Analysis of Site-Directed Mutants Locates a Non-Redox-Active Metal near the Active Site of Cytochrome *c* Oxidase of *Rhodobacter sphaeroides*

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ABSTRACT: Substoichiometric amounts of Mn are bound by the aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides* and appear in the EPR spectrum of the purified enzyme as signals that overlay those of Cu_A in the $g = 2.0$ region. The Mn is tightly bound and not removed by a high degree of purification or by washing with 50 mM EDTA. The amount of bound Mn varies with the ratio of Mg to Mn in the growth medium. Oxidase containing no EPR-detectable Mn can be prepared from cells grown in low Mn/Mg, while high Mn/Mg in the growth medium gives rise to near stoichiometric levels (0.7 mol/mol of aa_3). Incubation of purified Mn-deficient oxidase with 1 mM Mn does not allow incorporation into the tight binding site, indicating that this site is not accessible in the assembled protein. When bound Mn is depleted by growth in high Mg, there is no change in electron transfer activity, suggesting that Mg may substituted for Mn and maintain protein structure. Analysis of site-directed mutants in an extramembrane loop close to the active site of cytochrome oxidase identifies His-411 and Asp-412 of subunit I as probable ligands of the Mn. Mutation of either residue leads to lower activity and loss of Mn binding, even in cells grown in elevated concentrations of Mn. Since Mn binding correlates with the [Mn] to [Mg] ratio in the culture medium, we propose that Mn competes for the site that normally binds a stoichiometric Mg ion in aa_3-type cytochrome *c* oxidases.

Cytochrome *c* oxidase is the terminal member of the energy-transducing electron-transfer chain of mitochondria and many aerobic bacteria. The mitochondrial enzyme is composed of 13 subunits: the three largest, encoded by mitochondrial DNA, comprise the catalytic core, while 10 smaller, nuclear-encoded subunits play as yet undefined roles (Kadenbach et al., 1991). Bacterial cytochrome *c* oxidases are composed of 3 or 4 subunits, the three largest of which are homologs of the mitochondrially encoded subunits of the eukaryotic enzyme (Ludwig, 1987; Saraste, 1990). In cytochrome *c* oxidase electrons from cytochrome *c* are transferred to Cu_A in subunit II (Pan et al., 1991a; Hill, 1993), then to heme *a* in subunit I, and finally to the heme a_3−Cu_B center of subunit I, where oxygen reduction takes place and where the majority of the energy is derived for proton translocation.

The aa_3-type oxidases of *Rhodobacter sphaeroides* and *Paracoccus denitrificans* are closely related and appear to be the bacterial enzymes most similar to mitochondrial cytochrome *c* oxidase (Raitio et al., 1987, 1990; Steinrucke et al., 1987; Cao et al., 1991, 1992; Shapleigh & Gennis, 1992; Hosler et al., 1992). This is expected to be the case, since the ancestor of mitochondria has been genetically traced to the α-subgroup of the purple bacteria (Yang et al., 1985). Biochemical, spectroscopic, and genetic analyses show that cytochrome aa_3 of *Rb. sphaeroides* is an excellent model of the mitochondrial enzyme (Cao et al., 1991, 1992; Hosler et al., 1992; Shapleigh & Gennis, 1992). Studies of site-directed mutants in subunit I of this bacterial enzyme have identified histidines in transmembrane helices II, VI, VII, and X as ligands of heme *a* and the heme a_3−Cu_B center (Shapleigh et al., 1992; Hosler et al., 1993; Calhoun et al., 1993; Hosler et al., 1994a). Since hemes *a* and a_3 are both ligated by histidines in helix X, all three redox-active metals of subunit I are closely associated (Hosler et al., 1993).

In addition to the redox-active metals, cytochrome *c* oxidase isolated from beef heart mitochondria contains one Zn and one Mg atom per monomer (Einarsdottir & Caughey, 1985; Bombelka et al., 1986; Steffens et al., 1987, 1993; Moubarak et al., 1987; Buse & Steffens, 1991; Pan et al., 1991b). The structural and functional roles of these metals are not known. Biochemical studies, including EXAFS,[1] indicate that the Zn^{2+} ion is ligated by three cysteines and one nitrogen, with the binding site located in one or more nuclear subunits (Naqui et al., 1988; Scott, 1989; Pan et al., 1991c). Bacterial oxidases, without subunits homologous to the nuclear-encoded ones, do not bind stoichiometric Zn (Steffens et al., 1987; Buse & Steffens, 1991; A. Bresser & G. Buse, personal communication; J. A. Fee, personal communication), consistent with this assignment. One of the ligands of the Mg atom of beef heat cytochrome oxidase has recently been postulated to be a carboxylate residue in subunit IV (Lin et al., 1993). However, bacterial oxidases that contain Mg (Steffens et al., 1987; Buse & Steffens, 1991; A. Bresser and G. Buse, personal communication) have no

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[1] Abbreviations: EXAFS, extended X-ray absorption fine structure; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation.

