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Homotropic and heterotropic cooperativity in the tetrahaem cytochrome c_3 from *Desulfovibrio vulgaris*

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Abstract

The thermodynamic parameters which govern the homotropic (e^-/e^-) and heterotropic (e^-/H^+) cooperativity in the tetrahaem cytochrome c_3 isolated from *Desulfovibrio vulgaris* (Hildenborough) were determined, using the paramagnetic shifts of haem methyl groups in the NMR spectra of intermediate oxidized states at different pH levels. A model is put forward to explain how the network of positive and negative cooperativities between the four haems and acid/base group(s) enables the protein to achieve a proton-assisted $2e^-$ step.

Key words: Cytochrome c_3; Heme protein; NMR, two-dimensional; Cooperativity; Redox-Bohr effect; Redox mechanism

1. Introduction

The chemistry of life is the result of highly sophisticated organization, one of the most impressive examples of organization at the molecular level being the functional cooperativity between different regions of a protein. This has been demonstrated for the mechanism of the consecutive O_2 binding to the four haems of haemoglobin as well as for the allosteric regulation of several enzymes [1]. In the case of haemoglobin, the structural basis for both homotropic (O_2/O_2) as well as heterotropic cooperativity (O_2/H^+, Bohr effect) are well understood. *Desulfovibrio* spp. cytochrome c^3 is a tetrahaem protein which also exhibits cooperativity, but with the differences that: (i) the four haems are linked by a single peptide chain; and, (ii) they are redox active. Furthermore, being a small protein (13.5 kDa) it is well suited to be studied in solution by high-resolution NMR [2–8]. Indeed, NMR studies on this protein have shown that the four haems and acid/base group(s) constitute an interdependent consortium [3,9]: the haem redox potentials are pH dependent (redox-Bohr effect) [10] and each haem redox potential is dependent on the oxidation state of the other three haems (redox interaction potentials). The simplest model which may explain this cooperative behaviour therefore requires a network of redox interactions between the four haems and at least one protonation site.

Two previous analyses of thermodynamic properties used the same set of NMR data obtained for cytochrome c_3 from *Desulfovibrio gigas* [3,9]. There are essentially two differences between those studies: in the first case only relative haem redox potentials were determined and the redox interaction potentials were fixed according to the maximum concentration reached by the intermediate oxidation stages in redox titrations followed by NMR [3,6]; the second study used potentiometric titration followed by visible spectroscopy in order to fix both the redox interaction potentials and the absolute haem redox potentials [9]. Both studies considered a model in which the four haem redox potentials and six redox interaction potentials could change on protonation, and the data were fitted by grid searching. Thus 18 thermodynamic parameters were

Abbreviations: NOESY, nuclear Overhauser effect spectroscopy; RMSD, root mean square deviation; Dv c_3, *Desulfovibrio vulgaris* (Hildenborough) cytochrome c_3; Dg c_3, *Desulfovibrio gigas* cytochrome c_3.

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obtained in the first case [3], and the full set of 21 parameters, which includes the absolute redox potentials of the acidic and basic forms and the pK_a, in the second [9]. The assumption that the redox interaction potentials change on protonation is now shown to be unnecessary, thereby reducing the number of thermodynamic parameters to 15: four haem redox potentials and one pK_a for the fully reduced state, plus the six redox interaction potentials and the four redox-Bohr interactions.

In this work the chemical shifts for a number of haem methyl groups of *D. vulgaris* (Hildenborough) cytochrome c_3 (Dv c_3) in each oxidation stage as a function of pH have been fitted to a simple model which shows that the cytochrome has the ability to couple proton transfer with a concerted two-electron transfer. Since the resonances were assigned to specific groups within the three-dimensional structure, a methyl group from each haem which is furthest removed from the other haems was chosen so that the errors which would result from extrinsic dipolar shifts caused by nearby oxidized (paramagnetic) haems could be minimized [6].

