Redox-related conformational changes in *Rhodobacter capsulatus* cytochrome c_2

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Abstract

WEFT-NOESY and transfer WEFT-NOESY NMR spectra were used to determine the heme proton assignments for *Rhodobacter capsulatus* ferricytochrome c_2 . The Fermi contact and pseudo-contact contributions to the paramagnetic effect of the unpaired electron in the oxidized state were evaluated for the heme and ligand protons. The chemical shift assignments for the ^1H and ^{15}N NMR spectra were obtained by a combination of ^1H – ^1H and ^1H – ^{15}N two-dimensional NMR spectroscopy. The short-range nuclear Overhauser effect (NOE) data are consistent with the view that the secondary structure for the oxidized state of this protein closely approximates that of the reduced form, but with redox-related conformational changes between the two redox states. To understand the decrease in stability of the oxidized state of this cytochrome c_2 compared to the reduced form, the structural difference between the two redox states were analyzed by the differences in the NOE intensities, pseudo-contact shifts and the hydrogen–deuterium exchange rates of the amide protons. We find that the major difference between redox states, although subtle, involve heme protein interactions, orientation of the heme ligands, differences in hydrogen bond networks and, possible alterations in the position of some internal water molecules. Thus, it appears that the general destabilization of cytochrome c_2 , which occurs on oxidation, is consistent with the alteration of hydrogen bonds that result in changes in the internal dynamics of the protein.

Keywords: ferricytochrome c_2 ; NMR; pseudocontact shift; *Rhodobacter capsulatus*

Rhodobacter capsulatus cytochrome c_2 is a member of an extended family of heme proteins, the class I c -type cytochromes, and participates in both the photosynthetic and respiratory electron transfer pathways (Baccarini-Melandri et al., 1978; Meyer & Kamen, 1982; Cusanovich et al., 1988; Meyer & Cusanovich, 1989; Moore & Pettigrew, 1990; Cusanovich & Caffrey, 1991). Based on its small size, extensive characterization, and important functional interactions, this 116-residue protein is an attractive subject for studies of structure–function relationships in cytochromes by NMR spectroscopy. NMR studies of *R. capsulatus* cytochrome c_2 have been reported, including the assignment of the ^1H , ^{13}C , and ^{15}N NMR chemical shifts of the reduced form of the protein (Gooley

et al., 1990; Caffrey et al., 1994), the measurement of hydrogen–deuterium exchange rates for the amide protons of the reduced and oxidized states (Gooley et al., 1991b), and the determination of the g -tensor for this protein in solution (Zhao et al., 1996).

Crystal and solution structures have been reported for a number of cytochromes. For example, the crystal structure has been determined for cytochromes c from tuna (reduced and oxidized) (Takano & Dickerson, 1981a, 1981b), yeast-iso-1 (reduced and oxidized) (Louie & Brayer, 1990; Berghuis & Brayer, 1992), horse (oxidized) (Bushnell et al., 1990), and several cytochromes c_2 (reduced): *Rhodospirillum rubrum* (Bhatia, 1981), *R. capsulatus* (Benning et al., 1991), *Rhodopseudomonas viridis* (Sogabe & Miki, 1995), and *Paracoccus denitrificans* (Benning et al., 1994). Similar secondary structures have been found for those cytochromes where solid-state structures have been determined for both oxidation states [e.g., tuna (Takano & Dickerson, 1981a, 1981b); yeast iso-1-cytochrome c (Louie & Brayer, 1990; Berghuis & Brayer, 1992)]. A comparison of the solution structures by NMR has also shown that the two oxidation states have similar structures with only minor differences with respect to the solid state; for example, horse cytochrome c (Qi et al., 1994, 1996; Banci et al., 1997b,

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1999) and *Saccharomyces cerevisiae* iso-1-cytochrome c (Baistrocchi et al., 1996; Banci et al., 1997a). The solution structure of the reduced cytochrome c_2 from *R. capsulatus* has been determined using nuclear Overhauser effect (NOE) distance constraints (Cordier et al., 1998), showing no major differences from the crystal structure of this protein (Holden et al., 1987; Benning et al., 1991) and sharing common structural motifs with other class I c -type cytochromes (Salemme et al., 1973; Bhatia, 1981; Axelrod et al., 1994; Miki et al., 1986). Mutants of *R. capsulatus* cytochrome c_2 (Caffrey et al., 1991, 1992) have also been characterized by NMR spectroscopy, particularly with regard to the destabilization of the hydrogen bonds of Gly34 and His17 in the P35A mutant (Gooley & MacKenzie, 1990) and the structural and dynamic effects of single amino acid substitutions (Gooley et al., 1991a, 1992; Caffrey et al., 1997; Zhao et al., unpubl. data). Recently *R. capsulatus* cytochrome c_2 has been shown to be an excellent model for folding studies, because its only histidine and methionine residues constitute the native heme ligands (Sauder et al., 1996).

