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Effect of dimethyl sulfoxide on the structure and the functional properties of horseradish peroxidase as observed by spectroscopy and cyclic voltammetry

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Abstract

Electrochemical biosensors have found wide application in food and clinical areas, as well as in environmental field. A large number of articles focused on horseradish peroxidase (HRP)-based biosensors have been published in the last decade, due to the capability of HRP to quantitatively detect the presence of hydrogen peroxide produced in a reaction. At present a large body of multi-enzymatic amperometric biosensors are realized by entrapping HRP together with other enzymes into a polymeric matrix; these systems represent a promising example of simple, low-cost electrochemical tools for the analysis of bioanalytes in solution, such as glucose, choline and cholesterol. Due to the fact that polymers used for HRP entrapping are soluble in organic solvents and that many solvents are strong denaturants of aquo-soluble proteins, in this paper we investigate (in particular, by circular dichroism and electron paramagnetic spectroscopies) the effect of dimethyl sulfoxide, one of the organic solvents employed for polymer solubilization, on the structure and the functionality of HRP, in order to determine the effect induced by the solvent concentration on the structure and activity of the hemoprotein. This is relevant for basic and applied biochemistry, HRP being largely employed in bioinorganic chemistry and sensor area.

Keywords: horseradish peroxidase; protein denaturation; aquo-organic solvent; biosensor; EPR spectroscopy

1. Introduction

In recent years, extensive investigation performed on the structure and the reactions of metalloproteins important for biological electron transfer (eT) have clarified some basic properties of the redox chemistry involved. Electrochemical techniques revealed crucial to investigate eT between a redox protein and the electrode surface (and, thus, to study heterogeneous eT involving a biosystem as one of the partners), since allow to mimic in vitro redox reactions occurring in nature.

Electrochemical investigation of soluble proteins is complicated by a variety of problems, mostly related to the heterogeneous nature of the eT process [1,2]; among others, complexities are due to: (i) the slow diffusion of the biological molecules, which leads to low faradaic currents; (ii) the strong tendency of

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macromolecules to adsorb on the electrode surface once they approach to, with consequent structural degradation; (iii) the buried nature of the active site, which lowers heterogeneous eT rate significantly. Thus, immobilization of redox proteins onto the electrode surface has become a well-established area of research, since it avoids many of these problems and emphasizes the importance of engineering enzyme-modified electrodes. This justifies the big development of immobilization techniques in the recent years (see, for example, [3,4]), of importance for the realization of systems to be employed in sensor area.

At present, much effort is devoted to the realization of 'third-generation' amperometric biosensors [5,6]; being based on *direct* (unmediated) eT, these systems ensure a superior selectivity. Engineering electrodes with enzyme-entrapping solid matrices able to enhance, once in contact with the electrode surface, the electrochemical properties of a protein is of potential value for basic and applied bio-inorganic chemistry. In particular, a large number of articles describing the properties of horseradish peroxidase (HRP)-based electrochemical biosensors have been published in the last decade (see, for example, [7–13]), in view of the potentialities that these systems offer in biotechnological field; HRP, in fact, quantitatively detects the presence of hydrogen peroxide produced in a reaction.

HRP is an enzyme (formula weight approx. 44 000) widely used in electrochemical biosensors, since this enzyme catalyzes the hydrogen peroxide-dependent one-electron oxidation of a wide variety of substrates [16]. The protein contains the heme as active site; in the resting state, the heme–iron oxidation state is Fe(III). The HRP catalytic mechanism is carried out through the rapid reaction with hydrogen peroxide to give a two-equivalent oxidized form, called compound I, in which the active site contains an oxyferryl center (Fe(IV) = O) and a porphyrin π-cation radical; the rapid reaction of compound I with the substrate then regenerates the Fe(III)-ground state form via an intermediate called compound II. Recently, the X-ray structure of recombinant HRP isoenzyme C and that of its adducts with benzhydroxamic and ferulic acid, respectively, has been reported [17–19]. These studies have shown the presence of a well defined hydrophobic pocket for the organic substrate, and (like lactoperoxidase [20]) a relatively high dynamism and flexibility of the aromatic donor binding site in the enzyme.

