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Redox thermodynamics of cytochrome *c* in mixed water–organic solvent solutions

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Abstract

Bovine heart cytochrome *c* was studied through cyclic voltammetry in mixed water–organic solvent solutions under different conditions of temperature and the thermodynamic properties ΔS°_{rc} and ΔH°_{rc} calculated by the dependence of E° by temperature. The effect of the organic fraction of the solvent on the E° values of the native cyt *c* was found to be determined mainly by the decrease in dielectric constant of the medium. Specific interactions on the protein surface do not seem to play a remarkable role. The thermodynamic properties changes induced by the organic fraction have been interpreted tentatively in terms of solvation properties of cytochrome *c* and structural features of the protein environment.

Keywords: Cytochrome *c*; Cyclic voltammetry; Thermodynamic properties

1. Introduction

The reduction potential of redox metalloproteins strongly depends on electrostatic solvent–solute interactions [1]. The nature of the medium in which the protein performs its role could heavily affect the structure of the polypeptide chain, and therefore, its effectiveness on the metallic centre [2–4]. In particular, a decrease in the dielectric constant has been proposed to occur near the surface of the cell membrane and to give rise to local modifications in pH value and protein transitions to non-native states [2,4]. Mitochondrial cytochromes *c* are heme containing metalloproteins that shuttles electrons in the vicinity of the inner mitochondrial membrane [5–7]. The redox potential (E°) of ferro–ferricytochrome couple is critical to the physiological role of these proteins in that it determines the thermodynamics and influences kinetic features of electron exchange reactions with physiological partners [8]. The understanding of the molecular factors determining E° has been subject of much debate. The interactions of the heme group with the surrounding polypeptide chain and the solvent are among the main factors affecting E°, in particular it is sensitive to medium effects such as the pH and the nature and ionic composition of the solvent [8–13]. In spite of that, few data are available on the role of the solvent composition in determining the value of the redox potential of cytochrome *c*, and therefore, in the electron transfer process involving this protein in physiological conditions. The effect of a mixed water–organic solvent medium on the E° value of native cytochrome *c* was attributed mainly to the decrease in dielectric constant while no relevant specific organic solvent–protein interactions were observed [9,10], even if alcohols have been proposed to perturb the interactions between the heme and the protein moiety forming the heme crevice [14,15]. Instead, the presence of the organic solvent seems to affect conformation and stability of the alkaline form of cytochrome *c* [9,16,17].

To give a contribution in clarifying the topic, the enthalpy and entropy changes associated with the protein reduction have been determined for bovine heart cytochrome *c* in aqueous mixed solutions containing a collection organic solvents in different concentrations through variable temperature direct electrochemical experiments. The data have been interpreted tentatively

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in terms of solvation properties and structural feature of the protein and represent an help to understanding of the factors controlling the redox potential of the cytochromes *c*.

2. Experimental

2.1. Materials

Bovine heart cytochrome *c* (Sigma) was passed through a Superdex 75 column (Pharmacia) before use. The purity was tested through PAGE electrophoresis (Bio-Rad Miniprotean) and spectrophotometrically. Nanopure water was used throughout. The high purity solvents were purchased by Fluka and used as received. Solvent composition is expressed in per cent by volume.

2.2. Electrochemical measurements

Cyclic voltammetry experiments were performed with a Potentiostat/Galvanostat PAR model 273A using a cell for small volume samples ($V = 0.5$ ml) under argon. A 2 mm diameter gold disc and a Pt sheet were used as working and counter electrode, respectively.

All the potentials were measured using a saturated aqueous NaCl calomel electrode contained in a glass tube. The calomel electrode was separated from the solution contained in the tube by a Vycor set as well as the glass tube from the protein (working) solution. The glass tube was filled by a solution with the same composition of the working one (without the protein) and located so that the liquid junction between SCE and the solution in the glass tube was maintained at 25 °C, so did not affect dE°/dT. Due to absence of any information about the liquid junction potentials, electrochemical experiments were performing using Ferrocene–Ferrocinium couple [18] (as ferrocinium picrate) as a standard in the different working mixed solvents.

