The highly conserved methionine of subunit I of the heme-copper oxidases is not at the heme-copper dinuclear center: mutagenesis of M110 in subunit I of cytochrome bo_3-type ubiquinol oxidase from *Escherichia coli*

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Received 10 April 1995; revised version received 7 June 1995

Abstract **A common feature within the heme-copper oxidase superfamily is the dinuclear heme-copper center. Analysis via extended X-ray absorption fine structure (EXAFS) has led to the proposal that sulfur may be bound to Cu_B, a component of the dinuclear center, and a highly conserved methionine (M110 in the *E. coli* oxidase) in subunit I has been proposed as the ligand. Recent models of subunit I, however, suggest that this residue is unlikely to be near Cu_B, but is predicted to be near the low spin heme component of the heme-copper oxidases. In this paper, the role of M110 is examined by spectroscopic analyses of site-directed mutants of the bo_3-type oxidase from *Escherichia coli*. The results show that M110 is a non-essential residue and suggest that it is probably not near the heme-copper dinuclear center.**

Key words: ????

1. Introduction

The origin of the magnetic coupling in the heme-copper dinuclear center of cytochrome *c* oxidase has been a subject of controversy for over 20 years. Several bridging ligands, both endogenous and exogenous, have been proposed to bridge between the iron atom of heme a_3 and Cu_B. Candidates have include histidine, sulfide, chloride, μ-oxo, tyrosinate, and carboxylate bridges [1]. A sulfur or chloride ligand has been suggested based upon analysis of EXAFS data [2,3]. A sulfur atom has also been proposed in the bo_3-type oxidase, another member of the heme-copper oxidase superfamily [4], based upon curve-fitting of EXAFS data [5].

Sequence alignments of heme-copper oxidases from over 75 species reveal that subunit I, which binds the dinuclear center, contains no invariant cysteine residues [6]. One highly conserved methionine (M110 in the *E. coli* oxidase) is found in subunit I of the heme-copper oxidases. This methionine, which is present in all but three species, has been proposed as a ligand to Cu_B in the binuclear center [5]. However, this is incompatible with recent models of subunit I based on site-directed mutagenesis studies of two bacterial heme-copper oxidases, the bo_3-type oxidase from *E. coli* and aa_3-type oxidase from *Rhodobacter sphaeroides* [4,7]. In these models, M110 is located four residues below the histidine (H106) identified as a ligand for the low spin heme *b* component of the *E. coli* oxidase [8,9], which corresponds to the heme *a* component of the aa_3-type oxidases [4,7,10,11]. In this work, the role of M110 in the bo_3-type oxidase from *E. coli* was examined by site-directed mutagenesis. The results clearly show that M110 is not an essential residue and is unlikely to be near the dinuclear center, consistent with the current models of subunit I of the heme-copper oxidases [4,7,10].

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Abbreviations: EPR, electron paramagnetic resonance spectroscopy; EXAFS, extended X-ray fine structure spectroscopy; FTIR, Fourier transform infrared spectroscopy.

2. Materials and methods

Mutants were constructed and confirmed by DNA sequencing according to previously published methods [8]. To test enzyme function, complementation analysis was performed in strain GO105 and strain RG129, as previously outlined [8]. These strains contain mutations in the *cyo* gene and the *cyd* gene and are recombination-defective (*rec*A) [8,12] Expression of the mutants were performed in strain GL101 (*cyo sdh rec*A) or in strain GO105 (*cyo Δcyd rec*A).

Under anaerobic conditions, expression of the bo_3-type oxidase is greatly repressed [13,14]. To grow *E. coli* aerobically on non-fermentable substrates, a functional terminal oxidase must be present [15]. Expression of mutants which grow poorly under aerobic conditions requires a functional form of the *bd*-type oxidase, which has three heme components, b_{558}, b_{595}, and *d* [16–18]. Two mutants were examined in this study. M110A is fully functional, and was examined in host strain GO105 with no other oxidase present. M110I, however, resulted in slow growth of GO105, and therefore this mutant was examined in host strain GL101, which contains the alternate *bd*-type quinol oxidase.

EPR and visible spectroscopies were performed with membrane preparations as previously described [8]. Activity assays were performed spectrophotometrically by measuring the oxidation of NADH [19]. Cryogenic Fourier transform infrared absorption difference spectroscopy of CO adducts of the *E. coli* oxidase was performed as previously described [19–21].