The assignment of ferricytochrome c_2 provides a basis for comparison of the oxidized and reduced solution structures and determination of the pseudo-contact shifts (Williams et al., 1985; Feng et al., 1990a; Gao et al., 1991; Turner & Williams, 1993; Zhao et al., 1996) resulting from the presence of the paramagnetic iron in the oxidized cytochrome c_2 . Here we report the ^1H and ^{15}N resonance assignments of *R. capsulatus* ferricytochrome c_2 , including the ^1H resonances of the heme protons, obtained by a combination of two-dimensional (2D) ^1H - ^1H and ^1H - ^{15}N NMR spectra. This near complete chemical shift assignment is an expansion of the unpublished data, some of which were used in the redox evaluation of the hydrogen-deuterium exchange rates for the amide protons (Gooley et al., 1991b) and the determination of the $\mathbf{g}$ -tensor from the paramagnetic shifts (Zhao et al., 1996). The electron distribution of the heme and the attached ligands are examined using the pseudo-contact and Fermi contact shifts. Finally, in this paper, we summarize and compare the conformational changes that occur with oxidation of *R. capsulatus* cytochrome c_2 as determined by the differences in the NOE intensities between the reduced and the oxidized states, the observed and calculated pseudo-contact shifts, and the differences in the hydrogen-deuterium exchange rates of the amide protons between the two redox states (Gooley et al., 1991b).

Results and discussion

Heme assignments

The use of ^1H - ^1H and ^1H - ^{15}N 2D NMR including the WEFT-NOE spectroscopy (NOESY) and the transfer WEFT-NOESY spectra proved to be a valuable approach for the complete assignment of the ^1H and ^{15}N chemical shifts of *R. capsulatus* ferricytochrome c_2 . The chemical shift assignments of the NH and αH for the residues that are close to the heme, and especially for the heme and ligand protons, are not straightforward, due to the chemical shift effect of the unpaired electron spin of the heme Fe. For example, the NH of His17 is shifted downfield by 4.2 ppm, the αH of Met96 is shifted upfield 3.6 ppm, the C(5)H (labeled CD2 in the crystal structure) of His17 is shifted downfield by 7.67 ppm, and the ϵH_3 of Met96 is shifted upfield 5.46 ppm with respect to the reduced form (Gooley et al., 1990). Near the helix I region, the αH s of Lys14, Cys16, and Ser18 are shifted downfield by ~ 2 ppm, as has been shown for the analogous residues Ala15, Cys17, and

Thr19 in horse ferricytochrome c (Feng et al., 1989), presumably because these residues are adjacent to the heme and/or on the external face of the protein. Sections of the WEFT-NOESY and the transfer WEFT-NOESY spectra are shown in Figure 1 with connectivities traced from the methyl-1 to the methyl-5 of the heme, passing through the thiomethyl-2, thiomethine-2, thiomethyl-4, and thiomethine-4 in the heme group. The proton chemical shift assignments as well as the contact shifts and the pseudo-contact shifts calculated from the $\mathbf{g}$ -tensor (Zhao et al., 1996) are given in Table 1 for the heme and iron ligand protons for the *R. capsulatus* ferricytochrome c_2 .

Several studies have used ^1H and ^{13}C NMR to examine the electronic structure of the heme and to evaluate the Fermi contact and pseudo-contact contributions to the paramagnetic effect of the unpaired electron in the oxidized state (Turner, 1993, 1995; Banci et al., 1995, 1998; Kolczak et al., 1999). Where there is no significant change in the heme molecular structure of the oxidized state (Turner, 1993; Takano & Dickerson, 1981a, 1981b), the Fermi contact contribution may be determined from the observed redox-dependent chemical shift and the calculated pseudo-contact shift. Because the Fermi contact chemical shift is essentially determined by the π -electron spin density on the observed atom and the immediate neighbors (Karplus & Fraenkel, 1961), the values given in Table 1 demonstrate that the delocalization of the heme unpaired electron in the oxidized state is restricted to the heme, the ligated Met96 side chain and the ring of the axial His17 ligand, although there is still a significant contact shift for the His17 βH protons.

Fig. 1. Sections of the WEFT-NOESY and the transfer WEFT-NOESY spectra for the *R. capsulatus* ferricytochrome c_2 traced from the methyl-1 (M1) to the methyl-5 (M5) pass through the thiomethyl-2 (M2), thiomethine-2 (M2H), Methyl-3 (M3), thiomethyl-4 (M4), and thiomethine-4 (M4H) of the heme porphyrin group. The WEFT-NOESY with 160 ms relaxation time and 40 ms mixing time and the transfer WEFT-NOESY with 160 ms relaxation time and 100 ms mixing time were used. In the transfer WEFT-NOESY, the cross peaks between the reduced thiomethyl-2 (M2(R)) to thiomethine-2 (M2H(R)) and the thiomethyl-4 (M4(R)) to thiomethine-4 [M4H(R)] are labeled. Also in the WEFT-NOESY cross peaks between the methyl-1 to δ -meso proton (δm) and methyl-5 to β -meso proton (βm) as well as between the methyl-3 to βH of Phe98 (βF98), and methyl-5 to propionate-6 α (6α), -6 β (6β) protons, and αH (αM96) and βH (βM96) of Met96 are assigned.

