

# The thermodynamic characteristic of four-heme cytochrome *c* in *Rhodopseudomonas viridis* reaction centers, as derived from a quantitative analysis of the differential absorption spectra in $\alpha$ -domain\*

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A method of decomposing of the absorption spectrum of four-heme cytochrome of a *Rhodopseudomonas viridis* reaction center preparation into spectra of individual components was used to estimate the degree of the reduction of hemes as a function of redox potential in the medium. The method enables an evaluation of the shape of redox-titration curves of each heme. The redox-titration curves derived by this approach are approximated well by a Nernst equation with  $n=1$  and  $E_m$ -values of 360 mV, 312 mV, 20 mV and less than –50 mV. For all of the redox species the values of midpoint potential estimates by the above method are in good agreement with those determined earlier using another procedure [Dracheva et al. (1988) Eur. J. Biochem. 171, 253–264]. The accuracy of deconvolution of data is within the experimental errors of the redox potential measurement.

Reaction center; Four-heme cytochrome; Redox titration; Deconvolution method; (*Rhodopseudomonas viridis*)

## 1. INTRODUCTION

Optical studies of *Rhodopseudomonas viridis* RCs provided evidence that each of the four hemes of cytochrome *c* has its own absorption spectra in the  $\alpha$ -band domain [1–5]. This conclusion has also been made from low-temperature absorption and linear dichroism measurements on oriented RCs [6]. EPR studies of RCs and chromatophores have shown that the hemes may be distinguished also by the value of the  $g_z$ -peak [7,8]. The redox midpoint-potentials of individual hemes have been determined on the basis of absorption [2–4] and EPR [7,8] measurements. The crossing of the optical spectra of the hemes in  $\alpha$ -domain [2] as the coincidence of their  $g_z$ -factors (except for the high-potential heme) did not allow to observe the redox-titration curve of individual hemes.

An analysis of differential spectra taken at different redox potentials and the consideration of the individual molecular heme planes in the crystals of RC lead to the following potential sequence of heme groups: P960-high-low-high-low [9] in agreement with earlier results [2,7].

In this work we used the individual spectra of each

heme and linear least-squares method to elucidate the fracture of every heme from the absorption spectra of cytochrome *c* in the  $\alpha$ -band region as function of redox potential. For the four redox species, the values of the midpoint-potentials estimated by the decomposition method are in good agreement with those determined earlier [2].

## 2. MATERIALS AND METHODS

RCs from *Rps. viridis* were isolated as described elsewhere [2]. Absorption spectra were recorded with a Aminco DW-2a spectrophotometer. The redox potential was measured with Pt, Ag/AgCl electrodes. The redox-titrations of cytochrome *c* hemes were carried out as in [2]. Data storage, processing and curve-fitting were carried out on an IBM AT computer using the self-made program system GIM [10].

In the following we use the numbering of hemes which is defined according to their appearance along the amino acid chain [9], i.e. P960,  $c_3$ ,  $c_4$ ,  $c_2$ ,  $c_1$ , as well as their designations, introduced in [2] according to their maxima in  $\alpha$ -domain, i.e. P960, c559, c552, c556 and c554.

To obtain the  $E_h$  dependence of the concentration of a reduced form of a heme we used the following method of spectral decomposition (see also [11,12] and references within them to consider similar methods). Let  $A_i$  be the differential absorption of the cytochrome at wavelength  $\lambda_i$ , then

$$A_i = \sum_{j=1}^4 \epsilon_{ij} c_j^r \quad (i=1,2,\dots,N)$$

where  $\epsilon_{ij}$  is a differential reduced minus oxidized coefficient of extinction of  $j^{\text{th}}$  heme ( $j=1,2,3,4$ ) at wavelength  $\lambda_i$ ;  $c_j^r$  is a concentration of reduced form of  $j^{\text{th}}$  heme.

It is clear that measuring the differential absorbance spectrum at only four wavelengths ( $i=4$ ) gives a system of 4 equations for  $c_1^r$ ,  $c_2^r$ ,  $c_3^r$  and  $c_4^r$ . This is sufficient to calculate their concentrations. Using more than four wavelengths ( $i>4$ ) decreases the instability of the

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Abbreviations: RC, reaction center;  $E_m$ , midpoint redox potential;  $E_h$ , redox potential of medium;  $c_j^r$ , the concentration of the reduced state of  $j^{\text{th}}$  heme

\* In a preliminary form the data were presented in XIII International Congress on Photosynthesis, Stockholm

determination of  $c_j^i$  caused by random errors of measuring the absorbance at fixed wavelengths. Employing the least-squares method that minimizes the sum of squares of the differences between the actual

differential absorbance  $A_i$  and  $\sum_{j=1}^4 \epsilon_{ij} c_j^i$  for all wavelengths, one

can obtain the following system of four linear equations for  $c_j^i$ :

$$\sum_{j=1}^4 c_j^i \left( \sum_{k=1}^N \epsilon_{ik} \right) = \sum_{k=1}^N A_i \epsilon_{ik} \quad (k=1, \dots, 4)$$

If the spectra of the individual hemes  $\epsilon_{ij}$  are known, solving this system of four equations will yield  $c_1^i$ ,  $c_2^i$ ,  $c_3^i$  and  $c_4^i$ . Values of the capacity of the basic spectra  $\epsilon_{ij}$  were those estimated in [2]. Utilizing this procedure at each redox potential of medium one can estimate the shape of redox-titration curve of the heme.

