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High-resolution ^{1}H- and ^{15}N-NMR studies of *Rhodospirillum rubrum* cytochrome c_2

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Rhodospirillum rubrum **cytochrome c_2 was uniformly enriched in ^{15}N and studied by ^{1}H- and ^{15}N-NMR spectroscopy. Relaxation and NOE data allowed determination of the rotational correlation time and indicated more rapid side-chain motion in the native protein and increased segmental motion in the base-denatured protein. The π nitrogen of the ligand histidine and the indolic nitrogen of the invariant tryptophan both remain protonated and act as proton-donors in hydrogen bonds over a wide pH range and therefore do not contribute to pH-related changes in the midpoint potential. pK values identified by numerous methods in the ferrocytochrome at pH 6.9 and in the ferricytochrome at pH 6.2 arise from His-42. At pH values below the pK, the imidazolium group participates in a salt bridge or in a hydrogen bond with the carboxylate group of the inner propionate of the heme. Loss of the proton causes a local conformational change which alters the midpoint potential. The pK values of the amino terminus and lysines were also determined from pH titrations monitored by ^{15}N-NMR. Similar titrations of the ferricytochrome monitored by ^{1}H-NMR showed structural heterogeneity in that the resonance of heme ring methyl 8 split into a doublet as the pH was raised.**

Cytochromes *c* were among the first proteins to be studied in detail using high-resolution NMR spectroscopy (e.g., Refs. 1–4), not only because of their intrinsic importance in biochemistry, but also because they were accommodating candidates for study by the method. They are small, soluble, and (often) readily available, they have an interesting and informative optical spectrum, a structurally responsive midpoint potential, and interesting and informative chemical shift mechanisms. Bacterial cytochromes have the added advantage of being readily enriched in ^{13}C, ^{15}N or ^{2}H and present the potential for convenient genetic manipulation. Early NMR studies of bacterial cytochromes (e.g., Ref. 5) indicated that the NMR spectra of different cytochromes were indeed different from one another and from spectra of mitochondrial cytochromes and could therefore be used profitably to study their structures.

Some of the initial information provided by NMR studies of cytochromes *c* included the following observations. Methionine is the sixth ligand in both oxidation states [4,5]. The unpaired electron of ferricytochromes *c* is delocalized into the heme ring and into the extraplanar ligands (e.g., Refs. 1, 4–7). The methionine is displaced as ligand in the ferricytochrome at high pH (the 'alkaline isomerization') [7] and upon addition of azide [8]. There is a neutral-pH ionization that affects the NMR spectrum and midpoint potential of cytochromes c_2 that is not observed in the mitochondrial cytochrome [9]. Ferri- and ferrocytochrome *c* exchange electrons at an appreciable rate and the exchange can be used to assign resonances [4,6,9]. Some more recent contributions include the observation that the iron–sulfur bond length [10] and the stereochemistry about the sulfur (reviewed by the original authors in Ref. 11) can alter the midpoint potential.

Some of the important questions remaining include the following: What interactions are important in maintaining or altering the E_m of cytochromes *c* and c_2? What groups are responsible for the pH-dependent behavior of the midpoint potential and for triggering the alkaline isomerization? Although the latter question may appear trivial because it occurs at nonphysiological pH, it is actually very important in terms of the stability of the cytochrome structure, since the single ionization alters the structure significantly. Protonations and de-

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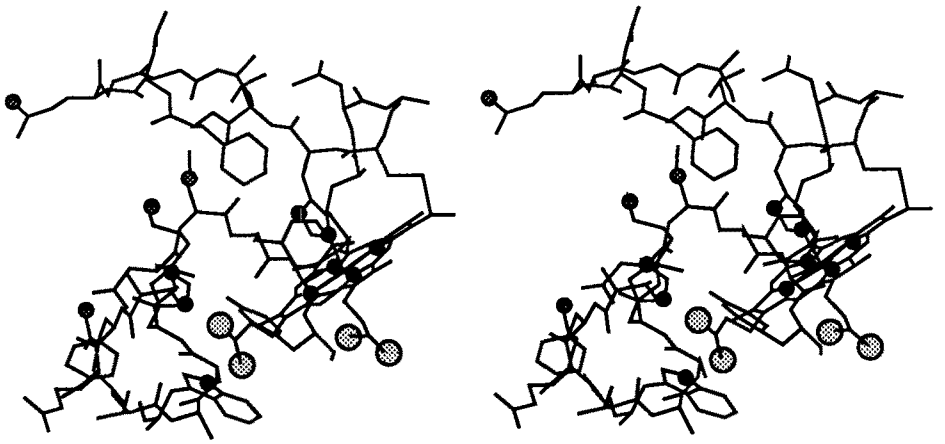