The working electrode was cleaned by first dipping it in ethanol for 10 min and then polishing it with alumina (BDH, particle size about 0.015 μm) water slurry on cotton wool; finally the electrode was treated in an ultrasonic pool for about 10 min. To remove residual adsorbed impurities, the electrode was first set at a potential of +1 V (vs. SCE) for 180 s and then subjected to ten voltammetric cycles between +0.7 and −0.6 V at 0.1 V s^{-1}. Modification of the electrode surface was performed by dipping the polished electrode into a 1 mM solution of 4-mercaptopyridine for 30 s, then rinsing it with nanopure water. Unbuffered protein solutions were freshly prepared before use and their concentration, varying over 0.15–0.25 mM, was checked spectrophotometrically. Protein samples were made up either in H_2O or H_2O–organic solvent (DMF, acetonitrile, ethanol, methanol, n-propanol) mixtures. The base electrolyte was 0.1 M $NaClO_4$. The experiments were carried out several times and the redox potentials were found to be reproducible within ±2 mV. All the redox potentials reported in this paper are referred to the standard hydrogen electrode (SHE). Temperature dependent E° measurements were carried out with a non-isothermal cell [18,19], in which the reference electrode is kept at constant temperature (25 °C), while the temperature of the working electrode is varied. For such an experimental setting, the standard entropy change of the reaction centre upon reduction (ΔS°_{rc}) is obtained from the slope of the plot of E° versus temperature, according to equation [18,19]:

$$\Delta S^\circ_{rc} = S^\circ_{red} - S^\circ_{ox} = nF(dE^\circ/dT) \quad (1)$$

The enthalpy change (ΔH°_{rc}) was obtained from the Gibbs–Helmholtz equation, namely, from the slope of the E°/T versus $1/T$ plot. The nonisothermal behaviour of the cell [18,19] was carefully checked by determining the ΔS°_{rc} value of ferricyanide–ferrocyanide couple (found 175 ± 4 vs. 173.6 J K^{-1} mol reported in ref. [18]).

The voltammetric response and the temperature dependence of E° for bovine cytochrome were studied as a function of solvent composition in binary water–organic solvent mixtures containing up to about 50% organic solvent (Fig. 1). At higher concentrations of the organic solvent, the protein solubility decreases dramatically and the CV signal is no more detectable. In all the cases, the electrochemical process appears to be reversible, monoelectronic and diffusion controlled. The redox behaviour of cytochrome *c* in solutions containing less than 10% organic solvent is very similar to that in water ('neutral' form) [8] and only one wave is observed (wave I). The increase in organic solvent content of the solution above 10% modifies the electrochemical behaviour of cytochrome *c*, in particular the redox potentials of wave I (related to the 'neutral' form) shift cathodically (Fig. 2, Tables 1–5) while a new, irreversible CV signal (wave II) due to an alkaline form of cytochrome *c* [8,9,20,21] is observed (Fig. 1b) at a solvent composition depending on the nature of the organic solvent. It increases in intensity to the detriment of wave I decreasing the water concentration. Their irreversibility is due to the fact that the reduced alkaline form of cytochrome *c* is unstable and converts spontaneously to the native reduced form [20,21] and prevents, in our experimental conditions, the calculation of the corresponding E° values.

The slope of the E° versus T plot (Fig. 3) for wave I in the low temperature range (about 4–30 °C) decrease at increasing the organic solvent concentration, while at higher temperatures, a break point is observed. This behaviour, already observed in water solutions at increasing the pH value [20] and in the presence of DMSO [9], was attributed to a change of the protein

