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Change in charge of an unvaried heme contact residue does not cause a major change of conformation in cytochrome *c*

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The structure of the Ala38 variant of yeast iso-1-cytochrome *c*, in which the previously unchanged Arg38 has been replaced, has been characterised by NMR. The NMR data indicate that the structure of the Ala38 variant is very similar to that of the wild type protein. In particular, the heme environment and interactions of the heme macrocycle are shown to be preserved. Analysis of the chemical shift perturbations to the resonances of Ile35 is shown to be consistent with the change in charge at position 38.

The only significant area of conformational change detected was at residues 39 and 58, close to the site of modification. Therefore the redox potential change accompanying the modification [1988, Biochemistry 28, 3188–3197] appears to be a direct consequence of the altered side-chain of residue 38 and not a result of secondary conformational changes induced by the modification.

Yeast cytochrome *c*; Mutagenesis; ^{1}H-NMR; COSY; NOE

1. INTRODUCTION

Cytochrome *c* is a small (molecular weight of about 12 500) monoheme protein that transfers electrons from cytochrome *c* reductase to cytochrome *c* oxidase in the mitochondrial respiratory chain. The iron switches between the ferric and ferrous states with a mid-point redox potential at pH 7 of 260 ± 20 mV (for recent reviews see references [1] and [2]).

In class I cytochromes *c* [3] the heme propionates may change ionisation state over the physiological pH range of 5–9, depending on their interactions with surrounding amino acid residues [2,4,5]. Heme propionate 7 (HP-7) ionises in this pH range in some prokaryote cytochromes *c*, the ionisation being accompanied by a substantial decrease in redox potential. In most class I cytochromes *c* however, including those from eukaryotes, HP-7 has a low pK_a [2,6,7]. The ionised form of HP-7 thus stabilises the oxidised form of the heme iron. This effect is modulated by various interactions of the propionate with polar and charged amino acids in the protein. For example, X-ray crystallography for the tuna protein [8] has shown that the Arg38 side chain is located in close proximity to HP-7 with an NH_2 group of Arg38 hydrogen bonding with the carboxylate of HP-7.

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To investigate further the role of Arg38, a residue that is unvaried amongst the 96 eukaryotic cytochromes *c* that have been sequenced ([2] and references therein), Cutler et al. [9] used site directed mutagenesis to replace it by the amino acids Lys, His, Gln, Asn, Leu, and Ala. Although the pK_a of HP-7 was not raised significantly by the mutations, the redox potentials were considerably altered; in all cases the variants had a lower redox potential than the wild type protein. The largest decrease, 47 mV, was observed for the Ala38 variant and therefore we have selected this for further study in order to determine the origin of the redox potential change. In the present paper we report high resolution NMR studies that reveal the modification is accompanied by only a small conformation change at the surface of the protein.

2. MATERIALS AND METHODS

Samples of the Ala38 variant of yeast iso-1-cytochrome *c* were obtained as described previously [9].

The amino acid numbering system is based on the alignment of the yeast iso-1-cytochrome *c* primary sequence with that of higher eukaryotic cytochromes *c* [2]. The crystal structure of yeast cytochrome *c* has recently been determined [10], although the coordinates are not yet in the public domain. However, it was found that yeast cytochrome *c* has structural homology with tuna cytochrome *c*, and consequently this crystal structure has been used to indicate which protons are expected to be close to mutation sites or take part in through space interactions with the haem macrocycle.

The Thr102 variant of yeast iso-1-cytochrome *c* is better behaved chemically than the wild type protein [11]. Similarly, the structure and

properties of the Thr102 variant are typical of eukaryotic cytochromes *c* in general [12]. Throughout this work all the proteins under study have had Cys102 replaced by threonine, and hereafter the Thr102 variant is referred to as the wild type protein.

