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Mutagenesis of residues 27 and 78 modulates heme orientation in cytochrome b_5

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Abstract A comparison of the primary sequences of the heme binding domains of bovine and rat microsomal cytochrome reveal differences at only six residues. These residues must therefore provide the origin for the observed variation in the ratio of the heme orientational isomers, the equilibrium constant of which ranges from ~9 in the bovine protein to ~1.6 for rat cytochrome b_5. Residues 7, 20, 21, and 30 are distant from the exposed heme edge whilst Leu^{27} and Phe^{78} are located close to different parts of the porphyrin macrocycle. ^{1}H NMR spectra of the heme and heme ligand resonances of a recombinant tobacco cytochrome b_5 extending from Gly^1 to Lys^{89} suggest, in combination with NMR data acquired for other forms of cytochrome b_5 and an inspection of their sequence homology, that the identity of residue 78 influences the relative ratios of heme isomers. The Gly^1-Lys^{89} domain of tobacco cytochrome b_5 has two equally abundant heme orientational isomers but retains the leucine side chain at position 27 whilst phenylalanine 78 is replaced by tyrosine. A more direct role for residue 78 in modulating the heme ratio is shown by site directed mutagenesis of bovine microsomal cytochrome b_5 where the mutation Phe^{78} > Tyr shifts the equilibrium constant for the heme orientational isomers from 9 to 3.5. Whilst the ratio is clearly shifted towards that exhibited by the rat protein the incomplete transition suggested the involvement of other residues. The mutation of Leu^{27} > Val was shown to result in a slightly smaller change in ratios of each isomer (from 9 to 4.0). Together these results point to the importance of these residues in modulating the ratio of heme isomers.

Key words: Cytochrome b_5; Site directed mutagenesis; ^{1}H NMR spectroscopy; Heme

1. Introduction

The primary sequences of many members of the cytochrome b_5 superfamily have been determined and are defined by the observation that all proteins contain a non-covalently bound heme group, with histidine ligands at the fifth and sixth coordination sites and the presence of a characteristic motif beginning FXXXHPGG. The most widely studied group of proteins within this superfamily are the heme binding domains isolated from the endoplasmic reticulum membrane of a wide range of eukaryotic cells. These domains, often obtained from limited proteolysis of membrane fractions [1], contain approximately 90 amino acid residues and have been characterised from yeast, insects, avian and mammalian species to reveal significant sequence homology [2]. Alongside the microsomal form of cytochrome b_5 sequentially homologous forms of the protein can also exist within the same cell. These related forms of cytochrome b_5 include flavocytochrome b_2 [3], sulphite oxidase [4] and nitrate reductase [5,6] as well as more closely related forms of the protein found in other cell compartments such as outer mitochondrial membrane cytochrome b_5 [7]. A comparison of some of these protein sequences enabled Guiard and Lederer [8] to propose a common 'b_5-fold' within these domains and this has been supported by crystallographic studies of bovine microsomal cytochrome b_5 [9] and yeast flavocytochrome b_2 [10]. For example, both proteins have four helices that provide a hydrophobic pocket for the non-covalently bound heme group. In general the heme has limited solvent accessibility and for microsomal cytochrome b_5 fourteen amino side chains make van der Waals contact with the porphyrin ring [11]. The structure of a large, but soluble, domain of bovine microsomal cytochrome b_5 extending from Ala^1 to Ser^{104} has also been derived using 3D heteronuclear NMR methods [12]. Although the solution and crystal structures show many similarities small, but significant, differences are apparent. Additionally ^{1}H NMR studies of the tryptic fragment of porcine cytochrome b_5 have shown, from the pattern of NOEs observed between the polypeptide and heme resonances,shown that the orientation of the protoporphyrin IX group originally suggested from crystallographic data was not found in solution [13].