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homologous peptide to subunit IV of eukaryotic oxidases (Saraste, 1990).

Unlike Zn^{2+} and Mg^{2+}, Mn^{2+} is paramagnetic and thus visible by EPR spectroscopy. EPR-detectable Mn is clearly present in bacterial cytochrome *c* oxidases, including those of *Rb. sphaeroides* (Hosler et al., 1992), *P. denitrificans* (Seelig et al., 1981; Haltia, 1992), *Nitrosomonas europaea* (Numata et al., 1989), and the caa_3-type cytochrome *c* oxidases of *Bacillus subtilus* (Lauraeus et al., 1991) and *Thermus thermophilus* (Fee et al., 1986). Analysis of a two-subunit preparation of *P. denitrificans* cytochrome aa_3 by EPR spectroscopy shows that the substoichiometric Mn atom is tightly bound and that the coordination geometry of the metal changes upon reduction of the redox-active metal centers (Seelig et al., 1981). Haltia (1992) has further determined that reduction of heme *a* and Cu_A, but not the heme a_3-Cu_B center, is sufficient to produce a change in the EPR signal of the Mn in *P. denitrificans* cytochrome oxidase.

In this study, we have examined and quantified Mn in the aa_3-type cytochrome *c* oxidase of *Rb. sphaeroides* using X-band EPR spectroscopy. By varying the Mn and Mg content of the cell culture medium, we have purified oxidases with Mn contents ranging from undetectable to nearly stoichiometric. Analysis of site-directed mutants in an extramembrane loop connecting transmembrane helices IX and X of subunit I identifies two consecutive residues, His-411 and Asp-412, as probable ligands of the bound Mn, while growth studies suggest that Mn and Mg may compete for this site. We have previously concluded that His-411 and Asp-412 are close to the heme a_3-Cu_B center of the enzyme (Hosler et al., 1994b). These results, then, identify a binding site for a non-redox-active metal on the periplasmic side of the membrane that is close to the active site of the cytochrome *c* oxidase.

EXPERIMENTAL PROCEDURES

All materials were prepared or purchased as in Hosler et al. (1992). Oxidase purification and electron transfer activity measurements followed the methods of Hosler et al. (1992). EDTA is present throughout the purification procedure at a concentration of 1 mM.

Bacterial Cell Growth. *Rhodobacter sphaeroides* strain CY91 was grown in Sistrom's medium A as described previously (Hosler et al., 1992), except that the $MnSO_4$ and $MgSO_4$ concentrations were varied as noted in the text. The CY91 cells grow well in concentrations of $MnSO_4$ up to 2 mM. However, at concentrations above 1.0 mM $MnSO_4$, increasing amounts of a dark brown precipitate appear in the medium during growth.

Electron Paramagnetic Resonance Spectroscopy (EPR). Measurements were performed as in Hosler et al. (1992) with the exceptions noted below and in the figure captions. Adventitious Mn was removed from the purified oxidases prior to EPR spectroscopy by incubating the sample in 50 mM EDTA, 10 mM KH_2PO_4, pH 7.6, 250 mM KCl, and 0.2% lauryl maltoside for 30 min at 4 °C, followed by concentration and equilibration of the sample into 0.1 mM EDTA, 50 mM KH_2PO_4, pH 7.6, 0.5 mM KCl, and 1% lauryl maltoside in a Centricon-30 concentrator (Amicon).

Quantitation of the Bound Mn. A sample from the preparation of oxidase that contained the greatest amount of EPR-detectable Mn (see Figure 1E) was acidified overnight with 1.0 N HCl. The concentration of Mn in the HCl solution, following removal of denatured protein by centrifugation, was determined at room temperature from a standard curve of $MnSO_4$ standards prepared in 1.0 N HCl and analyzed by EPR spectroscopy. The sample and standards were contained in a flat cell sample holder with a volume of 250 μL in a Varian TM_{001} cavity. The amplitude of the third peak from the low-field side of the spectrum was plotted vs the Mn concentration to create the standard curve. The EPR-determined Mn concentration was compared to the optically determined concentration of cytochrome aa_3 prior to acidification to determine the stoichiometry of bound Mn per oxidase monomer. The various stoichiometries of Mn incorporation into cytochrome *c* oxidase purified from cells grown in different concentrations of Mn and Mg (Table 1) were estimated by comparing the amplitudes of the $g = 1.85$ Mn^{2+} signals (peak to trough) from EPR spectra of the oxidized enzyme obtained at 10 K (not shown), using the amplitude of the heme *a* g_z signal at $g = 2.83$ (peak to baseline) as an internal standard.