Table 1. Chemical shifts of the heme moiety and iron ligand protons as well as their separated contact and pseudocontact contributions to the hyperfine chemical shift for *R. capsulatus* cytochrome *c*₂

Residue	Proton	Chemical shift		Hyperfine shift (ppm)	Calculated pseudocontact shift (ppm) ^b	Contact shift (ppm) ^c
		Oxidized (ppm)	Reduced (ppm) ^a			
Heme	α -meso	4.44	9.13	-4.69	-6.86	2.17
	β -meso	1.87	9.67	-7.80	-11.26	3.46
	γ -meso	8.98	9.57	-0.59	-7.01	6.42
	δ -meso	1.87	9.15	-7.28	-10.69	3.41
	Methyl-1	14.88	3.50	11.38	-5.24	16.62
	Methyl-3	33.57	3.03	30.54	-2.21	32.75
	Methyl-5	20.10	3.72	16.38	-5.11	21.49
	Methyl-8	33.07	2.20	30.87	-3.18	34.05
	Thiomethyl-2	0.17	2.28	-2.11	-3.01	0.90
	Thiomethine-2	0.44	5.44	-5.00	-4.90	-0.10
	Thiomethyl-4	2.45	2.26	0.19	-1.98	2.17
	Thiomethine-4	1.96	6.35	-4.39	-2.14	-2.25
	Propionate-6 α	10.81	4.30	6.51	-3.96	10.47
		1.14	4.67	-3.53	-4.60	1.07
	Propionate-6 β	0.62	3.57	-2.95	-1.76	-1.19
		1.84	2.67	-0.83	-1.96	1.13
	Propionate-7 α	10.57	4.38	6.19	-1.65	7.84
		13.60	3.70	9.90	-2.05	11.95
	Propionate-7 β	-0.26	3.01	-3.27	-2.98	-0.29
		0.86	2.72	-1.86	-2.27	0.41
His17	α H	8.46	3.19	5.27	5.32	-0.05
	β H	14.38	1.13	13.25	7.69	5.56
		8.43	0.69	7.74	6.61	1.13
	C(2)H	-0.53	0.41	-0.94	31.78	-32.72
	C(5)H	7.73	0.06	7.67	7.56	0.11
Met96	N _H	4.01	9.88	-5.87	18.39	-24.26
	α H	0.88	3.39	-2.51	-2.13	-0.38
	β H	5.99	-0.04	6.03	7.63	-1.60
		2.39	-2.58	4.97	9.62	-4.65
	γ H	-1.29	-3.62	2.33	13.70	-11.37
		0.23	-1.51	1.74	4.61	-2.87
	ϵ H	-8.39	-2.93	-5.46	13.13	-18.59

^aThe shifts of the reduced species are taken from Gooley et al. (1990).

^bThe pseudocontact shifts are calculated from the **g**-tensor (Zhao et al., 1996) and the coordinates of reduced X-ray structure (Benning et al., 1991).

^cThe contact shifts are obtained subtracting the pseudocontact shift from the hyperfine shift.

The chemical shifts of the heme and ligand protons as well as their apparent contact and pseudo-contact contributions (Table 1) to the hyperfine shifts provide a basis for the analysis of the electronic structure of the heme. The relative values of the Fermi contact contributions of the heme methyl groups appear to depend on the orientation of the axial His17 and Met96 ligands (Turner, 1995; Kolczak et al., 1999, and references therein). The bisector of the angle between the His17 nodal plane and the Met96 sulfur p-orbital nodal plane is approximately along the N_I-Fe-N_{III} heme axis. This is consistent with an asymmetrical distribution of the unpaired electron over the heme, with the larger spin density in pyrroles II and IV, as shown by the larger Fermi contact chemical shifts at methyl-3 and methyl-8. For the β and δ meso protons (Table 1), the pseudo-contact and the Fermi contact shifts are almost the same; for the α and γ meso pro-

tons, the pseudo-contact shifts are the same, but the contact shift is larger for the γ meso proton. Because the γ meso proton has the largest contact shift, it appears that there is a significant unpaired electron density in this region of the heme, possibly as a result of the Met96 sulfur lone pair, if the lone pair does not have pure p _{π} symmetry and thus points more directly at the γ than the α meso proton. The contact shifts for both thioether bridge protons are small, suggesting little unpaired electron spin density is delocalized into this region. For both axial ligands, the contact contributions on the protons decrease with the number of bonds from the iron. Although the three ring protons of His17 exhibit smaller hyperfine shifts than the horse protein (Feng et al., 1990a), for both *R. capsulatus* and horse cytochromes, the Fermi contact contribution is larger for C(2)H, which indicates that they have similar imidazole ring bond struc-

ture, with a much larger electron density at C(2) than C(5). This is consistent with the finding of Chacko and La Mar (1982) that the mechanism of spin delocalization of imidazole to Fe is using the highest energy filled π orbital of the imidazole ring, which has large electron density at the bonding nitrogen, N_7 , and also at the adjacent carbon, C(2), but a much smaller electron density at C(4) and C(5).