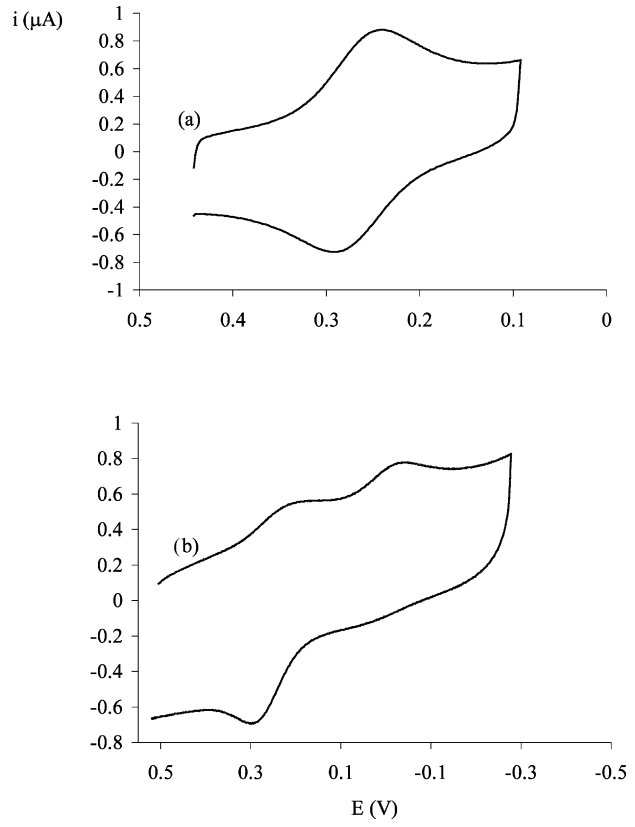

Fig. 1. Cyclic voltammograms for bovine heart cyt *c* at 4-mercaptopyridine surface-modified gold electrode: (a) in 5% ACN; (b) in 45% ACN. Scan rate 50 mV s^{-1}. Potentials are expressed vs. SHE. Protein concentration (a) 0.20 mM; (b) 0.18 mM. $T = 20$ °C.

conformation near the heme or to a temperature-induced residue ionisation, anyway phenomena involving only the 'neutral' form. The thermodynamic parameters of the redox process of the native form of cytochrome *c* have been calculated by the plot of E° versus T (ΔS°_{rc}, Fig. 3) and E°/T versus $1/T$ (ΔH°_{rc}, Fig. 4) and are reported in Tables 1–5.

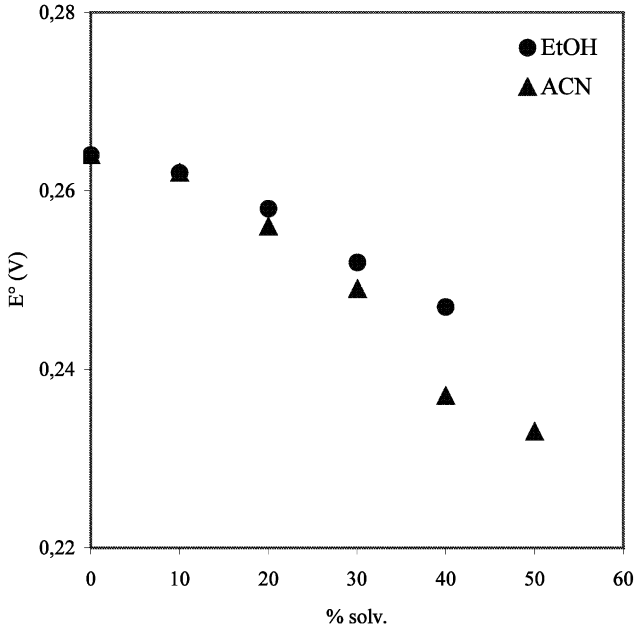

Fig. 2. Plot of the reduction potential against percentage of solvent for wave I. $T = 20$ °C. Error bars have the same dimensions as the symbols.

Table 1
Redox potentials for the native form of bovine cytochrome *c* in different ACN concentration, and $\Delta H^{\circ\prime}_{rc}$ [kJ mol^{-1}] and $\Delta S^{\circ\prime}_{rc}$ [J mol^{-1} K^{-1}] values determined from the temperature dependence of the redox potential

%	$E^{\circ\prime}$ [a]		$\Delta S^{\circ\prime}_{rc}$ [b]	$\Delta H^{\circ\prime}_{rc}$ [b]
0	0.264		−45	−38.7
5	0.263		−44	−38.3
10	0.262		−42	−37.6
15	0.258		−40	−36.6
20	0.256		−36	−35.3
25	0.252		−33	−34.0
30	0.249		−29	−32.5
35	0.244	< 45 °C	−26	−31.2
	0.237	> 45 °C	−95	−53.1
40	0.237	< 40 °C	−23	−29.6
	0.232	> 40 °C	−106	−55.6
45	0.235	< 35 °C	−19	−28.2
	0.232	> 35 °C	−118	−58.7
50	0.233	< 35 °C	−18	−27.8
	0.231	> 35 °C	−126	−61.0

[a] $T = 20$ °C.
[b] Average errors in the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are ±0.9 kJ mol^{-1} and ±3 J mol^{-1} K^{-1}, respectively.

