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Investigation of the solution structures and mobility of oxidised and reduced cytochrome b_5 by 2D NMR spectroscopy

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Two-dimensional ^{1}H NMR spectroscopy is used to examine the structure and mobility of cytochrome b_5 in solution. The assignment of many residues and the interpretation of nuclear Overhauser effects (NOEs) in both redox states allow definition of secondary structural elements. Comparison with X-ray diffraction data shows that differences between crystal and solution structures are small. The dynamics of the protein are examined and the protein is shown to be more mobile than cytochrome *c*. The relationship of the structure and dynamics to the electron transfer function of cytochrome b_5 is discussed.

Cytochrome b_5; 2D NMR; Protein mobility; Electron transfer

1. INTRODUCTION

Cytochrome b_5 is a small heme protein which transfers electrons in several redox systems [1–5]. Although the microsomal form of the protein is membrane bound, mild proteolysis releases a soluble fragment of up to 90 amino acid residues which contains the heme and retains activity with respect to electron transfer. In this paper the structure of this protein fragment in solution is examined using NMR spectroscopy. The proton assignments of more than a third of the main chain resonances in both redox states are presented. Of particular interest are the relationship between crystal and solution structures, the existence of any structural changes dependent on redox state change and the dynamics of protein side chains. In another heme protein, cytochrome *c*, X-ray diffraction and NMR studies show that there is little structural difference between crystal and solution states [6–8]. Again, only minor changes have been detected in association with the redox state change of this protein and furthermore, the interior of the protein is highly immobile. This contrasts with information relating to heme proteins such as hemoglobin, where changes of spin or oxidation state of the iron atom cause considerable perturbation of protein conformation [9]. In section 4, we shall consider the functional significance of the similarities and differences between cytochrome b_5 and these two proteins.

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2. MATERIALS AND METHODS

2.1. *Protein preparation*

Cytochrome b_5 from bovine liver microsomes was isolated following published procedures and the tryptic fragment of 84 amino acid residues prepared [10,11]. Samples were concentrated for NMR experiments using an Amicon microconcentrator with a molecular mass cut-off of 10 kDa. The solution conditions employed were either 90%/H_2O:10%/^2H_2O or 99.8 atom% ^2H_2O, both in 20 mM potassium phosphate buffer at pH 7.0. pH adjustments were made with small aliquots of ^2HCl and NaO^2H with no correction being made for the small isotope effect in ^2H_2O solutions. The concentration of samples was assessed spectrophotometrically using an extinction coefficient of 117000 $M^{-1} \cdot cm^{-1}$ for the oxidised protein at 412.5 nm [12]. To avoid possible aggregation, protein concentrations were

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kept below 6 mM. Reduced cytochrome b_5 was prepared by admitting a small aliquot of saturated sodium dithionite in buffered solution to the sample in a sealed NMR tube previously flushed with nitrogen.

2.2. *NMR measurements*

NMR spectra were recorded using either an AM500 or 600 MHz Bruker spectrometer, or the home built 500 MHz spectrometer of the Oxford Enzyme Group, equipped with a GE/Nicolet 1280 data acquisition system.

Resonance assignments in both redox states of the protein were made using phase sensitive *J*-correlated spectroscopy (COSY) [13,14], single relayed coherence transfer spectroscopy (RELAYED COSY) [15,16] and nuclear Overhauser enhancement spectroscopy (NOESY) [17] experiments. These were performed using the method of States et al. [18] for the home-built instrument or the method of Marion and Wüthrich [19] for the Bruker spectrometers. Standard phase cycling schemes were used. The data sets consisted of 512 by 4096 complex data points, each 2D experiment lasting between 10 and 20 h. The offset was set in the middle of the spectrum and the water signal suppressed by presaturation. Sweep widths of 12500 or 10000 Hz were used for experiments with the oxidised protein and corresponding sweep widths of 10000 or 8064 Hz for the reduced protein. For data acquired with the Bruker spectrometers, resolution enhancement in both dimensions using an unshifted sine bell function was employed and data sets zero-filled twice in t_1 to give a final size of 2048 by 2048 real data points. Data sets collected on the home built 500 MHz instrument were resolution enhanced in t_2 by trapezoidal multiplication (TM) and double-exponential multiplication (DM) and in t_1 by TM or a cosine bell function. All 2D NMR spectra are presented as contour plots. In COSY spectra both positive and negative levels are shown whereas in NOESY spectra only