3. Results

Two mutants were constructed for this study, in which M110 is substituted by alanine (M110A) and isoleucine (M110I). Both the M110I and M110A mutants complement oxidase-deficient strains RG129 and GO105, demonstrating that both mutant

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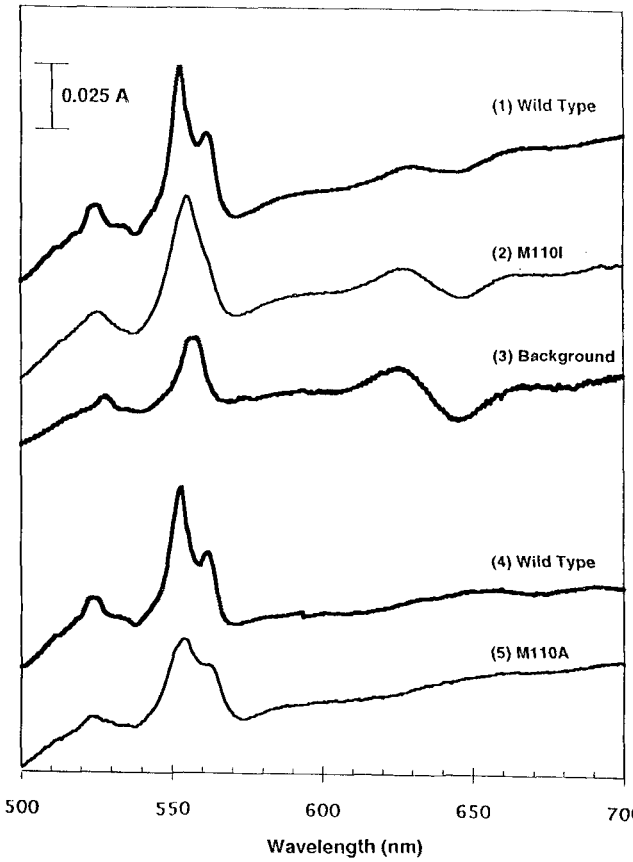

Fig. 1. The α- and β-bands of wildtype cytochrome bo_3 and M110 mutant oxidases. Samples contain 8 mg membrane protein/mL. Spectra were recorded at 77 k and are presented as dithionite-reduced *minus* air-oxidized visible spectra. The split α-band at 556 nm and 564 nm is characteristic of the wildtype enzyme. This split is nearly absent from the M110I mutant, but this is somewhat variable in different preparations. The top three spectra contain a minor amount of the cytochrome *bd* quinol oxidase from the background strain GL101. The bottom two spectra are in background strain GO105.

enzymes are functional in vivo. However, the M110I mutant grows more slowly than the wildtype control on minimal agar plates as well as on rich media supplemented with lactate. Activity assays show that the specific activity of M110I is 45% of the wildtype enzyme.

Visible dithionite-reduced minus air-oxidized spectra of the membranes containing the wildtype and mutant oxidases are shown in Fig. 1. In each case, the intensity of the absorbance clearly indicates that the oxidase is overproduced. The low spin heme *b* component of the wildtype enzyme has a split α-band with features near 556 nm and 564 nm. The spectrum of M110A is very similar to that of the wildtype oxidase, with perhaps a slightly broadened split α-band. The spectroscopic properties of M110I vary somewhat in independent membrane preparations. In most membrane preparations, M110I has a relatively diminished absorbance at 564 nm in the difference spectrum, and the split α-band is much less pronounced. However, in some preparations of M110I, the split α-band is much more apparent.

The EPR spectrum of wildtype cytochrome bo_3 contains features of heme *b*, which is low spin, with *g* values at 2.98, 2.22, and 1.47. EPR spectroscopy of M110I revealed small changes in the low spin heme signal (not shown), with the g_z value at 3.00, while the g_y remains at 2.22.

The EPR spectrum of the wildtype enzyme also contains a small signal at $g = 6$ from the high spin heme o_3 [22]. Magnetic interaction of Cu_B with heme o_3 results in a quenched signal at $g = 6$, and the intensity of this attenuated signal corresponds to much less than one heme per enzyme molecule. This spin coupling also renders Cu_B^{2+} EPR silent. If the sulfur of M110 formed a bridge between heme o_3 and Cu_B, the loss of this bridging atom might be expected to remove the spin coupling. The result should be a large increase in the high spin signal at $g = 6$, as well as the appearance of an EPR signal from the copper. However, the amount of the $g = 6$ signal in the EPR spectrum of M110I is comparable to that in the wildtype (not shown), demonstrating that the spin coupling still exists. No copper EPR signals are present near $g = 2$ in the EPR spectrum of M110I. By this criterion, the heme-copper binuclear center is unperturbed by the M110A and M110I mutations.

