EPR studies of wild-type and several mutants of cytochrome *c* oxidase from *Rhodobacter sphaeroides*: Glu^{286} is not a bridging ligand in the cytochrome a_3–Cu_B center

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Received 25 September 1995

Abstract **Wild-type and several mutants of cytochrome *c* oxidase from *Rhodobacter sphaeroides* were characterized by EPR spectroscopy. A pH-induced *g*12 signal, seen previously in mammalian cytochrome oxidase and assigned to the presence of a bridging carboxyl ligand in the bimetallic cytochrome a_3–Cu_B site, is found also in the bacterial enzyme. Mutation of glutamate-286 to glutamine inactivates the enzyme but does not affect this signal, demonstrating that the carboxyl group of this residue is not the bridging ligand. Three mutants, M106Q, located one helix turn below a histidine ligand to cytochrome *a*, and T352A as well as F391Q, located close to the bimetallic center, are shown to affect dramatically the low-spin heme signal of cytochrome *a*. These mutants are essentially inactive, suggesting that these three mutations result in alterations to cytochrome *a* that render the oxidase non-functional.**

Key words: Cytochrome oxidase; Cytochrome *a*; Bimetallic cytochrome a_3–Cu_B; EPR spectroscopy; *Rhodobacter sphaeroides*

1. Introduction

Electron paramagnetic resonance spectroscopy has been extensively used as a sensitive probe of the molecular environment of the metal centers of cytochrome *c* oxidase [1]. The EPR spectrum of oxidized 'fast' form of cytochrome oxidase in the absence of exogenous ligands reveals only low-spin heme signals from cytochrome *a* and signals originating from the Cu_A site [2]. The high-spin cytochrome a_3 and Cu_B, being antiferromagnetically coupled, are not observed by EPR in the oxidized enzyme [2,3]. Loss of Cu_B or partial reduction of the bimetallic center, however, introduce a large high-spin heme signal at *g*6.0, due to the uncoupling of cytochrome a_3 and Cu_B [2]. Upon induction of the 'slow' form of cytochrome oxidase by incubation at low pH, in which a carboxylic acid side chain was proposed to bind to the bimetallic center [4], a broad *g*12 signal, originating from the bimetallic center, also appears. This has also recently been observed in studies on the *E. coli* cytochrome bo_3 quinol oxidase [5].

The EPR characteristics of cytochrome oxidase have been largely determined using enzyme isolated from bovine heart. With the increased availability of bacterial oxidases, and the capability of creating site-directed mutants of these enzymes, it is of increasing value to characterize the EPR properties of these bacterial systems. Having done so, a number of site-directed mutants can be examined for effects on the magnetic properties of the metal centers. Such a model system, developed in *R. sphaeroides*, has been described by Hosler et al. [6]

In this report, we present EPR spectra of wild-type and mutant cytochrome oxidase from *R. sphaeroides*. In the wild-type enzyme a pH-induced conversion to the 'slow' form, as observed in bovine enzyme [4], is demonstrated by the appearance of the *g*12 signal. This conversion has not previously been demonstrated in any other aa_3-type oxidase of bacterial origin. In addition, several mutants are shown to induce significant shifts in the low-spin heme signals. These include mutations in transmembrane helices 2, 8 and 9 of subunit I. The changes observed in cytochrome *a* correlate with the loss of function, since all the mutants have turnover rates that are < 5% of the wild-type.

2. Materials and methods

2.1. Construction of site-directed mutants

The mutants M106Q, E286Q, T352A and F391Q were constructed using multiple site-directed mutagenesis methods on a template plasmid (pJS3) containing the *cta*D gene which codes for subunit I of cytochrome oxidase [7]. T352A and E286Q were constructed by an extension of a mutagenic single-stranded DNA primer on a single-stranded DNA template, using the method of Vandeyar et al. [8] M106Q was constructed using a single-step polymerase chain reaction (PCR) method in which the mutagenic primer overlapped a unique *Bam*HI restriction site, which allowed direct cloning of the PCR product. F391Q was constructed using a two-step PCR method, described by Landt et al. [9]. Mutagenic primers were as follows: M106Q, 5′-CCACGGGATCCTGCAGATGTTCTTCG-3′; F391Q, 5′-CTCTTCCTGCAGACCGTGGGCGGCG; T352A, 5′-GATGGCGGCGATGGTGAT-3′; E286Q, 5′-CCACCCCCAGGTCTACAT-3′. The mutations were verified by DNA sequencing methods.