Table 2
Redox potentials for the native form of bovine cytochrome *c* in different DMF concentration, and $\Delta H^{\circ\prime}_{rc}$ [kJ mol^{-1}] and $\Delta S^{\circ\prime}_{rc}$ [J mol^{-1} K^{-1}] values determined from the temperature dependence of the redox potential

%	$E^{\circ\prime}$ [a]		$\Delta S^{\circ\prime}_{rc}$ [b]	$\Delta H^{\circ\prime}_{rc}$ [b]
0	0.264		−45	−38.7
5	0.262		−45	−38.5
10	0.259		−44	−37.9
15	0.256		−40	−36.4
20	0.253		−35	−34.7
25	0.250	< 40 °C	−30	−32.9
	0.245	> 40 °C	−84	−49.9
30	0.247	< 30 °C	−27	−31.7
	0.244	> 30 °C	−88	−50.2
35	0.243	< 30 °C	−24	−30.5
	0.241	> 30 °C	−106	−55.4
40	0.238	< 25 °C	−22	−29.4
	0.237	> 25 °C	−113	−56.5
45	0.236	< 25 °C	−20	−28.6
	0.235	> 25 °C	−122	−59.0
50	0.235	< 20 °C	−19	−28.2
	0.235	> 20 °C	−133	−66.4

[a] $T = 20$ °C.
[b] Average errors in the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are ±0.9 kJ mol^{-1} and ±3 J mol^{-1} K^{-1}, respectively.

Table 3
Redox potentials for the native form of bovine cytochrome *c* in different MetOH concentration, and $\Delta H^{\circ\prime}_{rc}$ [kJ mol^{-1}] and $\Delta S^{\circ\prime}_{rc}$ [J mol^{-1} K^{-1}] values determined from the temperature dependence of the redox potential

%	$E^{\circ\prime}$ [a]		$\Delta S^{\circ\prime}_{rc}$ [b]	$\Delta H^{\circ\prime}_{rc}$ [b]
0	0.264		−45	−38.7
5	0.264		−44	−38.4
10	0.263		−43	−38.0
20	0.260		−39	−36.5
30	0.254	< 45 °C	−35	−34.8
	0.245	> 45 °C	−78	−45.4
35	0.252	< 40 °C	−32	−33.7
	0.245	> 40 °C	−96	−53.7
40	0.249	< 35 °C	−31	−33.1
	0.244	> 35 °C	−108	−56.8

[a] $T = 20$ °C.
[b] Average errors in the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are ±0.9 kJ mol^{-1} and ±3 J mol^{-1} K^{-1}, respectively.

Table 4
Redox potentials for the native form of bovine cytochrome *c* in different EtOH concentration, and $\Delta H^{\circ\prime}_{rc}$ [kJ mol^{-1}] and $\Delta S^{\circ\prime}_{rc}$ [J mol^{-1} K^{-1}] values determined from the temperature dependence of the redox potential

%	$E^{\circ\prime}$ [a]		$\Delta S^{\circ\prime}_{rc}$ [b]	$\Delta H^{\circ\prime}_{rc}$ [b]
0	0.264		−45	−38.7
5	0.263		−44	−38.3
10	0.262		−43	−37.9
20	0.258		−38	−36.0
30	0.252	< 45 °C	−34	−34.2
	0.242	> 45 °C	−81	−49.1
35	0.251	< 40 °C	−31	−33.3
	0.244	> 40 °C	−102	−55.5
40	0.247	< 35 °C	−28	−32.0
	0.242	> 35 °C	−116	−59.1

[a] $T = 20$ °C.
[b] Average errors in the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are ±0.9 kJ mol^{-1} and ±3 J mol^{-1} K^{-1}, respectively.

3. Discussion

Solvent medium–protein interactions play a relevant role in determining the E° values of redox metalloproteins, affecting both the enthalpic and entropic contributions to the free energy change of the redox process [9]. As found at increasing the organic solvent concentration, a cathodic shift of E° has also been observed increasing temperature and ionic strength of the solution [11]. This last effect is not unexpected since the shielding of the protein charge by the surrounding 'ionic atmosphere' is greater for the redox state carrying the larger charge, namely the oxidised form. As a consequence, this state will be preferential stabilised over the other. The decrease in E° of cyt *c* with increasing temperature and organic solvent concentration, on the contrary, is

Table 5
Redox potentials for the native form of bovine cytochrome *c* in different PropOH concentration, and $\Delta H^{\circ\prime}_{rc}$ [kJ mol^{-1}] and $\Delta S^{\circ\prime}_{rc}$ [J mol^{-1} K^{-1}] values determined from the temperature dependence of the redox potential