Recently, multi-enzymatic amperometric biosensors based on HRP isoenzyme C entrapped into a polymeric matrix (in the form of a membrane) on a pyrolytic graphite electrode, have been realized in one of our laboratories. These systems revealed able to detect the presence of soluble glucose, choline and cholesterol even in the absence of mediators or promoters [10,11,14,15], thus acting as 'third-generation' biosensors. Therefore, they may be considered as a promising example of simple, low-cost electrochemical systems for the analysis of biocompounds in solution, of interest in food and clinical areas.

Although the electrocatalytic properties of HRP adsorbed onto an electrode surface have been widely investigated under physiological-like conditions [7,10,11], relatively few studies have been devoted to the effect that organic solvents exert on the structure, the stability and the activity of the enzyme (see [21] and references therein). Organic solvents are often used for chemical modification of electrodes or for solubilization of polymers in which HRP will be entrapped. Since many organic solvents are strong protein denaturants, it may happen that an expected HRP-based sensor really acts as heme-based biosensor, due to an undesired protein denaturation. Dimethyl sulfoxide (DMSO), a well-known denaturant of water-soluble proteins, is one of the organic solvents employed for solubilization of polymers used for protein-entrapping [10,11,14,15,22,23]. This renders the enzyme-embedding procedure critical, since mixing of the polymer and the enzymes solutions (with formation of a mixed aquo-organic solvent) must occur without enzymes denaturation and/or precipitation phenomena.

Aim of the present paper is investigating the effect of DMSO on the structure and the functional properties of HRP. Particular care has been devoted to determine the solvent concentration range in which HRP retains both structural compactness and biological activity, since many polymers employed for enzyme entrapment are poorly soluble even in organic solvents as DMSO. This renders the enzyme-entrapment procedure very critical. If not enough polymer is present in the mixed aquo-organic solvent, then the solid matrix (i) will trap a too low amount of en-

zyme, and (ii) will not bind strongly to the electrode surface (thus sensibly reducing the sensor sensitivity and stability); on the other hand, a higher polymer concentration in the mixed solvent will require a higher amount of DMSO, thus sensibly increasing probabilities for enzyme denaturation. This makes data in the present work relevant for basic and applied biochemistry, since HRP is an enzyme widely employed in bioinorganic chemistry and in biosensor area due to its prompt reaction with hydrogen peroxide produced in a large body of chemical and biochemical reactions.

2. Materials and methods

2.1. Materials

Horseradish peroxidase (type VI-A), hemin and DMSO (ACS reagent, purity $\geq 99.9\%$) were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

2.2. Sample preparation

Samples were prepared by mixing a buffered solution of HRP (0.1 M phosphate buffer, pH 7.0) with DMSO at the desired (v/v) ratio. Protein concentration ranged from 5.5 to 200 μM, depending on the technique employed. In the range explored, no concentration effect was observed.

The pH_{app} of the mixed solvent varied as a function of DMSO concentration (percentage is expressed as v/v): 20% DMSO, pH_{app} 7.7; 30% DMSO, pH_{app} 8.3; 50% DMSO, pH_{app} 9.4; 70% DMSO, pH_{app} 10.5; 80% DMSO, pH_{app} 11. Under the same conditions, an identical pH was measured for the HRP and hemin solutions. The pH, however, does not affect the spectral properties of the protein, as demonstrated by the following procedure: small amounts of DMSO were stepwise added to a buffered HRP solution (up to a final 80% DMSO solution), and the pH was readjusted to pH 7.0 after each addition. In the presence of the same DMSO concentration, the spectral properties of the sample at pH_{app} 7.0 and of the other samples were practically identical, indicating that the changes observed are induced by DMSO.

2.3. Absorbance measurements

Optical spectra were recorded at 25°C on a double beam Uvikon 930 spectrophotometer.