RESULTS

Stoichiometry of Mn Binding. We have previously demonstrated that highly purified *Rb. sphaeroides* cytochrome *c* oxidase, like the enzyme from *P. denitrificans*, contains a tightly bound, non-redox-active Mn atom that is present in substoichiometric amounts (Hosler et al., 1992). This metal gives rise to strong EPR signals in the $g = 2.0$ region of the EPR spectrum, the most prominent of which appears at $g = 2.15$ (see Figure 1). The bound Mn is not released upon washing the enzyme with 50 mM EDTA, a process that does not alter the activity of the enzyme or the spectroscopic characteristics of the heme and copper centers (data not shown).

The final level of Mn incorporation can be altered by changing the concentration of $MnSO_4$ in the bacterial growth medium (Figure 1). When Sistrom's medium A is modified to include only 0.5 μM $MnSO_4$, rather than the usual concentration of 9 μM $MnSO_4$, the Mn content of the purified oxidase falls below the level of detection by EPR spectroscopy. Under these conditions, the Cu_A EPR spectrum of the *Rb. sphaeroides* oxidase is essentially identical to that of beef heart cytochrome *c* oxidase (Figure 1, A vs D). Conversely, when cells are grown in media containing a high concentration of $MnSO_4$ (0.7−1.0 mM), the Mn signals of the oxidase spectrum dominate the $g = 2.0$ region and mask the Cu_A signal (Figure 1E).

The incorporation of Mn is also strongly influenced by the level of Mg in the growth medium, as illustrated in Table 1. It is apparent that neither decreasing nor increasing the concentration of Mn alone in the growth medium is sufficient to maximally influence incorporation of Mn into the oxidase. It is only when Mg is varied in an inverse manner that complete loss or maximal incorporation of Mn (0.7 mol/mol of aa_3) is observed (Table 1). Note that the EPR technique is capable of detecting small amounts of oxidase-bound Mn, as low as 0.004 mol of Mn/mol of aa_3, or 0.4% (data not shown).

Identification of Two Probable Ligands of the Oxidase-Bound Mn by EPR Analysis of Site-Directed Mutants. Four conserved residues (His-411, Asp-412, Thr-413, Tyr-414)

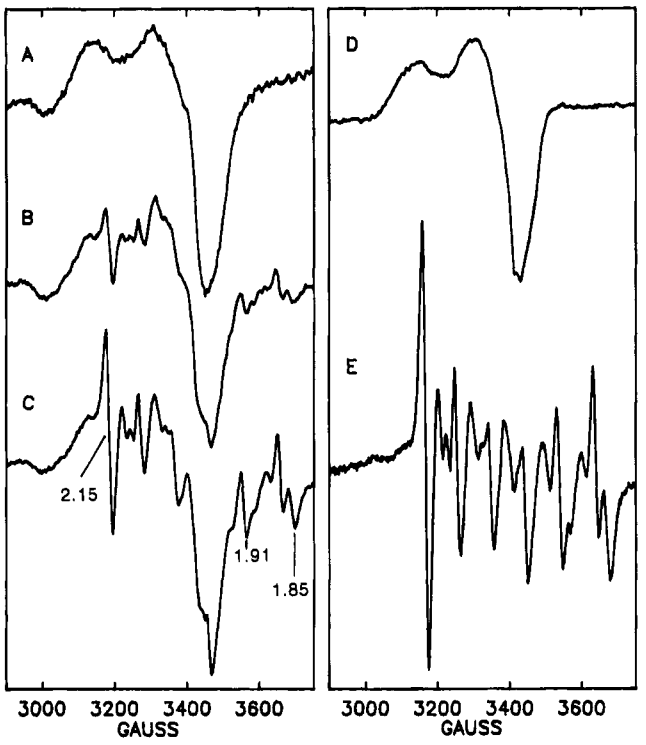

FIGURE 1: EPR spectra of purified *Rb. sphaeroides* cytochrome *c* oxidase grown in different concentrations of Mn^{2+} and Mg^{2+} and beef heart cytochrome oxidase. EPR spectra of 50−100 μM *Rb. sphaeroides* (A, B, C, E) or beef heart oxidase (D) samples were recorded at X-band, 110 K, as described under Experimental Procedures. Growth media for the cells from which the *Rb. sphaeroides* oxidases were isolated included 0.5 μM $MnSO_4$, 1.2 mM $MgSO_4$ (A); 9 μM $MnSO_4$, 1.2 mM $MgSO_4$ (B); 27 μM $MnSO_4$, 1.2 mM $MgSO_4$ (C); and 0.7 mM $MnSO_4$, 50 μM $MgSO_4$ (E). The labels on spectrum C indicate the *g* values of three of the signals arising from oxidase-bound Mn^{2+}. In spectrum E, of oxidase purified from cells grown in media containing high [Mn]/low [Mg], the Mn^{2+} signals obscure the Cu_A signals. The scale of spectrum E is approximately 8 times smaller than that of spectra A−D. Beef heart cytochrome oxidase (D) was purified by the method outlined in Suarez et al. (1984).