2. Results and discussion

The model established to explain the variation in the paramagnetic shifts of the haem methyl groups, obtained from NMR redox titrations of Dv c_3 (Fig. 1), is most easily understood in terms of free energy. The protonated, fully reduced protein may donate a proton or any one of the four haems may donate an electron and these processes define one pK_a and four microscopic redox potentials. The energies of the microstates are given by sums of parameters, which therefore define their relative concentrations at any pH and solution potential. At any stage, the paramagnetic shift of each methyl group is proportional to the percentage oxidation of the relevant haem [3,6]. For each pH, sixteen NMR observations were used (Fig. 1), four for each of the four methyl groups (one per haem). A severe restriction results from not knowing the solution redox potential of each NMR sample and hence being unable to give absolute values for the microscopic redox potentials and the redox interaction potentials. Therefore, using the Marquardt method, the NMR data are first fitted for relative values, by arbitrarily setting to zero one redox potential and one redox interaction. Finally, the absolute values of the redox potentials and interactions are fixed using data from redox titrations monitored by visible spectroscopy (Fig. 2). The potential for the first oxidation step, which involves the fully reduced protein and is obviously independent of the interactions, immediately fixes the four microscopic redox potentials. The redox potential

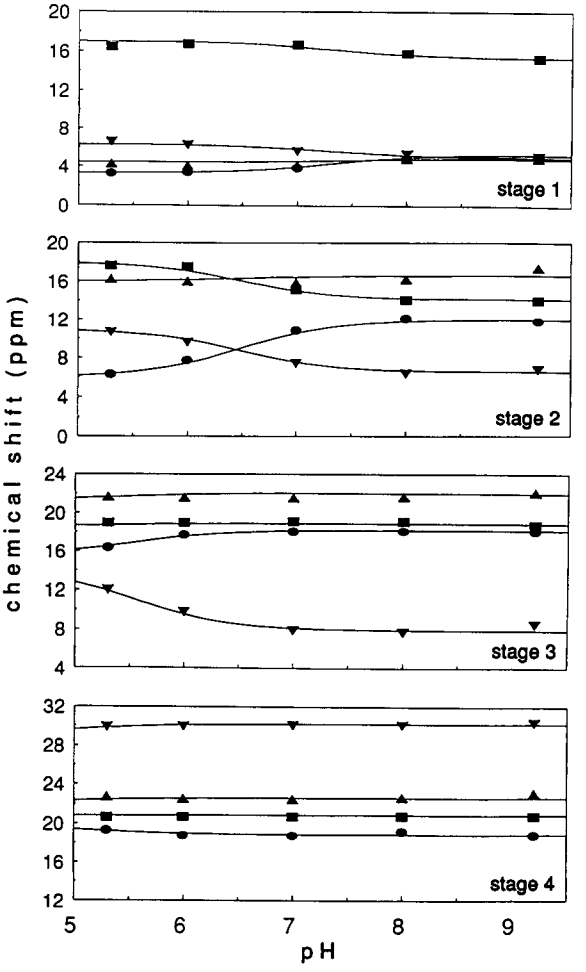

Fig. 1. Chemical shifts of the haem methyl group resonances (numbered according to IUPAC – IUB nomenclature) $M2^1_I$ (●), $M18^1_{II}$ (▲), $M12^1_{III}$ (■) and $M18^1_{IV}$ (▼), of Dv c_3 at different pH levels and oxidation stages. Roman numbers refer to the haems in order of attachment to the polypeptide sequence. Stage i ($i = 1$ to 4) is the oxidation stage with i haems oxidized [3,6]. NOESY spectra were obtained in a 500 MHz Bruker AMX Spectrometer from 2 mM samples in ^2H_2O at 298 K as previously described [6]. The full lines represent the best fit (RMSD = 0.25 ppm) of the shifts in the range pH 5.3–9.2 to the model of five interacting centres.

of the last step then fixes the haem-haem redox interaction potentials. The results presented in Table 1 were fitted to NMR data at five pH levels in the range 5.3–9.2 (Fig. 1). The values obtained for pK_a are 7.9 and 5.1 for the reduced and oxidized form, respectively.