Another probe of the integrity of the dinuclear center is the FTIR spectrum of CO bound to the oxidase. CO forms a stable complex with heme o_3 under ambient conditions. Upon photolysis of the iron–carbon bond, CO is free to interact with Cu_B [23], and will form a stable Cu_B–CO adduct at cryogenic temperatures [23,24]. The CO adduct of the M110I mutant exhibits an FTIR difference spectrum which is indistinguishable from that of the wildtype oxidase (Fig. 2).

4. Discussion

Both genetic complementation and the in vitro quinol oxidase assays demonstrate that M110 can be substituted by

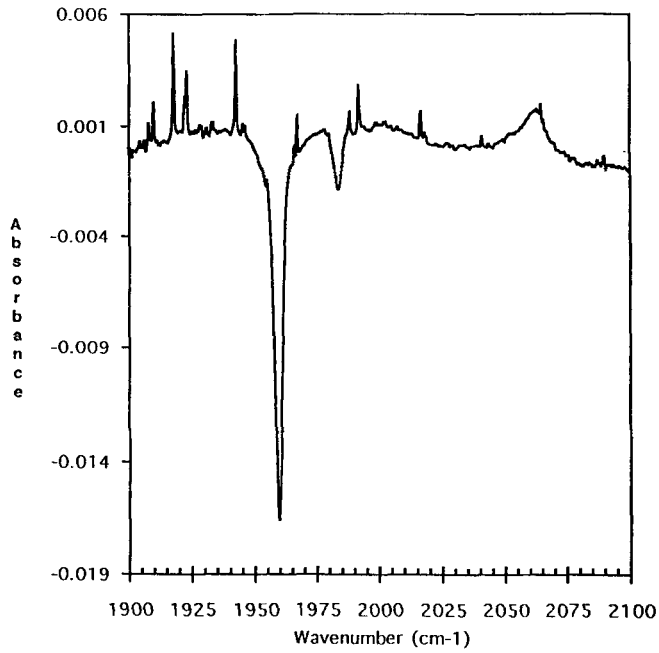

Fig. 2. Low temperature FTIR difference spectrum of the CO adduct of the M110I mutant oxidase, recorded using a membrane preparation containing the mutant. The difference spectrum is identical to that of the wildtype, with the Fe–CO band at 1959 cm^{-1} and the Cu_B–CO envelope near 2063 cm^{-1}. The Fe–CO band at 1984 cm^{-1} arises from cytochrome *bd*, which is also present in the membranes [26]. The spikes in the spectrum are water vapor.

another residue without elimination of enzymatic activity. M110A is indistinguishable from the wildtype oxidase, whereas M110I has about 45% of the specific activity of the wildtype control. Therefore, although this menthionine is very highly conserved among the heme-copper oxidases, it is not essential for the activity of the bo_3-type quinol oxidase.

In the current model of the heme-copper oxidases, M110 is one α-helical turn below H106, placing the sulfur of the methionine side chain near the periphery of the low spin heme *b* [4,7]. This model is incompatible with the suggestion that the sulfur atom of M110 is within the ligation sphere of the high spin heme o_3 or Cu_B [5]. The data presented definitively demonstrate that the substitutions for M110 do not perturb the integrity of the heme-copper dinuclear center. Hence, the sulfur atom of M110 is very unlikely to be a bridging ligand in cytochrome bo_3. By analogy, the equivalent methionines in the other members of the heme-copper superfamily are also unlikely to be located at the dinuclear center of these enzymes.

Whereas the M110A mutant has virtually identical enzymatic and spectroscopic properties as the wildtype, the isoleucine substitution at this position (M110I) has subtle affects on the visible and EPR spectra of the low spin heme *b* component of the oxidase. These changes may be due in part to the substitution of heme O for heme B at the low spin heme site, resulting in an oo_3-type oxidase, as has been previously reported [25]. Heme O causes narrowing and a blue-shift of the α-band, apparently due to the restoration of the symmetry of the heme electronic transitions [25]. This explanation would also be consistent with the variability of the spectroscopic properties of M110I, since this phenomenon is sensitive to growth conditions [25]. It should be noted that the perturbation caused by M110I cannot be simply ascribed to the substitution of heme O at the low spin site, since this substitution does not explain the reduced specific activity of this mutant [25]. A more detailed analysis would require the purification and full characterization of the M110I oxidase, which is beyond the scope of the present work.

In conclusion, the results presented here show that M110 is not required for the quinol oxidase activity of cytochrome bo_3 and does not appear to be in the immediate vicinity of the dinuclear center.

Acknowledgements: This work was supported by grants from the U.S. Department of Energy (R.B.G.) (DEFG02–87ER13716) and from the National Science Foundation (J.O.A.) (DMB89–04614).

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