Whilst structural similarities exist between members of the b_5 superfamily, microsomal cytochromes b_5 are observed to possess heterogeneity in the orientation of the heme group. This arises from rotation about the C5/C15 axis (Fig. 1) and leads to two conformations for the porphyrin within the holoprotein. In bovine microsomal cytochrome b_5 heme orientational heterogeneity is reflected in a major isomer representing approximately 90% of the total protein [14]. Interconversion between the major and the minor isomeric forms can be followed using NMR spectroscopy and it is observed that freshly reconstituted bovine protein contains heme orientations which revert over several hours to a form of the protein containing a ratio at equilibrium of 9:1 [15]. The rate of interconversion is modulated by pH and each heme orientation possesses a different redox potential although the functional significance of this, if any, remains obscure [16]. Unlike the bovine microsomal protein the corresponding form of recombinant rat cytochrome b_5 exhibits an equilibrium constant of 1.6 [17] despite a primary sequence that is restricted to six differences within the heme binding domain (residues 7–90) isolated via proteolytic digestion. Further NMR studies have reported that the isomeric ratio in chicken cytochrome b_5 is ~20:1 [17] whilst 2D NMR spectra of the rabbit and porcine proteins indicate one preferred or major orientation [18]. More recently 1D ^{1}H NMR spectroscopy of outer mitochondrial membrane (OMM) cytochrome b_5 has suggested an equilib-

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rium constant (K_{eq}) of 1.0 for the isomeric ratio [19]. Studies aimed at understanding the nature of heme orientational heterogeneity have focused on the effect of changing the methyl and vinyl substituents found at positions 2,3,7 and 8 of the macrocycle [15,17]. Using synthetic hemins where the vinyl side chains are replaced by hydrogens it has been suggested that a major determinant of heme orientation is a repulsive interaction between one vinyl group and a cluster of hydrophobic side chains belonging to Leu[27] and Leu[29] [20]. These workers showed that orientational preferences could be correlated with the size of the side chain at residues 27 and 29 for the rat, bovine and chicken forms of ferricytochrome b_5. Further NMR studies have suggested on the basis of relative NOE intensities that in rat cytochrome b_5 the two orientations also involve a slight counterclockwise angular displacement in going from orientation A to B [21]. However, since the heme group is identical in all cytochromes b_5 it seems clear that the protein matrix dictates the variation in the ratio of isomers observed in the different variants. A study of the protein component will therefore increase our knowledge of prosthetic group recognition as well as highlighting important amino acid residues involved in stabilising the overall structure.

In this paper we focus on role of the polypeptide in determining the heterogeneity exhibited in the orientation of heme groups in microsomal cytochromes b_5. Using wide sweep width 1D ^{1}H NMR spectroscopy we show, from the observed integrated areas of the heme resonances of recombinant forms of bovine and tobacco microsomal cytochrome b_5, together with site specific mutants of the bovine protein, that a strong correlation exists between the observed heme ratio and the identity of residues 78 and 27.

2. Materials and methods

The Gly[1]–Lys[89] domain of tobacco cytochrome b_5 was amplified from a full-length cDNA [19] and expressed in pET21d (Mortuza and Whitford 1996 unpublished results). Site directed mutagenesis of wild type bovine cytochrome b_5 used the method outlined by Barettino et al. [22]. This method requires the 'target' DNA in two different vectors and in this study bovine cytochrome b_5 (Ala[1]–Ser[104]) was cloned into pBluescriptII and ptrc99A. The first PCR involved synthesis and amplification of a 'megaprimer' from a pBluescript construct using a forward primer (KSfwd 5′-TCG AGG TCG ACG GTA TC-3′) and a mutagenic primer (for Phe[78]Tyr 5′-TCC AAA ACG TAC ATC ATT GGG-3′ and Leu[27]Val 5′-AGC ACG TGG GTG ATC CTG CAC-3′) which contains a mismatch that generates the required mutation. After purification from a 2% agarose gel the 'megaprimer' was used in a second PCR with a linearised ptrc99A construct and two 'universal' primers -KSfwd and a primer specific for the plasmid ptrc99A (5′-AAT CTG TAT CAG GCT GAA AA-3′). PCR reactions were performed in a total volume of 100 μl containing 5mM dNTPs, 15 μM of each primer, 2.5 units *Pyrococcus furiosus* exo$^-$ DNA polymerase (Stratagene), 10 μl of ×10 buffer and ~10 ng DNA template. Thirty amplification cycles were performed with denaturation at 94°C for 75 s, annealing at 51°C for 75 s and extension at 72°C for 140 s. The single PCR products were verified by DNA sequencing with the final product ligated, after digestion with *Nco*I and *Hin*dIII, into the vector pET21d cut with identical restriction endonucleases. Transformation of *E. coli* BL21 (DE3) and selection of a single colony containing the recombinant plasmid (pET-F78Y, pET-L27V) subsequently allowed the isolation and purification of the mutant protein using the methods described previously [23]. ^{1}H NMR spectra were recorded on 11.7 and 14.1 T spectrometers. Each sample was extensively exchanged against ^2H_2O and lyophilised before resuspending to a protein concentration of 2 mM in 20 mM phosphate pH 7.0. Wide sweep width spectra of 30–40 kHz were recorded using quadrature detection and between 8 and 32k data points at a temperature of 303 K.

