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Assignment of the redox potentials to the four haems in *Desulfovibrio vulgaris* cytochrome c_3 by 2D-NMR

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Using 2D-NMR the four haems of *Desulfovibrio vulgaris* (Hildenborough) cytochrome c_3 within the X-ray structure were fully cross-assigned according to their redox potential. The strategy used was based on a complete network of chemical exchange connectivities between the NMR signals obtained for all oxidation levels to the corresponding ones in the fully reduced spectrum [1992, Eur. J. Biochem., in press]. This unequivocal cross-assignment disagrees with earlier results obtained for the similar protein from *Desulfovibrio vulgaris* (Miyazaki F) [1991, FEBS Lett. 285, 149–151].

Cytochrome c_3; Multihaem protein; 2D-NMR; ROESY; NOESY; X-ray Structure

1. INTRODUCTION

Multiredox centre proteins are involved in several crucial steps of reactions in which fundamental energy transduction phenomena take place. However, the complexity of these proteins has hindered the determination of the precise physico-chemical parameters necessary to explain the intricate mechanisms involved, the architecture of the different redox centres, and the indispensable cross-assignment between each of the redox centres within the structure and the measurable parameters. Cytochrome c_3, a tetrahaem protein isolated from *Desulfovibrio* spp. [1], being a small (13 kDa) soluble protein for which X-ray structures are available [2–4], has been subjected to several physicochemical and spectroscopic studies [5–12]. Thus, a wealth of information is available for several homologous proteins from different *Desulfovibrio* spp. and much effort has been dedicated to the cross-assignment of the redox potentials of the individual haems to their position in the 3D structure [8–15].

During a redox titration, the 16 possible combinations of redox states of the individual haems can be grouped in a series of five *macroscopic* oxidation states [7], which we shall refer to as *stages*, and which are connected by four one-electron *steps*. Each of the five *stages* (numbered from 0 to 4, according to the number of oxidised haems) comprises a number of intermediate oxidation states, each having the same number of oxidised haems [7]. Since the haem iron atoms are in the low-spin state they are diamagnetic in the reduced form and paramagnetic when oxidised. Thus, NMR spectroscopy has been shown to be a useful technique to follow the redox *stages* of this protein as well as to measure the degree of oxidation of each haems, which have different redox potentials (individual *microscopic* redox potentials) at every *stage* [7,9]. As the interconversion between oxidation states belonging to the same *stage* (*intra*molecular electron exchange) is fast on the NMR time scale and the interconversion between states belonging to different *stages* (*inter*molecular exchange) is slow, a separate set of signals is observed for the protons of the haems in each different *stage*. Indeed, under these conditions the distribution of paramagnetic shifts observed for each *stage* is governed by the relative *microscopic* redox potentials [7,8].

The comparatively simple NMR spectra of the fully reduced or fully oxidised protein provide suitable starting points for assignment. Earlier work [8,9] has concentrated on the fully oxidised form (*stage* 4), but few specific assignments could be obtained. We have used 2D-1H-NMR to assign all of the haem methyl groups of ferrocytochrome c_3 (*stage* 0) unequivocally to individual haems in the three dimensional structure [16]. Since 2D-1H-NMR techniques can also be used to detect exchange processes between redox *stages* of cytocgrome c_3 [17] it is then possible to follow each haem

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Abbreviations. c3DvMF, *Desulfovibrio vulgaris* (Miyazaki F.) cytochrome c_3; c3DvH, *Desulfovibrio vulgaris* (Hildenborough) cytochrome c_3; c3DbN, *Desulfovibrio baculatus* (Norway) cytochrome c_3; ROESY, Rotating Frame NOE Spectroscopy, NOESY, NOE Spectroscopy; Mi, haem methyl groups (i = 2^1, 7^1, 12^1, 18^1) according to the IUPAC–IUB nomenclature.

