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Complexity in the redox titration of the dihaem cytochrome c_4

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Redox titration of the dihaem, two domain cytochromes c_4 from *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Azotobacter vinelandii* showed complex behaviour indicative of the presence of two redox components. In the case of the *P. stutzeri* cytochrome c_4, two spectroscopically distinct components were present during the redox titration. In contrast, cytochrome *c*-554(548) from a halophilic *Paracoccus* species is a stable dimer of a monohaem cytochrome which shows close homology to cytochrome c_4, but does not show complexity in its redox titration. The presence of chemically distinct haem environments or anti-cooperative interactions between identical haem groups are two possible explanations for the redox complexity of cytochrome c_4. The simple redox titration of cytochrome *c*-554(548) shows that haems situated relatively close together need not interact, but direct cleavage, separation and study of the domains will be necessary to decide whether they do or do not interact in the case of cytochrome c_4.

Introduction

Cytochrome c_4 is a dihaem cytochrome of an approx. molecular weight of 20000. The amino acid sequence shows evidence of a gene duplication event [1,2] giving rise to a two domain protein [3], each domain resembling a typical small monohaem cytochrome *c*.

Cytochrome c_4 is poorly characterised with respect to distribution, cellular location and electron-transfer function. This may be due in part to its probable location in the membrane and also to the fact that its characteristic properties have not always been clearly recognised; cytochrome c_4 is distinguished by a molecular weight of 20000 and a low α/β absorption ratio. On the basis of these properties, members of the cytochrome c_4 group have been isolated from several denitrifying bacteria [4–7]. The suggestive correlation with the denitrification process, however, is complicated by the presence of cytochrome c_4 in the strict aerobe *Azotobacter vinelandii* where it is proposed to form a terminal cytochrome oxidase complex with cytochrome *o* [10,11]. Such a complex may be widespread [12–15] and the apparent correlation between cytochrome c_4 and denitrification may simply be a consequence of the induction of cytochrome *o* under conditions of limiting oxygen.

The dihaem nature of cytochrome c_4 raises interesting questions regarding its role in electron transfer. Terminal oxygen reductases seem to be designed in such a way as to avoid single electron-reduction steps [16], and cytochrome c_4 may be a device for ensuring coordinated delivery of two electrons. A second possibility is that one domain of cytochrome c_4 may bind to a membrane reductase, while the other domain binds to an oxidase. In such a mode, cytochrome c_4 would

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Abbreviations: PMS, phenazine methosulphate; DAD, diaminodurol; Cyt, cytochrome.

provide a conduction route via intramolecular electron transfer.

Redox complexity in a dihaem system may be due to intrinsic redox potential differences or to haem : haem interactions giving rise to deviations from simple Nernstian behaviour. Such deviations are present in cytochrome c_4 titrations in the literature (see for example Fig. 1(a) of Ref. 10), but have not been noted and commented on. This paper describes the redox complexity of three cytochromes c_4 from *Pseudomonas aeruginosa, Pseudomonas stutzeri* and *A. vinelandii.*

Methods

Organisms and cytochromes

The organisms used were *P. aeruginosa* (P6009), *P. stutzeri* (Stanier 224, ATCC 17591) and *A. vinelandii* (strain *o*, ATCC 12837). Cytochrome c_4 was extracted from membranes using butanol [8] and purified by methods to be described separately (unpublished data). All three purified cytochromes c_4 gave single bands after SDS polyacrylamide gel electrophoresis and in the cases of the *P. aeruginosa* and *A. vinelandii* cytochromes the amino acid compositions closely resembled those of the published amino acid sequences [1,2]. The amino acid sequence of the cytochrome from *P. stutzeri* has not yet been determined.

Cytochrome *c*-554(548) from a halophilic *Paracoccus* species (originally described as *Halotolerant micrococcus* [17]) was a kind gift from Dr. T.E. Meyer. Horse cytochrome *c* was from Sigma (Type VI).

Redox titrations

Reductive and oxidative titrations were performed in an anaerobic cuvette constantly bubbled with argon and magnetically stirred (Cell stirrer, Bel-Art). The cuvette contained approx. 5 μM cytochrome in 0.02 M sodium phosphate (pH 7), 20 μM phenazine methosulphate (PMS, Sigma), Diaminodurol (DAD, Aldrich) and ferric ammonium sulphate and 0.4 mM EDTA.

