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Pro→Ala-35 *Rhodobacter capsulatus* cytochrome c_2 shows dynamic not structural differences

A ^{1}H and ^{15}N NMR study

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Comparative analysis of nuclear Overhauser effects show that the time average conformation of the wild-type and mutant Pro→Ala-35 *Rhodobacter capsulatus* cytochrome c_2 are indistinguishable. The ring resonances of Phe-51 and Tyr-53 show that their ring flip rates increase in P35A. NH proton exchange studies show that the exchange rates of the NH of Gly-34 and the $N_\pi H$ of His-17 increase by $\approx 10^2$ in P35A suggesting that their respective hydrogen bonds are destabilized in this protein. However, $^3J_{\alpha NH}$, ^{1}H and ^{15}N chemical shift data argue that these bonds are intact. These data are compatible if the replacement of a Pro with an Ala residue forms a cavity or increases local flexibility thus reducing steric hinderance and increasing solvent accessibility.

Cytochrome; Cytochrome c_2; NMR

1. INTRODUCTION

The midpoint reduction oxidation potential of the *c*-type cytochromes is believed to be determined by a number of factors including the nature of the Fe ligands, imidazole orientation, ionization of the heme propionates and the relative hydrophobicity of the heme environment ([1] and references therein). The sixth heme-Fe ligand is always His and is found at the C-terminal end of the N-terminal helix. The His ring N_τ is coordinated with the Fe while the proton of the ring $N_\pi H$ is hydrogen bonded with a strictly conserved Pro, located at position 35 in *Rhodobacter capsulatus* cytochrome c_2 (fig.1). Evidence for this hydrogen bond includes the X-ray structures of tuna cytochrome *c* [2] and *Rhodospirillium rubrum* cytochrome c_2 [3,4] and in ^{1}H NMR experiments of *R. rubrum* cytochrome c_2 where the $N_\pi H$ slowly exchanges for deuterium [5]. This Pro is believed to be invariant as it would constrain the protein backbone and orient its carbonyl to form this hydrogen bond. The recent cloning and over-expression of *R. capsulatus* cytochrome c_2 and its Pro→Ala-35 (P35A) mutant has initiated an investigation of the role of this Pro in structure and function. In that regard, the near complete assignment of the ^{1}H and ^{15}N NMR spectra of *R. capsulatus* cytochrome c_2 have been achieved [6]. Circular dichroism, NMR and near-infra-red spectroscopies show that P35A is structurally indistinguishable from the wild type (Gooley et al., in preparation). The mutantt is functional in vivo and has a midpoint redox potential only 10 mV less than the wild-type protein. However, both the oxidized and reduced forms of the mutant protein are 1 kcal/mol less stable than the respective wild-type forms (M.A. Cusanovich and M.S. Caffrey, personal communication).

This paper shows that while the time average structures of the wild-type and P35A cytochrome c_2 are indistinguishable, the rings of Phe-51 and Tyr-53 increase in flip rates.

In addition, the NH exchange rates for the $N_\pi H$ of His-17 and NH of Gly-34 increase in P35A suggesting destabilization of their respective hydrogen bonds. However, $^3J_{\alpha NH}$, ^{1}H and ^{15}N chemical shift data argue that these hydrogen bonds are intact. The data are compatible if there is a cavity created or an increase in local flexibility that both reduces steric hinderance and increases solvent accessibility.