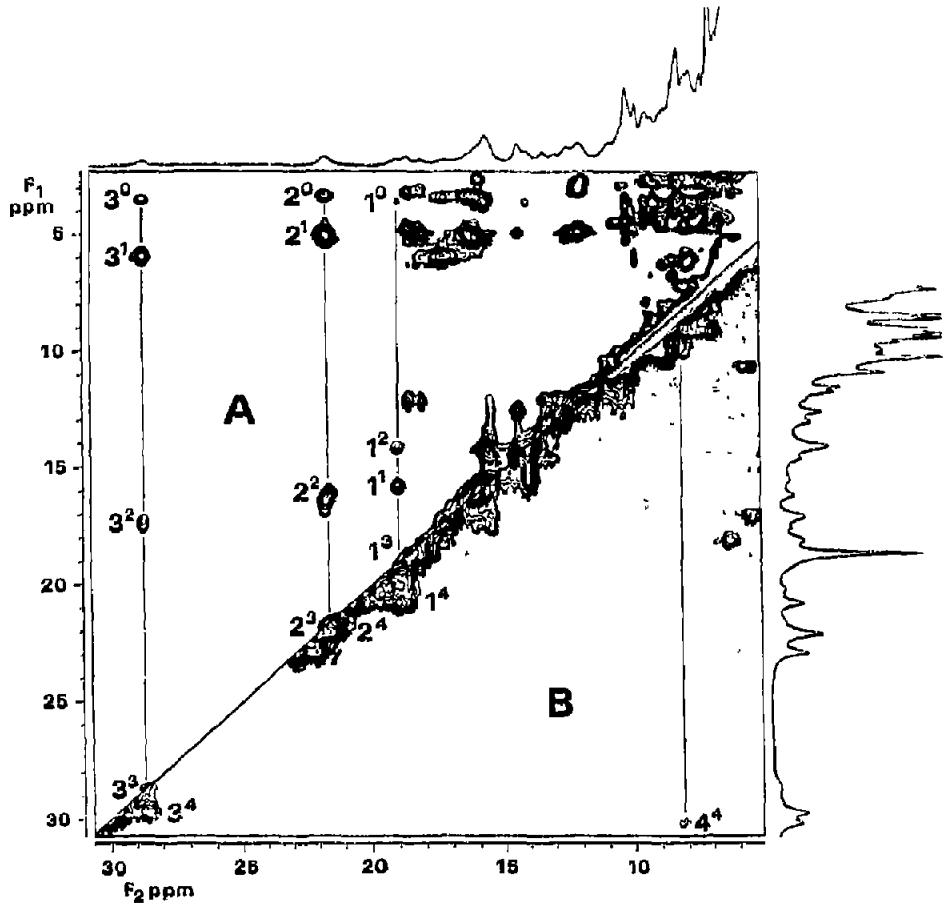

Fig. 1 - Combined NOESY (A) and ROESY (B) contour plots for the different levels of oxidation in c3DvH at 298 K and p²H=8.9. The spectra were obtained in a 500 MHz Bruker AMX-500 spectrometer from a 2 mM solution of c3DvH previously lyophilized and resuspended in ^2H_2O [16]. The fully reduced stage was achieved by addition of catalytic amounts of *D. gigas* hydrogenase in a hydrogen atmosphere [16]. The intermediate oxidation levels were obtained by first washing out the hydrogen from the reduced sample with nitrogen and then adding controlled amounts of air into the NMR tube with a Hamilton syringe through serum caps. The NOESY spectrum was collected (512 × 2048 data size) with a mixing time of 25 ms and transformed with a Gaussian apodisation (lb = −15 Hz, gb = 0.037) in F_2 and a shifted sine bell multiplication (cosine) in F_1 with zero filling to 1,024 data points in F_1. In the ROESY spectrum the same type of transformation was used (lb = −15 Hz, gb=0.045) and the pulse for spin lock was 10 ms. Chemical shifts are relative to 3-(trimethylsilyl) propane sulfonic acid (sodium salt). The NOESY spectrum emphasizes the first three *stages* of oxidation (0–3) and the ROESY spectrum shows the last *step* of oxidation (*stage* 3–4). Cross peaks which connect the chemical shifts of selected methyl groups in *stages* 0–4 to their shifts in *stage* 3 have been labelled. The numbers (1–4) identify the haems according to the redox potential (starting from the lowest one, thus corresponding to haems III, II, I, and IV, respectively). The superscripts (0–4) indicate the particular *stage* Thus, the shifts of these groups (cf Table I) should be read from the left hand (F1) scale. Negative peaks are indicated with dotted lines. 1D spectra of the samples are plotted at the edge of the corresponding half-spectra.

methyl group throughout every oxidation *stage*. This strategy is used here to obtain the full cross-assignment of the four haems within the X-ray structure according to their redox potential.

2. RESULTS AND DISCUSSION

Fig. 1 shows the NOESY (A) and ROESY (B) spectra of c3DvH samples (see experimental conditions in the figure legend) poised at intermediate oxidation levels for which populations belonging to redox *stages* 0–3 (Fig. 1A) and 3–4 (Fig. 1B) co-exist. The signals for each haem methyl group at individual oxidation *stages* give rise to a unique network of cross-peaks due to intermolecular electron transfer *steps* (Table I). The cross-assignment of the paramagnetically shifted resonances (*stages* 1–4) to those due to the fully reduced *stage* 0 [16] allows an unequivocal cross-assignment to each haem in the three-dimensional structure (see Table I). ROESY spectra [18] were used throughout the redox titration in order to differentiate cross peaks due to chemical exchange (positive intensities) from those due to NOE's (negative intensities), which are both positive in the NOESY spectra.