2.4. Specific activity measurements

The enzyme solutions were prepared by adding 10 μl of a 7.8 μM HRP solution to 490 μl of water or DMSO/water mixture. After a 10-min incubation, 10 μl of the HRP solution were added to a mixture containing 1.5 ml of 0.1 M phosphate buffer (pH 6.5), plus 1 ml of a 5.17 mM DMAB buffered solution, plus 50 μl of MBTH (2.1 mM), plus 50 μl of hydrogen peroxide (12.7 mM) [24]. The initial reaction rate was measured following the increase in absorbance at $\lambda = 590$ nm ($\varepsilon = 47\,600\ M^{-1}\,cm^{-1}$) [25].

Specific activities are expressed as micromoles of substrate oxidized by 1 mg of enzyme in 1 min of reaction (units/mg).

2.5. EPR measurements

EPR spectra were recorded at 4 and 77 K on a Bruker EMX spectrometer. An Oxford Instruments liquid helium transfer line was used for the measurements at 4 K. Instrumental parameters: receiver gain 100 000; modulation amplitude 10 G; modulation frequency 100 kHz; microwave frequency 9.502 GHz; power 10 mW; number of scans 5.

2.6. Circular dichroism (CD) measurements

Measurements were carried out using a Jasco J-710 spectropolarimeter (Tokyo, Japan) equipped with a PC as data processor. The molar ellipticity (deg cm^2 $dmol^{-1}$) is expressed as [θ] on a molar heme basis.

2.7. Electrochemical measurements

Protein embedding into the TBMPC membrane and membrane immobilization on a pyrolytic graphite (PG) electrode surface were achieved as previously described [10]. DC cyclic voltammograms were run in a (previously degassed) 30% and 80% (v/v) DMSO/0.1 M Tris–HCl buffer (pH 7.0) mixed

solvent. Measurements were carried out at 25°C in a glass microcell (sample volume: 1 ml); during measurements, the anaerobic environment was maintained by a gentle flow of high-purity grade N_2 just above the surface of the solution. A (properly modified) PG electrode (AMEL, Milan, Italy) was the working electrode, a saturated calomel electrode (AMEL) was the reference and a Pt ring the counter-electrode. An Amel 433/W multipolarograph (Milan, Italy) interfaced with a PC as data processor was employed for voltammetric measurements. The potentials reported in the text are referred to the Standard Calomel Electrode (SCE).

3. Results and discussion

3.1. Absorbance measurements

Optical measurements were carried out to determine the effect of DMSO concentration on the structure of HRP; DMSO, in fact, is a well known denaturant of aqua-soluble proteins. As shown in Fig. 1a, in aqueous medium and neutral pH the Soret spectrum of HRP is characterized by an asymmetric band centered at 403 nm, and a shoulder at approx. 380 nm; at higher wavelength range (shown in the inset of Fig. 1a), two weak absorption (charge-transfer) bands centered at approx. 500 and 641 nm, are present. In the 30–70% (v/v) DMSO solution range, the high-spin geometry of the heme–iron(III) is retained, as the unchanged λ_{max} of the Soret band (see Fig. 1) indicates; this excludes axial coordination of the solvent to the heme–iron. On the other hand, the band intensity increases with DMSO concentration (together with a slight blue-shift of the 641 nm band), suggesting progressive unpacking of the heme microenvironment; in the absence of λ-shifts, in fact, the increase of the Soret band is assumed diagnostic for a larger exposure of the heme group to the solvent without enzyme dissociation [21] (see also [26] for cytochrome *c*). In an 80% DMSO solution, the optical spectrum of HRP changes significantly; the Soret band becomes narrower and highly symmetric (see Fig. 1), while the 641-nm band blue-shifts to 625 nm (see insets of Fig. 1). Such a spectrum, typical of free Fe(III)-porphyrins, suggests that HRP may undergo denaturation under the conditions employed, with splitting of the prosthetic group from the protein matrix.