The ambient redox potential (E_{obs}) was monitored by a Pt pin electrode in combination with an Ag|AgCl reference (Russell pH Ltd., Auchtermuchty, U.K.) and the potential with reference to the standard hydrogen electrode (E_h) was obtained by adding 198 mV to E_{obs} [18].

Oxidative and reductive titrations were performed by addition of 0.02 M potassium ferricyanide and 0.02 M sodium dithionite, respectively. The ambient potential reading stabilised within a minute of an addition and the spectrum was then recorded. Full reduction was achieved by addition of solid dithionite. Isosbestic points were maintained throughout the titration except after addition of solid dithionite, where the reduction of PMS gives rise to a small contribution to the cytochrome spectrum. This spectrum was therefore offset to match the isosbestic point established during the titration.

Redox titrations were also performed by the method of mixtures in known concentrations of potassium ferro- and ferricyanide without a redox electrode present. Cytochromes were reduced with a 100 × excess of sodium ascorbate, and the reducing agent was removed by passage down Sephadex G-25 equilibrated in 0.5 mM sodium phosphate (pH 7) containing 10 mM NaCl and 0.5 mM EDTA. Oxidative, aerobic titrations were carried out by additions of small portions of 2.5 mM potassium ferricyanide to a solution containing 8 μM cytochrome in 1.7 mM sodium phosphate/1 mM EDTA/0.5 mM potassium ferrocyanide. The ambient redox potential was calculated for each addition after accounting for ferricyanide reduced during oxidation of the cytochrome. The ionic strength was approx. 0.02 M and did not alter significantly during the ferricyanide titration permitting the use of an E_m value of 385 mV for the ferro-ferricyanide couple given in Ref. 19 for this ionic strength.

Results

Horse cytochrome c and cytochrome c-558(548) from halophilic Paracoccus sp.

In both cases, isosbestic points and the position and shape of spectral peaks did not change throughout the redox titration (Fig. 1a shows the titration of the cytochrome *c*-554(548)). The experimental Nernst data fitted a slope of 60 mV (Fig. 2) with midpoint potentials at pH 7.0 of 261 mV for horse cytochrome *c* and 195 mV for cytochrome *c*-554(548) (Table I). In some samples of horse cytochrome *c*, downward deviations from