%	$E^{\circ\prime}$ [a]	$\Delta S^{\circ\prime}_{rc}$ [b]	$\Delta H^{\circ\prime}_{rc}$ [b]	
0	0.264		−45	−38.7
5	0.260		−43	−37.7
10	0.258		−41	−36.9
20	0.255	< 40 °C	−36	−35.2
	0.248	> 40 °C	−106	−57.1
30	0.252	< 30 °C	−32	−33.7
	0.249	> 30 °C	−133	−64.3
35	0.250	< 25 °C	−28	−32.3
	0.248	> 25 °C	−205	−85.0
40	0.246	< 25 °C	−25	−31.1
	0.245	> 25 °C	−320	−119.0

[a] $T = 20$ °C.
[b] Average errors in the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are ±0.9 kJ mol^{-1} and ±3 J mol^{-1} K^{-1}, respectively.

opposite to the effect expected on purely electrostatic grounds [22,23]. In fact, this should induce a destabilisation of the oxidised state owing to the strengthening of the repulsion between the heme iron and the positively charged residues located near the heme crevice. This seeming discrepancy has been attributed to the presence of one additional shielding water molecule in the oxidised state of cyt *c* [9,24]. Indeed, a greater shielding effect of the 'ionic atmosphere' (stabilising the ferri-form of cyt *c*) due to the lowering of the average dielectric constant of the mixed solvent at increasing the organic fraction could give a basic contribution to the observed cathodic shift of E°.

Recent circular dichroism, resonance Raman, ^{1}H NMR studies on bovine and horse cytochromes [9,10] indicate that the solvent composition does not alter appreciably the conformation of native cyt *c* [2,9,10] and the effects on the E° value can be attributed primarily at the characteristics of the solvent medium (composition and/or average dielectric constant). In this context, as already proposed for water–DMSO system [9], the organic solvent induced decrease in the temperature of the break point in the E° versus T plot for native cyt *c* (Fig. 3) can be simply related to the different solvent polarity obtained increasing the organic solvent concentration. The reason of this break point has been suggested to be a temperature change in protein conformation in the surroundings of the heme [25,26] or a deprotonation of a lysine [23]. Anyway, in both the cases, the decrease in the average dielectric constant of the solvent medium would reasonably strengthen the electrostatic interactions involving the charged centres on the cytochrome surface. Therefore, differences in electrostatic (and solvation) properties of cyt *c* in the two temperature ranges could give reason of the stabilisation induced by the organic solvent of the