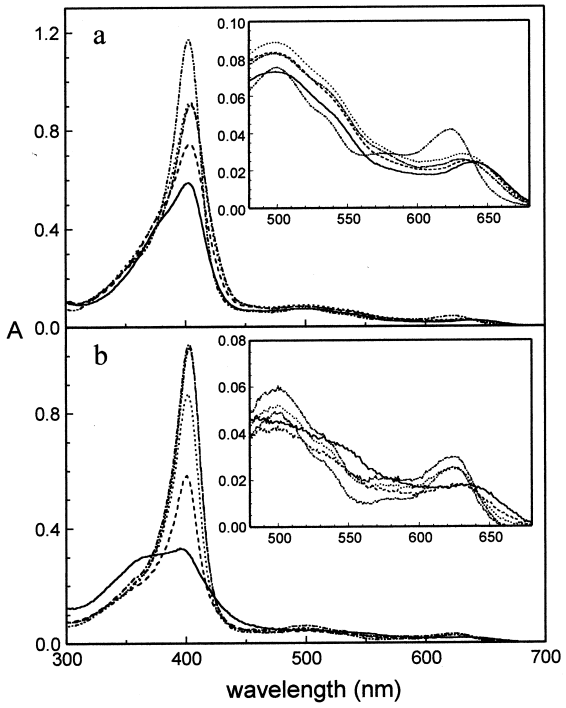

Fig. 1. Absorbance spectra of HRP (a) and hemin (b) recorded in the 300–700 nm wavelength range. (———) aqueous medium; (- - -) 30% DMSO; (- - -) 50% DMSO; (-.-.-.) 70% DMSO; (-..-..-..) 80% DMSO solution (v/v). HRP concentration: 5.5 μM; hemin concentration: 5.1 μM. The temperature was 25°C. The insets show the absorbance spectra in the 480–680 nm wavelength range, on enlarged scale.

To get deeper insight on this point, absorbance spectra of hemin were carried out under the same conditions. As shown in Fig. 1b, the spectrum of the free heme is significantly affected by the solvent composition; the broad spectrum observed in aqueous medium gradually becomes sharper and more intense as the DMSO concentration increases, and the λ_{max} is red-shifted (from approx. 398 nm to 402–403 nm). This indicates that the organic solvent affects the properties of the heme group, free or inserted in the protein matrix; further, the close similarity of the spectra recorded in an 80% DMSO solution strongly suggests that the prosthetic group may split out from the polypeptide in HRP. Ultrafiltration of a 70% DMSO and an 80% DMSO HRP solution performed on a Millipore ultrafree-CL membrane, cut-off 10 kDa, confirms this view. As illustrated in Fig. 2, in the latter case the absorbance

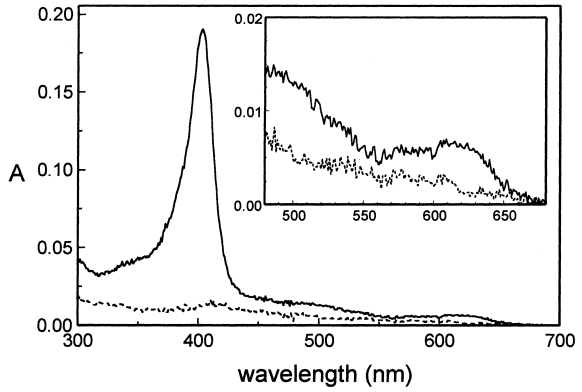

Fig. 2. Absorbance spectra of samples resulting from ultrafiltration of 70% (v/v) (broken line) and 80% (v/v) (continuous line) HRP-containing DMSO solutions recorded in the 300–700 nm wavelength range. The inset shows the absorbance spectra in the 480–680 nm wavelength range, on an enlarged scale. Ultrafiltration of the two solutions was performed by means of an ultrafree-CL membrane (Millipore, molecular cut-off: 10 kDa). Temperature: 25°C.

spectrum of the sample resulting from ultrafiltration shows a well defined absorption band centered at approx. 402 nm to be ascribed to the heme group, whereas no appreciable absorbance is observed in the former case. This unequivocally indicates that the prosthetic group splits from the polypeptide at DMSO concentration $\geq 80\%$ (v/v).