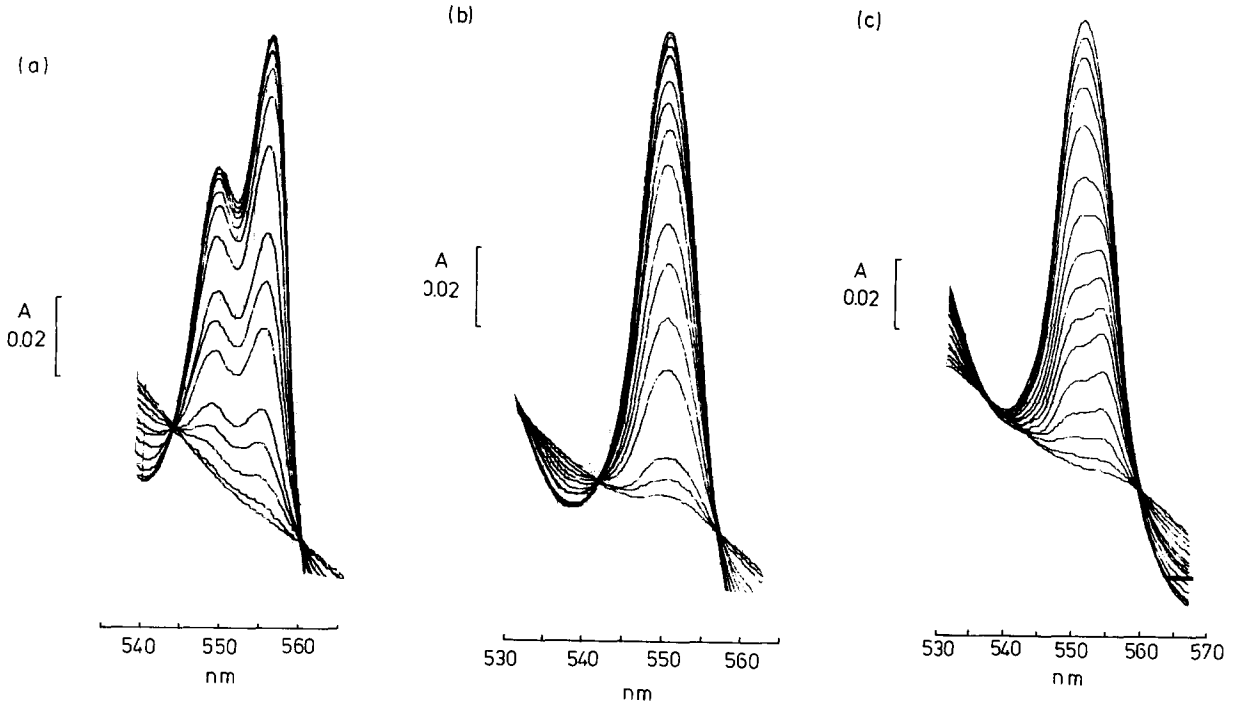

Fig. 1. Cytochrome spectra were recorded on a Unicam SP1800 spectrophotometer adapted to take a stirred anaerobic cuvette with a redox electrode. The cuvette contained approx. 5 μM cytochrome in 0.02 M sodium phosphate (pH 7), 20 μM PMS, DAD and ferric ammonium sulphate, and 0.4 mM EDTA. The redox titration was performed at 23°C. Each spectrum corresponds to a particular ambient redox potential (not shown) and is used to calculate log $Cyt._{ox}/Cyt._{red}$. Titration by the method of mixtures gave very similar results (experimental points not included). (a) Cytochrome *c*-554(548) from halophilic *Paracoccus* sp.; (b) cytochrome c_4 from *A. vinelandii*; (c) Cytochrome c_4 from *P. stutzeri* 224.

TABLE I

ANALYSIS OF THE NERNST PLOTS OF CYTOCHROMES

The analyses are for the theoretical curves shown in Figs. 2 and 3. The percentage contribution in the case of *P. stutzeri* cytochrome c_4 was calculated for the absorbance change at 552 nm, but the figures are little different if the absorbance changes at the α-peak maxima are used.

Cytochrome		Midpoint potentials (mV)	Percentage contribution
Horse cyt. *c*		261 (single)	100
Cyt. *c*-554(548)		195 (single)	100
Cyt. c_4	*A. vinelandii*	317	56
		263	44
	P. aeruginosa	322	50
		268	50
	P. stutzeri	300	41
		190	59

the 60 mV line were observed at logarithmic oxidation/reduction values more negative than −1 indicating the presence of small amounts of cytochrome with a more negative midpoint potential. This problem could be overcome by passage down Sephadex G-75 superfine (1.5 × 90 cm in 0.02 M Tris-HCl (pH 8.0) containing 0.1 M NaCl).

Cytochrome c_4 from A. vinelandii and P. aeruginosa

Isosbestic points and spectral characteristics did not alter during the titrations (Fig. 1b shows the titration of *A. vinelandii* cytochrome c_4), but in both cases the experimental data clearly lay off the 60 mV line expected for a single redox component (Fig. 3a and b).

The simplest model to account for this deviation is that the two haems differ in redox potential. Theoretical curves can be drawn for the case where two different components contribute to a

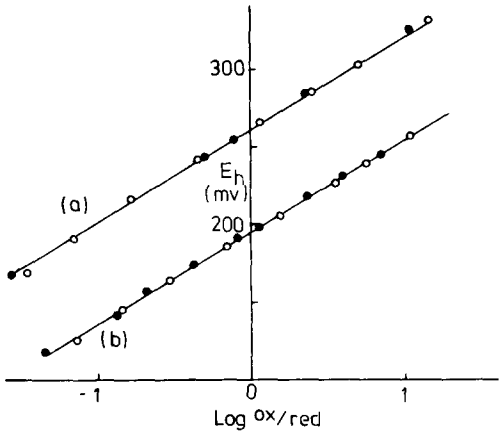

Fig. 2. Nernst plots for horse cytochrome *c* and cytochrome *c*-554(548) from halophilic *Paracoccus* sp. ○, ●, Experimental points from oxidative and reductive titrations respectively. Values of log $Cyt._{ox}/Cyt._{red}$ were obtained from the absorbance changes at the α-peak maximum. The lines are slopes of 60 mV. (a) Horse cytochrome *c*; (b) cytochrome *c*-554(548) from halophilic *Paracoccus* sp.