Samples of the ferricytochromes were prepared for NMR by exchanging H_2O for D_2O using Centricon-10 (Amicon) devices and 3 cycles of concentration and dilution in D_2O to effect the exchange of NH protons for deuterons. The final protein concentrations for NMR were approximately 8 mM, with a sample volume of 400 μl. Samples of ferrocytochrome *c* were obtained by reduction of ferricytochrome samples as described above with stoichiometric amounts of sodium dithionite, the final pH being adjusted to approximately 7.0 by the addition of either NaOD or DCl. 1,4-Dioxane was used as an internal standard, but all chemical shifts are quoted in parts per million (ppm) downfield from the methyl resonance of 2,2-dimentyl-2-silapentane-5-sulfonate.

All the NOE difference spectra and COSY data sets described in this paper were acquired on a JEOL GX-400 FT-NMR spectrometer at the University of East Anglia. NMR data were collected at temperatures of 25°C and 37°C. For each of the NOE difference spectra 16384 data points were collected with 1000 scans on and off resonance. Resolution-enhanced spectra were obtained with the standard Gaussian multiplication routine of the JEOL PLEXUS programme. The data sets for the COSY spectra consisted of 2048 × 512 data points. 160 scans were collected per column point, resulting in a 12 h acquisition time. Resolution enhancement was achieved using a sine bell window function.

NOESY spectra were collected using a home-built 500-MHz spectrometer at Oxford, equipped with a GE/Nicolet 1280 data acquisition system. Samples were investigated in H_2O/D_2O (9:1) at 37°C with a pH meter reading of 7.0, the protein concentrations being 8 mM. Phase-sensitive NOESY data sets (mixing time 133 ms) consisted of 1024 × 1024 data points, and data collection required 12 h. Data sets were zero filled in both F1 and F2 dimensions to give a final matrix size of 2048 × 2048 real points. Resolution enhancement used a trapezoidal window function followed by a cosine bell function. The processed data were then transferred via magnetic tape to the microVax using software written in Oxford. The data sets were scaled relative to each other prior to overlaying using the intensity of the side chain trimethyl group of Me_3Lys72 which is a single resonance of 9 magnetically equivalent protons.

Protein studies were augmented using a Silicon Graphics Iris 4D-20 workstation incorporating Polygen software. Possible structures of the variant protein were generated based upon the crystal structure coordinates of tuna cytochrome *c* [8]. Thus proton-proton interactions observed in the NOE studies could be identified and confirmed by inspection of the possible proton-proton distances in the generated structures.

3. RESULTS AND DISCUSSION

Assignments of the resonances of the wild type and variant proteins were made by a combination of NOE difference, COSY, NOESY, and structural prediction methods. This phase of the work was aided by the previously described assignments of the wild type protein [12]. A summary of some of the assignments made are shown in Tables I and II for the ferro- and ferricytochromes *c* respectively. The NMR studies reveal that the structural characteristics of the mutated and wild type proteins are virtually identical. However, spectroscopic anomalies were observed and this discussion deals with the implications of these differences.

3.1. *The Protein Backbone*

As the samples were run in 90% H_2O the amide protons of the protein backbone had not exchanged and consequently the secondary structure was monitored by the through space interactions observed in the NOESY spectra. A comparison of the chemical shifts of the amide and α protons with those of the wild type protein indicates that no major changes have occurred (except where shown in Table I). Similarly, the patterns of nuclear Overhauser enhancements to the backbone fold for the 2 proteins are almost identical.