For methylene and methine protons attached to the heme, the Fermi contact shift depends on the spin density on the heme atom to which the group is attached and on the dihedral angle θ between the methylene (or methine) C–H bond and the normal to the heme plane. This angular dependence, which is linearly related to $\cos^2 \theta$ (Turner, 1993; Nguyen et al., 1999), would only be observed where there is restricted rotation and there is a specific orientation of the proton(s) with respect to the heme plane. If the C–H bond dihedral angle θ is 0° , that is, parallel to the heme normal, the Fermi contact contribution would be maximum for a particular electron density; if the C–H bond dihedral angle θ is 90° , that is, perpendicular to the heme normal, then the Fermi contact contribution would be minimum. For the methylene protons in propionate-6 and propionate-7, there is restricted rotation because the propionates are a part of a hydrogen bonding network. For the two propionate-7 α protons, there is a significant Fermi contact shift and they have a similar contribution (Table 1) to the chemical shift, which indicates that for the α methylene group in solution the two α protons have almost the same orientation with respect to the heme plane, as is found in the crystal structure (Benning et al., 1991). For the propionate-6 α protons, the Fermi contact contributions are quite different. For the α -proton with the smaller value, the contact shift indicates that, in solution, the α proton dihedral angle would orient the C–H close to the heme plane (perpendicular to the heme normal) and the second α -proton nearly parallel to the heme normal, again as observed in the crystal structure (Benning et al., 1991).

Protein assignments

The assignments for the main-chain sequential connectivities were determined from NOESY and ^1H – ^{15}N heteronuclear multiple quantum coherence (HMQC)-NOESY spectra based on the sequential approach (Wüthrich, 1986) and the main chain directed (MCD) analysis (Englander & Wand, 1987). The majority of spin systems were assigned to residue type with total correlation spectroscopy (TOCSY) and double-quantum filtered correlation spectroscopy (DQF-COSY) spectra either in H_2O or D_2O . Because the ^1H – ^{15}N coupling constants are large (90 Hz), and result in strong cross peaks for the amide group in the ^1H – ^{15}N spectra, some spin systems that were not observed in the TOCSY spectrum were present in the ^1H – ^{15}N HMQC spectrum (such as Met96, Gly80, Cys16, Gly46, and Gly34). All the ^{15}NH resonances were identified by ^1H – ^{15}N HMQC spectroscopy, except for the amino terminus and the prolines (the ^1H – ^{15}N HMQC spectrum is available in Supplementary material in the Electronic Appendix, Fig. S1). The NOE connectivities of NH_i – NH_{i+1} in the ^1H – ^{15}N HMQC-NOESY spectrum were compared with those of the NOESY spectrum to confirm the main-chain sequential assignments.

The chemical shifts of the side-chain protons were obtained with a combination of DQF-COSY, TQF-COSY, TOCSY, and NOESY NMR experiments in H_2O and D_2O . The complete ^1H and ^{15}N chemical shift assignments for *R. capsulatus* ferricytochrome c_2 are available in Supplementary material in the Electronic Appendix (Table S1). The majority of side-chain assignments were readily

resolved in the TOCSY spectra in H_2O with the assistance of the NOESY spectra in H_2O . The αH of Met96 was only observed in the 30 ms TOCSY, due to the short T_2 transverse relaxation time, which results from the proximity of the heme paramagnetic center. The NH – αH – βH_2 fragments of Tyr48, Tyr75, and Phe82, which were not observed in the TOCSY spectrum acquired in H_2O , were resolved from the correlation obtained by a comparison of the NOESY spectrum acquired in H_2O with the TOCSY and DQF-COSY experiments in D_2O . There are 12 aromatic substituents in this protein (5 Phe, 5 Tyr, 1 His, 1 Trp), and correlations for all residues, except for Tyr75, are observed in the DQF-COSY and TOCSY experiments. Also, intraresidue NOEs from the phenyl H(2,6) protons to the NH – αH – βH_2 fragments of these residues are observed in the NOESY experiment in D_2O , thus specifically assigning these ring spin systems. The TQF-COSY spectrum facilitated the assignment of Gly and Val residues. The αH of Gly and the βH protons of Val residues were obtained in the DQF-COSY spectrum acquired in D_2O , but the αH protons of Gly95 overlapped with one of the βH protons of Phe51, and the αH of Gly104 overlapped with one of the βH protons of Phe10. However, in the TQF-COSY spectrum acquired in D_2O , correlations between the two αH protons of Gly and between the αH and βH of Val are filtered out, thus allowing an unambiguous assignment.

As the oxidized form of this cytochrome c_2 is less stable than the reduced form by $\sim 3 \text{ kcal mol}^{-1}$, based on guanidine-HCl denaturation experiments (Caffrey & Cusanovich, 1991), a comparison of the ^1H spectra of the less rigid oxidized state of the protein with that of the more stable reduced form should reflect dynamic differences. In Figure 2, the aromatic regions of the TOCSY spectra of reduced (Fig. 2A) and oxidized (Fig. 2B) cytochrome c_2 are shown. The symmetry-related aromatic proton resonances in the Phe and Tyr residues generally collapse to a single time-averaged resonance upon oxidation of cytochromes (Feng et al., 1989; Banci et al., 1997a), such as Trp67, Tyr48, and Tyr38 and Tyr53, which is indicative of an increased rotational rate of the phenyl group in the oxidized state, as would be expected for a protein with an overall “loose” structure. It was difficult to observe the aromatic proton resonances of Tyr53 in the oxidized state because of the line broadening of the resonances due to the increase of the flipping rate of the phenyl ring. The NOE connectivities of Tyr53 (Fig. 2A) indicate that phenyl ring of this residue is flipping slowly on the NMR timescale for the reduced protein (Gooley et al., 1990), but the broad resonances (Fig. 2B) indicate that this flipping rate has increased to an intermediate rate for the oxidized form. The aromatic protons of Tyr75 were not assigned for the oxidized state, even though they are clearly observed in the reduced state (Gooley et al., 1990), possibly due to an increase in the flipping rate to an intermediate rate where the proton resonances of the phenyl group are very broad. There is one exception for this observed increase in flipping rate in the oxidized state, however, because the connectivities of the aromatic protons for Phe98 were found to be just the opposite, becoming more complex on oxidation, which will be discussed below.