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Abbreviations: NMR, nuclear magnetic resonance spectroscopy; 1D, one-dimensional; 2D, two-dimensional; HMQC, 2D heteronuclear multiple quantum correlated spectroscopy; TOCSY, 2D total correlated spectroscopy; DQF-COSY, 2D double quantum filtered correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy

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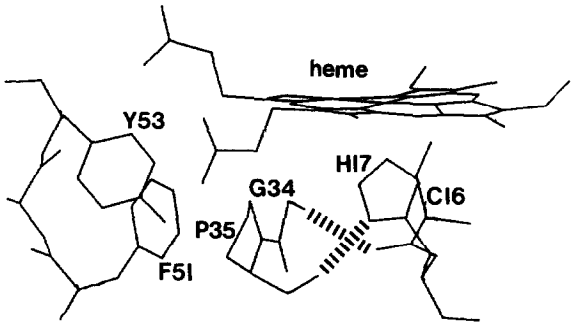

Fig.1. Model of the local environment of Pro-35 in *R. capsulatus* cytochrome c_2 based on the X-ray coordinates of *R. rubrum* [4]. The hydrogen bonds between the NH of Gly-34 and CO of Cys-16 and between the $N_\pi H$ of His-17 and CO of Pro-35 are shown.

2. EXPERIMENTAL

Recombinant bacteria were grown by respiration (aerobic, dark) on RCVB minimal media [7] supplemented with tetracycline (2.5 μg/ml) in a 16 liter fermentor. ^{15}N-enriched cytochrome c_2 was obtained by replacing the nitrogen source in the media with $(^{15}NH_4)_2SO_4$ (Isotech). Cytochrome c_2 was purified according to the method of Bartsch [8] except that cell extracts were prepared by sonication or french press and cell debris was removed by centrifugation at 130 000 × *g* for 2 h. Samples of ferrocytochrome c_2 for NMR were prepared by reducing the protein with dithionite, dialyzing and concentrating to 1–3 mM in 50 mM phosphate, 0.5 mM dithiothreitol, pH 6 at 4°C.

NMR spectra were recorded on a Bruker AM-500. Conventional pulse sequences for ^1H-^{15}N HMQC [9] and TOCSY [10,11] were used. Competing ROE effects in the TOCSY experiment were minimized with a delay 2.4 times the 90° pulse inserted between each pulse of each block of the MLEV-17 spin lock [12]. To reduce baseplane distortions, the phases of the receiver and of the signal were optimized [13].

3. RESULTS

In fig.2 the aromatic regions of TOCSY spectra of reduced P35A and wild-type cytochrome c_2 are shown. For the wild-type protein the connectivities of Tyr-53 show that the ring of this residue is slowly flipping on the NMR time scale. Similarly, the broad resonances of Phe-51 show that this ring is flipping at an intermediate rate. For P35A, under the same conditions, the connectivities for these rings show that the ring of Phe-51 flips at a fast rate and Tyr-53 at an intermediate rate. In both proteins the H4 ring proton of Phe-51 shows NOEs to both αH protons of Gly-34 indicating that this ring is near Pro-35. Indeed based on the X-ray structure of *R. rubrum* cytochrome c_2 [4] the ring of Phe-51 and the sidechain of Pro-35 are expected to be oriented parallel to each other in *R. capsulatus* (fig.1). Similarly NOEs between Phe-51 and Tyr-53 show these residues are near each other in both wild-type and P35A. The OH group of Tyr-53 is observed in NOESY spectra of P35A and wild-type indicating that it is in slow exchange. This observation is consistent with the OH being hydrogen bonded to the rear heme propionate [2–4]. These data show that the time average orientations of the rings of Phe-51 and Tyr-53 along their C(1)–C(4) axes are the same in both proteins.

To further observe the consequences of the mutation on protein dynamics, the proton-deuterium exchange of the resolved $N_\pi H$ of His-17 was followed by 20 min 1D ^1H acquisitions over a time course of 30 days. Rate constants were calculated exponential least-squares analysis. This proton exchanges with a rate constant of $0.58 \pm 0.06 \times 10^{-5}$ min^{-1} in wild-type and $0.22 \pm 0.01 \times 10^{-3}$ min^{-1} in P35A. Similarly, exchange of peptide NH protons were followed by 25 min ^1H-^{15}N HMQC acquisitions recorded over a 5-day period. The most significant observation in these spectra is the exchange rate of the NH of Gly-34. In wild-type this proton shows an exchange rate of $0.44 \pm 0.04 \times 10^{-3}$ min^{-1} while in P35A the proton exchanges faster than can be detected by these experiments ($> 0.5 \times 10^{-1}$ min^{-1}).