3. Results and discussion

The rat and bovine forms of microsomal cytochrome b_5 have been extensively studied using NMR spectroscopy and this has yielded a large number of sequence specific assignments [11,18,24]. 1 and 2D NMR methods reveal that the ratio of heme orientations differ in each form of the protein ranging from approximately 9:1 in bovine cytochrome b_5 [14] to 1.6:1 in the form isolated from rat [17]. The magnitude of these differences is surprising in view of the very high sequence homology exhibited between each domain (Fig. 2) but the origin of this heterogeneity may be inferred from the nature and extent of the differences between these cytochromes b_5. The sequences include only the region of the protein obtained after limited proteolysis and omit, in the case of the bovine protein, six residues at the amino terminus together with the longer membrane binding tail. Previous ^{1}H NMR studies have shown that longer domains of recombinant bovine cytochrome b_5 retain similar ratios of heme isomers when compared with those isolated by limited trypsinolysis [21,25,26]. Within this region (7–90) there are six amino acid residues that differ between the bovine and rat proteins: Ala[7](Asp[7]), Asn[20](Lys[20]), Asn[21](Asp[21]), Leu[27](Val[27]) Tyr[30](His[30]) and Phe[78](Tyr[78]). In the crystal structure [8,11] and the recently determined NMR derived structures of cytochrome b_5 [11] residues 20 and 21 are found in a turn region whilst Tyr[30] is located in a four residue turn between two β strands that provide the base to the heme binding pocket. In view of their proximity to the heme group Leu[27] and Phe[78] are better candidates for a role of the protein matrix in modulating the heme isomeric ratio in cytochromes b_5. The side chain of Leu[27] makes van der Waals contact with pyrrole ring 2

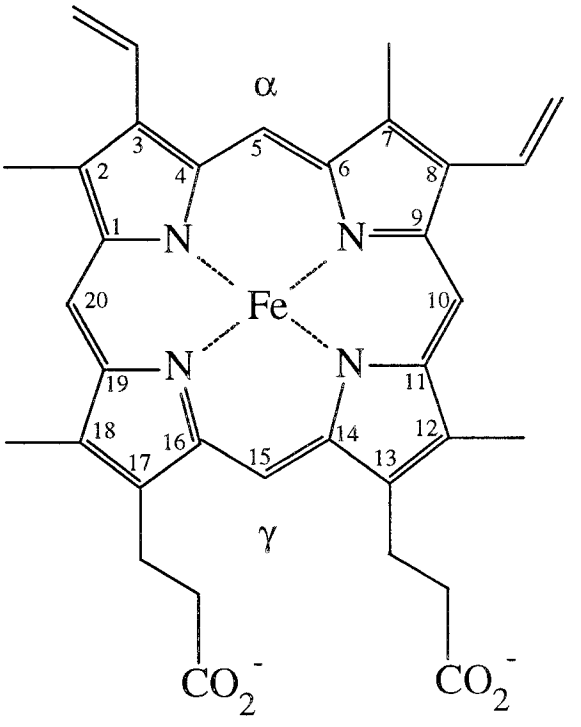

Fig. 1. The heme macrocycle showing the positions of the methyl, vinyl and propionate side chains. The two heme isomers arise as a result of rotation about the C5/C15 axis (sometimes called the α/γ axis).

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                10          20          30          40          50
Rat       ......DVKY  YTLEEIQKHK  DSKSTWVILH  FKVYDLTKFL  EEHPGGEEVL
Bovine    ......AVKY  YTLEEIQKHN  NSKSTWLILH  YKVYDLTKFL  EEHPGGEEVL

                60          70          80          90
Rat       REQAGGDATE  NFEDVGHSTD  ARELSKTYII  GELHPDDRSK
Bovine    REQAGGDATE  NFEDVGHSTD  ARELSKTFII  GELHPDDRSK
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Fig. 2. The primary sequences of soluble domains of rat and bovine microsomal cytochromes b_5. The highlighted regions show the differences between bovine and rat cytochromes b_5 from residues 7 to 90. The numbering system for amino acid residues accounts for the additional four residues found at the amino terminus in the recombinant bovine microsomal protein[22].