Table I also shows the fraction of oxidation observed for each of the four haems in each *stage*, calculated from the relevant chemical shifts observed for one methyl group chosen for each haem. This calculation is simply

Table I

Chemical shifts of one methyl group for each haem in the five oxidation stages and corresponding oxidation fractions, x_i

Stage	Chemical shifts (ppm)				x_i				Σx_i
	$M12^{\downarrow}_{III}$	$M7^{\downarrow}_{II}$	$M18^{\downarrow}_{I}$	$M18^{\downarrow}_{IV}$	$M12^{\downarrow}_{III}$	$M7^{\downarrow}_{II}$	$M18^{\downarrow}_{I}$	$M18^{\downarrow}_{IV}$	
0	3.41	3.13	3.27	3.26	0 0	0.0	0.0	0.0	0.0
1	15 61	4.93	5.72	6.09	0.705	0.095	0.093	0.105	0.998
2	13.98	16.10	17.20	7.01	0.611	0.687	0.527	0.139	1.964
3	18.96	21.54	28.57	8.20	0.899	0.975	0.957	0 183	3.014
4	20.71	22.00	29.69	30.13	1.0	1 0	1 0	1.0	4.0

based on the ratio of the paramagnetic shifts of the methyl groups at each *stage* to their total shifts in the fully oxidised protein [7,8]. In order to minimize errors arising from extrinsic paramagnetic shifts (i.e. dipolar shifts caused in the proton signals of one haem by any of the other haems [7]) the haem methyl groups chosen to probe the degree of oxidation of each haem in the successive oxidation *stages* and depicted in Fig. 1, were those of groups pointing towards the protein surface and therefore far from the other haems: $M12^{\downarrow}_{III}$, $M7^{\downarrow}_{II}$, $M18^{\downarrow}_{I}$ and $M18^{\downarrow}_{IV}$ (Roman numbers indicating their position in the primary sequence [19]). It is also assumed that the distribution of Fermi contact shifts does not vary between *stages*. Analysis of Table I shows that neither the extrinsic shifts nor any variation of the Fermi contact ones can contribute significantly since the sums of the oxidation fraction at each *stage* are very close to integers.

3. CONCLUSIONS

Table I shows that haem III becomes largely oxidised in *step* 1 (*stage* 0–1), and is followed by haems II and I which both oxidise to a similar extent in *steps* 2 and 3, and finally by haem IV in *step* 4. The fraction of oxidation for each step can also be used to calculate the relative mid-point redox potentials as well as the interacting potentials [7,8] of the haems at each *stage* (manuscript in preparation).

Although the X-ray structure of c3DvH cytochrome c_3 has recently been obtained [4] its coordinates are not yet available and so those of c3DvMF [3] were used for the assignment of *stage* 0 [16]. The two proteins are closely homologous, as is the relative architecture of their four haems, which is also maintained in the much less homologous protein c3DbN [20]. Furthermore, comparison of the 1D-NMR spectra of c3DvMF [9] and c3DvH [6] shows that the redox patterns are quite similar, though not identical, as indeed are the haem methyl group shifts for the fully oxidised state.

A previous identification of the four haems of c3DvMF was attempted by 1D-1H-NMR using a few specific assignments made in *stage* 4 [8–10]. The assignment obtained differs from ours in that the earlier work assigned the largest change in oxidation in the first *step* to haem II whereas we find that the major change for haem II occurs in *step* 2 and that it is haem III which becomes largely oxidised in *step* 1. The present work relies on an extensive network of chemical exchange patterns connecting *all* the redox *stages*. Comparison of the spectra shows that methyl D of Ref. 8 which was assigned to haem III is in fact $M7^{\downarrow}_{II}$. The earlier assignment is unconvincing since it was made solely on the basis of the proportional change of the paramagnetic shift in the first reduction *step* which is comparable [8] with that of the firmly assigned $M2^{\downarrow}_{III}$ [8–10]. Indeed, the magnitude of the extrinsic shifts may be such that a comparison of the proportional changes in a single *step* (cf. Table I) could be quite misleading, particularly in this case, since $M2^{\downarrow}_{III}$ gives a strong NOE (i.e. it is very close) to a methyl group which belongs to the haem most reduced in that same step, $M12^{\downarrow}_{IV}$ [8–10]. There may be small differences in the relative redox potentials of the four haems in the Miyazaki and Hildenborough proteins, but the overall pattern appears to be the same. However, a different order of redox potentials has been proposed for the much less homologous c3DbN on the basis of single crystal EPR studies [14]. Such a difference would reflect the influence of the polypeptide chain. Studies to elucidate the physicochemical and physiological significance of the control of redox potentials are currently in progress.

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