NOE analysis

A summary of the short-range NOEs for *R. capsulatus* ferricytochrome c_2 , as shown in Figure 3, indicates that the secondary structural units for the oxidized form of cytochrome c_2 in solution include five long stretches of α -helices between residues 3–17, 55–65, 69–78, 80–88, and 103–115, three reverse turns including

Fig. 2. Section of a TOCSY spectrum showing the connectivities for the aromatic resonances of (A) reduced and (B) oxidized forms of *R. capsulatus* cytochrome c_2 . The connectivities for Trp67, Tyr38, Tyr48, Tyr53, and Phe98 are labeled. The spectra were acquired with a mixing time of 60 ms on 2–3 mM cytochrome c_2 in D_2O , 100 mM phosphate buffer at 30 °C and pH 5.5. Assignments were determined from a combination of DQF-COSY, NOESY, and TOCSY spectra recorded at 30 °C.

residues 37–40, 40–43 (type II), and 89–92 (type I), and an Ω loop in the region of residues 19–28, which is consistent with the solution structure of the reduced protein (Cordier et al., 1998).

The comparison of the NOE intensities of the oxidized (Fig. 3) with the reduced (Gooley et al., 1990) *R. capsulatus* cytochrome c_2

establishes that both redox states have similar secondary structures in solution. The similarity in secondary structure of the two redox states has also been reported for horse cytochrome c (Qi et al., 1994, 1996; Banci et al., 1997b, 1999) and other homologous cytochromes, as previously discussed. Because it has been shown

Summary of Short-Range NOEs (i to $i+5$)

Fig. 3. Summary of the short-range NOEs (i to $i+5$) observed in 1H - 1H NOESY and 1H - ^{15}N HMQC-NOESY spectra of *R. capsulatus* ferricytochrome c_2 . Spectra were acquired with a mixing time of 100 ms in H_2O at 30 °C and pH 5.5. Black bars indicate NOEs observed in 1H - 1H NOESY or 1H - ^{15}N HMQC-NOESY spectra, dark stippled bars indicate NOEs ambiguous in 1H - 1H NOESY, which are resolved in 1H - ^{15}N HMQC-NOESY spectra. Light stippled bars indicate $d_{\alpha\delta i+1}$ or $d_{\beta\delta i+1}$ from Pro residues. Qualitative intensities are described by the thickness of the bar. Several long-range NOEs (i to $>i+5$) are indicated for the segment 19–28, which appears to span Ω loops. Protons that show slow, intermediate, and fast NH exchange rate constants (k_m , min^{-1}) are categorized as follows: <0.0001 (○), 0.0001 – 0.001 (■), and >0.001 (●) (Gooley et al., 1991b).

that the reduced solution (Cordier et al., 1998) and crystal structures (Benning et al., 1991) are also similar, it is clear that no major changes in secondary structure occur on going from crystal to solution or oxidized to reduced. Nevertheless, differences exist that suggest subtle backbone/side-chain structural changes that occur on going from the solid state to solution or on changing the redox state.

For example, in a comparison of the redox states in solution, the connectivities of the aromatic group for Phe98 become more complex on oxidation in the TOCSY spectra (Fig. 2), which suggests that in the oxidized state Phe98 has a slower rate of rotation than in the reduced cytochrome. It is possible that this complex NMR pattern in the oxidized state could be explained by larger chemical shift differences between the protons on the two sides of the phenylalanine ring for the oxidized protein compared to the reduced. However, a large chemical shift effect would involve some specific interresidue interaction, which normally would also result in an increased barrier to rotation of the phenyl group and thus a lower rotational rate. In the NOESY spectra, the intensities of the NOE cross peaks between the β protons of Lys12 and aromatic protons of Phe98 in the oxidized form are stronger than those in the reduced form, and the cross peaks between γ and β protons of Leu100 and the H(3,5) of Phe98 were clearly observable in the oxidized form. This suggests that hindrance to rotation for the aromatic group of Phe98 in the oxidized protein is caused by the β protons of Lys12 and the side-chain protons of Leu100, which are apparently closer to the phenyl group of Phe98. This is consistent with the redox-related conformation studies, which have shown that Asn11, Lys12, Lys99, and Leu100 experience significant local conformational changes with a change in oxidation state (Zhao et al., 1996). The increased constraints on Phe98 in the oxidized cytochrome are surprising because Phe98 is in the hinge region (positions 88–102, Dumortier et al., 1998), which rotates away from the heme ~ 30 time/s. However, the equilibrium lies strongly toward the closed conformation with $<1\%$ of the hinge rotated to its open position at any time. Apparently, the increased constraints on Phe98 are minor relative to the overall destabilization in the hinge region in the oxidized cytochrome relative to the reduced form. Unfortunately, hydrogen–deuterium exchange rates are too fast to measure by NMR in the hinge region (Gooley et al., 1991b) in both redox states; hence, they do not clarify the situation.