whilst the aromatic (HD2 and HE2) protons of Phe^{78} lie ~4 Å from the C2 methyl and C3 vinyl groups. Further evidence supporting the importance of residues 27 and 78 is seen by comparing other cytochrome b_5 sequences for which NMR spectroscopic data is available. The equilibrium constant in chicken microsomal cytochrome b_5 is ~20 (the largest ratio yet reported) [17] and contains a His residue found at position 30. This is identical to that found in the rat protein suggesting this side chain does not modulate the ratio of heme isomers. Elsewhere in the sequence of chicken cytochrome b_5 Phe 78 is unchanged whilst a conservative replacement of isoleucine for leucine occurs at residue 27. Similarly in porcine cytochrome b_5 Phe^{78} and Leu^{27} remain unchanged and a ratio of major to minor isomers of approximately 9 exists [18].

Evidence supporting the importance of Phe^{78} is also seen from the ^{1}H NMR spectrum of recombinant tobacco cytochrome b_5 (Fig. 3). The form of tobacco cytochrome b_5 used in this study contains the first 89 amino acid residues and was amplified from a full-length cDNA template [20]. In contrast to mammalian cytochromes b_5 similar proteins from plants have not been extensively characterised. The 1D ^{1}H NMR spectrum shown here is the first for a plant cytochrome b_5. The NMR spectrum is typical of a low spin ferric heme and resembles, in terms of the number and linewidth of resonances, the profiles exhibited by many other cytochromes b_5. Although the number and linewidth of resonances shown by the 89 residue domain of tobacco cytochrome b_5 are similar their relative intensities deviate significantly from that seen for wild type bovine microsomal cytochrome b_5 (see below). In the absence of definitive assignments for the heme resonances of tobacco cytochrome b_5 integration of the areas of peaks of the methyl groups representing the two isomeric orientations leads to an estimation of the equilibrium constant (K_{eq}) for the two conformers of 1.1 ± 0.1. For example, the resonance at 30.5 ppm is, by analogy to the bovine protein, attributable to heme methyl C7 in what is normally the minor orientation. It can be clearly seen that this peak is of similar intensity to the resonance at 15.5 ppm representing the other orientation and leads to a ratio close to unity. A comparison of the areas of the C2 vinyl α proton and the heme C12 methyl resonances reveals a value of 3 and this is again consistent with each heme orientation being found in approximately equal ratios. Since tobacco cytochrome b_5 has Leu and Ile side chains at residues 27 and 29 it is unlikely that the orientational preference of the heme group can be explained solely on the basis of the side chain volume since this remains equivalent to that found in the bovine protein. The value of 1.0 determined for the equilibrium constant suggests that this protein belongs to the class of cytochromes b_5 showing two heme orientations of approximately equal stability.

Taken together these results suggest that rat and tobacco microsomal cytochrome b_5, along with the OMM form [19], can be assigned to a class of proteins containing approximately equal isomeric ratios. In contrast the bovine, rabbit, porcine and chicken variants form a second class of cytochromes b_5 containing predominantly one heme orientation. It is also likely that human microsomal cytochrome b_5 falls within the latter group since a recombinant form of the erythrocyte protein, with which it appears to possess an identical soluble domain, has an equilibrium constant of ~9 [27]. With due consideration to the limitations imposed by the restrictive set of sequence and NMR data available for cytochromes b_5 it is clear that the identity of residue 78 correlates exactly with the ratio of heme isomers observed for each protein whilst that at position 27 offers no clear correlation. This residue is phenylalanine in the bovine, human, porcine and chicken cytochromes b_5 but is changed to tyrosine in rat, tobacco and OMM proteins. The alignment of protein sequences for which NMR spectroscopic data exist emphasise the correlation between Phe^{78} and heme orientation (Fig. 4).