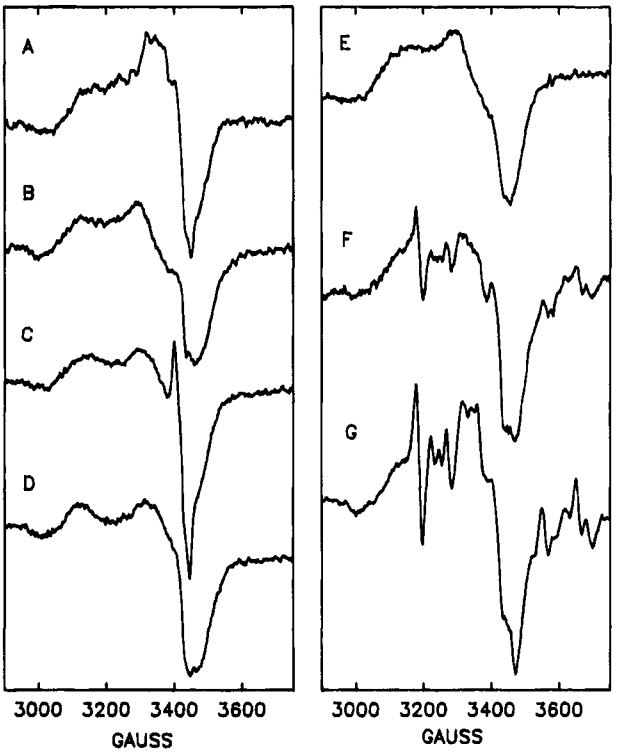

FIGURE 2: EPR spectra of purified cytochrome *c* oxidases of *Rb. sphaeroides* containing alterations in the IX−X extramembrane loop of subunit I. Spectra of 35−50 μM oxidase samples were recorded at X-band, 110 K. Oxidase samples H411A (A), D412N (B), H411Y (E), T413N (F), and Y414F (G) were purified from cells grown in media containing 9 μM $MnSO_4$ and 1.2 mM $MgSO_4$. Mutant oxidases H411A and D412N also fail to bind Mn when grown in media containing three times this level of Mn (27 μM $MnSO_4$ and 1.2 mM $MgSO_4$; spectra C and D, respectively). The radical signal seen at $g = 2.0$ in spectrum C appears from time to time in these preparations, but it does not obscure the Mn signals if present.

in an extramembrane loop connecting transmembrane helices IX and X of subunit I of the *Rb. sphaeroides* oxidase have been shown to form part of a structural "cap" over the heme *a* and heme a_3−Cu_B centers (Hosler et al., 1994b). Purified oxidases containing site-directed alterations of these four residues were examined for tightly bound Mn by EPR spectroscopy. Three mutant oxidases, H411A, H411Y, and D412N, show no Mn incorporation. This is true even when the mutant oxidases are isolated from cells grown in elevated concentrations of Mn (Figure 2). This result identifies His-411 and Asp-412 as probable ligands of the oxidase-bound Mn. In contrast, mutant oxidase T413N binds a level of Mn similar to the wild-type enzyme, while mutant oxidase Y414F binds an even greater amount (Figure 2). Of the eighteen mutant oxidases that we have thus far examined by EPR spectroscopy, all of which contain alterations in

Table 1: Stoichiometry of Mn Binding and Electron-Transfer Activities of Wild-Type (wt) and Mutant *Rb. sphaeroides* Cytochrome *c* Oxidases Isolated from Cells Grown in Differing Concentrations of Mn and Mg

	Mn and Mg content of the culture medium				
oxidase	Mn (μM)	Mg (μM)	initial [Mg]/[Mn]	Mn content/aa_3 (% saturation)[a]	electron-transfer activity (TN; s^{-1})[b]
wt 1	0.5	1200	2400	0	1490
wt 2	9.0	1200	133	1.3	1470
wt 3	27	1200	44	4.1	1450
wt 4	1000	1200	1.2	48	1430
wt 5	700	50	0.07	70	1550
wt 6	9.0	10 000	1111	0.4	1600
H411A	9.0	1200	133	0	700
H411A	27.0	1200	44	0	750
D412N	9.0	1200	133	0	640
D412N	27.0	1200	44	0	580
H411Y	9.0	1200	133	0	<10

[a] The content of Mn was determined as in Experimental Procedures. [b] From a representative polarographic experiment, performed as in Hosler et al. (1992), but using 38 μM horse heart cytochrome *c*.