Table I

NMR assignments for non-exchangeable protons in the ferrocytochromes *c* which exhibit a chemical shift difference of greater than 0.05 ppm at 298K, pH 7.0

Proton	Wild type	A38 T102	Stereospecific NOEs
Leu32			
α	3.96	4.04	L32NH
NH	7.78	7.69	L32α, δ_2
Ile35			
α	3.73	4.04	I35δ_1
Phe36			
NH	7.96	8.03	I35γ_2, F36o
His39			
α	5.63	5.71	I35γ_1; H39β_1, β_2, NH; L58α, δ_2; W59C2, C6, C7; S40NH
β_2	2.98	3.06	H39α
Ser40			
NH	8.71	8.64	H39α; V57NH, γ_2
Val57			
α	3.76	3.69	V57NH; L58NH
Leu58			
α	3.79	4.04	H39α; L59NH
NH	8.17	7.96	V57γ_1, γ_2, α
Trp59			
C2	6.95	6.86	I35γ_1, δ_1; H39α; M64ϵ; W59C7
C4	7.62	7.54	W59C6
NH	8.08	8.02	H39α; V57γ_2; L58δ_1, δ_2, α

Table II

NMR assignments for non-exchangeable protons in the ferricytochromes *c* which exhibit a chemical shift difference of greater than 0.05 ppm for the non-contact shifted resonances at 298K, pH 7.0

Proton	Wild type	A38 T102	Stereospecific NOEs
Val28			
β	1.34	1.20	G29α_1; P30δ_1, δ_2
Gly29			
α_1	−0.54	−0.40	V28β; P30δ_2; HM5
α_2	−3.21	−3.27	P30δ_1, δ_2
Pro30			
γ_2	−0.75	−0.67	P30δ_1, δ_2
δ_1	−1.51	−1.63	V28β; G29α_1; P30γ_2, δ_2; HM5
δ_2	−5.54	−5.75	V28β; G29α_1, α_2; P30γ_2, γ_1, δ_2
Val57			
γ_1	0.34	0.26	V57β
Trp59			
C7	7.54	7.60	HP7; HM8
Leu68			
δ_2	−3.21	−3.31	M80ϵ; F82o, m; L85δ_2; TE2-M

3.2. *The Heme Environment*

One indication of the nature of the heme environment is the through space interactions of the heme with the groups packed around it. It was found that despite differences in the chemical shifts of the heme resonances the NOE effects of the heme macrocycle with the rest of the protein remained the same. Thus it may be concluded that the protein fold around the heme is not significantly altered by the amino acid substitution made.

It was found that all the chemical shifts of the heme and axial ligand resonances of the ferricytochromes *c* were affected by the mutations even though the NOE patterns indicate that the protein fold around the heme is unaltered. Consequently, the chemical shift differences for the heme resonances must be due to paramagnetic effects.

HP-7 β_1 has been particularly affected with a chemical shift difference of 1.20 ppm between the wild type and Ala38 variant. In the latter protein the interaction between Arg38 and HP-7 is broken, which leads to a redistribution of electron density away from the propionate carboxylate group back into the heme plane. In general, however, it is not possible to quantify all these effects since the parameters of the contact shift equation have not been fully evaluated.

3.3. *Residues 28–30*

The resonances of Val28 β, Gly29 α_1 and α_2 protons and the Pro30 protons are all affected in the oxidised form of the Ala38 variant. Proline 30 interacts with the heme axial ligand His18 via a hydrogen bond between the Pro30 carbonyl and His18 NH. Gly29 is also very close to the heme and interacts with Pro30. Consequently both Gly29 and Pro30 experience very strong pseudocontact shifts, so therefore may be expected to be sensitive to any perturbation of the heme macrocycle. Hence electron density redistribution within the heme and axial ligands would affect Pro30 and Gly29. Similarly, the Val28 β proton resonance has a 0.16 ppm shift difference from the wild type protein, and since this proton is close in space to both Pro30 and Gly29 it too must experience a change in its pseudocontact interaction with the heme macrocycle.

3.4. *Isoleucine 35*

In general the chemical shifts and NOE patterns of the resonances of Ile 35 are hardly perturbed by the substitution of arginine for an alanine at position 38. However, the αCH proton of Ile35 in the reduced Ala38 protein exhibits a 0.31 ppm difference in chemical shift from the wild type protein. From the X-ray crystal structure it is known that the inter-proton distance of Ile35 α to Arg38 ζ carbon atom is 4.17 Å [8] and consequently this places the Ile35 proton close to the positive charge situated on Arg38.