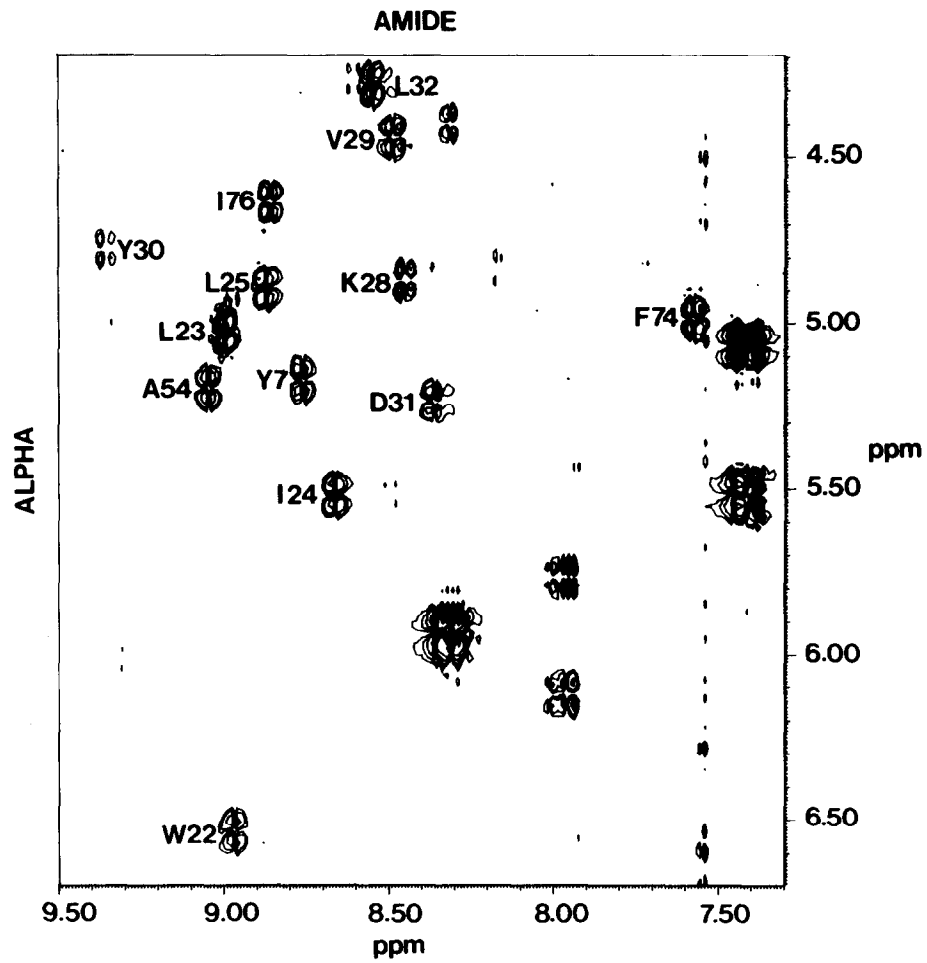


Fig.1. Phase-sensitive 500 MHz COSY spectrum of ferrocytochrome b_5 (5 mM) showing non-exchanged amide-α cross-peaks. The spectrum was acquired in ^2H_2O/20 mM potassium phosphate buffer at 30°C and pH 7.0. The three large cross-peaks to the right of the figure represent coupling between the CH and CH_2 protons of heme vinyls 2 and 4 [23].

positive levels are shown. Mixing times of either 120 or 140 ms were used in the latter. In the spectra presented here, the vertical and horizontal axes represent F_1 and F_2, respectively. All spectra were collected at 30°C. Chemical shift values are quoted to ± 0.02 ppm. 1,4-Dioxane was used as an internal standard with a resonance at 3.74 ppm relative to 2,2-trimethyl-2-silapentane-5-sulfonate.

3. RESULTS AND DISCUSSION

3.1. *Assignments*

Resonance assignments for greater than 40% of all protons in both oxidised and reduced cytochrome b_5 were obtained using standard 2D NMR methodology. The sequential assignment procedures developed by Wüthrich and co-workers [20] were used to aid this process. Previous NMR work on this protein has resulted in the assignment of resonances both of the heme group and also of a few amino acid side chains in close proximity to the heme [21–23]. In a more recent study, a number of aromatic side chain resonances were also assigned using 2D NMR techniques [24]. There are no major disagreements between these assignments and our more extensive set. In a short paper it is not possible to give a detailed description of the manner in which these assignments were made and this will appear subsequently. However, some particular features of the data will be presented to illustrate the application of the results so far obtained.

