Volume 217, number 1, 57–61 FEB 04792 June 1987

EPR redox study of cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki

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Received 26 March 1987

We report the results of an EPR potentiometric titration of cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki: the EPR spectral features of the four hemes are identified. The four midpoint redox potentials, which are deduced from the integrated intensity variations as a function of the redox potential, are within the range −230 to −360 mV with two nearly equal intermediate values, in agreement with previous electrochemical measurements. A structural change of the environment of the heme with the most negative potential is observed during the first step of the reduction. The correspondence between the redox sites as characterized by the EPR potentiometric titration, and the hemes in the tridimensional structure, is discussed.

Cytochrome c_3; EPR; Redox potentiometry; (*Desulfovibrio vulgaris*)

1. INTRODUCTION

Cytochromes c_3 are multihemic proteins functioning as electron carriers in the sulfate-reducing bacteria. Each molecule (M_r 13000) contains four hemes covalently bound to the peptide chain with two histidines as axial ligands. The midpoint redox potentials which have been reported are generally different and largely negative [1–4]. The X-ray diffraction structure has been established for two species, namely *Desulfovibrio vulgaris* Miyazaki [5] and *D. desulfuricans* Norway [6]. The relative arrangement of the hemes is very similar in both cases in spite of the weak homology between the sequences. Some of their physico-chemical properties present marked differences. In particular in *D. vulgaris* Miyazaki cytochrome c_3, the range which is covered by the midpoint redox potentials measured by electrochemical methods [1,2] is narrower than in the case of *D. desulfuricans* Norway [3,4]. The NMR spectra recorded at different steps of reduction indicate a fast intramolecular electron exchange for *D. vulgaris* Miyazaki cytochrome c_3 [7] whereas a slow exchange is observed for *D. desulfuricans* Norway cytochrome c_3 [8].

An important aspect of the current works concerns the possible existence of interactions between the hemes. These interactions can lead to a modulation of the redox potential of a given heme depending upon the oxidation state of the other redox centers of the molecule [1,9]. Progress on this subject needs a development of comparative approaches by different techniques. In the case of *D. vulgaris* Miyazaki cytochrome c_3, the main data on the redox properties have been deduced from electrochemical or optical measurements. These techniques are only sensitive to the macroscopic redox parameters which are not well adapted for a discussion of the problem of interacting potentials. In this note we present the results of an EPR study of *D. vulgaris* Miyazaki cytochrome c_3 at different steps of redox equilibrium. The main interest of this technique comes from the fact that the

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

amplitude and the shape of the spectra are directly sensitive to the microscopic redox parameters relative to each heme and to their direct environment which can possibly be modified during the titration.

2. MATERIALS AND METHODS

The purification method of cytochrome c_3 is different from that previously reported [10,11]. A new purification procedure entirely composed of milder processes has been developed. The supernatant of the bacterial sonicate prepared from *D. vulgaris* Miyazaki was made 40% saturated with ammonium sulfate and centrifuged. The supernatant was then made 70% saturated and centrifuged to remove most proteins including desulfoviridin. The supernatant was finally made 90% saturated and centrifuged. The supernatant was passed through a column of DE 32 (Whatman) to adsorb cytochrome c_3 and cytochrome *c*-553, which were eluted with 10 mM Tris-HCl, pH 7.4, as a deep-red solution. The cytochrome was gel-chromatographed on a column of Sephadex G-50 (fine) (50 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl, as an eluting buffer) to separate cytochrome c_3 from cytochrome *c*-553. The cytochrome c_3 solution was dialysed against H_2O, adsorbed on a column of CM-cellulose (NH_4^+ form), and eluted by concentration gradient of aqueous NH_3 from 20 to 100 mM, cytochrome c_3 being eluted at an ammoniacal concentration of about 30 mM. The purity index (A_{552} of the ferro form/A_{280} of the ferri form) of the final preparation is 3.2. The experimental procedures for the potentiometric titration and the EPR measurements are identical to those used previously for *D. desulfuricans* Norway cytochrome c_3 [4]. The concentration of cytochrome c_3 was 0.45 mM in 0.1 M Tris-HCl at pH 8.1. The redox potentials were adjusted at 21°C by small amounts of $Na_2S_2O_4$ at 100 mM in the presence of the following mediators at 10 μM: 2-hydroxy-1,4-naphthoquinone, phenosafranine, benzyl viologen, methyl viologen.

