Electrochemical study of the redox properties of [2Fe-2S] ferredoxins Evidence for superreduction of the Rieske [2Fe-2S] cluster

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Abstract Direct, unmediated electrochemistry has been used to compare the redox properties of [2Fe-2S] clusters in spinach ferredoxin, *Spirulina platensis* ferredoxin and the water soluble fragment of the Rieske protein. The use of electrochemistry enabled, for the first time, the observation of the second reduction step, [Fe(III),Fe(II)] to [Fe(II),Fe(II)], in a biological [2Fe-2S] system. A water-soluble fragment of the Rieske protein from bovine heart bc_1 complex exhibits two subsequent quasi-reversible responses in cyclic voltammetry on activated glassy carbon. In contrast the ferredoxins from spinach and *Spirulina platensis* only show one single reduction potential. These results support a seniority scheme for biological iron-sulfur clusters relating cluster size to electron transfer versatility. Electrochemical reduction of spinach ferredoxin in the presence of $NADP^+$ and ferredoxin:$NADP^+$ oxidoreductase results in the generation of NADPH. The second order rate constant for the reaction between the ferredoxin and the reductase was estimated from cyclic voltammetry experiments to be $>3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$.

Key words: Rieske; Iron-sulfur cluster; Spinach ferredoxin; Cyclic voltammetry; Superreduction

1. Introduction

Direct unmediated electrochemistry has evolved in the last decade in a valuable technique for the study of redox properties of predominantly small redox proteins. Not only is it possible to study redox potentials and their dependence on parameters like temperature and pH, but also kinetic data in the form of rate constants can be obtained [1]. An important advantage of the use electrochemistry is the possibility to extend the potential window beyond the values which can be obtained in solution with chemical reductants or oxidants. This advantage has already led to the discovery of a presumably superreduced state in [3Fe-4S] clusters [2]. The 'superreduction' in this case is, however, associated with an adsorbed species and it is uncertain if this state can also be obtained in solution.

One of the major assets of iron as a bio-inorganic element is the fact that its ferric/ferrous transition is conveniently fine-tuned through its coordination chemistry over the better part of the redox potential range available to life [3]. This property is frequently used in iron-sulfur proteins, which act in electron transfer, Lewis-acid catalysis, redox catalysis, and (redox linked) regulation [4]. It is at present still poorly understood what determines the reduction potential(s) of these proteins, and what determines whether their functioning is associated with the transfer of no, one, one pair, or multiple pairs of electrons.

By a fortunate coincidence of circumstances we have found it possible to study the complete redox behaviour of a biological [2Fe-2S] system: (i) the Rieske protein can be purified as a water-soluble fragment with no observable change in its iron-sulfur cluster [5]; (ii) the fragment exhibits a direct, unmediated electrochemical response on glassy carbon [6]; (iii) the first reduction potential of the Rieske cluster is unusually high, namely $E_{m,7} \approx +0.3$ V [7]; (iv) carbon electrodes have a high overpotential for H_2 evolution, therefore, their use allows for aqueous-solution bio-electrochemistry down to $E \approx -1$ V [8]. The voltammetric characterization of the Rieske cluster and the comparison of its properties with other (i.e. low-potential) biological [2Fe-2S] clusters are the subject of this study.

2. Materials and methods

The water-soluble fragment of the Rieske protein of bovine heart bc_1 complex was isolated and purified as described previously [5,6]. Ferredoxin:$NADP^+$ oxidoreductase from spinach leaves and [2Fe-2S] ferredoxin from *Spirulina platensis* were obtained from Sigma Chemie (Bornem, Belgium) as the salt-free lyophilizate. The redissolved ferredoxin was found to be homogeneous in SDS-PAGE and isoelectric focusing, and was used without further purification. Spinach ferredoxin was a gift from Drs. M. Hirasawa and D.B. Knaff (Texas Tech at Lubbock). Both the ferredoxin from *Spirulina platensis* and from spinach were measured in the presence of 2 mM neomycin. Direct cyclic voltammetry in 15 μl volume was performed at the nitric acid activated glassy carbon electrode with the electrochemical cell described in [9] The electrode preparation for the spinach ferredoxin was slightly modified from this procedure. After treatment of the working electrode with nitric acid and washing with 0.1 M dipotassiumhydrogenphosphate and water, the electrode was held in a methane flame until it became red-hot. After cooling down 20 μl of a 20 mM neomycin solution was applied onto the electrode. Subsequently the electrode was dried with a tissue and mounted in the electrochemical cell. The sample was applied and the cyclic voltammetry was started using a BAS CV 27 potentiostat (BioAnalytical Systems Indiana, USA) connected to a X-Y recorder (Kipp & Zonen, NL). After one scan neomycin was added to the sample in a final concentration of 2 mM. Potentials were measured with reference to the saturated calomel electrode ($E = +244.4$ mV at 25°C) and re- calculated with reference to the standard hydrogen electrode ($E = 0$ mV). The pH dependence of the different proteins was studied using MES, HEPES, CHES, CAPS buffers of the appropriate pH all in a final concentration of 50 mM.

