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ELECTROCHEMICAL APPROACH TO THE MECHANISM OF UREA DENATURATION OF HORSE HEART CYTOCHROME *c*

Raymonde PILARD [a], Jean HALADJIAN [a,*], Pierre BIANCO [a], Paule-Andrée SERRE [a] and Viktor BRABEC [b]

[a] *Laboratoire de Chimie et Electrochimie des Complexes, Université de Provence, Place Victor Hugo, 13331 Marseille Cedex 3, France, and* [b] *Institute of Biophysics, Czechoslovak Academy of Sciences, 612 65 Brno, Czechoslovakia*

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The effect of urea denaturation on the electroactivity of horse heart cytochrome *c* has been studied by differential pulse polarography and cyclic voltammetry at a gold electrode; the gold electrode was activated by 4,4'-bipyridine. Essentially, two redox couples with $E'_{01} \approx 0.25$ V and $E'_{02} \approx -0.05$ V (vs. normal hydrogen electrode) have been detected. The experimental results have been interpreted on the basis of the existence of equilibria between native and denatured electroactive forms; transitory species have been assumed to appear on reduction. The scheme that we have proposed agrees well with the conclusions obtained previously by other authors on conformational changes. Moreover, the advantage of electrochemical techniques in investigating the denaturation process has been underlined.

1. Introduction

Different reasons can be put forward to explain the relatively high redox potential value of 0.255 V vs. a normal hydrogen electrode (NHE) for cytochrome *c* [1–3] in comparison with the potentials of simpler monohemic systems (−0.050 to −0.210 V/NHE) [4–6]: the hemic coordination configuration [7], hydrophobicity of the heme environment [8] and/or low fraction of heme surface exposed to the aqueous solvent [9]. Consequently, it can be expected that any modification of one of these factors, e.g., the unfolding of a proteinic moiety, is able to affect the redox potential value.

One of the fruitful ways of investigating redox properties of proteins is the electrochemical approach to electron-transfer exchange. In this respect, it appears that the electroreactivity of a protein is dependent on several factors such as the nature of the electrode, type of medium used, structure of the electroactive molecule and extent of adsorption onto the electrode material. The latter factor, in particular, seems to be essential to explain the overall exchange process [10]. In the case of cytochrome *c*, it has been well established that the protein denatures irreversibly when it adsorbs on the electrode surface by forming a layer of flattened molecules [11,12]. Unfolding can be considered as a prerequisite step of the electron exchange.

From these considerations it emerges that the study of the folding-unfolding process of cytochrome *c* is fundamental to a better understanding of the electron transfer and, moreover, to the elucidation of interrelationships between protein structure and biological function. Horse heart cytochrome *c* is particularly suitable for this kind of study; this protein is easily available in a highly purified form and has been studied extensively

* To whom correspondence and reprint requests should be addressed.

[13]. Considerable research has been done on the unfolding of horse heart cytochrome *c* with urea [14–18], guanidinium chloride [19–22], alcohols [14,23,24] and heat [14,25], but, to our knowledge, no investigation using electrochemical techniques, except potentiometric measurements of redox potential in the presence of urea [15], has been performed to this end. Thus, polarography and voltammetry, which have been used successfully in previous investigations on native ferricytochrome *c* [26,27], have been useful in the present work for obtaining thermodynamic and kinetic data on the electron-exchange process and for characterizing the redox behavior of denatured species as identified by other techniques.

2. Experimental

2.1. Materials

Horse heart cytochrome *c* (type VI) from Sigma Chemical Co. was used without further purification. 4,4'-Bipyridine (purum) and urea (purum p.a.) were obtained from Fluka. Urea was used after evacuation for 12 h and as fresh solutions. All other chemicals used were reagent grade. Mercury was triply distilled.

Solutions were prepared with demineralized water.

2.2. Methods and apparatus

The working electrodes used in the present work were either a mercury or a gold electrode. The gold electrode was constructed in our laboratory by inserting a gold wire into a resin casing. Its geometric area was 0.0079 cm^2. The gold surface was polished before each experiment with ultrafine emery paper until the surface was brought to a mirror finish.