2.2. Purification of cytochrome oxidase

The purification of wild-type and mutant cytochrome oxidase was facilitated by the genetic fusion of a six-histidine affinity tag to the C-terminus of subunit I, as recently described [10]. This allowed a single-step purification of the enzyme using a nitrilotriacetic acid-agarose affinity column. This modification has been shown previously to have no observable effect on enzyme structure or function. All purification steps were performed at pH 8.0. The purified enzyme was first concentrated, then diluted into the appropriate buffers (0.1 M MES, pH 6.0, or 0.1 M HEPES, pH 7.0), and finally concentrated again to 50–80 μM for EPR analysis. In the case of pH 6 samples, the sample was incubated at room temperature for 2 h before freezing.

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Abbreviations: EPR, electron paramagnetic resonance; PCR, polymerase chain reaction; FTIR, Fourier-transform infrared.

SSDI 0014-5793(95)01149-8

2.3. EPR spectroscopy

EPR spectra were recorded with a Bruker ER 200D-SRC X-band spectrometer equipped with a standard TE102 rectangular cavity and an Oxford Instruments ESR-9 helium-flow cryostat. Experimental conditions: temperature, 10K; microwave power, 2 mW; microwave frequency, 9.45 GHz; modulation amplitude, 2 mT; time constant, 200 ms; recording time, 100 s.

3. Results

The EPR spectra of wild-type cytochrome oxidase from *R. sphaeroides* and the E286Q mutant are shown before and after a low pH incubation, demonstrating the pH-induced conversion to the 'slow' form, as indicated by the *g*12 signal (Fig. 1). EPR spectra of several mutants are shown in Fig. 2, and the modeled positions of the mutated residues with respect to the metal centers are illustrated in Fig. 3.

All spectra contain variable amounts of manganese impurity demonstrated by a 6-line signal around the Cu_A signal at *g*2 [6]. The signals at *g*6 and *g*4.3 are mostly due to denatured material and correspond to negligible concentrations of high-spin Fe^{3+} [2]. All mutants display a heterogeneity in the low-spin cytochrome *a* revealed by the appearance of two different EPR signals. This is most clearly seen in F391Q where the peaks at *g*2.91 and *g*2.59 must arise from two different species. Fairly dramatic effects on the cytochrome *a* low-spin signal are observed in all the mutants shown in Fig. 2. All three *g*-values are shifted from the wild type positions at $g_z = 2.81$, $g_y = 2.30$ and $g_x = 1.62$. In T352A only the peaks at $g_z = 2.94$ and $g_y \sim 2.3$ can be observed whereas the peak at g_x must be too broad to be seen.

4. Discussion

The EPR spectrum of wild-type cytochrome *c* oxidase from *R. sphaeroides* has very similar characteristics compared to the mammalian enzyme, and the enzyme effectively undergoes the pH-induced conversion leading to the appearance of a *g*12 signal (Fig. 1). The highly conserved E286 residue has been proposed to play a role in the formation of this *g*12 signal [11]. However, we have clearly demonstrated that a mutation at this residue has no effect on the formation of the *g*12 signal, so that the carboxyl group of this residue cannot be the bridging ligand in the low-pH form of the bimetallic center. This conclusion is consistent with a similar study with *E. coli* cytochrome bo_3 [5], but our results with the *Rhodobacter* enzyme are not simply confirmatory. First, in our case the *g*12 signal was induced by a lowering of the pH rather than forming spontaneously. Second, the mutant of the *E. coli* oxidase is functional [12], whereas the *Rhodobacter* mutant shows a complete lack of turnover. In the recently determined structure of the homologous oxidase from *P. denitrificans* [13], E286 is clearly not in a location where it could readily bridge cytochrome a_3 and Cu_B. E286 is located within what appears to be a proton pumping channel, and the lack of turnover of the E286Q mutant attests to the importance of this residue in the *R. sphaeroides* oxidase.

It is of interest to note that preliminary results indicate that incubation of the T352A mutant at low pH does not result in generation of the *g*12 signal. However, additional studies are needed to explore whether this residue might be directly responsible for the EPR characteristics associated with the slow form of the oxidase.