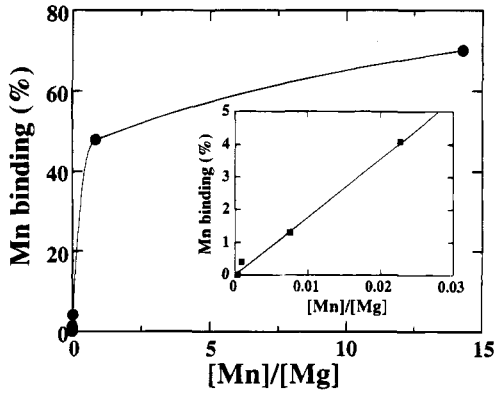

FIGURE 3: Effect of the Mn to Mg ratio during growth on the incorporation of Mn into the fully assembled, purified bacterial oxidase. Data derived from Table 1 is plotted with the ratio of the $MnSO_4$ and $MgSO_4$ contents of Sistrom's medium A plotted on the abscissa and the percent incorporation of Mn plotted on the ordinate. 100% is equal to 1 mol of Mn/mol of aa_3 in the purified enzyme. The Mn content was determined as described in Experimental Procedures. The inset shows an expanded scale of the first four data points in order to demonstrate the linear relationship between the Mn to Mg ratio and the incorporation of Mn into the enzyme.

various regions of subunit I, only the His-411 and Asp-412 mutants fail to bind Mn.

Mg Competition for the Mn Site. The ability of Mg to occupy the Mn binding site of the *Rb. sphaeroides* oxidase is of obvious interest, since the two metals can often substitute for each other in metalloproteins (Reed & Markham, 1984; Declercq et al., 1991; Tanier et al., 1991, 1992; Jenkins et al., 1992). An initial approach to the question of Mn–Mg substitution is to vary the Mn and Mg content of the bacterial growth medium.

Experiments 1–4 of Table 1 show that the stoichiometry of oxidase-bound Mn increases with the concentration of Mn present during cell growth, as shown by the EPR spectra of Figure 1. Experiments 5 and 6 of Table 1, however, show that the concentration of Mg is also an important factor. High [Mn]/low [Mg] conditions (expt 5 of Table 1; Figure 1E) increase the amount of bound Mn, while low [Mn]/high [Mg] during growth suppresses Mn incorporation (expt 6 of Table 1). In fact, Figure 3 shows that Mn binding is directly related to the Mn to Mg ratio in the culture medium. Manganese incorporation appears to saturate at high [Mn]/[Mg] values, indicating that one or more additional factors limit the extent of Mn binding. This limitation may be the maximum steady-state concentration of free Mn within the cell, or in the growth medium. In fact, at 1 mM Mn and above, a dark precipitate is observed in the growth vessel, suggesting that we are not keeping these higher concentrations in solution.

Since the bacterial oxidase binds detectable amounts of Mn even when the concentration of $MgSO_4$ in the growth medium is 1–3 orders of magnitude greater than that of $MnSO_4$ (Table 1), it may be that this metal binding site has a higher affinity for Mn than for Mg. However, aqueous Mn will not incorporate into the purified Mn-deficient protein (Figure 4), indicating that the binding site is not accessible to metal ions in the external solvent once the protein is fully assembled. The experiment of Figure 4 also demonstrates the ability of the EDTA wash to completely remove adventitious Mn.

Effect of Mn Content on Oxidase Activity. Altering the Mn content of the wild-type enzyme by growth conditions has no effect on the electron-transfer activities of the purified oxidases (Table 1, expts 1–6). In light of the fact that Mg^{2+} and Mn^{2+} prefer similar coordination environments (Hughes, 1981; Glusker, 1991; Tanier et al., 1991, 1992) and replacement of Mg by Mn often results in native or near-native protein structure (Reed & Markham, 1984; Declercq et al., 1991; Tanier et al., 1991, 1992; Jenkins et al., 1992), these results are consistent with our proposal of Mn substitution at the Mg binding site during growth under high [Mn]/low [Mg] conditions.