It is known that the local diamagnetic shielding of a hydrogen atom may be influenced by an electric field. In a protein, such fields can arise from the existence of dipoles or as in this case, a formal charge. The effect of charges within a molecule on the local diamagnetic shielding of a proton is given by:

$$\Delta\delta = 12.5 \times 10^{-6} \frac{q_i \cos\theta_i}{R_i^2}$$

where $\Delta\delta$ is the shift due to the charge, q_i is the charge on the i^{th} atom, R_i the distance of the proton from the charge in Å, and θ_i the angle between the charge and the C–H bond of the proton [12].

Figure 1 shows the orientation of Ile35 with respect to Arg38 in tuna cytochrome *c*. In solution, side chain positions are subject to dynamic change and therefore we have calculated the electric field effect as a function of the angle θ_i (Fig. 2). By virtue of the approximate orientation of the 2 groups the range of $\theta = 90$–$180°$ was used to calculate $\Delta\delta$ which gave values of between 0 and −0.72 ppm (here the negative sign indicates an upfield chemical shift). These values correlate well with the observed upfield chemical shift (from the primary position) of 0.50 ppm seen for Ile35 α in the wild type protein. Consequently the observed downfield shift of 0.31 ppm for Ile35 α seen in the Ala38 variant is consistent with a removal of charge from the vicinity of Ile35. Thus the chemical shift difference for Ile35 α in the Ala38 variant with respect to the wild type protein can in part be attributed to the absence of the positive charge of Arg38.

3.5. *Leucine 58*

The resonances arising from the protons of Leu58 α, δ_2, and NH show significant differences in chemical

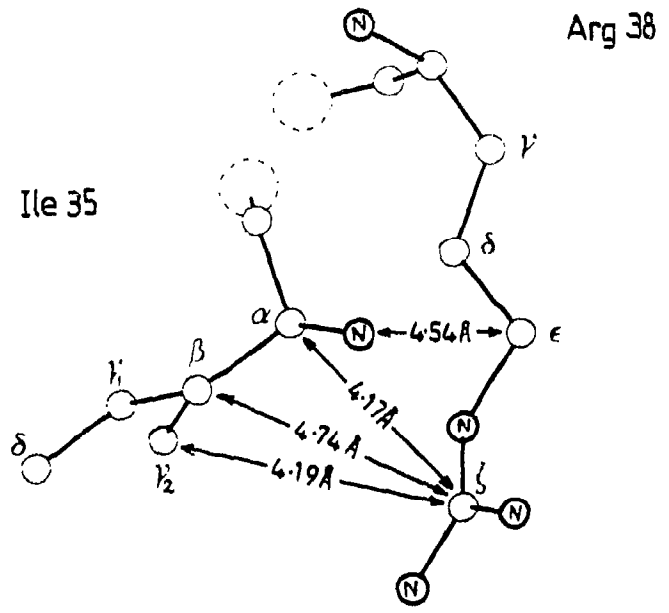

Fig. 1. Orientation of Ile35 with respect to Arg38 adapted from the tuna cytochrome *c* X-ray crystal structure [8].