redox titration; in constructing such a theoretical curve, the relative contributions of the two components to the absorption spectra are estimated from the anti-logarithm of the logarithmic values on the total oxidation/total reduction scale at the middle of the sigmoidal portion of the data. A simple programme written in BASIC for a BBC microcomputer, and available on requests from the authors, allowed rapid visual assessment of trial fits to the experimental data. The solid curves in Fig. 3a and b are theoretical lines, defined by the parameters in Table I, which provide a good visual fit to the data. In all three titrations in Fig. 3, the experimental points at greater than 90% reduction fall slightly off the theoretical curves for two components. This can be corrected by proposing the presence of a small amount of cytochrome (approx. 5%) of lower redox potential. Since the samples are not contaminated by other cytochromes as judged by electrophoresis, this probably represents a small proportion of cytochrome c_4 damaged perhaps as a consequence of butanol extraction. Indeed cytochrome c_4 prepared in low yield from the soluble fraction of cells disrupted by the French press does not show this deviation from a two-component curve at high levels of reduction.

Cytochrome c_4 from P. stutzeri

In contrast to the other spectroscopic titrations, that of cytochrome c_4 from *P. stutzeri* clearly shows the presence of two spectrally distinct components having different isosbestic points (Fig. 1c). The contribution of the higher potential component with the asymmetric, red-shifted α-peak to the fully reduced spectrum is detectable as an asymmetry on the long-wavelength side of the

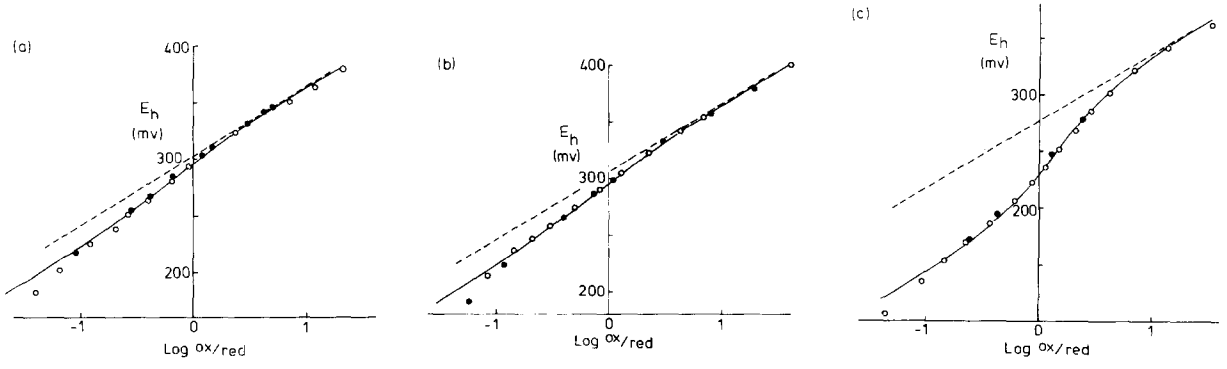

Fig. 3. Nernst plots for cytochrome c_4. ○, ●, Experimental points from oxidative and reductive titrations respectively. Values of log ox/red were obtained from the absorbance changes at the α-peak maximum for the cytochromes c_4 from *P. aeruginosa* and *A. vinelandii*. In the case of *P. stutzeri* cytochrome c_4, log ox/red was calculated from the absorbance change at 552 nm, equidistant between the α-peak maxima of the two components. However, there was very little difference in the analysis if log ox/red was calculated from the absorbance at the α-peak maximum at each stage of the titration. The broken lines are slopes of 60 mV. The solid lines are theoretical curves for two components with midpoint potentials and percentage contributions as defined in Table I. Cytochrome c_4 from (A) *A. vinelandii*; (b) *P. aeruginosa*; (c) *P. stutzeri*.