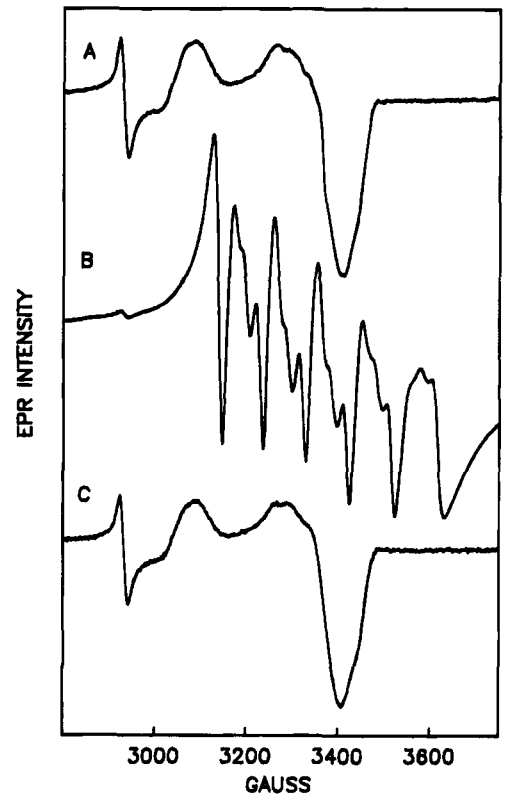

FIGURE 4: Manganese cannot be introduced into the fully assembled, purified bacterial oxidase. All spectra were recorded at 10 K. Spectrum A is of 40 μM *Rb. sphaeroides* cytochrome *c* oxidase, purified from cells grown in low [Mn]/high [Mg], such that it contains no EPR-detectable Mn. The signal at $g = 2.31$ is g_y of heme *a*. Spectrum B is the same sample during a 2-h incubation in 1.0 mM $MnSO_4$ and shows the 6-line spectrum of hexaqua Mn^{2+} and Mn bound to the surface of the oxidase. To obtain spectrum B, aqueous Mn was added to the EPR tube, and the sample incubated at 4 °C for 2 h and then frozen for spectroscopy. The scale of spectrum B is approximately 20 times smaller than that of spectra A and C. Spectrum C shows the same sample after it was thawed; removed from the EPR tube; washed with 50 mM EDTA as described in Experimental Procedures; chromatographed on a 0.5 × 15 cm Sephadex G-50 column preequilibrated with 50 mM KH_2PO_4, pH 7.6, 1 mM EDTA, and 0.2% lauryl maltoside; and reconcentrated in a Centricon-30 microconcentrator (Amicon). It is clear that no EPR-detectable Mn has been introduced into the purified Mn-deficient oxidase by incubating the enzyme in a high concentration of $MnSO_4$.

In contrast, the oxidase mutants that have lost the ability to bind Mn show activity reductions of 50–60% (Table 1). These altered oxidases may have diminished Mg as well as Mn binding at this site, resulting in a structural alteration that leads to lower electron-transfer activity.

DISCUSSION

Previous analysis of the His-411 and Asp-412 mutants of the IX–X extramembrane loop by optical and resonance

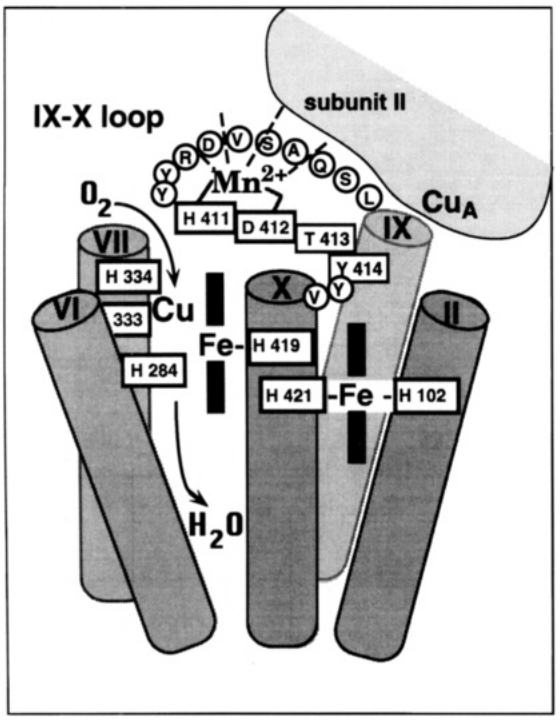

FIGURE 5: Model of five of the transmembrane helices of subunit I and the extramembrane loop between helices IX and X. This model illustrates our proposed role for the Mn (Mg) binding site in coordinating the interaction of subunits I and II in the region of the redox-active metal centers. The assignment of the ligands of heme *a* and the heme a_3−Cu_B center, as well as the proximity of His-411 and Asp-412 to these metals, has been previously reported [see Shapleigh et al. (1992) and Hosler et al. (1993, 1994b)]. The ligation of Mn^{2+} by Asp-412 (D412) is drawn as a bent line, since we argue in the accompanying paper (Espe et al., 1995) that Asp-412 is likely to be coordinating a water molecule that functions as a direct ligand to the metal. Four of the six ligands of the Mn remain to be identified; some or all of these are suggested to be located in subunit II.

Raman spectroscopy places these residues close to the heme a_3−Cu_B binuclear center where oxygen is reduced to water (Hosler et al., 1994b). The results presented here show these residues to be part of an extramembrane metal binding site, whose function is not clear but whose location near the active site of cytochrome *c* oxidase makes it of considerable interest.