### 3. RESULTS AND DISCUSSION

Fig.1a shows experimental differential spectra in the  $\alpha$ -band region of cytochrome *c* observed under different redox potentials from 256 to 410 mV, plotted as absorbance-wavelength- $E_h$ -surface. In the given wavelength region, the data obtain information on the  $E_h$  profile of each high-potential heme. It is evident from surface that the position of the maximum is shifted to shorter wavelengths, as  $E_h$  is lowered. One can examine the redox potential dependence of cytochrome absorbance for a fixed wavelength, for example, for 559 nm, as is shown in fig.1b. The  $E_m$  of the high-potential hemes in this case can be derived by a computer analysis of the redox titration curve based on the assumption that it consists of two Nernst curves (see e.g. [2,7]). It is important to outline that this assumption is not true for cooperative interactions between the hemes (see e.g. [13,14]). The approach based on the spectral decomposition allows one to obtain more detailed information about the titration curves of hemes without using any assumptions concerning the curve shape.

Shown in fig.2 is the  $E_h$  dependence of the fraction of the reduced high-potential hemes obtained from fig.1a by the decomposition method performed as described above. The points were determined using the four (a) or only two high-potential (b) spectra of the hemes taken from work [2]. The use of all the spectra of individual components shows that at high redox-potentials the low-potential hemes, c552 and c554, make no contribution to the absorbance in the  $\alpha$ -band region. The errors in the region between 250 and 300 mV (see fig.2a) may be due to insufficient accuracy of the spectra of individual hemes used by us as a basis for the decomposition. It is seen that the procedure using only c559 and c556 heme spectra give similar results (fig.2b). Utilization of only two spectra of high-potential hemes gives points which are approximated well by one-electron Nernst curves with  $E_m = 362$  mV and 300 mV, respectively (fig.2b). These values are in good agreement with those obtained from points presented in fig.1b. All three types of the mathematical treatments of the data presented in fig.1b and fig.2 give 360 mV for  $E_m$  of the

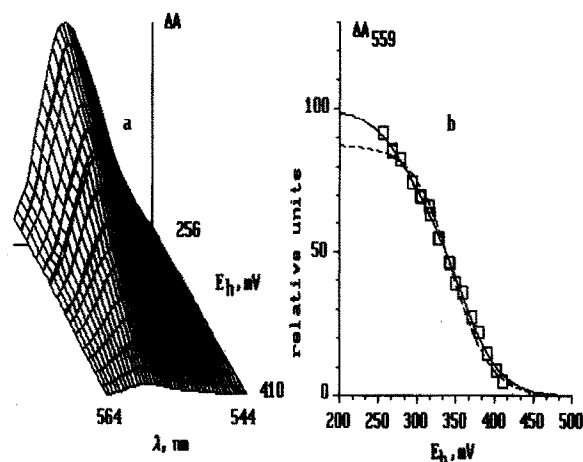


Fig.1. (a) Experimental absorbance-wavelength- $E_h$  surface for high potential redox titration of cytochrome *c* in *Rps. viridis* RCs. Experimental conditions as in [2]. The reference sample was poised at  $E_h = 470$  mV. (b) Redox titrations of absorbance changes at 559 nm, as derived from the data given in (a); (---) single component approximation of the data with  $E_m = 345$  mV,  $n = 1$ ; (—) two component approximation ( $n = 1$ ) with  $E_m = 360$  mV (66%) and with  $E_m = 285$  mV (34%). The value of SD is 5 times larger for one-component approximation than in the case of two.

high-potential heme. The  $E_m$ -value of the other high-potential heme (c556) is dependent essentially on the mathematical procedure and varies from 285 for simple procedure (fig.1b) to 312 mV (fig.2a). Thus the traditional method of estimation of  $E_m$  from computer deconvolution of the one redox titration curve obtained at fixed wavelength gives values of  $E_m$  close to those obtained by method of spectral decomposition although the difference of  $E_m$  obtained by these two methods may reach 30 mV. The choice of method of decomposition is the presentation of the shape of redox titration curve of individual heme.

As seen from fig.2b, at low redox potentials the concentration of the reduced c559 heme is less than the concentration of the reduced c556 heme. This means that the extinction coefficient of heme c559 at 559 nm is 1.2 times smaller than that for heme c556 at 556 nm.