3. Results

3.1. Spinach ferredoxin

Spinach ferredoxin on a nitric acid activated glassy carbon electrode gives rise to completely irreversible voltammograms

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Abbreviations: SHE, Standard Hydrogen Electrode; SCE, Saturated Calomel Electrode.

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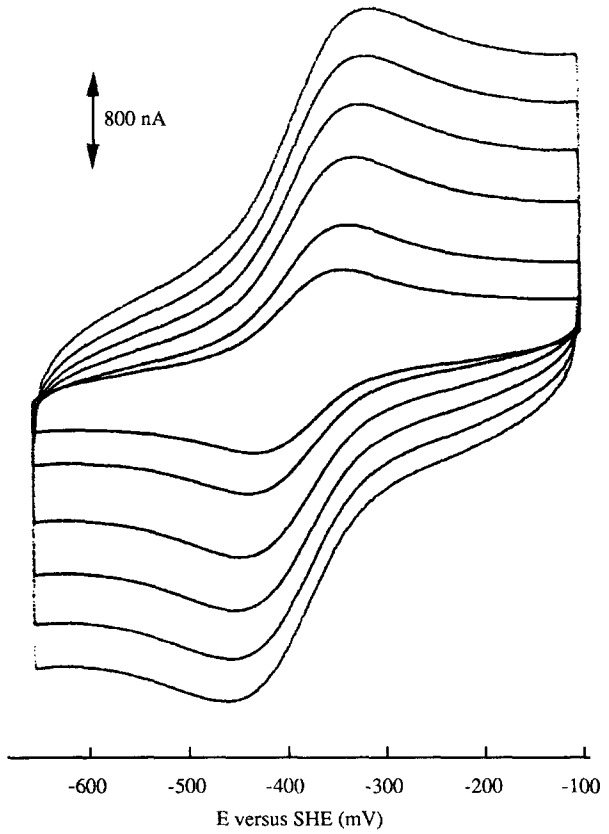

Fig. 1. Cyclic voltammograms of spinach ferredoxin in the presence of 2 mM neomycin at scan rates of 10, 20, 40, 60, 80 and 100 mV/s. Ferredoxin concentration was 280 μM in 50 mM HEPES, pH 7.0. Working electrode/counter electrode/reference electrode; glassy carbon/platinum/saturated calomel.

even in the presence of promotor, i.e. 2 mM neomycin. Only the electrode treatment in which the electrode has been heated in a flame and neomycin has been applied to the electrode after cooling down makes it possible to obtain quasi reversible voltammograms as shown in Fig. 1. Estimation of the heterogeneous rate constant from the voltammogram, using the procedure of Nicholson [10] results in a value of $2.2 \cdot 10^{-3}$ $cm^{-1} \cdot s^{-1}$. This value, which remains unchanged in the range of scan rates between 10 and 100 $mV \cdot s^{-1}$, is ten times higher than the value reported by Crawley and Hawkridge using viologen modified gold electrodes [11]. This is remarkable since the viologen is expected to facilitate the electron transfer between the electrode and the protein. The redox potential of spinach ferredoxin, estimated from the voltammogram as the average of the anodic and cathodic peak potentials, is found to be −405 mV versus SHE. This is slightly less negative than the potentials between −428 and −420 mV, obtained from earlier reported mediated voltammetry studies, redox titrations and equilibration studies with H_2 and hydrogenase [12,13]. The pH independence of the redox potential in the range pH 6.0 and 10.5 is consistent with previous determinations [14,15].

In the presence of ferredoxin:$NADP^+$ oxidoreductase and $NADP^+$ a catalytic reduction current develops due to the continuous oxidation of the ferredoxin by $NADP^+$. This is shown in Fig. 2. The shape of the voltammogram, however, indicates that the electron transfer between the ferredoxin and the electrode is the rate limiting step. Therefore, it is only possible to determine the minimum value for the second order rate constant between the ferredoxin and the reductase. The value for the second order rate constant calculated as described in [16] was found to be $>3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. This value is similar to the values reported by Dasgupta and Ryan for the electron transfer between spinach ferredoxin and reductants like Eu-DTPA and Fe-EDTA, which gave the highest values in the series of reductants tested [17]. This indicates that the electron transfer between the enzyme and the ferredoxin indeed is very efficient.