While the above results suggest that the respective hydrogen bonds are destabilized, analysis of chemical shift data does not agree with this conclusion. For example, in the ^{15}N spectrum of P35A the N_π resonance shifts by 1 ppm further downfield and the N_τ resonance shifts by 0.26 ppm further upfield, relative to their respective resonances in the wild-type (fig.3). Previous studies on the ^{15}N chemical shift of His residues correlates chemical shift with protonation state and hydrogen bond formation [14,15]. The chemical shifts of the His-17 ring nitrogens agree that the $N_\pi H$ is hydrogen bonded [5]. Furthermore the differences in chemical shift between wild and P35A argue that the hydrogen bond of the $N_\pi H$ of His-17 and CO of residue 35 is slightly shorter in P35A. In support of a shorter hydrogen bond, the $N_\pi H$ resonance shifts 0.1 ppm further downfield in the ^1H spectrum of P35A relative to this resonance for wild-type [16]. These arguments can be extended to the hydrogen bond between Gly-34 and Cys-16. The ^1H chemical shift of the NH resonance of Gly-34 is 7.1 ppm in both wild-type and P35A suggesting that the length of this hydrogen bond is the same in both proteins. The chemical shift of this NH proton is consistent with a long hydrogen-oxygen distance [17]. Assuming no ring current effects and using the formula $\Delta\delta_N = 19.2 d_N^{-3} - 2.3$, where $\Delta\delta_N$ is the observed ^1H chemical shift of the NH proton minus the random coil value and d_N^{-3} is the distance between the NH proton and oxygen acceptor, the hydrogen bond between the NH of Gly-34 and CO of Cys-16 is calculated to be 2.7 Å. This value compares favourably to 2.7 Å for tuna cytochrome *c* [2] and 2.9 Å for *R. rubrum* cytochrome c_2 [4]. In fig.4 cross-sections parallel to the ^{15}N dimension of a high resolution ^1H-^{15}N HMQC spectrum [18] for a number of ^{15}NH resonances of P35A and wild type cytochrome c_2 are shown. Measured $^3J_{\alpha NH}$ show that the dihedral angle ϕ is the same for Gly-34 of P35A