The auxiliary electrode was a platinum wire and the reference electrode was a Metrohm Ag/AgCl (saturated NaCl solution) electrode. Throughout this paper, all potentials are given vs. the Ag/AgCl reference electrode, unless otherwise specified. We have verified that the potential of this reference electrode was independent of the change of medium when the urea concentration increased from 0 to 9.5 M, by measuring simultaneously the value of the redox potential of the system ferrocene/ferrocinium (0.18 V) which has been adopted as a common reference system for various solvents [28].

Buffer solutions were prepared using 0.05 M KH_2PO_4/K_2HPO_4 + 0.20 M KCl + varying concentrations of urea, then adjusted to pH 7.0 with HCl. Buffer solutions served also as supporting electrolyte. For each urea concentration studied, a known weight of lyophilized cytochrome *c* was dissolved in buffer solution just before the experiment. The concentration of cytochrome *c* solutions was calculated after measuring the absorption of cytochrome *c* in the reduced form at 550 nm ($\epsilon_{max} = 29\,500\ M^{-1}\ cm^{-1}$) [29].

Oxygen was purged from solutions by bubbling with U grade nitrogen for 1/2 h before experiments.

Temperature was maintained constant at 25 ± 0.05°C.

Differential pulse (DP) polarograms at the dropping mercury electrode were recorded on a Sefram X-Y recorder coupled with a PAR 174 A Polarographic Analyzer equipped with an M 174/70 drop timer, using a drop time of 2 s, a pulse amplitude of −50 mV and a scan rate of 2 mV s^{-1}. Cyclic voltammograms (CV) were performed with a PAR 175 Universal Programmer coupled to a PAR 173 Potentiostat, using a Metrohm hanging mercury drop electrode or the gold electrode described above. Capacitance measurements with the mercury electrode were obtained by phase-selective a.c. polarography. Alternating current polarography was carried out with a PARC Model 174A analyzer coupled with a PARC Model 174/50 a.c. polarographic interface and a PARC Model 5204 lock-in analyzer. A phase angle of 90° with respect to the applied alternating voltage was employed. For a.c. polarography a d.c. ramp of 0.002 V s^{-1} and a modulation voltage of 80 Hz and 0.030 V peak-to-peak were employed; drop time was 2.0 s.

A Cary 14 spectrophotometer served for all optical measurements.

3. Results

3.1. DP polarography

The effect of urea concentration on the DP polarogram of cytochrome c has been studied over the range 0–9.5 M for a cytochrome c concentration of 150 μM (fig. 1). At low urea concentrations only one peak is observed (peak 1) at $E_{p1} \approx -0.30$ V; when the urea concentration increases above 3 M, the height of peak 1 decreases and E_{p1} is shifted toward more negative values. A new small peak at $E_{p2} \approx -0.65$ V appears at a urea concentration of about 5 M, then a third smaller peak at $E_{p3} \approx -0.8$ V when the urea concentration rises above about 7 M. The dependence of peak heights on urea concentration is shown in fig. 2.

A factor which can be put forward in the observed phenomena is the adsorption of urea on mercury [30,31]. The effect of urea adsorption on the electrochemical behavior of cytochrome c was followed by means of electrocapillary and a.c. polarographic measurements. It was found that urea is adsorbed in the medium used throughout this work at the mercury electrode within a broad range of potentials including those at which polarographic signals of cytochrome c were observed (fig. 1). However, the quadrature component of alternating current measured at −0.3, −0.65 and −0.8 V was changed only if urea was present in the concentration range 0–approx. 3 M. The increase in urea concentration above this level had only a negligible effect on the quadrature component of alternating current. Thus, it is concluded that the native proteinic form (peak 1) [26] progressively disappears when the urea concentration is increased; new peaks (peaks 2 and 3) can be reasonably assigned to new electroactive forms resulting from the conversion of the native protein. We have verified that the dependence of peak 1, 2 and 3 heights on cytochrome c concentration was linear in the range investigated.