On the basis of the above observations we have changed residue 78 in bovine microsomal cytochrome b_5 using site directed mutagenesis from a phenylalanine to tyrosine. The mutation of Phe^{78} to Tyr yields a holoprotein with very similar uv-vis absorbance spectra in the reduced and oxidised states to that found in the wild type protein (results not

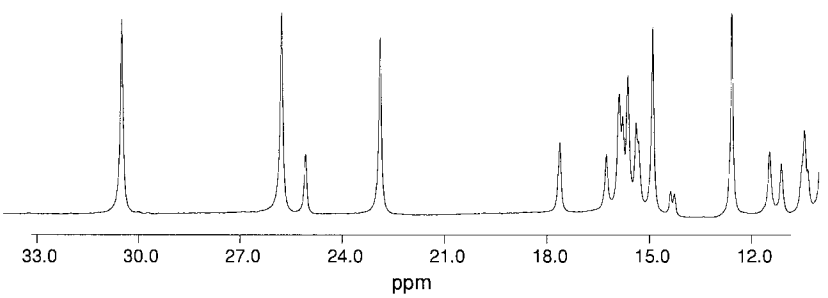

Fig. 3. 1H NMR spectrum of tobacco ferricytochrome b_5. The spectrum shows the heme and heme ligand resonances between ~10 and 34 ppm for the Gly^1–Lys^{89} domain of tobacco cytochrome b_5. The sample contained ~2 mM protein in 20 mM phosphate pH 7.0 and was recorded at 303 K.

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                    10        20        30        40        50
Bovine      ......AVKY YTLEEIQKHN NSKSTWLILH YKVYDLTKFL EEHPGGEEVL
Chicken     .........RGRY YRLEEVQKHN NSQSTWIIVH HRIYDITKFL DEHPGGEEVL
Rabbit        ..DVKY YTLEEIKKHN HGKSTWLILH HKVYDLTKFL EEHPGGEEVL
Pig         ......AVKY YYLEEIQKHN NSKSTWLILH HKVYDLTKFL EEHPGGEEVL
Human       ......AVKY YTLEEIQKHN HSKSTWLILH HKVYDLTKFL EEHPGGEEVL
Tobacco     ......ETKV FTLAEVSQHN NAKDCWLVIS GKVYDVTKFL DDHPGGEEVL
Rat         ........DVKY YTLEEIQKHK DSKSTWVILH HKVYDLTKFL EEHPGGEEVL
OMM         ........AVTY YRLEEVAKRN TAEETWMVIH GRVYDITRFL SEHPGGEEVL

                    60        70        80        90
Bovine      REQAGGDATE NFEDVGHSTD ARELSKTFII GELH.PDDRSK
Chicken     REQAGGDATE NFEDVGHSTD ARALSETFII GELH.PDDRPK
Rabbit      REQAGGDATE NFEDVGHSTD ARELSKTFII GELH.PDDRSK
Pig         REQAGGDATE NFEDVGHSTD ARELSKTFII GELH.PDDRSK
Human       REQAGGDATE NFEDVGHSTD AREMSKTFII GELH.PDDRPK
Tobacco     LSATGKDATD DFEDVGHSSS ARAMLDPYYV GDIDSATIPTK
Rat         REQAGGDATE NFEDVGHSTD ARELSKTYII GELH.PDDRSK
OMM         LEQAGADATE SFEDVGHSPD AREMLKQYYI GDVHPNDLKPK
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Fig. 4. The primary sequences of the soluble domains of microsomal cytochromes b_5 from cow, chicken, rabbit, pig, human, tobacco and rat together with the sequence of the isolated rat outer mitochondrial membrane cytochrome b_5. The lightly shaded regions represent sequence identity and include only the regions of the protein isolated, in the case of the bovine protein, via limited proteolysis (residues 7–90). The darker shading indicates the position of residues 78 and 27

shown). However, ^{1}H NMR spectra of this mutant show a change in the relative intensities of the heme methyl resonances when compared with the wild type protein and reveal an alteration in the NMR spectroscopic properties of this protein (Fig. 5). The mutation decreases the relative stability of the major isomer as reflected in the change in K_{eq} from 9 ± 0.1 to 3.5 ± 0.1. The decrease in the isomeric ratio upon mutation highlights the involvement of the side chain of residue 78 in modulating this transition. Different mutations of this residue (Tyr^{78} > Lys) have been described for recombinant rat cytochrome b_5 but the heme isomeric ratios in these proteins were not reported although the uv-vis absorbance spectra of Tyr > Lys mutants did not alter [28]. It is interesting to note that although the transition observed in the Phe^{78}Tyr mutant is significant it does not reach the values observed in either rat or tobacco cytochrome b_5. The observed change in the ratio of isomers suggests that although Phe^{78} may have a major role in modulating heme orientation other residues contribute to these effects. The identity of residue 78 is clearly important since the mutation of Phe^{78} > Leu results in a cytochrome with virtually identical ratios to the wild type protein (results not shown). In this context Leu^{27} would appear to be a valid additional candidate and the mutagenesis of Leu^{27} > Val leads to a change in the isomeric ratio from 9 to 4.0, less than that observed for the residue 78 transition.