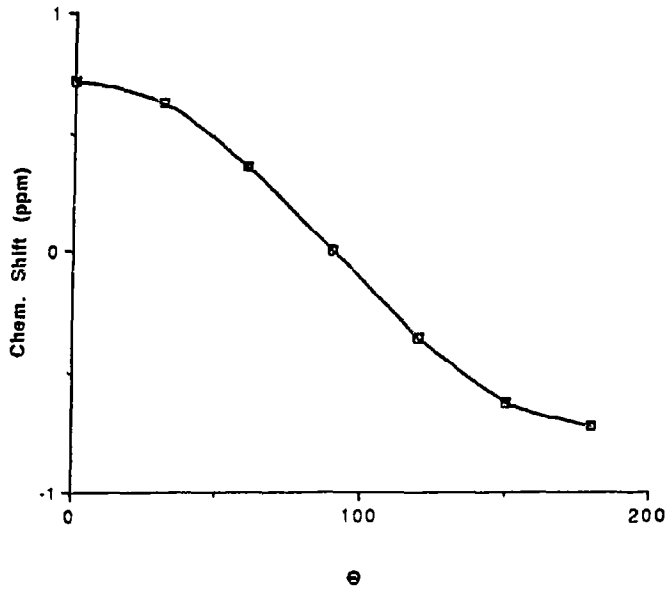

Fig. 2. Graph of $\Delta\delta$ versus θ for electrostatic shielding effects, where $\Delta\delta$ is the chemical shift due to electrostatic shielding, and θ the angle between the charge and the CH bond of the proton in question. R_i was taken to be 4.17 Å.

shift from the wild type values. The secondary shift analysis is shown in Table III. It can be seen that the α and δ_2 protons move towards their primary positions, whereas the amide proton is shifted even further upfield. Figure 3 shows the orientation, adapted from the tuna crystal structure, of His39 relative to Leu58 for the wild type protein. The chemical shift differences observed above may be attributed to the His39 side chain moving towards the Leu58 NH proton and away

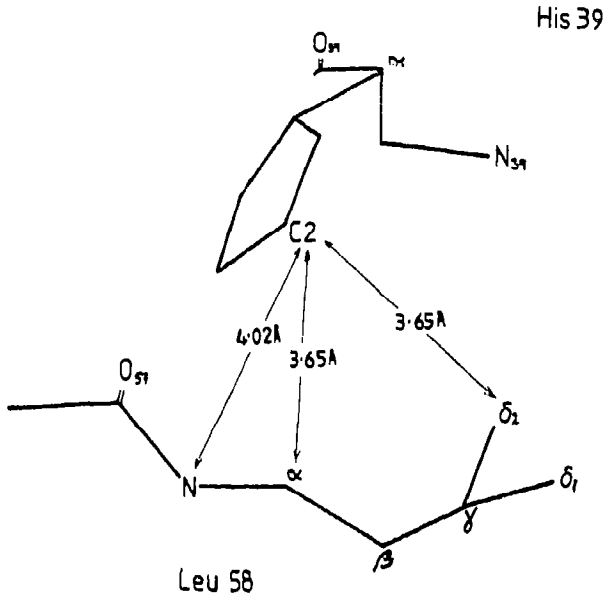

Fig. 3. Orientation of His39 with respect to Leu58 adapted from the tuna cytochrome c X-ray crystal structure [8].

Table III

Secondary chemical shifts of Leu58

Proton	$1°\delta$ (ppm)	Wild type δ_{obs} (ppm)	Ala38 variant δ_{obs} (ppm)	$2°\delta$ (ppm)
NH	8.42	8.17	7.96	0.21
α	4.38	3.79	4.04	−0.25
δ_2	0.92	0.37	0.49	−0.16

$1°\delta$ is the primary chemical shift [14], δ_{obs} the observed chemical shift, and $2°\delta$ the secondary chemical shift (wild type, Ala38 variant).

from the α and δ_2 protons. The mobility of His39 is indicated by its different chemical shift values and temperature dependent chemical shift of its α proton, and this motion not only accounts for Leu58 shift differences, but can also be applied to those differences observed for His39 α. Hence it can be concluded that substitution of Arg38 for an alanine results in a conformational reorganisation of His39 that affects the Leu58 environment.

4. CONCLUSIONS

The NMR data indicate that the replacement of Arg38 by Ala38 results in only a small conformational change close to the site of modification. Therefore the decrease in redox potential that accompanies the modification must arise largely from changes in the electrostatic interactions of the haem iron. Both the change in size of the residue at position 38 and the change in charge probably contribute to such altered interactions.

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