3.2. *Electrochemical superreduction of the Rieske protein*

Cyclic voltammetry of the water soluble fragment of the Rieske protein in the range of −100 mV to 700 mV versus SHE results in well defined voltammograms as described in ref [6]. Upon extension of the scan range to lower potentials a second redox transition was observed at −840 mV vs. SHE. This second redox transition appears to be unique for the Rieske type cluster since this cannot be observed with either the ferredoxin from *Spirulina platensis* or spinach. Fig. 3 shows the cyclic voltammograms of the Rieske fragment, the ferredoxin of *Spirulina platensis*, and a baseline.

In contrast with the first reduction the second reduction of the Rieske fragment is not pH dependent (data not shown). The independence of pH is consistent with the postulated coordination of these type of clusters. As described in earlier work the

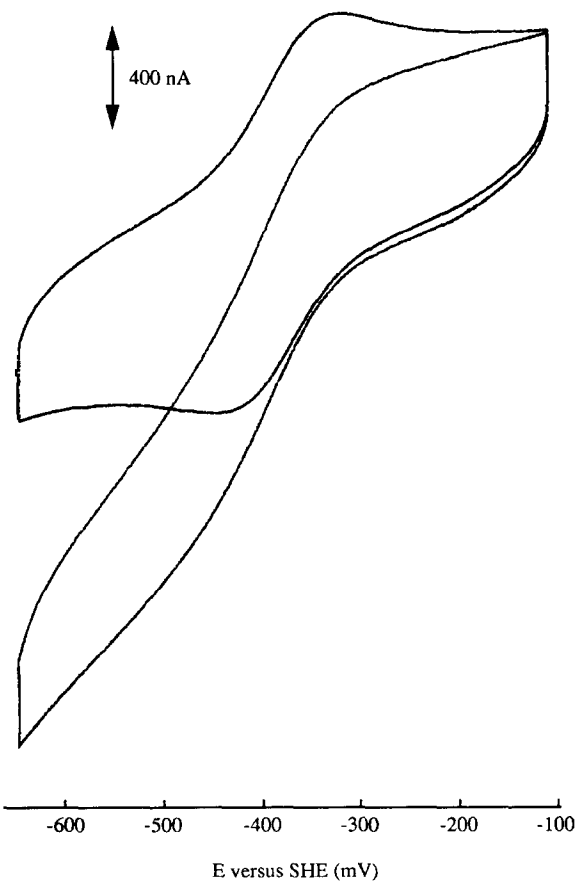

Fig. 2. Cyclic voltammograms of spinach ferredoxin in 50 mM HEPES, pH 7.0, in the presence of 2 mM neomycin. The sigmoidal shaped voltammogram develops after addition of 1 mM $NADP^+$ and 2.7 μM ferredoxin:$NADP^+$ oxidoreductase.