3. RESULTS AND DISCUSSION

The EPR spectrum of *D. vulgaris* Miyazaki cytochrome c_3 in the oxidized state is given in fig.1A. The weak components around $g = 2$ and 2.47 are due to an impurity which has a midpoint redox potential much higher than those of cytochrome c_3. They disappear before the beginning of the reduction of the protein. The general shape of the spectrum and the positions of the different features are reminiscent of those reported for *D. vulgaris* Hildenborough cytochrome c_3 [12] which has a high degree of sequence homology with *D. vulgaris* Miyazaki cytochrome c_3. However the poor quality of the spectrum presented in [12], probably because of fast passage effects under saturating conditions, and the absence of the high-field region do not allow a precise comparison. At high field, three resolved peaks can be seen at g_x = 1.425, 1.590 and 1.675. From the analysis of the spectral shape evolution as a function of the redox potential and by using the empirical rule generally verified for low-spin hemoproteins $\Sigma_i g_i^2 \sim 16$ (i = x,y,z), one may identify the corresponding features in the g_z region. They are respectively the narrow peak at 2.965, the broader peak at 2.81 and the shoulder at 2.72. The fourth heme gives a very broad peak at $g_z = 3.38$ and its contribution at high field is not observable. Assuming a g_y value of about 2, the corresponding g_x value would be about 0.7.

Spectrum B in fig.1 corresponds to a sample poised at a low potential. The peak at 1.675 disappeared completely and the peak at 1.590 has been shifted. This shift of about 75 G downfield occurs during the first step of cytochrome c_3 reduction (fig.1, inset) and reveals a structural change of the environment of the corresponding heme. A shift of the g_z peak of this heme of about 20 G upfield should be expected. This effect is difficult to appreciate because of the low resolution of the peak in the oxidized state of the sample.

We have measured the total intensity (double integration) of the EPR spectra of *D. vulgaris* Miyazaki cytochrome c_3 at each step of the reduction. The experimental results are reported in fig.2A as a function of the redox potential. The continuous curve represents the best fit obtained by adding the contribution of four Nernst curves of equal amplitude and centered at $e_1 = -230$ mV, $e_2 = -300$ mV, $e_3 = -310$ mV and $e_4 = -360$ mV.

When interacting potentials are negligible, these values correspond to the microscopic midpoint redox potentials which characterize the different