Fig. 1. The structure of cytochrome c_2 in the region near the heme as determined by X-ray crystallography. Side-chain nitrogen atoms are shown as small filled or shaded spheres; the propionate oxygen atoms are shown as grey spheres. Trp-62 is toward the bottom, His-42 is toward the left; the heme and ligand His-18 are toward the right.

protonations in the neutral pH range must involve nitrogen-containing groups or abnormally basic carboxylates (most cytochromes c_2 have no free cysteine residues). We therefore focused directly on nitrogen-containing groups near the iron or those associated with the heme, in particular, the ligand His-18, His-42 and the invariant Trp-62. The π nitrogen of the ligand histidine is said to be a proton donor in a hydrogen bond, the strength of which could alter the ligand strength of the the τ nitrogen [12–14]. His-42 and Trp-62 are near the inner heme carboxylate, and their participation in hydrogen bonding or ion-pair formation could affect the orientation of the heme. The relative location of the groups near the heme is shown in Fig. 1.

^{15}N chemical shifts, especially of sp^2-hybridized nitrogens, are quite responsive to changes in protonation state, hydrogen bonding and metal ligation. In addition, the magnitude and sign of the ^1H-^{15}N nuclear Overhauser effect (NOE) are sensitive to the rate of molecular motion in the range of molecular tumbling rates for small proteins. Therefore, ^{15}N can provide a completely nonperturbing reporter group which is sensitive to the interactions that must be measured in order to understand the structure, stability and E_m of cytochromes *c*. Unfortunately, ^{15}N occurs with a natural abundance of only 0.37% and has a gyromagnetic ratio (γ) of about one-tenth that of protons. Signal-to-noise in NMR is proportional to concentration and to γ^3, so it is necessary to enrich samples in ^{15}N even to obtain sensitivity 1/1000th that of protons. Signals from ^{15}N atoms that do not have an appreciable scalar coupling constant for a proton or those completely decoupled by exchange cannot be observed either using polarization transfer (INEPT or DEPT) or by reverse polarization transfer (RPT, indirect detection).

Materials and Methods

Cytochrome c_2 was enriched in ^{15}N by growth of the G-9 mutant of *R. rubrum* on [^{15}N]ammonium sulfate and subsequent purification of the protein as described previously [15]. ^{15}N- and ^1H-NMR spectra were recorded at 50.67 and 500 MHz, respectively, using a GE NT-500 spectrometer. Relevant parameters are given in the text. ^{15}N chemical shifts are reported relative to external 1 M $^2HNO_3$ in ^2H_2O.

Results and Discussion

The ^{15}N-NMR spectrum of ferrocytochrome c_2 is shown in Fig. 2. The resonances labeled 1, 2, 5 and 6 arise from the pyrrole nitrogens. Resonances 3 and 4, which are visible only at low pH, arise from the imidazolic protons of His-42. Resonance 7 and the doublet 8 + 9 arise from the ligand His-18 τ and π nitrogens, respectively. Resonances 10 and 11 are from proline imide nitrogens. The indolic nitrogen resonance of Trp-62 is unresolved at 246 ppm. Peptide resonances appear between 245 and 260 ppm; amides appear slightly upfield of 260 ppm. The amino-terminal nitrogen appears between 330 and 345, depending on the pH, and the ϵ-amino groups of lysine appear clustered near 340 ppm.

Theoretical plots of the dependence of relaxation times and NOE's on correlation time are given in Ref. 15. When the nuclear Overhauser effect is elicited by saturating the proton resonances between acquisitions, the resonances of the lysines and the free amino terminus are inverted, indicating that their correlation times are less than a nanosecond. The resonances of more rigid