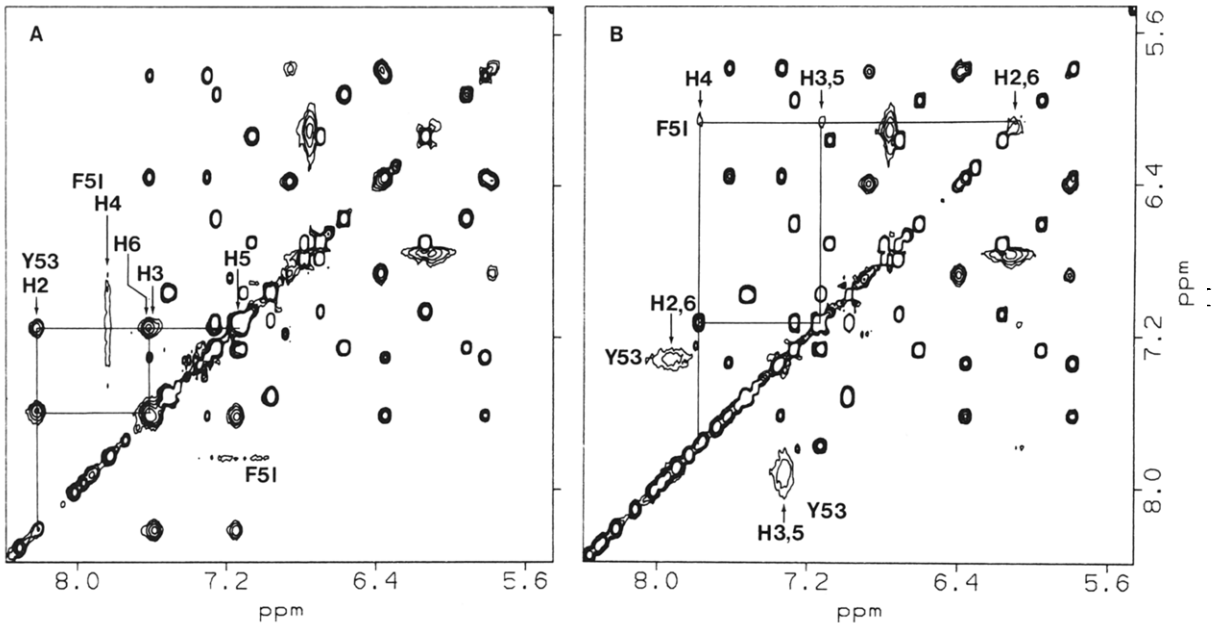

Fig.2. Section of a TOCSY spectrum showing the connectivities for the aromatic resonances of (A) wild-type and (B) P35A *R. capsulatus* ferrocytochrome c_2. The connectivities for Phe-51 and Tyr-53 are labelled. The spectra were acquired with a mixing time of 70 ms on 2–3 mM cytochrome c_2 in D_2O, 50 mM phosphate, 0.5 mM dithiothreitol at 30°C and pH 6. Assignments were determined from a combination of DQF-COSY, NOESY and TOCSY spectra recorded at 30 and 40°C.

and of wild-type. Similar measurement of $^3J_{\alpha NH}$ of His-17 suggests that the peptide unit of the NH of His-17 and the CO of Cys-16 has a similar orientation in both proteins. These data argue that the orientation and electronic environment of the NH proton of Gly-34 is the same in both proteins and that the hydrogen bond with Cys-16 is intact.

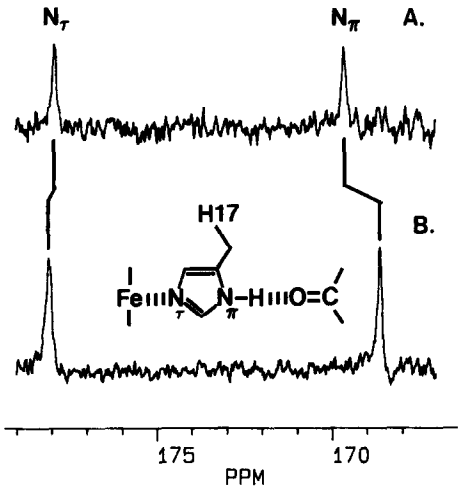

Fig.3. Part of the 1D ^{15}N spectra of (A) wild-type and (B) P35A *R. capsulatus* ferrocytochrome c_2. The ring nitrogens of His-17 are labelled. Spectra were acquired on 2–3 mM cytochrome c_2 in 90% H_2O/10% D_2O, 50 mM phosphate, 0.5 mM dithiothreitol at 30°C and pH 6. ^{15}N chemical shifts are referenced to $(^{15}NH_4)_2SO_4$ at 22.3 ppm.