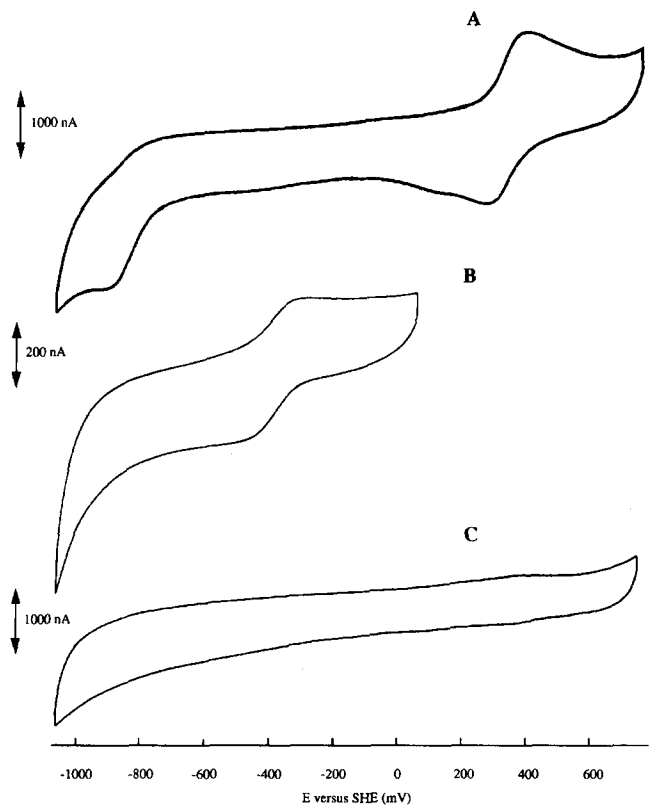

Fig. 3. Cyclic voltammogram of (A) Rieske fragment (3 mg/ml), (B) *Spirulina platensis* ferredoxin (2 mg/ml) in the presence of 2 mM neomycin, and (C) baseline. The voltammograms were recorded in 50 mM HEPES, pH 7.0, at a scan rate of 10 $mV \cdot s^{-1}$. Working electrode/counter electrode/reference electrode; glassy carbon/platinum/saturated calomel.

pH dependence of the first transition can be explained by the protonation of histidines coordinated to the one of the iron atoms in the cluster [6]. However the other Fe-atom is coordinated by cysteines. Such a coordination is not likely to give rise to pH dependence as is obvious from results with the all cysteine coordinated [2Fe-2S] cluster in spinach ferredoxin [14,15].

4. Discussion

In order to interpret the observation of superreduction in the Rieske fragment but not in spinach or *Spirulina platensis* ferredoxin it is necessary to consider the properties of iron sulfur clusters in proteins The biological function of iron-sulfur proteins is not limited to mediation of redox reaction through single-electron transfer. In recent times three other functions have been identified, namely, non-redox catalysis [18,19], regulation of gene expression [20,21], and (multi) electron pair associated redox catalysis. Evidence for the latter function is limited, e.g. by the lack of high-resolution structural data on the complex proteins involved. The electron-pair associated hydrogenase reaction is a case in point. The active center of Fe-only hydrogenase probably encompasses a single iron-sulfur cluster [22,23]. This cluster appears to contain some six iron atoms [24]. Spectroscopic, sequence data, and X-ray structural evidence points to the possibility of similar 'superclusters' in other iron-sulfur redox enzymes, viz sulfite reductase [25], CO dehydrogenase [26,27], and nitrogenase [28,29]. This information combined with what is known on the redox properties of small, electron transfer iron-sulfur proteins [4] has led us to hypothesize the following seniority scheme for the redox properties of biological iron-sulfur clusters [30–32].

The size of an iron-sulfur cluster is directly related to its electron transfer versatility; 'superclusters' (i.e. >4 Fe) are required for multiple electron transfer. This seniority scheme follows from three theses: (i) a biological iron-sulfur cluster of n iron atoms can – in principle – go through n subsequent redox transitions (formally n ferric/ferrous transitions), i.e. the cluster has n reduction potentials; (ii) with increasing value of n each individual reduction potential can be tuned (by protein coordination) over an increasingly wide potential range; 3) with increasing n the difference between subsequent reduction potentials decreases.

The first thesis is self-evident, when valence states other than ferric and ferrous are excluded. The second thesis reflects the range of observed reduction potentials for rubredoxins, [2Fe-2S] proteins, and [4Fe-4S] proteins [2]. The third thesis is based on the two different transitions, 3 +/2 + and 2 +/1 +, observed in [4Fe-4S] proteins (i.e. high-potential iron proteins vs. ferredoxins), and on the multiple transitions observed in the putative [6Fe-6S] protein [33] and in the P-cluster of nitrogenase [34].

Thus far, the lack of information on the second reduction potential of [2Fe-2S] proteins has been a serious weakness in the experimental support for the seniority scheme. In fact, the available cyclic voltammetry data on iron-sulfur model clusters in DMF are at variance with the scheme: the potential difference between two subsequent E_m's is small, namely ≈ 0.3 V, for $Fe_2S_2L_2$ clusters and larger, namely ≈ 0.7 V, for $Fe_4S_4L_4$ [35]. Also, the potential difference for other (i.e. non sulfur) biological 2Fe clusters is maximally 0.2 V [36], and is in other cases even negative [37,38]. We have now found that the Rieske [2Fe-2S] cluster can go through two subsequent reduction steps, that both these steps are not anomalous thermodynamically and kinetically, and that the potential difference is ≈ 1.1 V. We conclude from these results that the redox data on iron-sulfur models and on non-sulfur biological 2Fe clusters is not relevant to biological [2Fe-2S] systems, and that the redox properties of the latter do support the scheme.

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