Comparison of NMR parameters

Differences in the NOE maps between the oxidized (Fig. 3) and reduced (Gooley et al., 1990) *R. capsulatus* cytochrome c_2 can be used to qualitatively determine redox-related conformational changes. For example, some regions of *R. capsulatus* cytochrome c_2 have only minor differences between NOEs in the two oxidation states. These include residues 2–10 (helix I, N-terminal), 19–29 (Ω loop), 83–87 (helix IV), and 104–116 (helix V, C-terminal). As with horse cytochrome c (Feng et al., 1990a), the N- and C-terminal helices, although they play a critical role in the protein structure, apparently are not involved in the modulation of redox properties. The Ω loop (residues 19–29) and helix IV (segment 83–87) are insertions in *R. capsulatus* cytochrome c_2 compared to mitochondrial cytochrome c (Feng et al., 1990a; Bhatia, 1981; Takano & Dickerson, 1981a, 1981b) and are not likely to play a role in the redox properties.

Table 2 compares the various regions of *R. capsulatus* cytochrome c_2 where there is a significant conformational change with

oxidation based on the $\mathbf{g}$ -tensor pseudo-contact chemical shift calculation, the change in NOE maps of the two oxidation states, and the change in the hydrogen–deuterium exchange rate. Although the change in the exchange rates are included in Table 2, it should be noted that an increase with oxidation is not necessarily an indication of a conformational change in the protein, because changes in exchange rate generally indicate actual access to the solvent, which is not always due to a static structural change. For example, the similarity of the hydrogen–deuterium exchange rates for the amino acids in segment 83–87, irrespective of their position relative to the protein surface (Gooley et al., 1991b), is attributed to a perturbation of the cooperative unfolding equilibria (Wagner & Wüthrich, 1982; Englander & Kallenbach, 1984; Wand et al., 1986) rather than any significant static structural change. Neither the NOE nor the $\mathbf{g}$ -tensor calculation indicate any significant structural changes in these regions.

From Table 2 it is evident that considerable overlap occurs among the NOE, $\mathbf{g}$ -tensor and exchange data sets, and that the NOE and $\mathbf{g}$ -tensor data show general agreement for the structural regions that are perturbed with the change from the reduced to the oxidized state. These include residues 11–18, 32–36, 42–48, 53–69, 74–76, and the hinge region, 88–102. The structural region encompassing residues 11–18 and 32–36 includes the C-terminus of helix I with the ligated His17, and Cys13 and Cys16, which covalently bind the heme. It has been suggested that helix I (3–17) be separated into two motifs (3–9, 11–18), based on the nonhelical structure of Phe10 in the solution structure of the reduced *R. capsulatus* cytochrome c_2 (Cordier et al., 1998). Note that in Table 2 all observed structural changes in helix I are in the 11–18 region. The regions 11–18 and 32–36 could be considered as one structural region because of the hydrogen bonds between them. Based on the crystal structure, the likely hydrogen bonds are: Gly34(N) to Cys16(O), Cys16(O) to Thr33(N), Lys14(O) to Ala31(N), Ser18(OG1) to Lys32(NZ), His17(ND1) to Pro35(O) (Benning et al., 1991). Apparently, the change in oxidation state, which changes the heme electron density distribution, results in structural changes that alter the interactions between the 11–18 and 32–36 regions. Because oxidation results in a destabilization of the cytochrome c_2 , we assume that some or all of the hydrogen bonds are weakened somewhat. Figure 4 shows the 11–18 and 32–36 (dark ribbon, right side) regions. Also included in Figure 4 is Arg43, because the guanidine group NH1 and NH2 protons are hydrogen bonded to Asn36 (OD1, OD2) as well the rear heme propionate-7 (O2A), which provides an additional connection to the heme.

The second structural region to be considered, 42–47 and 53–69 (dark ribbon, bottom), is also connected by hydrogen bonds, including Phe65(O) to Ala45(N) and Trp67(O) to Gly42(N), and Trp67(N) to Arg43(O). The residues 42–47 and 53–69 comprise the bottom of the cytochrome c_2 (Fig. 4) and include the bulk of helix II and the regions (42–47 and 64–69) where there is no secondary structure. However, for this region, there are hydrogen bonds to three of the four internal water molecules and the heme propionates in the reduced cytochrome c_2 . In the region 42–48, residues Arg43(NE), Thr44(O), and Thr47(OG1 and N) are all within hydrogen bond distance with internal water 18H (Benning et al., 1991) and water 18H is hydrogen bonded to propionate-7 (O2A). For the 53–69 region, the key residues in this region include Trp67(NE1), which is also hydrogen bonded to the rear heme propionate-7 (O1A) and Tyr53(OH), which appears to interact with the rear heme propionate (O2A) and water 18H. Lys54(N), Ser56(OG), Ile57(N), and Tyr75(OH) are in hydrogen