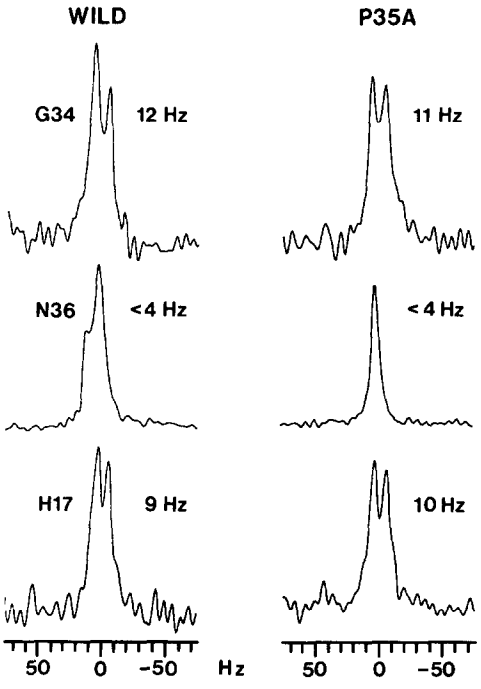

Fig.4. Cross-sections parallel to the ^{15}N axis of ^1H-^{15}N correlations from HMQC spectra of wild-type and P35A. The spectra were processed with no resolution enhancement in the ^{15}N dimension and zero-filled to 0.73 Hz/point. Spectra were acquired on 1–2 mM cytochrome c_2 in 90% H_2O/10% D_2O, 50 mM phosphate, 0.5 mM dithiothreitol at 30°C and pH 6. Measured $^3J_{\alpha NH}$ for the two neighboring residues of residue 35, Gly-34 and Asn-36, show no significant difference arguing that the mutation does not effect the orientation of the backbone. The shoulder on the side of the wild-type Asn-36 resonance has been assigned to the αNH of Lys-77.

4. DISCUSSION

In addition to reducing the constraint of the protein backbone the mutation Pro→Ala-35 in *R. capsulatus* cytochrome c_2 will introduce an additional NH proton and remove a CH_2-CH moiety. These changes may have direct effects on the protein's stability. The predicted destabilization for exchanging a proline for an alanine residue in an hydrophobic environment is approximately 0.24 kcal/mol [19]. Other workers, however, have shown that the loss of a -CH_2- group can reduce a protein's stability by 1 kcal/mol, suggesting that the loss of a CH_2-CH moiety should further destabilize a protein [20].

Guanidine-HCl titrations show that P35A is 1 kcal/mol less stable than wild-type cytochrome c_2. The above general observations suggest that the difference may be accountable by the simple replacement of the Pro for Ala residue. As the NOE data indicate that the time average structures of P35A and wild-type are indistinguishable, the mutation has either left a cavity or increased the flexibility of the protein in the region of the mutation. This alteration is reflected in the increased NH exchange rates which, as argued above, must be due to increased solvent accessibility and not hydrogen bond destabilization. The proposal of a cavity argues that NH exchange may occur by a solvent penetration model [21], whereas increase in conformer populations argues that NH exchange may occur by a local unfolding model [22].

Assuming a Van der Waal's radius of 2 Å for a tetrahedral -CH_2- group, the loss of a CH_2-CH moiety would leave a cavity of 30–40 $Å^3$ easily accommodating an H_2O molecule which requires 11.5 $Å^3$ [23]. Solvent access may be increased by additional side chain motions, as indicated by the increased ring flip rates of Phe-51 and Tyr-53. An internal water molecule requires the formation of three hydrogen bonds, either to other nearby water molecules or to polar groups of the protein. The NH of Ala-35 is available to form a hdyrogen bond and additional groups such as the CO of Ala-35 and Cys-16 may form partial hydrogen bonds with water and therefore accelerate the exchange of the NH of Gly-34 and the $N_\pi H$ of His-17. Equally compatible with the data is a model samples a larger number of conformers, including unfolded species, for P35A but where the average conformer is the same as the wild-type. In this model, instead of a cavity providing solvent access, the additional conformers would expose the NH protons to the solvent thus increasing their exchange rates.

It has recently been suggested that measurement of the exchange rate of the $N_\pi H$ of the His heme-ligand may correlate with the midpoint redox potential [5]. Clearly, in this paper, it is shown that the exchange rate of this proton does not necessarily correlate with the redox potential and argues that the protein can tolerate a more flexible and hydrated environment in this region. A more precise correlation with redox potential may be the chemical shift of the N_τ and N_π nuclei which would indicate strength or length of the hydrogen bond between the $N_\pi H$ of the His and the CO of the Pro. It may be observed for *c*-type cytochromes that a combination of the hydrogen bond strength and hydrophobicity of this region correlates with redox potential.

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