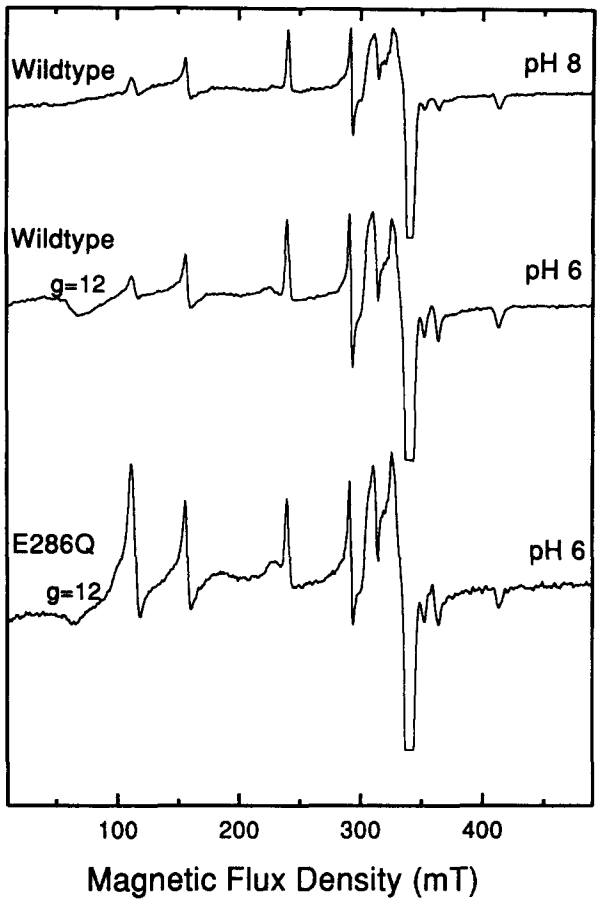

Fig. 1. EPR spectra demonstrating the pH-induced $g = 12$ signal in wild-type cytochrome *c* oxidase and the E286Q mutant after 2 h incubation at pH 6.

The other mutants examined in this study are in diverse regions of the subunit I sequence. M106 is a highly-conserved residue which is one turn below the cytochrome *a* ligand H102 in helix 2 (Fig. 3). According to recent crystal structure data from cytochrome aa_3 of *P. denitrificans*, this methionine side chain is positioned directly below F420, a residue which is stacked directly between the edges of cytochrome *a* and cytochrome a_3 (see Fig. 3) [13]. Nonpolar substitutions in the equivalent position in *E. coli* cytochrome bo_3 have recently been demonstrated to have relatively little effect on that enzyme [14]. F391 is also a highly-conserved residue located in helix 9 about 6.5 Å away from the porphyrin ring of cytochrome a_3 [13]. [T352] is a highly-conserved residue at the top of helix 8, very near to the binuclear center [13]. It is worth noting that mutations of the hydrophobic residues, F391 and M106, to glutamine are very non-conservative replacements of non-polar by polar side chains, and could be predicted to significantly disrupt the local environment. Replacement of T352 by alanine is more conservative, but replaces a polar by a non-polar side chain. The results of this small set of mutants examined (M106Q, F391Q, and T352A) demonstrate that the EPR spectrum of cytochrome oxidase can be significantly altered by single amino acid changes. Each of these mutants has very low steady state turnover (< 5% of wild-type), suggesting that the loss of function is due, at least in part, to a perturbation of cytochrome *a*. Possibly, then these mutations lead to small conformational

changes, which induce subtle changes on the exact structure of cytochrome *a*, thereby altering its the redox properties. Future studies can test this hypothesis.

In the cases of M106Q and F391Q, the EPR signal from the main species remains sharp, indicating the maintenance of a well-defined structure at the low-spin heme in the presence of these mutations. FTIR and resonance Raman studies on these mutants support this interpretation (see below). On the other hand, the T352A signal at *g*2.94 is much broader, suggesting a less ordered state. It is natural that the cytochrome *a* site has been changed in M106Q, since the mutation is right at this site (Fig. 3), but in the other two mutants the changes are closer to the bimetallic site. This is in agreement with other evidence that the two heme sites interact with each other [15–17].

These conclusions are supported by recent resonance Raman and FTIR characterization of these mutants in the fully reduced state (M. Pressler, G.T. Babcock, unpublished; D. Mitchell, J.O. Alben, unpublished). These techniques provide sensitive probes of the heme environment and the structure of the bimetallic center, respectively. In the Raman spectra of F391Q and M106Q, no changes in the heme-specific modes were observed. However, in T352A, all cytochrome a_3-specific modes were extensively broadened. FTIR difference spectroscopy of the reduced CO-enzyme adduct has demonstrated relatively stable CO binding states in F391Q and M106Q, whereas T352A shows a very disordered bimetallic center.