Structure of the Mn Binding Site. The bound Mn is clearly six-coordinate, based upon the line spacings of the EPR signal [see Misra and Sun (1991)]. Loss of Mn binding upon mutation of His-411 and Asp-412 identifies these residues as probable ligands and indicates that the Mn is bound at a single site. Manganese (and Mg) prefers oxygen ligation, although histidine does ligand Mn in several binding sites of known structure (Hardman et al., 1982; Stallings et al., 1985; Einsphar et al., 1986; Yamashita et al., 1989; McCracken et al., 1991). A detailed analysis of the coordination geometry of the Mn binding site using X-band and Q-band EPR and ESEEM spectroscopy of both the oxidized and the reduced enzyme is given in the accompanying paper (Espe et al., 1995).

Manganese ligation by His-411 and Asp-412 places this metal binding site close to the heme a_3−Cu_B center of subunit I (Figure 5), on the basis of our previous analysis of this region of the IX−X extramembrane loop (Hosler et al., 1994b). It is very possible that the remaining ligands are conserved carboxylate residues in subunit II, since a close approach of this Cu_A-containing subunit to the heme *a* binding region of subunit I is predicted by kinetic (Hill, 1993, 1994) and physical studies (Goodman & Leigh, 1985). Indeed, a preparation containing only subunit I of *P. denitrificans* does not contain Mn (Haltia, 1992). One role for the non-redox-active metal may be to coordinate the interaction of subunits I and II in order to achieve rapid electron transfer between Cu_A and heme *a*. Subunit III is not necessary for Mn binding: a two-subunit preparation of *P. denitrificans* cytochrome *c* oxidase lacking subunit III binds a high level of Mn (Seelig et al., 1981).

Localization of this non-redox-active metal binding site immediately above the heme a_3−Cu_B center suggests that Mn and heme *a* centers may be close enough for magnetic interaction (see Figure 5). This possibility was examined by determining the power saturation profiles of heme *a* in wild-type oxidase containing 0.7 Mn per monomer and in oxidase containing approximately 0.004 Mn per monomer. The power saturation characteristics of the g_z component of heme *a* in these two enzymes are nearly identical (data not shown), indicating no significant effect on the relaxation properties of heme *a* by Mn^{2+}. However, neither an upper nor a lower limit on the distance between the bound Mn and heme *a* could be assigned from these data, since the relaxation properties of Mn^{2+} have not been well characterized.

Does Mn Bind to a Conserved Non-Redox-Active Metal Binding Site? An important question is whether this site normally contains a metal ion or whether Mn fortuitously binds to a group of residues that are not a physiological metal site. We deem the former hypothesis more tenable than the latter for the following reasons. It is clear that this site is within the interior of the protein since bound Mn cannot be removed by high concentrations of EDTA, nor can Mn be introduced into the purified Mn-deficient enzyme. The presence or absence of a metal in an interior position would likely cause a significant change in structure, particularly in a region close to the active site of the oxidase. However, we observe no changes in the optical spectrum or the electron-transfer activity of the *Rb. sphaeroides* oxidase as the Mn content is varied from undetectable to nearly stoichiometric by altering the Mn/Mg ratio of the growth medium. Thus, it seems reasonable to propose that an existing metal binding site is occupied to a greater or lesser extent by Mn^{2+} and that EPR-silent Mg^{2+} is occupying the remainder of the sites, thus maintaining normal structure.

Low levels of Mn binding appear ubiquitous among bacterial aa_3 or caa_3-type cytochrome *c* oxidases, on the basis of examination of published spectra (see above) and metal analysis of the enzyme of *P. denitrificans* (Steffens et al., 1987; Steffens & Buse, 1991). The aligned sequences shown in Table 2 demonstrate that His-411 and Asp-412 are conserved among these enzymes. In contrast, bacterial oxidases that utilize ubiquinol as substrate rather than cytochrome *c*, and also lack Cu_A, do not bind Mn (Lauraeus et al., 1991; Puustinen et al., 1991; J. Hill and R. Gennis, personal communication). Each of these quinol oxidases contains asparagine instead of aspartate at the equivalent of position 412 (Table 2). This corroborates the lack of Mn binding by the D412N mutant of *Rb. sphaeroides* and emphasizes the importance of Asp-412 in Mn binding. The ba_3-type oxidase of *T. thermophilus* is an interesting outlier and is thus placed in a separate category in Table 2. Although this enzyme contains Cu_A and oxidizes cytochrome

Table 2: Alignment of the HDTY Region of the IX−X Interhelix Loop and the Top Residues of Transmembrane Helix X of Subunit I[a]