It is also worth considering how the conservative replacement of Phe^{78} with Tyr can bring about this change in the properties of the protein. Crystallographic studies show that the side chain of Phe^{78} points outwards into solution [9,12] whilst NMR studies also suggest the aromatic ring flips about the C_β-C_γ axis on the μs–ms time scale [24,25]. The addition of a single –OH group highlights the sensitivity of the protein to small changes in sequence. A structural basis for the observed transition is not obvious when one considers the ori-

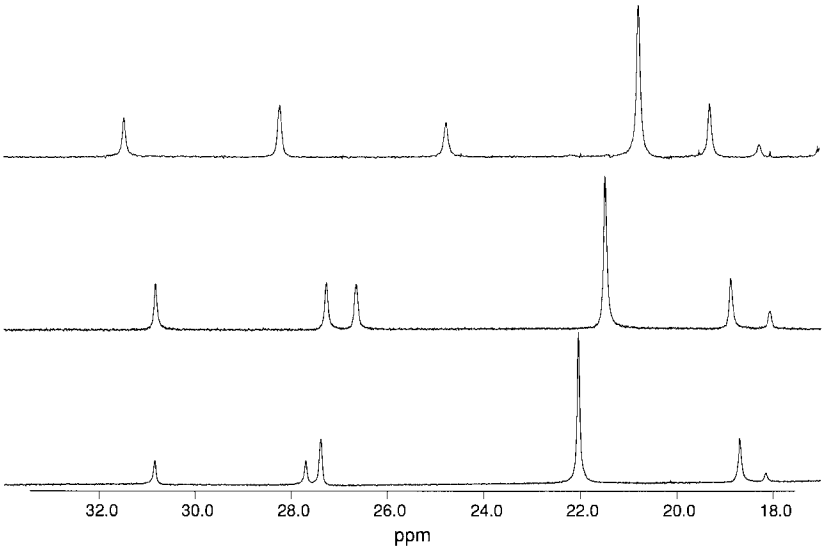

Fig. 5. ^{1}H NMR spectrum of bovine microsomal cytochrome b_5 (Ala^1–Ser^{104}) together with the Phe^{78}Tyr, Leu^{27}Val mutants of this protein. Each spectra was recorded on a sample containing ~2 mM protein in 20 mM phosphate pH 7.0, 303 K. Bottom: Wild type (Ala^1–Ser^{104}) cytochrome b_5. Middle: Phe^{78}Tyr mutant of cytochrome b_5. Top: Leu^{27} > Val mutant of (Ala^1–Ser^{104}) cytochrome b_5. For the wild type protein the assignments from left to right are: heme C7 methyl (orientation B), heme C18 methyl (orientation B), C3 vinyl α (orientation A), heme C12 methyl (orientation A), heme C17 propionate α (orientation A), C8 vinyl α minor isomer (orientation B).

entation of the Phe side chain in the crystal structure [8] where part of the aromatic ring, and in particular the HZ proton, is exposed to the solvent. In the structure derived using 3D NMR spectroscopy Phe^{78} lies not in a well defined β strand but in a turn region that extends from residues 76 to 79 yet still remains an integral of the heme binding pocket [11]. It is possible that the Phe side chain lies closer to the heme than predicted from the crystal structure and exerts an effect directly on the heme group. However, Phe^{78} is also close to Phe^{39} and may make van der Waals contact as part of an aromatic network that also includes His^{43}. This distance will alter upon addition of a hydroxyl group in the Tyr mutant with additional hydrogen bonding or disruption to the base of the heme binding pocket being obvious structural consequences. For the $Leu^{27} > Val$ substitution there is a clear decrease in side chain volume but the precise structural perturbations accompanying this mutation are currently unclear. The net result is the relative stabilisation of orientation B (the minor isomer in bovine cytochrome b_5) where the C8 vinyl occupies the position of the heme C2 methyl of orientation A. Together these data, particularly for the $Phe^{78} > Tyr$ substitution, point to the subtlety of prosthetic group-protein interactions in cytochrome b_5 and further high resolution structural and functional characterisation of these mutants will elucidate the molecular basis for these transitions.