3.2. CD measurements

CD measurements were carried out in the Soret (400–450 nm), a spectral region directly related to the heme microenvironment. Changes in the Soret dichroic spectrum reflect structural rearrangements occurring in the heme pocket; in particular, for hemoproteins in which the prosthetic group is not covalently bound to the polypeptide, the loss of the dichroic signal is considered diagnostic for protein unfolding (the free heme, in fact, is a symmetrical group). As shown in Fig. 3, the peak of the Soret CD spectrum recorded in a 20% (v/v) DMSO solution, is red-shifted of 4–5 nm with respect to water, indicating that the organic solvent perturbs the heme pocket conformation, which becomes less packed. Progressive increase of the organic solvent concentration, however, does not induce further perturbation in the active-site region, up to a 70% (v/v) DMSO solution; the CD spectrum, in fact, remains practically unchanged. On the other hand, full protein denaturation occurs in an 80% (v/v) DMSO solution, as clearly revealed by the absence of dichroic signal.

3.3. EPR measurements

Applied to HRP, low-temperature EPR provides important structural information, since it can probe solvent effects on the spin-state and geometry of the heme–iron(III) revealing, for example, if solvent penetrates into the heme crevice of the protein. In phosphate buffer and neutral pH, HRP exhibits the well known EPR spectral pattern due to the high-spin ferric heme, characterized by a rhombic splitting in the region $g \cong 6$ ($g_x = 6.3$, $g_y = 5.6$, $R = 4.37$) and by a $g \cong 2$ low intensity peak [27,28] (spectrum not shown). Rhombicity of the heme plane is calculated from the equation: $R = (\Delta g/16)\ 100$, where R is defined as percent of rhombicity, and Δg represents the splitting near $g = 6$. Fig. 4 shows the EPR spectra of HRP recorded at increasing DMSO concentration (from 5% to 80%, v/v), at 4 K. Spectra were well resolved and no low-spin peak was observed in the $g = 2$ region. In the 5–50% (v/v) DMSO concentration range, EPR spectra of HRP show the characteristic rhombic splitting near $g = 6$ ($g_x = 5.95$, $g_y = 5.53$; $R = 2.62$); however, a decreased percent of rhombicity is observed with respect to spectra in buffered aqueous medium (similarly to what is found in

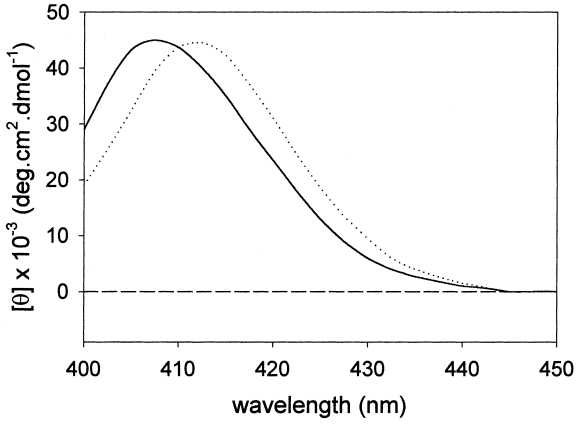

Fig. 3. Soret CD spectrum of HRP in: (———) phosphate buffer, pH 7.0; (- - -) 20–70% (v/v) DMSO solution. The dichroic spectrum in a 80% (v/v) DMSO solution overlaps the baseline. HRP concentration: 10 μM. Temperature: 25°C.

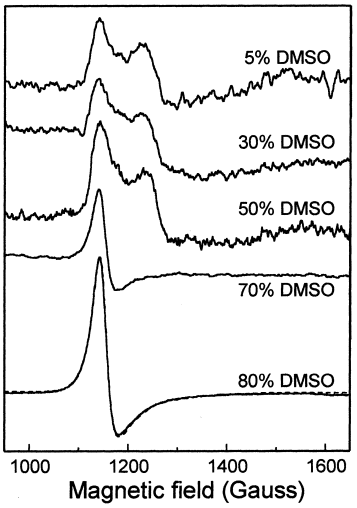

Fig. 4. X-band EPR spectra of HRP in a 5–80% (v/v) DMSO solution, at 4 K. Dotted line at 80% DMSO solution refers to the normalised spectrum of hemin. HRP and hemin concentration: 0.3 mM. Setting conditions were 9.50 GHz microwave frequency; 10 mW microwave power; 10 G modulation amplitude.