3. Conclusions

This work has established that the chemical shifts of the haem methyl groups in different stages of oxidation, and hence the populations of oxidized haem at each oxidation stage [6], are described adequately by a simple model of five interacting centres. All of the haems show negative energies of interaction with the

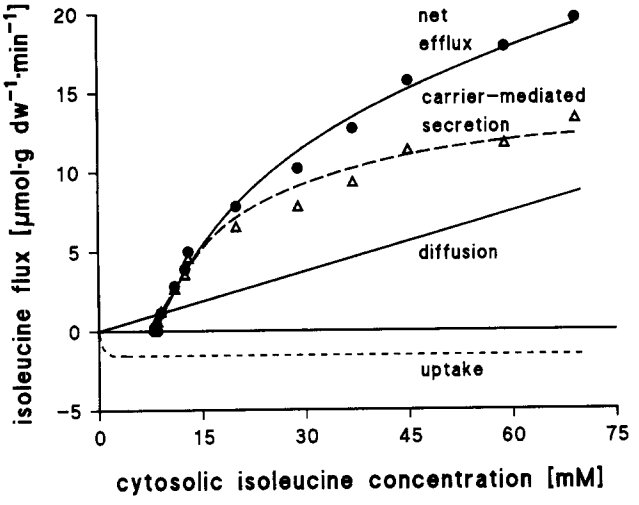

Fig. 2. Mole fraction of the total reduced haem determined by visible spectroscopy at pH 8.0 for various solution potentials, performed as previously described [13,14]. No sign of visible spectral cooperativity was detected. The full line represents the best fit to the model of five interacting centres using the energy parameters reported in Table 1.

protonation site, positive heterocooperativity, as expected on electrostatic grounds for transfer of particles with opposite charges. This positive redox-Bohr effect is the thermodynamic basis for electron/proton energy transduction. Some haem-haem redox interaction energies are also negative. In particular, the energy of interaction between haems I and II, which have previously been shown to be the intermediate haems to become oxidized [6], has a negative value, showing positive homocooperativity between these haems, which cannot be explained by electrostatic interactions and must involve some change in the protein conformation. The release (uptake) of one electron by one of these haems facilitates the release (uptake) of a second electron by the other haem, thus favouring a concerted $2e^-$ step.

Table 1
Energy parameters for the five interacting centres in Dv c_3

	H I	H II	H III	H IV	Proton(s)
H I	**−246**	−40	24	4	−76
H II	–	**−268**	−4	8	−40
H III	–	–	**−331**	33	−30
H IV	–	–	–	**−282**	−18
Proton(s)	–	–	–	–	**465**

Energy parameters (meV) for the five interacting centres were obtained by fitting the model described in the text to the NMR (Fig. 1) and visible data (Fig. 2). The energies, expressed in meV, are presented in a matrix with the diagonal elements (bold-face) representing the energy for oxidizing haems (i.e., reduction potentials E_i, in mV) and deprotonating the ionizable group in the fully reduced molecule (G_H^{red}). The off-diagonal elements represent the energy of redox interaction between the relevant oxidized haems and between these and the deprotonated group. The energy of each microstate is given by the sum of its diagonal term and the relevant interaction energies, i.e., the relevant off-diagonal terms. The pK_a of the reduced protein is:

$pK_a^{red} = G_H^{red}F/2.3RT = 5.1$

and the pK_a of the oxidized protein is:

$pK_a^{ox} = G_H^{ox}F/2.3RT = 7.9$

where G_H^{ox} is the energy for deprotonating the ionizable group in the fully oxidized molecule (i.e., the sum of all elements in the last column).

The quality and quantity of experimental data available for Dv c_3 far exceeds that used for *D. gigas* cytochrome c_3 (Dg c_3) in an earlier study [9]. The model used in that work assumed that the protein conformation, and hence the haem-haem redox interaction potentials, could change on protonation. The simpler model of five charge centres with fixed interactions is clearly sufficient to explain all of the data now reported for Dv c_3 as well as the earlier data for Dg c_3 [3]. However, analysis of the structural basis for the interactions and the significance of the redox linked conformation change must await further experimental data.

The results reported above unequivocally demonstrate that the interactions within this core structure interplay so that homotropic (e^-/e^-) as well as heterotropic (e^-/H^+) cooperativity is achieved. The thermodynamic parameters listed in Table 1 give the protein the intrinsic properties necessary to perform both a concerted $2e^-$ step as well as to work as an electron/proton energy transducer. However, it should be stressed that for the heterotropic cooperativity to be efficiently used the cytochrome c_3 redox cycle should generate separation of charge. This may be achieved by sending the electrons through the membrane system [11] to be used in the cytoplasm for sulphate reduction and leaving the protons in the periplasmic space, thus creating a protonmotive force which can activate the membrane ATPase [12].

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