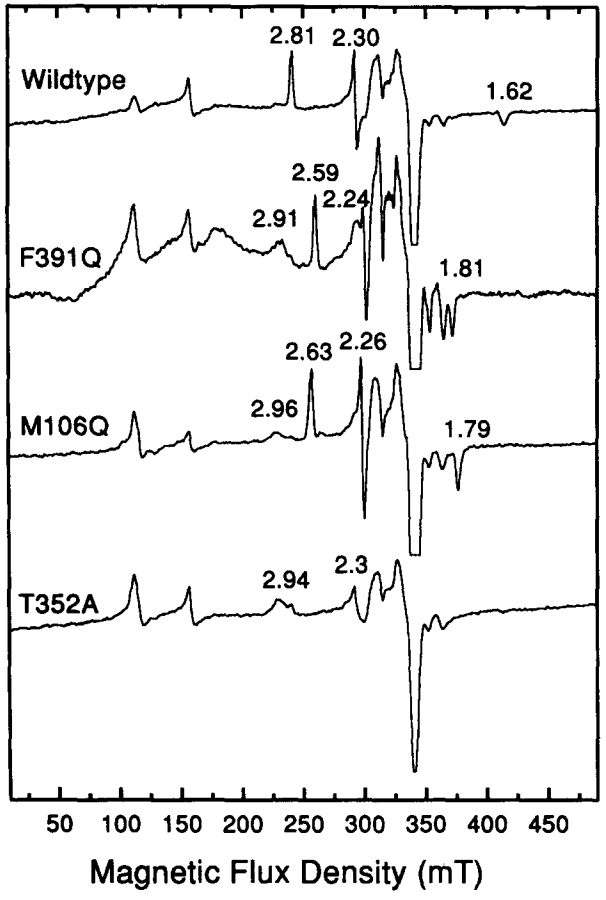

Fig. 2. EPR spectra of wild-type (pH 8), and F391Q (pH 8), M106Q (pH 8), and T352A (pH 7). g_x, g_y, and g_z values of the low-spin signals are labelled. In F391Q and M106Q, two low-spin signals are present, but only the g_z peak of the second species is clearly observed and labelled 2.91 and 2.96, respectively.

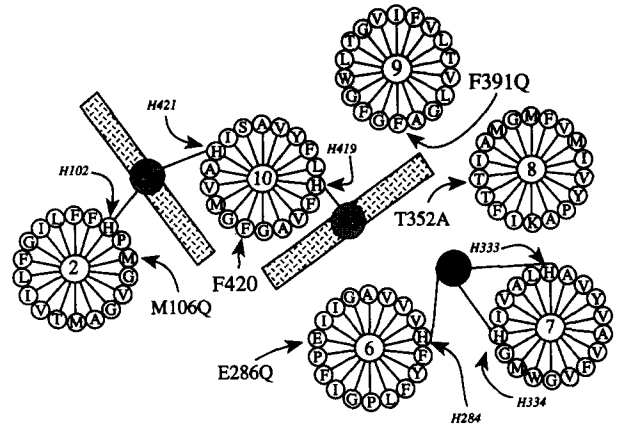

Fig. 3. Schematic helical wheel diagram of subunit I metal centers indicating the positions of metal ligands (small italics) and mutated residues (large print). The position of F420 is also indicated.

It is intriguing to find such a significant shift in the EPR spectra of F391Q and M106Q without observing any particular changes using Raman and FTIR methods. Interestingly, both FTIR and Raman experiments are characterizations of the fully reduced enzyme, whereas EPR examines the oxidized state. It is plausible that conformational differences exist between oxidized and reduced states of the enzyme, and that a mutation could potentially affect specifically one or the other conformation.

In conclusion, the EPR experiments have demonstrated that: (1) E286 does not provide the carboxylate presumed to be a bridging ligand in the pH-induced slow form of the oxidase; and (2) mutation of residues near the cytochrome a_3–Cu_B bimetallic center can result in changes in the spectroscopic properties of cytochrome *a*, thus illustrating conformational effects in this region of the protein. The loss of oxidase activity in F391Q, M106Q and T352A may be due, at least in part, to perturbations of cytochrome *a*.

Acknowledgements We wish to thank Dr. H. Michel for communicating his structural information prior to the publication of the crystal structure.

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