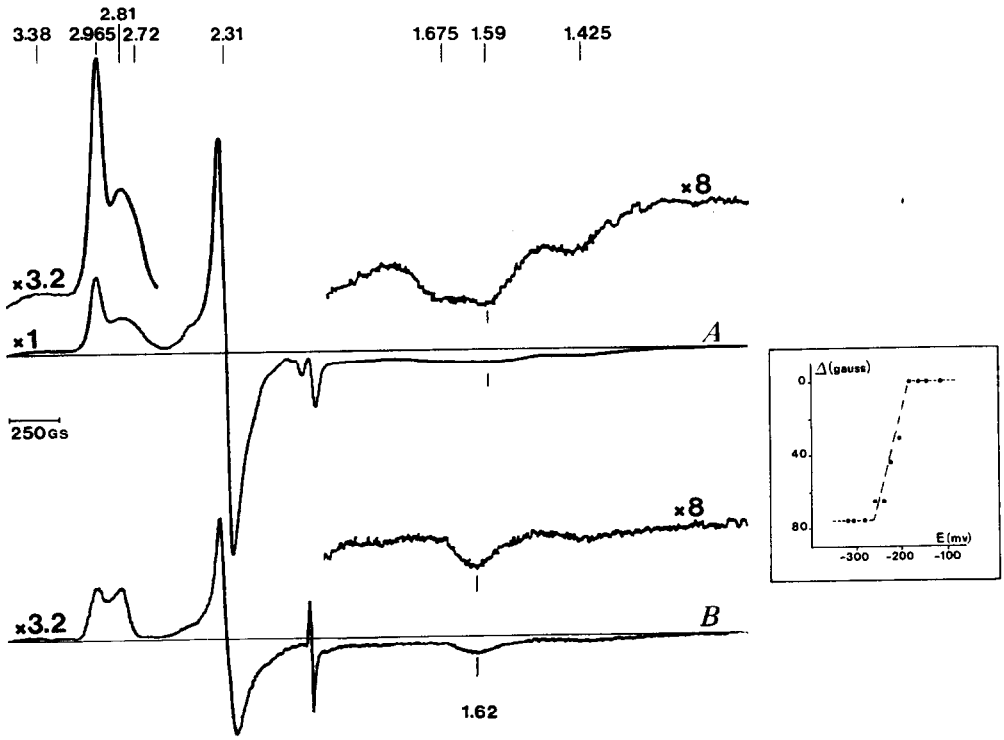

Fig.1. EPR spectra of *D. vulgaris* Miyasaki cytochrome c_3 in the oxidized state (A) and poised at −341 mV (B). EPR experimental conditions: T = 16 K, microwave power, 0.5 mW at 9300 MHz; field modulation, 20 G p.p. at 100 kHz. The relative gains are indicated on the spectra. In the inset is represented the shift downfield Δ of the peak g = 1.59 as a function of the redox potential.

hemes. The corresponding macroscopic midpoint redox potentials can then be calculated by using expressions such as those given in [1]. We obtained the values E_1 = −227 mV, E_2 = −287 mV, E_3 = −320 mV and E_4 = −366 mV which are comparable to those deduced from electrochemical measurements: −225, −278, −298, −339 mV [1] and −240, −297, −315, −357 mV [2].

The correspondence between the spectral features (g values) and the microscopic potentials was easily deduced from a qualitative examination of the amplitude of the different peaks of the spectra, as a function of the redox potential. We found: heme 1 (−230 mV, g_x = 1.675, g_z = 2.72), heme 2 (−300 mV, g_x = 1.425, g_z = 2.965), heme 3 (−310 mV, g_z = 3.38), heme 4 (−360 mV, g_x = 1.590, g_z = 2.81). For a more precise analysis of the amplitude variations it is necessary to take into account the overlap between the spectra of the different hemes. The relative contributions of the hemes to a given peak were adjusted so as to obtain the best fit of the redox titration curve deduced from the variation of the amplitude of this peak. As illustrated in fig.2B and C, we obtained a reasonable fit for heme 2 and heme 3 which have peaks well resolved and separated from heme 4.

On the other hand, a good fit could not be obtained for the peaks which were attributed to hemes 1 and 4. The main reason is likely the progressive shift of the peaks of heme 4 arising as heme 1 is reduced. Because of the strong overlap between the features of hemes 1 and 4, the resulting spectral shape is modified during the titration. A second reason could be an eventual contribution of interacting potentials. However, effects of these potentials on the redox titration of hemes 1 and 4 are expected to be weak as the values e_1 and e_4 are well separated from e_2 and e_3. In the g_y region, the fit of the peak-to-peak amplitude (not shown) indicates a nearly equal contribution