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References

[1] Strittmatter, P. and Velick, S.F. (1956) J. Biol. Chem. 221, 253–264.
[2] Lederer, F. (1994) Biochimie 76, 674–692.
[3] Guiard, B. and Lederer, F. (1977) Eur. J. Biochem. 74, 181–190.
[4] Guiard, B. and Lederer, F. (1979) Eur. J. Biochem. 100, 441–453.
[5] Lê, K.H.D. and Lederer, F. (1983) EMBO J. 2, 1909–1914.
[6] Johnson, J.L. and Rajagopalan, K.V. (1977) J. Biol. Chem. 252, 2017–2025.
[7] Lederer, F., Ghrir, R., Guiard, B., Cortial, S. and Ito, A. (1983) Eur. J. Biochem. 132, 95–102.
[8] Guiard, B. and Lederer, F. (1979) J. Mol. Biol. 135, 639–650.
[9] Durley, R.C.E. and Mathews, F.S. (1996) Acta Cryst. D 52, 65–76.
[10] Xia, Z.-X. and Mathews, F.S. (1990) J. Mol. Biol. 212, 837–863.
[11] Mathews, F.S., Czerwinski, E.W. and Argos, P. (1979) in: The Porphyrins (Dolphin, D., Ed.) Vol. 7, pp. 107–147, Academic Press, New York.
[12] Muskett, F.W., Kelly, G.P. and Whitford, D. (1996) J. Mol. Biol. 258, 172–189.
[13] Keller, R.M. and Wuthrich, K. (1980) Biochim. Biophys. Acta 621, 204–217.
[14] La Mar, G.N., Burns, P.D., Jackson, J.T., Smith, K.M., Langry, K.C. and Strittmatter, P. (1981) J. Biol. Chem. 256, 6075–6079.
[15] McLachlan, S.J., La Mar, G.N., Burns, P.D., Smith, K.D. and Langry, K.C. (1986) Biochim. Biophys. Acta 874, 274–284.
[16] Walker, F.A., Emrick, D., Rivera, J.E., Hanquet, B.J. and Buttlaire, D.H. (1988) J. Am. Chem. Soc. 110, 6234–6240.
[17] Lee, K.-B., La Mar, G.N., Kehres, L.A., Fujinari, E.M., Smith, K.M., Pochapsky, T.C. and Sligar, S.G. (1990) Biochemistry 29, 9623–9631.
[18] Guiles, R.D., Altman, J., Lipka, J.J., Kuntz, I. and Waskell, L. (1990) Biochemistry 29, 1276–1279.
[19] Rivera, M., Barillas-Mury, C., Christensen, K.A., Little, J.W., Wells, M.A. and Walker, F.A. (1992) Biochemistry 31, 12233–12240.
[20] Smith, M.A., Napier, J.A., Stymne, S., Tatham, A.S., Shewry, P.R. and Stobart, A.K. (1994) Biochem. J. 303, 73–79.
[21] Pochapsky, T.C., Sligar, S.G., McLachlan, S.J. and La Mar, G.N. (1990) J. Am. Chem. Soc. 112, 5258–5263.
[22] Barrettino, D., Feigenbutz, M., Valcarel, R. and Stunnenberg, H.G. (1994) Nucleic Acids Res. 22, 541–542.
[23] Hewson, R., Newbold, R.J. and Whitford, D. (1993) Protein Eng. 6, 953–964.
[24] Guiles, R.D., Basus, V.J., Kuntz, I. and Waskell, L. (1992) Biochemistry 31, 11365–11375.
[25] Veitch, N.C., Concar, D.W., Williams, R.J.P. and Whitford, D. (1988) FEBS Lett. 238, 49–55.
[26] Veitch, N.C., Whitford, D. and Williams, R.J.P. (1990) FEBS Lett. 269, 297–304.
[27] Lloyd, E., Ferrer, J.C., Funk, W.D., Mauk, M.R. and Mauk, A.G. (1994) Biochemistry 33, 11432–11437.
[28] Vergeres, G., Chen, D.Y., Wu, F.F. and Waskell, L. (1993) Arch. Biochem. Biophys. 305, 231–241.