3.2. *Main chain resonances*

In ferricytochrome b_5, analysis of the NOESY spectrum revealed NOE connectivity patterns characteristic of both β-sheet and α-helical regions. In conjunction with the COSY spectra, the β-sheet region of the protein was almost completely assigned in both this redox state and in the reduced form. Assignment of this region was aided by the slow exchange properties of the amide protons which allowed many of them to be observed in spectra measured in ^2H_2O. The 'fingerprint' region (HN-HA cross peaks) of the COSY spectrum of ferrocytochrome b_5 is illustrated in fig.1.

Comparison between main chain NOEs and those predicted to occur from the X-ray crystal structure revealed that there were no significant differences between crystal and solution state backbone conformations in those regions of secondary structure which could be assigned.

Inspection of main chain HA and HN resonances (listed in tables 1,2) in both oxidation states reveals that a correlation may be obtained with regard to the disposition of these in defined

Table 1

^{1}H NMR assignments of main chain protons in ferricytochrome b_5

Residue	δ (ppm)		Residue	δ (ppm)	
	HN	HA		HN	HA
Tyr 6	8.11	5.63	Val 45	7.14	3.97
Tyr 7	8.64	5.09	Ala 50	8.21	3.68
Thr 8	9.15	4.53	Ala 54	8.16	4.39[a]
Thr 21	8.75	5.40	Glu 56	8.43	3.92
Trp 22	8.76	6.16	Ala 67	8.27	4.38
Leu 23	8.38	4.43	Ser 71	8.36	3.86
Ile 24	8.03	4.98	Lys 72	7.60	4.03
Leu 25	8.36	4.36	Phe 74	7.24	4.79
His 26	9.20	3.75	Ile 75	6.80	3.45
Tyr 27	8.22	3.70	Ile 76	8.59	4.52
Val 29	8.27	4.04	Glu 78	8.96	4.90
Tyr 30	8.81	4.38	Leu 79	8.84	4.59
Asp 31	8.11	4.97	Asp 82	11.06	4.43
Leu 32	8.34	4.25	Asp 83	8.16	4.19
Lys 34	7.80	4.04	Arg 84	6.99	4.03
Phe 35	8.11	5.14			

[a] [23]

Table 2

^{1}H NMR assignments of main chain protons in ferrocytochrome b_5

Residue	δ (ppm)		Residue	δ (ppm)	
	HN	HA		HN	HA
Tyr 6	8.17	5.71	Leu 46	5.94	3.83
Tyr 7	8.75	5.17	Ala 54	9.03	5.20
Thr 8	9.23	4.64	Thr 55	8.57	3.30
Trp 22	8.95	6.51	Glu 56	8.69	3.82
Leu 23	8.98	5.03	Asp 60	8.08	4.19
Ile 24	8.65	5.52	Val 61	6.63	3.18[a]
Leu 25	8.85	4.91	His 63	6.20	2.57
His 26	9.47	3.83	Thr 73	7.87	4.01
Tyr 27	8.38	3.87	Phe 74	7.60	5.00
Lys 28	8.46	4.88	Ile 75	6.98	3.70
Val 29	8.48	4.45	Ile 76	8.85	4.65
Tyr 30	9.34	4.79	Glu 78	9.09	5.32
Asp 31	8.37	5.24	Asp 82	11.12	4.45
Leu 32	8.53	4.30	Arg 84	7.03	4.08
Leu 36	8.43	2.93			