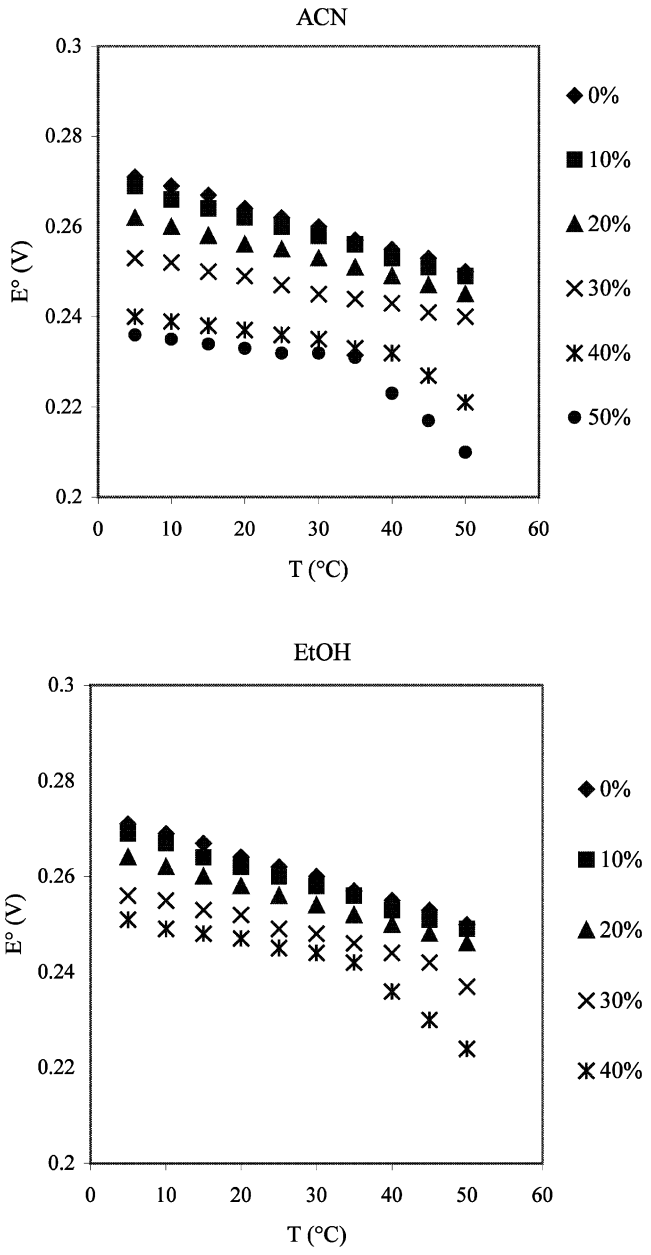

Fig. 3. E° vs. T plots at different ACN and EtOH concentrations. Similar trends are observed for the other solvents. Error bars have the same dimensions as the symbols.

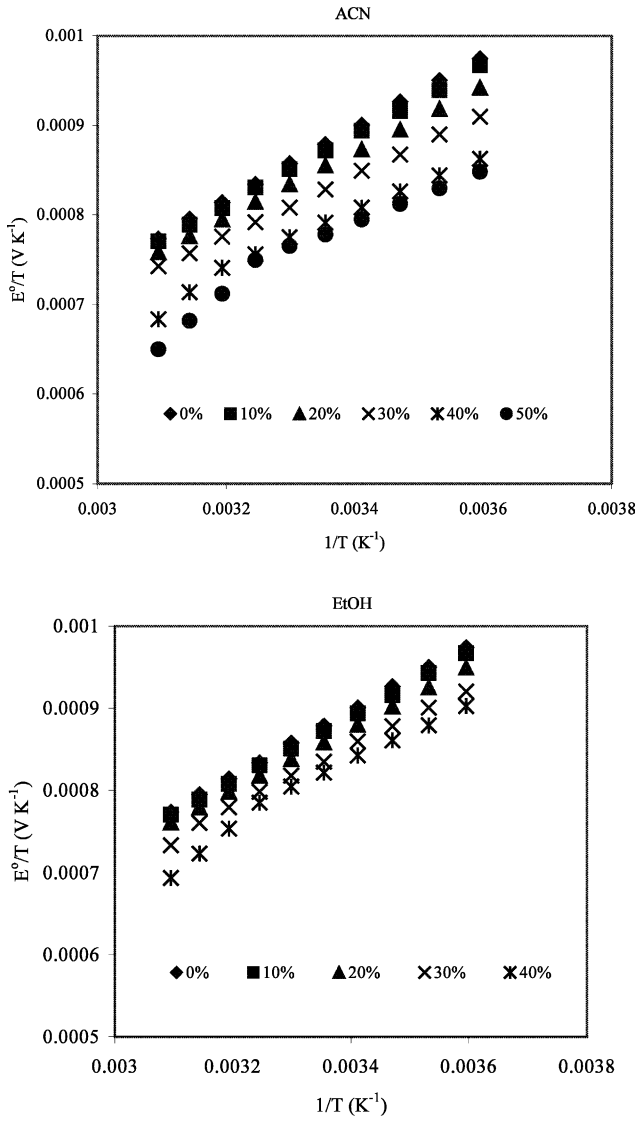

Fig. 4. E°/T vs. $1/T$ plots at different ACN and EtOH concentrations. Similar trends are observed for the other solvents. Error bars have the same dimensions as the symbols.

high-temperature conformer (namely, a decrease in the break point temperature).

The opposite trend of the plot E° versus T found for the low- and high-temperature conformers (Fig. 3, Tables 1–5) at increasing the organic solvent concentration agrees with the above hypothesis [9].

The fact that ΔS°_{rc} of the native low temperature conformer increases at increasing the organic solvent concentration (Tables 1–5) indicates that, due to the lowering of the average dielectric constant, the interaction with the solvent medium and the protein flexibility (the determinants of ΔS°_{rc} [8,9,27]) of the two redox states tends to be the same. It is noteworthy that a similar increase in ΔS°_{rc} (as well as a decrease in the temperature of the thermal transition of the native cyt c) has been observed previously in DMSO [9] and, most of all, by decreasing the ionic strength [11]. These facts suggest that the perturbation of the H-bonding network surrounding the protein due to the rearrangement of the ionic atmosphere induced by the oxidation–reduction process gives a basic contribution to ΔS°_{rc}.