80–95% (v/v) dioxan solution [21]). This indicates that HRP substantially retains the heme crevice integrity, even though the reduced rhombicity of the prosthetic group in DMSO suggests that the active site pocket is less packed. Further, the absence of low-spin peaks excludes axial coordination of the organic solvent to the heme–iron(III) and, thus, penetration of DMSO into the heme crevice.

In 70% and 80% DMSO solution, the EPR spectra appear considerably altered; the loss of the rhombic splitting in the $g = 6$ region, together with the presence of a single peak at $g = 5.94$, clearly indicate that the heme iron geometry is changing to an axial (tetragonal) geometry, which resembles that observed in myoglobin ($g = 5.97$) and in the free Fe(III)–porphyrin [29]. In particular, the spectrum of Fig. 4 which refers to an 80% DMSO solution, shows a signal as sharp as that of hemin chloride [21,30], suggesting that the heme group may split from the peptide. This is confirmed and better elucidated by the EPR spectrum of hemin run under the same conditions; as clearly shown in Fig. 4, the EPR spectrum of hemin and that of the protein in the same solvent matrix are practically overlapping.

As shown in Fig. 5, EPR spectra of the protein at 77 K are sufficiently resolved to show the unequivocal and critical evolution from a still large (in a 70% DMSO solution) spectral pattern to a final and shifted (in an 80% DMSO solution) sharper signal. On the other hand, EPR spectral pattern of hemin at 77 K remains unchanged (not shown).

3.4. Cyclic voltammetry measurements

Applied to proteins, this technique provides information on the heterogeneous eT kinetics between biomolecules and the electrode surface, and allows determination of the redox potential of the biosystem under investigation. Fig. 6 shows the cyclic voltammograms of HRP entrapped within a TBMPC membrane at a PG electrode, run in a 30% (upper panel) and an 80% (lower panel) DMSO solution, at a scan rate 100 mV/s. A well-defined electrochemistry is observed in both cases; in the upper panel, the cathodic and the anodic peak are symmetric and the i_a/i_c is about unity. The redox potential determined, $E_{1/2} = -394 \pm 5$ mV vs. SCE, is close to that estimated for the protein in phosphate buffer and neutral pH [10], indicating that the presence of DMSO at this concentration does not substantially alter the redox properties of the embedded protein. Similar results were obtained in 40% and 60% DMSO solutions (not shown).

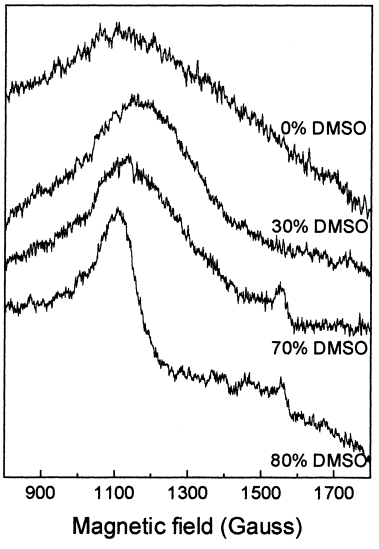

Fig. 5. X-band EPR spectra of HRP in aqueous medium and in a 30–80% (v/v) DMSO solution, at 77 K. HRP concentration: 0.3 mM. Setting conditions were 9.50 GHz microwave frequency; 10 mW microwave power; 10 G modulation amplitude.