From the shape of the curves in fig. 2 it is suggested that a significant change does take place at the urea concentration of about 5.5 M which corresponds to the common midpoint of curves 1 and 2, whereas alterations at lower concentrations seem to be relatively weak.

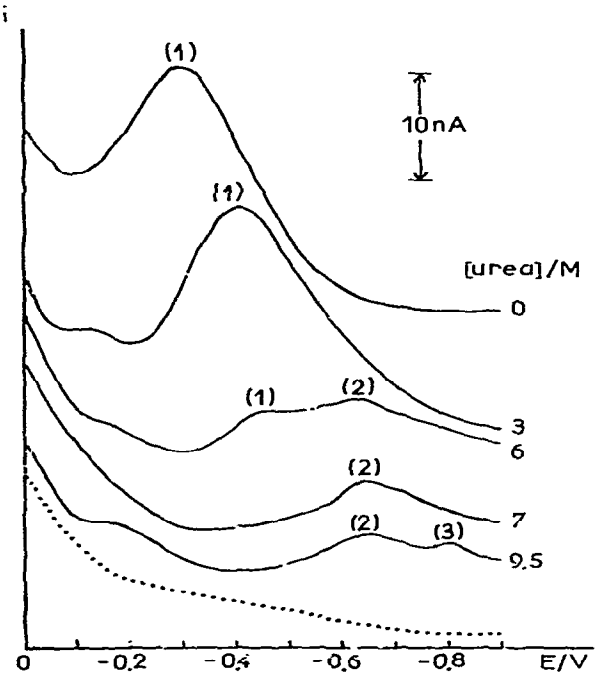

Fig. 1. DP polarograms of 150 μM cytochrome c in (0.05 M KH_2PO_4/K_2HPO_4 + 0.20 M KCl + varying urea concentrations, pH 7.0) medium. (·····) Background solution (9.5 M urea concentration).

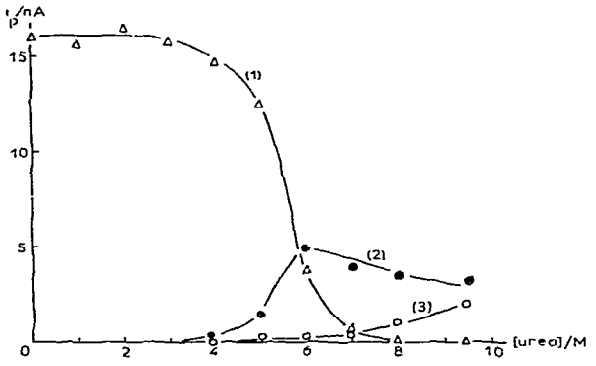

Fig. 2. Effect of increasing urea concentration on DP polarography peak heights (same medium as in fig. 1). (△) Peak 1, (●) peak 2, (○) peak 3.

3.2. CV at the hanging mercury drop electrode

In accordance with our foregoing results on cytochrome *c* [32], one cathodic peak at $E_{pc} \approx -0.40$ V is observed in the absence of urea but no anodic counterpeak. When the urea concentration is increased this peak disappears and one low, broad cathodic peak at $E_{pc} \approx -0.75$ V can be detected but no anodic counterpeak. This result suggests that cytochrome *c* in either the absence or presence of urea can be considered as a slow electrochemical system when studied at the mercury electrode. However, DP and CV experiments are useful for several reasons:

(1) From a theoretical point of view, the denaturation process leads to new electroactive forms,

(2) from an analytical point of view, electrochemical techniques are an efficient tool for detecting easily the quenching of the native form (fig. 2).

(3) for some similarity between the shape of curve 1 in fig. 2 and the shape of the dependences of redox potentials and absolute extinction of the 695 nm band on urea concentration, as observed previously by other authors [15].

3.3. CV at the gold electrode

It is known that cytochrome *c* is electroinactive at a gold electrode [33]. Fortunately, in the presence of the activator 4,4'-bipyridine, Eddowes and Hill [27] have shown that a rapid electron transfer to cytochrome *c* can take place, which is a convenient way to measure the value of the redox potential E_0'. Thus, in the present paper, the gold electrode has been used always in the presence of 0.01 M 4,4'-bipyridine.