α-peak. Of the systems studied in this paper (Fig. 3c, Table I) the two haems of *P. stutzeri* cytochrome c_4 show the greatest separation in midpoint potentials.

The Nernst data were calculated at 552 nm, which lies between the α-peak maxima of the two components. However, there was very little difference in the analysis if the absorbance at the α-peak maximum at each stage in the titration was used to calculate the logarithm of the total oxidation/total reduction value.

Discussion

Redox titration of the dihaem cytochrome c_4 indicates the presence of potentiometrically distinct haem groups with a separation varying between 54 and 110 mV. Such complexity could arise in two ways. Either the redox potentials of the haems are intrinsically different (Fig. 4a), or there is interaction between two identical haems such that the addition of the first electron is more favourable (more positive redox potential) than the addition of the second (Fig. 4b). In this respect we should note that the Nernst plot is the formal equivalent of the Hill plot used to analyse cooperativity in ligand binding [20]. Thus Nernst slopes less than 60 mV indicate positive cooperativity, while those greater than 60 mV (as seen here) show negative cooperativity. Such anti-cooperativity might be explained in electrostatic terms as due to charge repulsion of the ferric haems in the fully oxidised protein.

We propose two ways of distinguishing these possibilities. One is to study a related dihaem system with haem environments known to be chemically identical, and to ask if there is evidence of haem interaction. The cytochrome *c*-554(548) of halophilic *Paracoccus* sp. contains 83 amino acids and a single haem group [1]. The amino acid sequence is closely related to the first half of the cytochrome c_4 molecule [1] and the cytochrome is isolated as a very stable dimer [17]. We propose that this dimer of cytochrome *c*-554(548) may structurally resemble the two domains of cytochrome c_4 and that its potentiometric titration may be a test for anti-cooperative effects between haem groups. As can be seen in Fig. 2 no such effects are detected indicating that the haems of

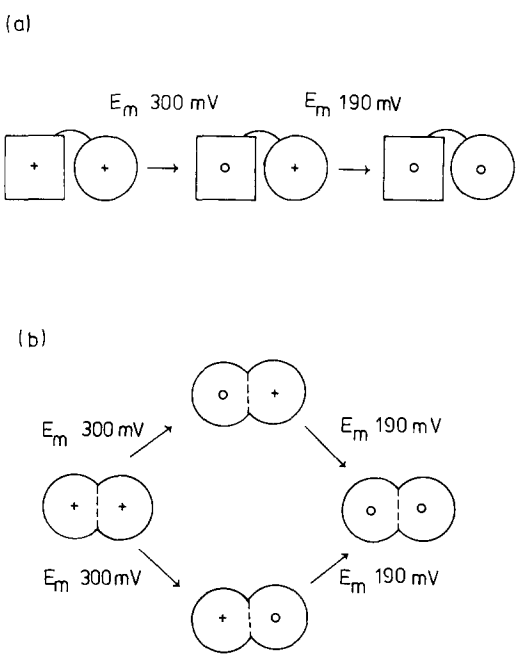

Fig. 4. Models for reduction of cytochrome c_4. In model (a) two domains with intrinsically different redox properties are shown as a square and a circle. The midpoint potential for reduction of the ferric haem (+) in the 'square' domain is 300 mV and for the ferric haem in the 'circular' domain is 190 mV. The domains do not interact. In model (b) the two domains are equivalent in the fully oxidised state. The addition of the first electron can occur to either domain, and alleviates an electrostatic repulsion. Thus the first electron reduction is a more favourable process (more positive redox potential) than the second.

the cytochrome *c*-554(548) dimer do not interact. A similar result was obtained (not shown) for the dimeric cytochrome c_5 of *Pseudomonas stutzeri*.

Although such evidence indicates that two haem groups, separated by the relatively small distance that will prevail in these dimers, need not interact, it does not demonstrate that they do not interact in cytochrome c_4. A more direct approach is to proteolytically cleave the two-domain cytochrome c_4 molecule and study the separated domains. Experiments of this kind are in progress.

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