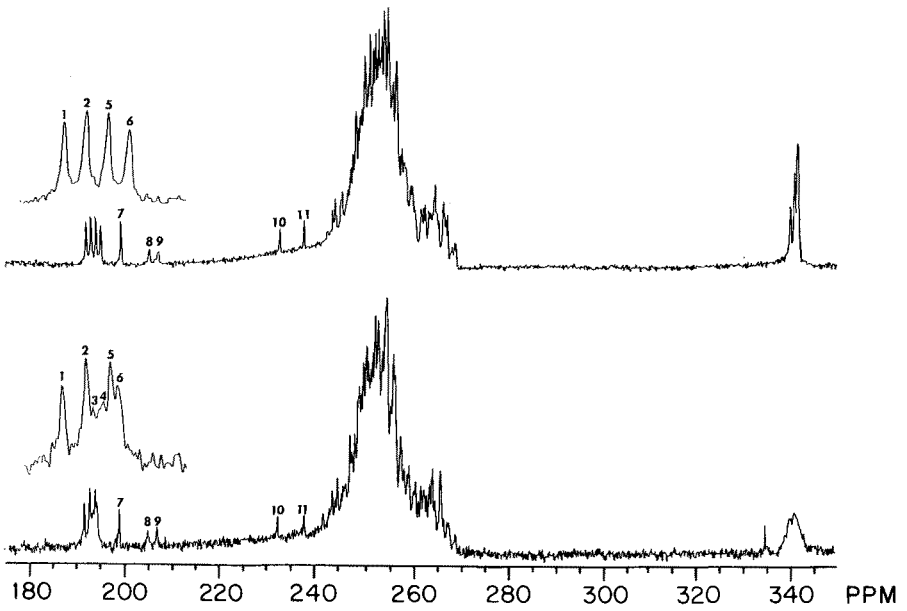

Fig. 2. The ^{15}N-NMR spectrum of cytochrome c_2 taken in H_2O without proton decoupling at pH 8.8 (upper trace) and at pH 4.9 (lower trace).

groups including the pyrrole nitrogens, ligand histidine side-chain nitrogens and the bulk of the peptide nitrogen resonances are reduced to about 60–80% of their normal intensity by the NOE, consistent with a correlation time of a few nanoseconds. The NOE's of base-denatured cytochrome indicate that much of the protein has enhanced mobility compared to the native protein [16]. Exchange-mediated NOE's in which only solvent protons are irradiated indicate enhanced access to solvent protons in the denatured cytochrome [16].

From the observed coupling to a proton and from its chemical shift, it is clear that the π nitrogen of the ligand histidine in both oxidation states is protonated over the neutral pH range and is hydrogen-bonded below pH 9 [17,18]. The proton exchanges extremely slowly [15–17]. Thus, changes in protonation state or hydrogen bonding involving the ligand histidine can not be involved in altering the midpoint potential. Likewise, the indolic nitrogen of Trp-62 does not lose its proton. By comparison to the chemical shifts of indoles in hydrogen-bonding versus non-hydrogen-bonding solvents, it is clear that this nitrogen is a proton donor in a hydrogen bond. The chemical shifts of the Trp-62 nitrogen and proton change with pH, but only by a small amount. In the ferrocytochrome, the pK of 7.0 determined from these changes is the same as that determined from the small change in chemical shifts of the pyrrole nitrogens. Several other resonances shift with a pK indistinguishable from this one and the shifts must therefore represent a local conformational change rather than a simple deprotonation. In the ferricytochrome, there appears to be a similar ionization, but with a pK of 6.2. The changes in chemical shift are larger in the ferricytochrome because they reflect changes in the hyperfine and pseudocontact shifts, suggesting changes in the electronic structure or orientation of the heme. Despite evidence from other cytochromes that ionizations having these pK values arise from the inner propionate, it is most reasonable to attribute the ionization to His-42. Neither ring nitrogen of His-42 can be observed in the ^{15}N-NMR spectrum of either oxidation state because of proton tautomerism occurring at an intermediate rate, which causes severe exchange broadening. The C2 proton of His-42 could not be observed in the ^{1}H-NMR spectrum of either oxidation state. However, a proton NOESY spectrum indicated the presence of a pH-dependent resonance close to the Leu-32, and an NOE-difference pH titration revealed that His-42 has a pK of 7.0 in the ferrocytochrome [18]. Its pK is lower in the ferricytochrome and appears to be responsible for the changes in midpoint potential and chemical shifts that occur with the pK of 6.2. If the proton lost by His-42 at pH 6.2 is one shared with the heme propionate in a hydrogen bond, it is not meaningful to assign the ionization to one group or the other. If the His-42-propionate interaction is a charge–charge interaction, a salt bridge, we assign the pK to His-42.

The pK_a values of the amino terminus (8.4 in the ferrocytochrome, 8.6 in the ferricytochrome) and the lysines were also measured. ^{1}H-NMR reveals that there are at least two distinct ionizations in the ferricytochrome that alter the chemical shifts of groups near the heme; they occur with pK values of 6.2 and 9.2. The origin of the latter transition remains obscure. Unlike other cytochromes studied by NMR, the resonance of the heme ring methyl 8 of the ferricytochrome splits at higher pH values, indicating structural heterogeneity.

In addition to providing direct structural informa-

tion, ^{15}N enrichment provides information that can be used to assign proton resonances via traditional spin decoupling or modern heteronuclear shift correlation experiments. In fact, the nitrogen chemical shift can be used as a third frequency domain to extend two-dimensional NMR to three dimensions. Currently, it appears that the entire proton and nitrogen NMR spectra can be assigned using a combination of COSY, NOESY, TOCSY and HSC experiments. Since, contrary to popular belief, NOE's can be observed in paramagnetic systems, the possibility exists that NOE-derived internuclear distances can be used to calculate the solution structure of both oxidation states of cytochrome c_2 as well as the alkaline-isomerized ferricytochrome if a single, well-defined structure exists.

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