In the absence of urea (fig. 3A), two peaks (peaks 1c and 1a) at $E_{p1c} = 0.02$ V and $E_{p1a} = 0.10$ V corresponding to a reversible redox system are observed. This result agrees with previous data on the electrochemical behavior of cytochrome *c* in the presence of 4,4'-bipyridine [27] and furnishes the value of 0.255 V/NHE for E_0', in accord with other authors [1–3]. When the urea concentration is increased (fig. 3B–D), the heights of peaks 1c and 1a decrease, then a new pair (peaks 2c and 2a) at $E_{p2c} \approx -0.30$ V and $E_{p2a} \approx -0.15$ V appears above 7 M concentration. The heights of these peaks are quite small even at the cytochrome *c* concentration of 300 μM investigated. At the highest urea concentrations, the first couple (1c–1a) is absent. The values of the redox potentials E_{01}' (≈ 0.25 V/NHE) and E_{02}' (≈ -0.05 V/NHE) corresponding to couples 1c–1a and 2c–2a, respectively, can be estimated from the CV data [34]. The effect of increasing concentrations of urea on the redox potential values E_{01}' and E_{02}' is shown in fig. 4; E_{01}' and E_{02}' decrease slightly when the urea concentration is increased in the ranges 0–7 and 7–9.5 M, respectively. The most striking observation is that the 6–7 M value seems to be a boundary concentration corresponding to the appearance of the negative couple 2c–2a; above this limit, the height of the positive couple 1c–1a is

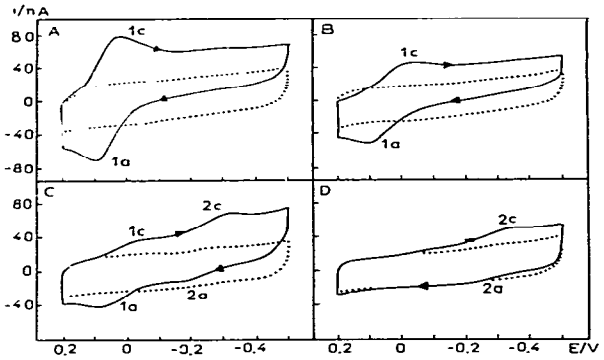

Fig. 3. Cyclic voltammograms at the gold electrode of 300 μM (A–C) and 450 μM (D) cytochrome *c* (same medium as in fig. 1 + 0.01 M 4,4'-bipyridine) at various urea concentrations: (A) 0 M, (B) 6 M, (C) 7 M, (D) 9.5 M. Scan rate $v = 0.005$ V s^{-1} (·····) Background solution.

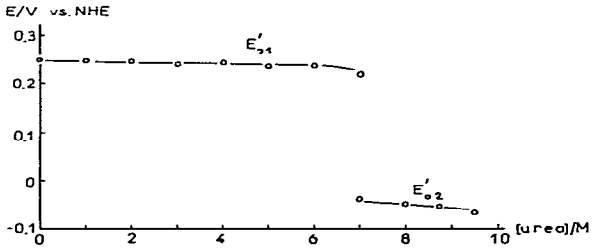

Fig. 4. Effect of increasing urea concentration on the redox potential of cytochrome *c* (same medium as in fig. 3) from CV data.

considerably lowered up to 9.5 M where it disappears (fig. 3).

The reversibility of the denaturation process has been ascertained by starting from the most concentrated (9.5 M) urea solution and by using decreasing urea concentrations. The cyclic voltammograms are superposable on the foregoing curves obtained when the urea concentration is increased.

In order to investigate the interdependence between the two redox couples 1c–1a and 2c–2a, the effect of scan rate v has been studied for a 300 μM cytochrome c solution at 7 M urea concentration. The interpretation of CV experiments can be more conveniently conducted when using slow scan rates. When v decreases, peak 2a tends to be quenched and peak 1a tends to be enhanced. At a scan rate of 0.001 V s^{-1} peak 2a disappears. This observation supports the idea of an interdependence between both couples and thus the existence of a kinetically controlled reaction.