[a] [23]

regions of secondary structure in the protein (fig.2a,b). It has been previously shown that there is a relationship between the secondary chemical shift of HA protons and the region of secondary structure in which they occur [25]. This was based on a theoretical analysis of the dipolar shift due to the peptide carbonyl groups in β-sheet and α-helical structures. Confirmation of this relationship can be seen in the pattern of shifts found for cytochrome b_5. The particularly large shifts in the reduced protein for Leu 46, Val 61 and His 63 are due to the proximity of these residues to the porphyrin ring. It is also important to note that the disposition of shifts in the oxidised form is also a function of the paramagnetic properties of the heme iron ($S = ½$). The similar correlation for HN protons (notably in the reduced form) has not been previously noted. Here, however, the chemical shifts are open to many other influences, especially hydrogen bonding, which may make the corresponding diagnostic use of HN secondary shifts more difficult. Two residues, Ile 75 and Asp 82, show particularly unusual shifts in both oxidation states. Although Ile 75 occurs in the β_2-strand of the pleated sheet structure, X-ray diffraction and NMR data suggest that it does not participate in this region of secondary structure but rather may be considered as a β-bulge. The apparently anomalous position of Ile 75 in the figures reflects this type of local environment. Two factors may

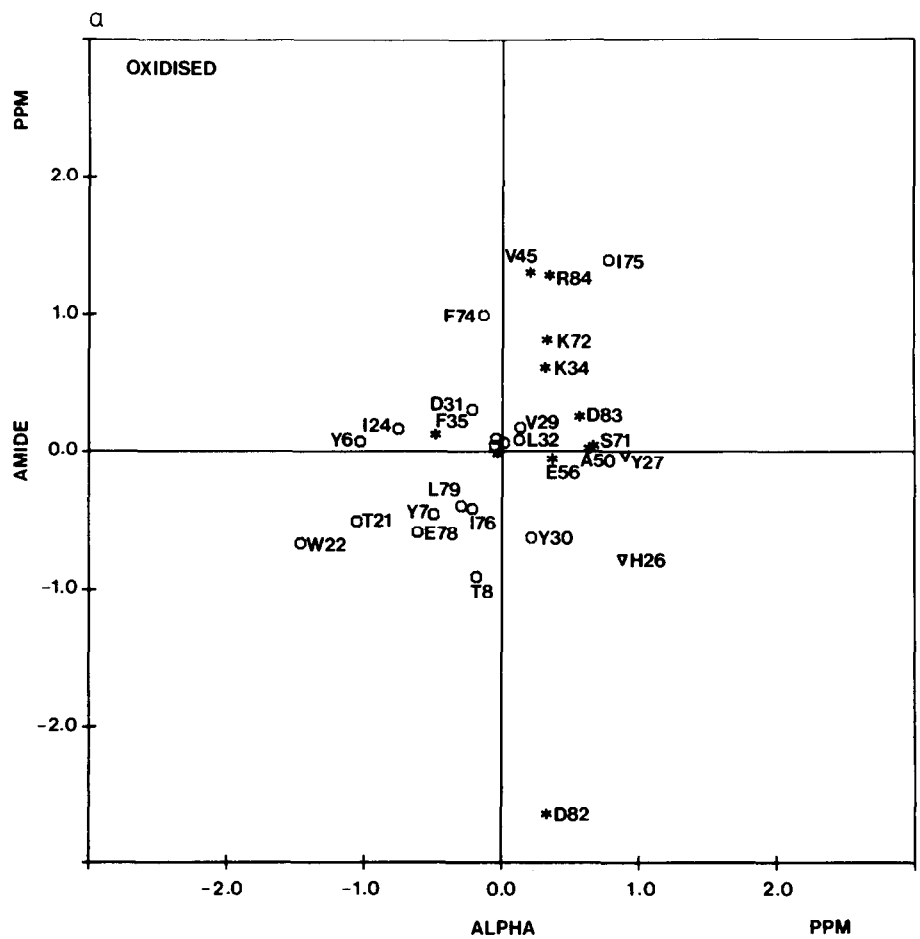


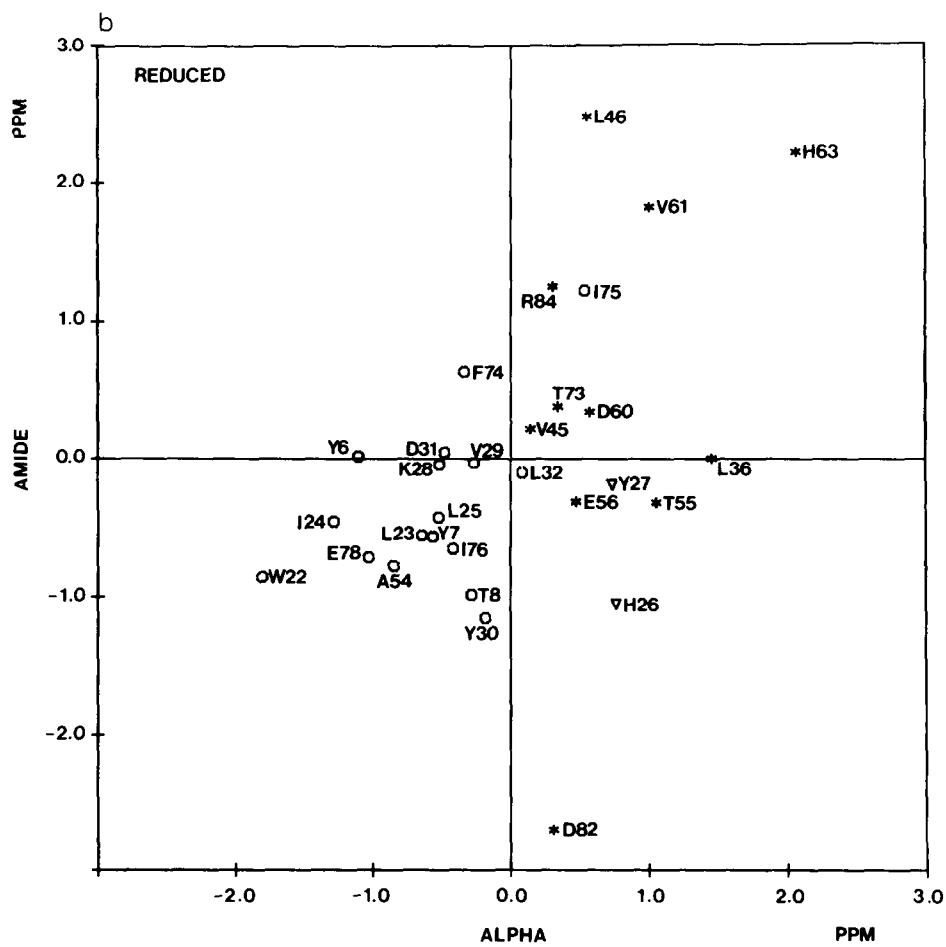