Table 2. Summary of structural changes from NMR data

POSITION	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
g-TENSOR ^a											N α	α			α	α								α	
NOE ^b										α	N α	N α	N	N	N	N								N	N
EXCHANGE ^c														N				N	N						
2° STRUCTURE ^d																									
POSITION	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
g-TENSOR											N						N α		α						
NOE							N	N α	N					N	N		N α	N	N		N	N			α
EXCHANGE										N		N						N			N	N	N		
2° STRUCTURE																									
POSITION	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
g-TENSOR				N		N	N				α			α	N α	N α	N							N	
NOE	N			N	α	N α	N α	N	N	N	N	N		α	N	N	N	N α	N						N
EXCHANGE							N	N		N	N						N					N			
2° STRUCTURE																									
POSITION	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	00
g-TENSOR																			N		N			N	
NOE	N α	N					N						N α	N	α	N		N	α	N	N α	N α	N		
EXCHANGE	N	N					N	N	N	N	N	N													N
2° STRUCTURE																									
POSITION	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16									
g-TENSOR	N		α																						
NOE	N α	N						α		N															
EXCHANGE																									
2° STRUCTURE																									

^ag-TENSOR is noted when significant differences in chemical shifts between oxidized and reduced cytochrome of the C α H(α) or NH(N) proton were identified from the calculated pseudocontact shifts utilizing the optimized g-tensor obtained previously (Zhao et al., 1996)
^bNOE selects for adjacent residues on the protein backbone that undergo a change in the NOE intensity consistent with a conformational change of the C α H(α) or NH(N)proton of one residue to its nearest neighbors on a change in redox state.
^cEXCHANGE represents those NH(N) protons that have altered hydrogen–deuterium exchange rate on oxidation (Gooley et al., 1991b).
^d2° STRUCTURE is the elements of secondary structure identified in the crystal structure (Benning et al., 1991).

bond network involving the front heme propionate and water 31H, 43H, and 45H. Presumably, oxidation results in some changes of the hydrogen bond strength, including those involving the propionates and internal water molecules, and results in changes in NOE strength, g-tensor, and exchange rates in these regions (Table 2). Changes in the orientation of the propionate-7 chain have been

observed with oxidation in the solution structures of iso-1-cytochrome *c* (Banci et al., 1997a; Baistrocchi et al., 1996) and horse cytochrome *c* (Banci et al., 1997b, 1999). For horse cytochrome *c* (Bushnell et al., 1990; Qi et al., 1996; Banci et al., 1997b; Bertini et al., 1997), it has been shown that the position of the internal water molecules in solution is not the same as in the crystal struc-

Fig. 4. The α -carbon model, with selected side chains, of *R. capsulatus* ferrocyclochrome *c*₂ from the X-ray crystal coordinates (Benning et al., 1991). Backbone (ribbon) and selected side chains. Bound waters, shown as spheres, are from left to right, 43H, 31H, 45H, and 18H (rear most). Dark ribbons are backbone regions, 74–76/88–102 (left), 42–47/53–69 (bottom), and 11–18/32–36 (right). The remainder of the backbone is light. The side chains shown are, from left to right, Y75, W67, P35, and R43.

ture and is dependent on the redox state. It has also been shown that there are changes in the internal water structure for yeast iso-1-cytochrome c with the oxidation state (Banci et al., 1997a). The determination of the position of the internal water(s) is important because it is possible that one molecule in particular, which has been termed the "catalytic" water, plays a central role in stabilizing both oxidation states in horse cytochrome c (Bertini et al., 1997).

The third domain (74–76 and 88–102) includes the so-called hinge region (Dumortier et al., 1998), which is able to move with high frequency ($20\text{--}30\text{ s}^{-1}$) in the oxidized cytochrome, and allows exogenous ligands access to the methionine coordination site. Both Tyr75(OH) and Thr94(OG1) are hydrogen bonded to an internal water (31H), which in turn is hydrogen bonded to the carboxyl of the front propionate-6 (Benning et al., 1991). As well, there is a hydrogen bond between internal water 43H and Lys93(O), which may in part be responsible for the transmission of perturbations between this domain and the 42–47 and 53–69 region.

Conclusions

In the evaluation of the decreased stability of the oxidized form of *R. capsulatus* cytochrome c_2 compared to the reduced form for this protein, we have found that the major differences between the two redox states are apparently subtle changes, including heme–protein interactions, the orientation of the heme ligands, rearrangements of some hydrogen bonds with possible alterations in the position of some internal water molecules surrounding the heme, as seen for other cytochromes c (Takano & Dickerson, 1981a, 1981b; Louie & Brayer, 1990; Berghuis & Brayer, 1992; Qi et al., 1994; Banci et al., 1997a, 1999).