Complementary experiments by differential pulse voltammetry (DPV) have shown that the heights of DPV peaks at a given urea concentration depend linearly on the cytochrome c concentration.

4. Discussion

The results obtained from electrochemical techniques when the urea concentration of a cytochrome c solution is increased show the progressive disappearance of the DP or CV peaks attributed to the native form N and the appearance at more negative potentials of new DP peaks and of a new CV couple. Two factors can be put forward to explain these voltammetric responses; first, the denaturation of the native molecules in the bulk solution, and secondly the adsorption of urea on the electrode surface.

The results of a.c. polarography of urea solutions not containing cytochrome c lead to the conclusion that the changes of polarographic signals of cytochrome c caused by urea additions cannot be connected only with adsorption of urea at the mercury electrode. The marked changes in the polarographic behavior of cytochrome c at the mercury electrode appeared only if the urea concentration was increased above 3 M (fig. 2). No significant changes in adsorption of urea at the electrode charged to potentials of -0.3, -0.65 and -0.8 V were observed at urea concentrations higher than about 3 M. The latter conclusion accords also with the stronger ability of cytochrome c to adsorb on the electrode in comparison with urea. Thus, it seems reasonable that the new DP and CV peaks should be assigned to some of the 'denatured' (= nonnative) forms which are known to appear with increasing urea concentration [14–18]. The data in fig. 2 which result from DP polarography are particularly significant, since the existence of at least two denatured forms corresponding to curves 2 and 3 can be considered; curve 1 is assigned to the disappearance of the native form. Thus, only from DP polarography experiments a scheme such as $N \rightleftharpoons D_1 \rightleftharpoons D_2$ might be proposed, where D_1 and D_2 represent two different denatured forms of cytochrome c.

From an electrochemical point of view, CV at the mercury electrode shows that either the native or the denatured forms correspond to slow electrochemical systems. However, it is important to note that denatured forms D_1 and D_2 are reduced at the mercury electrode at markedly more negative potentials than the native form N. Moreover, the redox potential value of the new couple (2c–2a) obtained in the range 7–9.5 M when the gold electrode is used (≈ -0.05 V/NHE) is less positive than the known value of 0.255 V/NHE [1–3] corresponding to N. This conclusion accords well with the results obtained by Myer et al. [17,35] from the study of the reduction of cytochrome c with ascorbate ($E_0' = 0.08$ V/NHE at pH 7 [36]) in the presence of urea: these authors have shown that their 9 M urea form, D, is an ascorbate-irreducible form.

The marked transition observed in fig. 4 can be compared with the denaturation profiles obtained by Myer et al. [15,16] from changes of the absolute extinction of the 695 nm band; a marked step is also obtained for the fluorescence efficiency of the tryptophan residue in the same urea concentration range. All these phenomena denote drastic modifications of the protein conformation.

Another important result lies in the fact that both couples 1c–1a and 2c–2a detected by CV at

the gold electrode seem to be interdependent. It has been reported above that a very slow scan rate tends to favor peak 1a to the detriment of peak 2a. This observation implicates the existence of interdependent equilibria between various forms which appear during the denaturation process. We propose to represent the overall denaturation process where oxidized and reduced forms have been included (and identified by subscripts ox and red) by the following scheme:

$$\begin{array}{ccccc} & & \xrightarrow[\text{(increasing urea concentration)}]{} & & \\ N_{ox} & \rightleftharpoons & D_{1ox} & \rightleftharpoons & D_{2ox} \\ \uparrow\downarrow e & & \uparrow\downarrow e & & \uparrow\downarrow e \\ N_{red} & \leftarrow & D_{1red} & \leftarrow & D_{2red} \end{array}$$

The most positive couple 1c–1a would reflect the fast redox process $N_{ox} \overset{e}{\rightleftharpoons} N_{red}$ which progressively disappears with increasing urea concentration. The negative couple (2c–2a would correspond to the redox process $D_{1ox} \overset{e}{\rightleftharpoons} D_{1red}$ and/or $D_{2ox} \overset{e}{\rightleftharpoons} D_{2red}$ where D_{1ox} and D_{2ox} are the oxidized forms present at high urea concentrations. To explain why peak 2a is very weak and disappears at the slowest scan rates, it can be proposed to regard D_{1red} and/or D_{2red} as unstable and that they rearrange to give N_{red} (or a very similar species). In this hypothesis it is evident that peak 1a is favored and its height may increase with time in comparison with peak 2a.