As has been found in [2] and supported by data in [9], the spectrum of c559 heme contains a shoulder at 552 nm. The deconvolution of the data presented in fig.1a into three gaussian spectra of equal widths with maxima at 559, 556 and 552 nm shows that the 559-nm component has the same midpoint as that at 552 nm (361 and 363 mV, respectively). The amplitude of the former, however, is 3 times larger than that of the latter. This is an independent piece of evidence that the maxima at 559 and 552 nm originate both from the same high-potential heme.

Fig.3 shows the dependence of the cytochrome differential spectra on the redox potential of the medium from -30 to 140 mV (a) and redox titration curves for c552 and c554 hemes (b) obtained by the decomposition method. For c552 heme,  $E_m$  is 23 mV. For c554 heme  $E_m$  is lower than -50 mV.

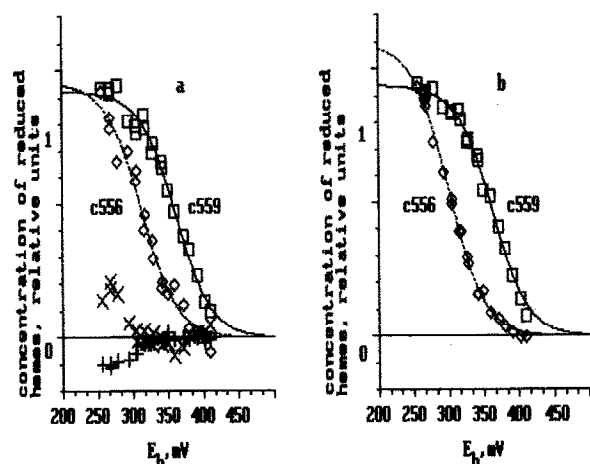


Fig.2.  $E_h$  dependence of the amount of high-potential hemes of cytochrome in *Rps. viridis* RCs, as obtained from data of fig.1a. The points were determined by the decomposition method using the spectra of: (a) four individual hemes; or (b) only two high-potential hemes. The spectra of the individual hemes were taken from [2]. ( $\square$ — $\square$ ), c559; ( $\diamond$ — $\diamond$ ), c556; ( $\times$ — $\times$ ), c552; (+ +), c554. The points are approximated by one-electron Nernst curves with  $E_m = 362$  mV and 312 mV (a), and with  $E_m = 362$  and  $E_m = 300$  mV (b), for c559 and c556 hemes, respectively.

The results of the decomposition largely depend on the spectra of individual hemes. The maxima of the individual hemes in the  $\alpha$ -domain obtained in [2] coincide well for those obtained in [9] for three hemes (c559, c556 and c552). However, they differ significantly for the heme with lowest redox potential (554 nm in [2], 552.5 nm in [9]). The reason for this is unclear. The values of widths are similar in both works.

It appears that although all the four hemes have  $\alpha$ -peaks located very close to one another, the above decomposition method enables a selective titration of each heme and the determination of the midpoint redox potential of each. Our data clearly demonstrate the spectral and redox heterogeneity of the hemes. The heterogeneity, established first in [1,2], was confirmed by optical and EPR spectroscopy [3–9]. By calculating the electrostatic potentials of the hemes within the protein by a numerical solution of the Poisson equation, it was shown [15,16] that their heterogeneity is due to the electrostatic interactions with amino acid residues of the protein. It is clear that  $E_m$  of the individual hemes may also be changed by electrostatic interactions between the hemes. In the latter situation, it is necessary to determine midpoint potentials of each heme for a given distribution of electrons among the other hemes. Therefore  $E_m$  values obtained above must be considered as a first approximation to the real ones when this interaction is small. A more general situation will be presented elsewhere.

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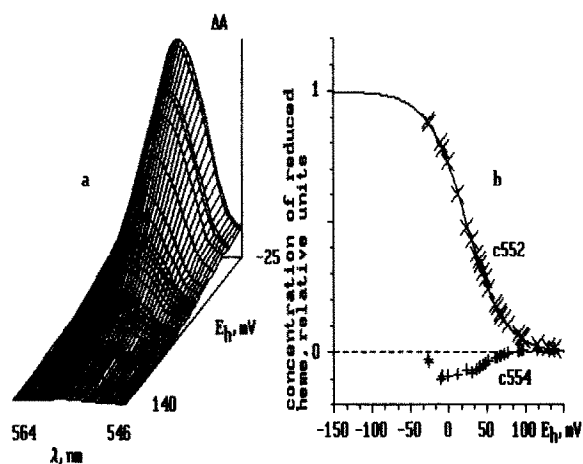


Fig.3. (a) The experimental absorbance-wavelength- $E_h$  surface for a low-potential redox titration of cytochrome c in *Rps. viridis* RCs. The reference sample was poised at  $E_h = 200$  mV. Experimental conditions as in [2]. (b)  $E_h$  dependence of low-potential hemes of cytochrome c obtained from data presented in (a). The points were determined by decomposition method using the spectra of two low-potential c552 ( $\times$ ) and c554 (+) hemes taken from [2]. The points were approximated by one-electron Nernst curve with  $E_m = 20$  mV for c552 heme.

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