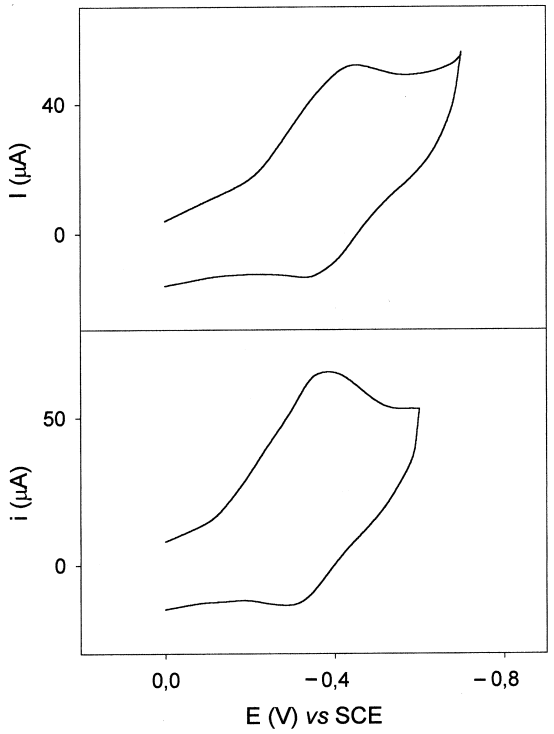

Fig. 6. DC cyclic voltammograms of TBMPC-entrapped HRP at a PG electrode, run in a 30% (v/v) DMSO solution (upper panel) and in an 80% (v/v) DMSO solution (lower panel). Scan rate: 100 mV/s. The temperature was 25°C.

By contrast, in an 80% DMSO solution a differently shaped cyclic voltammogram is achieved (see lower panel of Fig. 6), characterized by shorter peak-to-peak separation and a less negative redox potential ($E_{1/2} = -317 \pm 5$ mV vs. SCE). This value is very similar to that ($E_{1/2} = -311 \pm 7$ mV vs. SCE) determined from cyclic voltammograms of free hemin run under the same conditions (not shown), which proves that (i) the enzyme undergoes denaturation, and (ii) the heme is split from the protein matrix.

In aqueous medium and neutral pH, the redox potential of HRP is approx. 150 mV more negative than that of the free heme [31]; the redox potentials here determined in a 30% and an 80% DMSO solution, differ for approx. 77 mV. By assuming that in the latter case the redox potential is that of the free heme, the lower ΔE observed is consistent with a conformation of the protein in DMSO still compact but less structured than in aqueous medium, in which the active site is more exposed to the solvent (in agreement with spectroscopic data).

3.5. Activity measurements

The HRP activity in aqueous solution reported in the present paper (670 ± 10 units/mg), is comparable to that previously determined under similar conditions [24]. In DMSO, up to a 70% DMSO solution the protein retains its capability to catalyze the substrate oxidation by hydrogen peroxide; in this DMSO concentration range, the protein–substrate affinity results practically unchanged, indicating that the enzyme structure remains compact and the active site is still efficiently shielded by the polypeptide. In other words, the organic solvent seems not able to alter the enzyme's heme crevice significantly, during substrate oxidations. This provides evidence that HRP behaves differently from enzymes using water as co-reactant, which undergo full inactivation in the presence of organic solvents [32].

By contrast, no protein activity is observed in an 80% DMSO solution, consistent with full protein unfolding.

3.6. Temperature-induced denaturation

Stability of HRP in DMSO has been investigated by following the unfolding process induced by temperature. Fig. 7 shows the thermal denaturation profiles of the protein in the mixed aqua-organic solvent, obtained from ellipticity values at $\lambda = 412$ nm. In the 20–60% DMSO solution range, stability of HRP is virtually identical to that of the native-like form (not

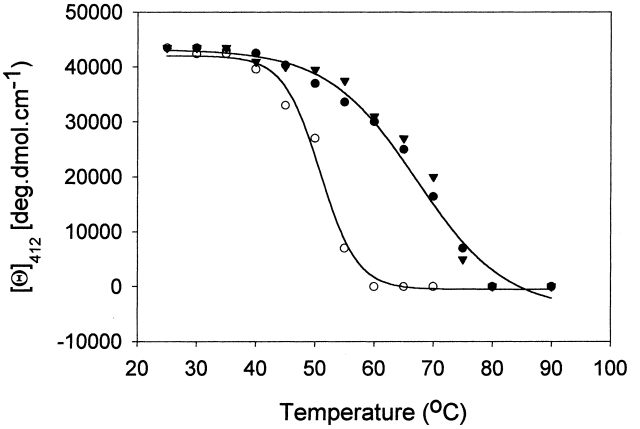

Fig. 7. Temperature-induced denaturation profiles of HRP dissolved in a (▼) 20%, (●) 60%, (○) 70% (v/v) DMSO solution, obtained from ellipticity values at $\lambda = 412$ nm.

