase from *H. mediterranei* (2.5 μg ml^{-1}).

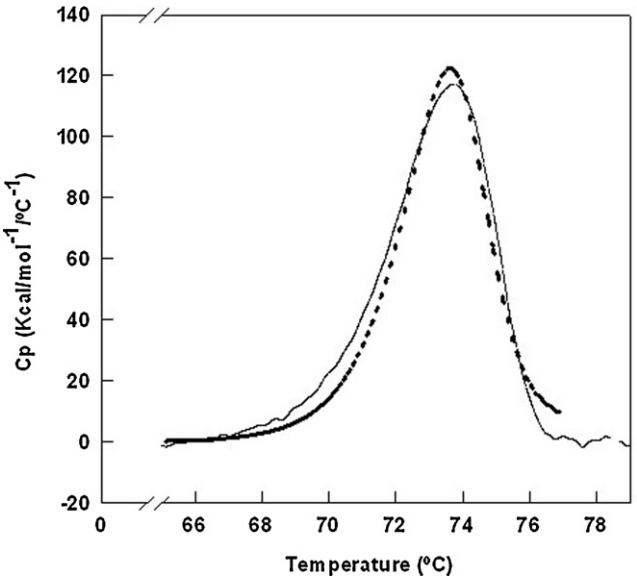

Fig. 5. DSC scan of D-2-hydroxyacid dehydrogenase (solid line) shown in comparison to the fit to a two-state dissociative model (dashed line). Total protein concentration: 0.8 mg/ml. Buffer Tris–HCl 20 mM, pH 8.0, 3 mM EDTA, 4 M NaCl.

where N is the native state, n is the number of dissociable subunits, K is the equilibrium constant, the brackets signify molar concentrations of species and where D is the denaturated state. The results show that n equal to 2 is the best fit.

The calorimetric parameters obtained were: $T_m = 75.59 \pm 0.02\,^{\circ}C$, $\Delta H = 397 \pm 2$ kcal mol^{-1}, where ΔH is the experimental calorimetric enthalpy change.

4. Discussion

Intrinsic fluorophores residues in native proteins are not found in identical locations. Every residue is characterized by a particular microenvironment which consists of different physico-chemical conditions such as polarizability, microviscosity, availability of charged groups, possible specific interactions, etc., which influence chromophore fluorescence. As a consequence, the protein fluorescence is conditioned by the sum of fluorescent contributions of individual tryptophan residues, which vary over a rather wide range [11].

When the emission maximum of a protein is close to 350 nm (340 nm in the case of D-2-hydroxyacid dehydrogenase) it is indicative of a relatively hydrophilic environment for the fluorescent residues [12]. This also implies that there are more tryptophan residues accessible to the solvent than buried ones. This point was verified when using the ionic quencher KI by the values of $(f_a)_{eff}$ obtained.

The unfolded state of the D2-HDH protein was achieved when the emission maximum reached a value over 350 nm. Unfolding of the enzyme in GdnCl shows a quenching of the fluorescence intensities as shown in Fig. 1(a). Proteins assume random-coil configurations in concentrated GdnCl solutions [13]. Fluorescence was quenched upon denaturation of D-2-hydroxyacid dehydrogenase from *H. mediterranei* in GdnCl accompanied by a shift in the fluorescence maximum to 350 nm, as has been described for other halophilic proteins, e.g. malate dehydrogenase from *Haloarcula marismortui* [14], aldolase and glyceraldehyde-3-phosphate dehydrogenase from *H. vallismortis* [10] and glutamate dehydrogenase from *H. mediterranei* [15].

The decrease of the fluorescence intensities could be a reflection of their subunit associations, since all these enzymes previously quoted have a multimeric structure.

When the enzyme is incubated with GdnCl, denaturation occurs. Calorimetric results suggest that this process is a single two transition state with subunit dissociation (Eq. (3)).

On the other hand, when the enzyme is incubated with urea, there is no change in the emission spectrum (Fig. 1(b)). In consequence, it is not a denaturing agent for D2-HDH in short periods of incubation. Polar solvents such as water, with a high polarizability value, provide a change in the microenvironment of the fluorophore by increasing solvation, reducing excited state complex energy and producing a red shift. Urea usually denatures proteins, decreasing the hydrophobic microenvironment and directly binding to the amide units via hydrogen bonds with overall exothermic interaction microenvironment. In our case, high urea concentrations do not unfold the D2-HDH protein before 46 h of incubation, what implies an unfolding process extremely slow, although it causes the loss of its activity after 5 min of incubation in the same conditions. Probably, the unfolding of the enzyme in urea is a two step process. The first step consists of a change of its native conformation, without unfolding, followed by a second step in which the polypeptide chain is unfolded and dissociation occurs. These processes could be described with the next equation:

$$N_2 \leftrightarrow X_2 \leftrightarrow 2D \quad (4)$$

where X_2 is a different conformation state or a dissociated state, in which there is not shift of the maximum wavelength emission peak of the protein but there is total loss of activity.