In the presence of a mixed aqueous solvent, the electrostatic interactions between protein and ionic atmosphere increase according to the decreasing of the average dielectric constant to give a stabilisation of the oxidate state and a cathodic shift of E° (Fig. 2). The organic fraction of the solvent, however, reduces the extent of the H-bonding network, so that the rearrange-

ment of the ionic atmosphere induced by the change of the protein charge produces a more limited effect on ΔS°_{rc}.

Mixed methanol and ethanol aqueous solutions show the smaller increase in ΔS°_{rc} values at increasing the organic solvent concentration (Tables 1–5), according to their ability in H-bond making and to the possibility that ferrocytochrome *c*, less susceptible to modification than ferri-form, is gradually deformed [15].

The remarkably negative ΔH°_{rc} values found for both the native conformers was ascribed to the stabilisation of the ferroheme by ligand binding interactions and the hydrophobicity of the heme environment [5,7,8]. In the case of the low-temperature conformer, the increase in ΔH°_{rc} value observed increasing the organic solvent concentration gives a contribution to E° opposite but of the same order to that due to the entropic term. Enthalpy–entropy compensation phenomena in the biophysics of biopolymers are well documented, in particular the hydration process, even if their origin is not completely clarified [28–34]. Indeed, the ΔH°_{rc} value could be (partially) related to the electrostatic effect of the negatively charged ionic atmosphere that surrounds cyt *c*, which should indeed stabilise the ferri form (which bears a greater positive charge than the reduced state). As a consequence, the decrease in average dielectric constant due to the increase in organic solvent concentration strengthens the electrostatic interaction between protein and ionic atmosphere most of all for the oxidised form of cyt *c* and induces an increase in the ΔH°_{rc} values.

The solvent dependence of ΔS°_{rc} and ΔH°_{rc} for transition–metal redox couples has been deeply investigated by Weaver and Sahami [35,36]. They studied carefully the factors involved in the thermodynamics of outer-sphere electron transfer reaction of metal–ion complexes. In spite of the remarkably structural difference existing among redox complexes and electron transfer metal proteins, some interesting common behaviours concerning the solvent effect could be observed. In particular, cytochrome *c* and transition–metal redox couples containing polypyridines (in which the inner coordination shell is unaffected by the redox state and the observed solvent effect on the redox thermodynamics is expected to be due to entirely to outer-sphere contributions) show small changes in E° values (ΔG°_{rc}) in the different solvent media due to large enthalpy–entropy compensative effects. The ΔS°_{rc} values of this kind of complexes were invariably found to be higher in non aqueous solvents (most of all in aprotic media) than in water. Analogously, ΔS°_{rc} of cytochrome *c* increases with increasing the organic fraction of the medium (also in this case, mostly for aprotic solvents) but the $\Delta(\Delta S^{\circ}_{rc})^{s-w}$ values (ranging from 14 J K^{-1} mol in 50% MetOH to 27 J K^{-1} mol in 50% ACN) are lower than those of the polypyridine complexes in the different organic solvents (80–170 J K^{-1} mol [35]). The positive values of $\Delta(\Delta S^{\circ}_{rc})^{s-w}$ were attributed to the enhancement of solvent polarisation around the molecule in non aqueous solvents compared with the same phenomenon in water [35]. In fact, the additional positive charge of the oxidised form induces a greater solvent ordering in the proximity of the redox complex for those solvents having minor intrinsic order and which are, therefore, more sensitive to the effect of the electric field [35]. The same arguments can be used also in the case of cytochrome *c* in mixed media but the extent of the solvent effect on ΔS°_{rc} is lower. This fact is probably due to the structural effect of the aqueous fraction in the bulk of the mixed solvent. Moreover, the presence of water molecules firmly bound onto the protein surface [37,38] makes less effective the electrostatic interactions between the redox centre and the solvent dipoles.

No structural data are available regarding the high-temperature conformer, so any comment on its thermodynamic behaviour at this stage would be purely speculative. It is worthy of note, however, that the decrease in the temperature of the break point in the E° versus T profiles for native cytochrome *c* with increasing the organic solvent concentration is always accompanied by the appearance of the alkaline form at lower temperature [9,20].

Acknowledgements

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