shown), and the thermal transition proved to be highly reversible (approx. 85% reversibility is detected, for a solution heated up to 80°C). However, in a 70% DMSO solution the protein stability markedly decreases (see Fig. 7), suggesting that DMSO concentration is enough to alter the protein structure (in good accord with EPR data), likely weakening (or breaking) intramolecular bonds essential for the enzyme compactness.

4. Conclusions

Spectroscopic data here reported clearly indicate the following.

(i) Up to a 60% DMSO solution, HRP retains a compact tertiary structure and substantial integrity of the active site microenvironment. However, under these conditions the decreased rhombicity of the heme–iron(III), which gradually converts into an axial (tetragonal) geometry as the organic solvent concentration is increased, suggests that the heme pocket is not as packed as in aqueous medium. On the other hand, spectroscopic data exclude that DMSO can penetrate into the heme crevice; the metal, in fact, remains pentacoordinated (or hexacoordinated, with a water molecule at the sixth axial position) as in the native form, which indicates that the organic solvent does not coordinate to the heme–iron(III) as axial ligand. Functional data strongly support spectroscopy. The enzyme activity in DMSO, for example, is very similar to that in aqueous medium. Water is known to play an essential role in enzymatic catalysis, since it enhances the active-site flexibility; thus, the unmodified enzyme-substrate affinity in DMSO indicates that the organic solvent does not perturb the active-site pocket significantly and that enough water is still present. Clearly, penetration of DMSO in the enzyme's active site would alter the local polarity and the H-bonding strength of the substrate to the enzyme. Further confirmation is provided by the HRP redox potential determined in DMSO; although less negative than under physiological conditions, the $E_{1/2}$ value is, however, considerably more negative than that of the free heme, indicating that HRP retains a compact (though less packed) tertiary structure.

(ii) In a 70% DMSO solution, the heme symmetry becomes preponderantly tetragonal; this is consistent with a more penetrating action of the organic solvent into the protein structure, which also affects the heme microenvironment. The decreased protein stability as well as the loss of enzyme activity indicate that the hydrogen-bonding network of the heme pocket is broken, even though the heme still remains inserted in the polypeptide.

(iii) In an 80% DMSO solution, the drastic spectroscopic changes observed, together with the loss of enzyme activity and the dramatic change of the enzyme redox potential, provide clear evidence for the occurrence of protein unfolding, proved by the splitting of the prosthetic group from the protein matrix.

With reference to the spectroscopic techniques employed, CD appears very sensitive to even small structural changes occurring in the enzyme at low DMSO concentration as, for example, the rearrangements taking place in the active-site pocket; on the other hand, EPR appears very efficient at any DMSO concentration, able to differentiate between the metal-site geometries and highly sensitive in detecting those structural changes in the heme crevice just preceding protein denaturation (see EPR spectrum recorded in a 70% DMSO solution). On the whole, data obtained underline the validity of a combined application of these techniques to gain deeper information on structural changes interesting biological macromolecules.

In conclusion, the results described here provide novel information on the effect of a strong denaturant, as DMSO, on the structure and the functional properties of HRP. Data obtained are of relevance for basic and applied bioinorganic chemistry, HRP isoenzyme C being a suitable 'marker' for investigating factors that affect the structural stability of the active site [33,34]; not only do they clarify some basic aspects of the effect exerted by the organic solvent on the active site microenvironment of the enzyme, but they also provide precious information on the conditions permitting a correct realization of HRP-based electrochemical biosensors in which the simultaneous presence of HRP and DMSO in solution (see, for example, [14,15]) without protein unfolding phenomena, is required.

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