It is worth to say that in some cases, e.g. some enzymes from thermophilic microorganisms, the use of urea as denaturing agent do not produce the unfolding of the enzyme since urea is a neutral molecule that does not compete with residues which are implied in the ion pairs present in these proteins [16,17]. However, the charged molecule of GdnCl could compete with those residues destroying ion pairs and forming new interactions with the denaturing agent, which leads to the unfolding of the protein. As a consequence, urea interacts with nonpolar groups to a higher extent than GdnCl does, producing substantially different effects on the conformational state of some proteins.

Ionic quenchers such as iodide are specific for fluorophores located on the surface of the protein. Iodide is negatively charged and hydrated and therefore it is likely to be limited to quenching only surface tryptophan residues, whereas the non-ionic quencher acrylamide can quench the emission of not only the external but also the buried tryptophan residues by penetrating into the protein matrix [18]. The results of our quenching studies indicate that D2-HDH from *H. mediterranei* contains exposed tryptophan residues together with internal ones, giving a characteristic value for the spectral maximum positioned close to the region of 340 nm. Furthermore, D2-HDH must contain a high amount of accessible tryptophan residues [$(f_a)_{eff} = 0.87 \pm 0.05$ at 40 °C], as shown by the results of the use of KI as fluorescence quencher. The collision rate depends on the molecular charge of the ionizable groups located in the vicinity of tryptophan residues as well as on the ionic strength [7]. This significant fraction suggests that negatively charged residues are not located in the vicinity of the exposed tryptophan residues. Moreover, the percentage of fluorophore available to the quencher at 40 °C is higher than at 25 °C, indicating that a conformational change has taken place with a partial unfolding of the protein (Table 1). The plot F_0/F versus [KI] (Fig. 3(a)) displays an increase in the slopes with the temperature, because of the increase in collision frequency according to a dynamic quenching. The linearity of modified Stern–Volmer plots suggests that only one type of tryptophan residues is affected (e.g. those exposed to the solvent in the native D2-HDH). In contrast, the nonlinear and upward-curving relationship for the D2-HDH protein shown by the acrylamide quenching experiments suggests that in this case this compound does not act as a pure dynamic quencher. This might

be due to a compact nature of the enzyme. On the other hand, this behaviour can also be explained by the formation of dark complexes [7]. In our case, the upward curve of the plot can be correlated with the juxtaposition of acrylamide molecules and tryptophan residues without the formation of actual dark complexes between the quencher and the chromophore. Our data show that, in this protein, acrylamide quenches by a combination of static and dynamic processes. A similar fact was described by Eftink and Ghiron [19]. They describe different models to explain the interaction between the acrylamide and the tryptophan residues. In every case using acrylamide as a quencher it can be estimated the presence of tryptophan residues and its relative location on the protein.

On the other hand, the *n* value equal to 2, obtained in the calorimetric denaturation assays, together with the crystals obtained in crystallization experiments have shown that the protein crystallizes as a dimer in two different space groups, $P2_1$ (crystals of form I) and *P*1 (crystals of form II) [20], strongly suggesting that the protein is a dimer. In contrast with the results we previously reported [1] in which it was said that it was a tetramer. Sequence similarity suggests that D2-HDH belongs to a family of proteins in which most of the members have a homodimeric structure [21,22]. However, some variations between their multimeric structures have been observed. For example, D-2-hydroxyisocaproate dehydrogenase from *L. casei* (1dxy) [4] is a hexamer in which three dimers arranged around a threefold axis and D-3-phosphoglycerate dehydrogenase from *E. coli* (1psd) [23] is a protein in which two dimers come together to form a tetramer. This could suggest that although D2-HDH crystallizes as a dimer, it might form a tetramer (a dimer of dimers) in the gel filtration conditions.

5. Conclusions

These results allow us to conclude that the tryptophan residues that are responsible for the fluorescence emission are located close to the surface of the protein, accessible to the solvent and that urea does not produce the unfolded state of the protein in short periods of time. Halophilic proteins are probably more compact than their mesophilic counterpads but less than thermophilic proteins where real ionic pairs networks exist.

Acknowledgements

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