In recent works, Myer et al. [15–18] give the following equilibria $N \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons D$ where X_1 and X_2 represent intermediary species between the native, N, and denatured, D, forms with respective midtransition urea concentrations of 2–2.5, 6.2 and 7 M; N to X_1 would correspond to the loosening of the frontal section of the heme crevice, X_1 to X_2 would be a conformational perturbation step leading to an unfolded form and X_2 to D would reflect the reorganization of the polypeptide backbone with the disruption of the Met-80 S linkage, loosening of the tryptophan heme domain and drastic modification of the polypeptide conformation of the protein. The foregoing interpretation of the denaturation process can accord also with our conclusions. In our scheme, only one intermediary form comes into play. But in the assumption of Myer et al. it seems that structural differences between N and X_1 are not too drastic; consequently, the electrochemical characteristics of N and X_1 could be rather similar and small differences could account for the slight decrease in E_0' and also for the shifting of E_{pl} toward more negative potentials (fig. 1). Thus, what we have called N could include in fact also the form X_1. We have noted previously that a significant change occurs at the 6–7 M urea concentration, since the couple 2c–2a in figs. 3 and 4 begins to appear above this value. Midtransition urea concentrations of 6.2 and 7 M correspond in the data of Myer et al. to the most drastic alterations, i.e., unfolding and structural reorganization. From our electrochemical data, it is apparent that the forms which appear at high urea concentration become reducible at more negative potentials. This observation has theoretical importance: among the hypotheses on the factors which govern redox potential values, it has been proposed by Stellwagen [9] that the heme exposure could be determining; a more exposed heme should lead to a more negative redox potential value. This is the case at the present time, since a more negative redox potential value is obtained when the protein becomes unfolded in the presence of urea.

Another observation which agrees with the scheme that we have proposed above is relative to the stability of the reduced form. As pointed out by Drew and Dickerson [23], it is known that methionine binds more tightly to reduced than to oxidized heme, and thus the intermediary conformation is more resistant to methanol unfolding in the reduced protein. The stabilization of the tertiary structure of cytochrome *c* on reduction has been estimated at 1.1 kcal/mol by McLendon and Smith [37]. It can be supposed that this is true when urea is used. This resistance to unfolding can explain why our D_{1red} and/or D_{2red}, where the Met-80 S-iron linkage is disrupted, have to be considered as unstable and as only transitory species which tend to be converted preferentially into a Met-80 S-iron bonded reduced form. This conclusion agrees with the fact that the folding-unfolding step has been found to be accomplished relatively quickly by several authors [18,38].

Comparison between the results obtained in the present work on urea denaturation and the effect of alkaline pH on cytochrome *c* behavior [39]

shows a marked analogy between both processes. In the case of the pH effect, results can be interpreted on the basis of the existence of two electroactive cytochrome *c* forms, in agreement with the scheme of Lambeth et al. [40] where the existence of transient species has been also postulated. In both processes a drastic alteration of the conformation occurs when the Met-80 S-iron linkage is disrupted either at high urea concentration or at alkaline pH. This observation has been also underlined by Kaminsky et al. [14] who think that the conformational alterations of ferricytochrome *c* induced by denaturants and by changes of pH are cooperative.

Besides, one of the advantages of the present work has been to prove that electrochemical techniques can furnish useful data on the redox behavior of denatured cytochrome *c* forms. In particular, the activator role of 4,4′-bipyridine must be put forward; its effect in activating electron exchange at a gold electrode [27] is positive with either native or denatured forms although less marked in the latter case.

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