Residue number (*Rb. sphaeroides*)	411	412	413	414	415	416	417	418	419	420	421
Mitochondrial cytochrome *c* oxidases											
Bovine *aa*3	H	D	T	Y	Y	V	V	A	H	F	H
Human *aa*3	H	D	T	Y	Y	V	V	A	H	F	H
*D. melanogaster aa*3	H	D	T	Y	Y	V	V	A	H	F	H
*S. cerevisiae aa*3	H	D	T	Y	Y	V	V	G	H	F	H
*T. brucei aa*3	H	D	T	Y	F	V	V	A	H	F	H
Bacterial cytochrome *c* oxidases											
*Rb. sphaeroides aa*3	H	D	T	Y	Y	V	V	A	H	F	H
*P. denitrificans aa*3	H	D	T	Y	Y	V	V	A	H	F	H
*B. subtilis caa*3-605	H	D	T	Y	F	V	V	A	H	F	H
*T. thermophilus caa*3	H	D	S	Y	F	V	V	A	H	F	H
PS-3 *caa*3	H	D	S	Y	F	V	V	A	H	F	H
Bacterial quinol oxidases											
E. coli bo	H	N	S	L	F	L	I	A	H	F	H
*B. subtilis aa*3-600	H	N	T	Y	F	L	V	S	H	F	H
*Sulfolobus acidocaldarius aa*3	H	N	S	Y	Y	V	V	G	H	F	H
Other											
*T. thermophilus ba*3	H	N	T	A	W	V	P	G	H	F	H

[a] Sequence alignments are taken from the compilation of Calhoun (1993).

c (Zimmermann et al., 1988), its primary sequence of subunit I is similar to that of the quinol oxidases with respect to the presence of asparagine at position 412. Indeed, a published EPR spectrum indicates that this enzyme does not contain Mn (Surerus et al., 1992). Thus, the sequence alignments are consistent with our experimental information that His-411 and Asp-412 are ligands of a non-redox-active metal in the IX−X loop.

Purified bovine cytochrome *c* oxidase also does not contain Mn (Steffens et al., 1987, 1993; Steffens & Buse, 1991). The reason for this is not apparent from sequence alignments (Table 2), since all the mitochondrial versions of cytochrome aa_3 conserve both His-411 and Asp-412. Other differences in the structure of the more complex mitochondrial enzyme (*e.g.*, the involvement of subunit IV; see below) may provide for greater specificity for another metal, possibly Mg, at this site. Alternatively, Mn may not be available in mitochondria during oxidase assembly.

Does Mn Occupy the Mg Binding Site of Cytochrome c Oxidases? Several observations suggest that both Mn and Mg can occupy the metal binding site. First, Mn and Mg appear to compete for binding to the enzyme (Figure 3; Table 1). Second, the retention of electron-transfer activity and optically normal heme centers in the presence or absence of Mn suggests that another metal, likely Mg, can occupy the site and maintain protein structure and stability. Third, preliminary metal analyses of two oxidases that do not bind Mn, cytochrome *bo* of *Escherichia coli* and cytochrome ba_3 of *T. thermophilus*, indicate that these enzymes also fail to bind significant amounts of Mg (J. Hill and R. Gennis, personal communication; J. A. Fee, personal communication). Finally, cytochrome aa_3 of *P. denitrificans*, which is closely related to cytochrome aa_3 of *Rb. sphaeroides*, does contain Mg. Several metal analyses of the *P. denitrificans* enzyme place the Mg content at close to stoichiometric, but variable (Steffens et al., 1987; Steffens & Buse, 1991). Some of this variability may arise from different amounts of Mn binding in different preparations of the enzyme. Conclusive evidence that this site binds both Mn and Mg will depend upon extensive metal analyses of preparations of the oxidase isolated from cells grown in various amounts of Mn and Mg.

A separate question is whether the Mn binding site of *Rb. sphaeroides* corresponds to the site that binds the stoichiometric Mg in mitochondrial cytochrome *c* oxidase. Chan and colleagues (Lin et al., 1993) have recently reported evidence that one of the ligands of the Mg atom of beef heart cytochrome *c* oxidase is located in subunit IV, one of the nuclear-encoded subunits not present in bacterial cytochrome oxidases. The glutamate residue designated by Lin et al. (1993) as a Mg ligand is located in the carboxy-terminus of subunit IV, which is predicted to be on the same side of the membrane as the IX−X loop of subunit I [Zhang et al. (1988)]. Thus, it is possible that subunits I and IV of eukaryotic oxidases have evolved to create a single buried

site that binds Mg, but not Mn. Definition of the remaining ligands of the Mn (or Mg) binding site of *Rb. sphaeroides* by genetic methods may help determine whether the bacterial metal binding site is conserved in mitochondrial cytochrome oxidase. If all of the ligands are conserved in the eukaryotic enzyme, retention of the bacterial form of the metal binding site would be highly likely.

ADDED IN PROOF

Mutant H411N described in Hosler et al. (1994b) was recently resequenced and found to be H411Y (J. Fetter, unpublished); it is so designated in this paper. This does not alter any previous conclusions.

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