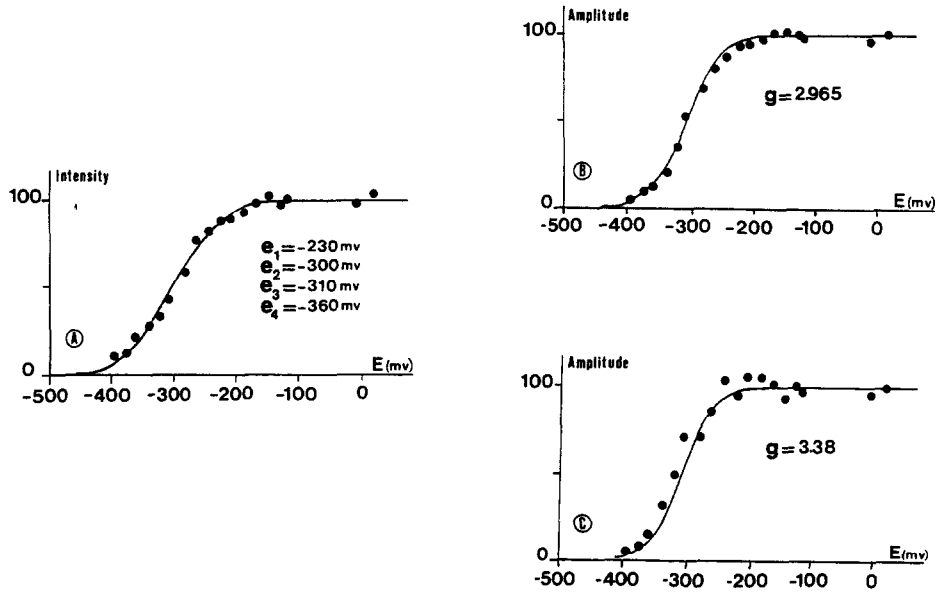

Fig.2. Redox titration curves: (A) intensity (measured by a double integration of the spectra); (B,C) amplitude at g = 2.965 and g = 3.38, respectively. The continuous curve in (A) is the best fit with Nernst curves as described in the text with the values of the microscopic midpoint redox potentials indicated in the figure. The best fits in (B,C) were obtained for the following contributions of the different hemes: peak at g = 2.965, 80% heme 2, 5% heme 3, 15% heme 4; peak at g = 3.38, 100% heme 3.

of the three hemes 1, 2 and 4. The contribution of heme 3, which has a very broad spectrum, is very weak.

This work must be considered as a first step of a more complete study where the interacting potentials will be taken into account in the analysis of the experimental results. However, at the present stage, several important points can be discussed:

(i) The variations of the total EPR intensity and of the amplitudes of the peaks attributed to heme 2 and heme 3 can be reasonably simulated without assuming an appreciable contribution of interacting potentials. This suggests that the interactions involving these two hemes are weak. In agreement with the previous results obtained with electrochemical methods, the four midpoint redox potentials are in a range which is narrower than in the case of *D. desulfuricans* Norway cytochrome c_3, and two of them, e_2 and e_3, are nearly equal.

(ii) Reduction of heme 1 is accompanied by a spectral change of heme 4, thus reflecting some structural modification of its environment. This effect could be related to the apparition of an intermediate observed by optical spectroscopy and kinetic measurements during the first steps of the reduction of *D. vulgaris* Miyazaki cytochrome c_3 [13,14].

(iii) As for *D. desulfuricans* Norway cytochrome c_3, one of the hemes (heme 3 with g_z = 3.38) is characterized by a g_z value well separated from the others and largely above 3. Such high g_z values observed in several ferrihemes have been attributed to a nearly axial configuration of the ligand field [15]. In the case of hemoproteins with two histidines as axial ligands this symmetry is expected when the dihedral angle between the two imidazole planes is large [16,17]. When the tridimensional structure is available, this model can be used to identify the magnetic redox sites [18]. In *D. vulgaris* Miyazaki cytochrome c_3, hemes I, III and IV (numbering as in [5] except for the Roman characters) have their axial imidazole planes nearly parallel, while in heme II this angle is about 64° [5]. Thus, the model predicts the correspondence heme II-redox site 3 (−300 mV, g_z = 3.38). Other arguments based on the correlation between potential and redox site exposure observed for some hemoproteins [19] have been used to identify the redox sites [2,6]. From these arguments, heme II, the most exposed heme, should be identified as the

redox site 4 which has the most negative potential, in contradiction with the identification given by the model based on axial ligand orientation. One must also note that the correspondence heme II-redox site 3 (−300 mV) is not in agreement with the model presented in [2] where redox sites 2 and 3 are identified as hemes III and IV.

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