The hydrogen bond networks and corresponding structural domains perturbed by oxidation can be separated into three general groups. First, a network of interactions centered around the front heme propionate (propionate-6) and waters 31H, 43H, and 45H. This ties together the 90s region (Lys93, Thr94, Gly95, and Met96), the 50s region (Ile57, Ser56, and Lys54) as well as Tyr75. This network of interactions is principally on the left side of the cytochrome relative to the usual orientation (Fig. 4). Second, a network of interactions centered on water 18H and the rear heme propionate (propionate-7), which involves Gly42, Arg43, Thr44, Ala45, Gly46, Thr47, Tyr48, Tyr53, Phe65, and Trp67, which defines the bottom portion of cytochrome c_2 . Finally, the right side of cytochrome c_2 (Fig. 4), which includes the ligated histidine at position 17 as well as hydrogen bonds involving Pro35, Thr33, Gly34, Cys16, Ser18, and Lys14, also forms a network of interactions. It is interesting that Asn36 is strongly hydrogen bonded to Arg43, and thus the rear propionate region described above is coupled to the right side of the molecule just discussed. Although the precise changes in the hydrogen bonding networks resulting from oxidation cannot be specified at this time, oxidation does result in a general destabilization of cytochrome c_2 , consistent with the weakening or breaking of some hydrogen bonds and thus altering the internal dynamics. The results presented here do point to key amino acids where mutations should have substantial effects on the hydrogen bond networks and protein stability. The NMR data establish that the principal features of the secondary structure of reduced *R. capsulatus* cytochrome c_2 are not altered on oxidation, consistent with what has been seen with other class I c -type cytochromes.

Materials and methods

Sample preparation

R. capsulatus cytochrome c_2 and ^{15}N -enriched samples were prepared as described previously (Gooley et al., 1990). The ferri-cytochrome c_2 samples were prepared by oxidizing the protein with a small excess of potassium ferricyanide, then dialyzing and concentrating to 2–3 mM in 100 mM phosphate buffer at pH 5.5. The samples contained either 10% D_2O for the experiments in H_2O or 99.996% D_2O for the experiments in D_2O . The sample for the magnetization transfer experiment was prepared by mixing 40% fully reduced (Gooley et al., 1990) and 60% fully oxidized cytochrome c_2 solutions in 100 mM phosphate D_2O buffer at pH 5.5.

NMR spectroscopy

Homonuclear $^1\text{H}\text{--}^1\text{H}$ and heteronuclear $^1\text{H}\text{--}^{15}\text{N}$ NMR experiments were performed at 20 or 30 °C on a Bruker AM-500 spectrometer (Bruker Instruments, Boston, Massachusetts) equipped with additional hardware as previously described (Gooley et al., 1990). TOCSY (Braunschweiler & Ernst, 1983; Davis & Bax, 1985), NOESY (Jeener et al., 1979), DQF-COSY (Rance et al., 1983), TQF-COSY (Wokaun & Ernst, 1977; Shaka & Freeman, 1983), $^1\text{H}\text{--}^{15}\text{N}$ HMQC (Bax et al., 1983; Bax & Marion, 1988), and $^1\text{H}\text{--}^{15}\text{N}$ HMQC-NOESY (Gronenborn et al., 1989) spectra were used for the assignment. TOCSY spectra were recorded with mixing times of 30 and 60 ms; NOESY and $^1\text{H}\text{--}^{15}\text{N}$ HMQC-NOESY spectra were recorded with mixing times of 50 and 100 ms. WEFT-NOESY spectra (Inubushi & Becker, 1983; Chen et al., 1994) were obtained with a relaxation time of 160 ms and mixing time of 40 ms to allow relaxation only for paramagnetically relaxed protons in this protein. The transfer WEFT-NOESY NMR spectra were obtained by using the same magnetization transfer technique developed by Feng et al. (1990b). In a mixture of the oxidized and reduced forms of the *R. capsulatus* cytochrome c_2 , the protein interconverts between the two oxidation states via electron transfer in the same way as horse cytochrome c (Feng et al., 1990b). The redox exchange reaction is sufficiently rapid that a selective saturation of one state will transfer saturation to the corresponding resonance of the other oxidation state in the transfer WEFT-NOESY experiment.

All 2D spectra were recorded in pure absorption with time-proportional phase incrementation (TPPI) (Redfield & Kunz, 1975). The solvent resonance was suppressed by selective irradiation during the preparation period. 2D $^1\text{H}\text{--}^1\text{H}$ spectra were usually acquired with a spectral width of 10 kHz, 900 t_1 points, 2,048 data points in t_2 , and 128 transients for each t_1 point. Two-dimensional $^1\text{H}\text{--}^{15}\text{N}$ spectra were acquired with spectral widths of 10 kHz for ^1H and 5,000 Hz for ^{15}N , 2,048 data points in t_2 , and 128 transients for each of 900 t_1 points. WEFT-NOESY and transfer WEFT-NOESY spectra were acquired with spectral widths of 31,250 Hz, 1,024 t_1 points, 4,096 data points in t_2 , and 256 transients for each t_1 point. The data of all 2D spectra were processed on a Silicon Graphics computer work station with the program Felix (Molecular Simulations, San Diego, California). Prior to 2D Fourier transformation, the data were multiplied by shifted sine bells in both t_1 and t_2 , and zero filled to yield a $2,048 \times 2,048$ or $4,096 \times 2,048$ matrix of real data points.

Supplementary material in the Electronic Appendix

Supplementary material in electronic appendix Table S1 gives the ^1H and ^{15}N chemical shifts of *R. capsulatus* ferricytochrome c_2 . Figure S1 is a section of the ^1H - ^{15}N HMQC spectrum of *R. capsulatus* ferricytochrome c_2 acquired in H_2O at 30°C and pH 5.5.

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