Fig.2. (a) Secondary chemical shifts (defined as the difference between random coil and observed shifts) of HN and HA protons in ferricytochrome b_5. Residues situated in the β-pleated sheet are represented by circles, those in α-helical regions by stars and two residues from a β-bend by triangles. The four points close to the origin correspond to A67, L23, A54 and L25 (clockwise from '*',

contribute to the unusual HN shift of Asp 82, a main chain–side chain hydrogen bond with His 80 ND and the proximity of the amide proton to the plane of the histidine ring.

3.3. *Side chain resonances*

The NOE data for side chain resonances give a greater depth of detail to the solution structure. As an illustration of this, fig.3 shows NOEs between amide/aromatic protons and methyl groups. For a comparison with the crystal structure, expected NOE observations (corresponding to interproton distances of <4.5 Å) were calculated. These showed that given the extent of current assignments and using over 150 defined NOEs the overall agreement was good in most parts of the structure, with only a few small crystal/solution state differences extant. These small differences are largely associated with residues Ala 50 and Ile 75 (oxidised state) and His 80 (reduced state).

All of the side chain resonances of the aromatic amino acids tyrosine and phenylalanine exhibited AA'XX' and AA'XX'M coupling patterns, respectively (noted from COSY spectra), showing that these rings undergo flipping by virtue of rotation about the CB–CG bond [26]. By way of contrast, the two methyl cross-peaks of valines and leucines were usually well separated and these residues are therefore maintained overwhelmingly in one conformation.



respectively). (b) Secondary chemical shifts of HN and HA protons in ferrocytochrome b_5. The region of secondary structure in which residues occur is indicated as in (a).

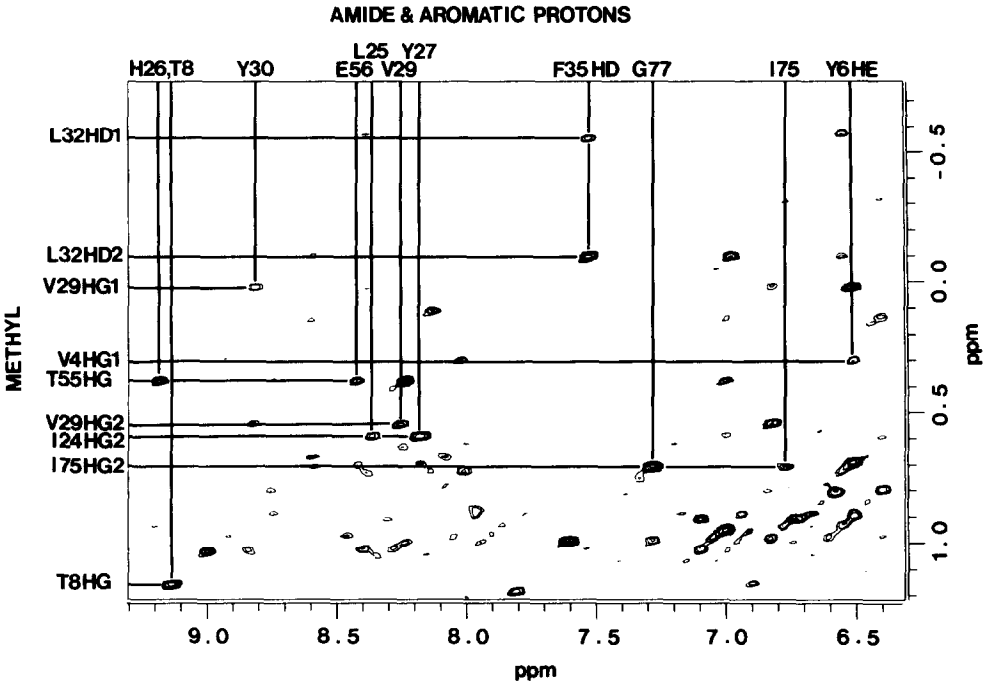


Fig.3. Phase-sensitive 500 MHz NOESY spectrum of ferricytochrome b_5 (4 mM) illustrating NOEs between amide/aromatic protons and methyl protons. Many of the residues shown occur in β-sheet regions. The spectrum was acquired in 90% H_2O: 10% ^2H_2O/20 mM potassium phosphate buffer at 30°C and pH 7.0. A mixing time of 120 ms was employed.

4. CONCLUSION

NMR techniques are employed in this study to define much of the solution structure of cytochrome b_5 in both redox states and to make comparisons with the crystal data, available at 2.0 and 2.8 Å for oxidised and reduced states, respectively [27,28]. It is clear from the many assignments made and the interpretation of NOE data that the overall fold of the protein is virtually unchanged between crystal and solution states. Some small differences in a few side chain orientations have been noted and will be analysed elsewhere.

The extent of conformational change linked to the redox state change is also small. This observation was substantiated by calculation of pseudocontact shifts for the oxidised state and comparison with the observed redox state shifts by similar methods to those used in the examination of cytochrome *c* [8]. Preliminary 2D NMR experiments examining the interprotein complex formed between cytochromes b_5 and *c* also indicate that the overall structures show almost no perturbation on binding (Concar et al., unpublished).

Side chain mobility determined from these NMR studies provides an insight into overall protein dynamics. For ring flipping to occur, cytochrome b_5 must be able to undergo small 'breathing' fluctuations. The considerable mobility of the heme-binding pocket, containing four fast flipping aromatic side chains (Tyr 30, Phe 35, Phe 58 and Phe 74) is in sharp contrast to the analogous region in cytochrome *c*. In evidence of this less stringent structural requirement in the heme pocket is the observation that heme binding in cytochrome b_5 is disordered, with two solution conformers existing [29]. Despite the higher mobility of cytochrome b_5 compared with cytochrome *c* the two systems are similar in that they show little conformational change associated with a change of state of the iron atom. This contrasts with the known case of conformational change linked to redox or spin state change which occurs in some other heme proteins, notably hemoglobin. Presumably, the β-pleated sheet in cytochrome b_5 acts to impart rigidity to the

protein structure in a similar fashion to the effect of cross-linking between heme and polypeptide found in cytochromes *c*. However, the additional thioether cross-linking in the latter is clearly more effective in reducing the configurational space available to the internal aromatic residues than is the β-sheet of cytochrome b_5. These observations imply that proteins such as cytochromes b_5 and *c* have structures within which the arrangement of secondary structural elements optimises electron transfer only. In contrast, some other heme proteins have secondary structural elements arranged so that processes such as redox state or spin state change at the iron atom give transmitted effects to comparatively distant parts of the protein thus coupling these changes with other events.

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