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Site-directed mutagenesis studies on subunit I of the aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides*: a brief review of progress to date

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Introduction

The mitochondrial cytochrome *c* oxidase is closely related to a wide variety of bacterial respiratory oxidases [1,2]. Taken together, these oxidases comprise a superfamily of heme-copper containing oxidases. These oxidases are characterized by a unique bimetallic center consisting of a heme and a copper which is the site where molecular oxygen is reduced to water [1,3,4]. In addition, all the oxidases in this superfamily contain a six-coordinate heme which is the immediate electron donor to the heme-copper binuclear center [5,6]. The mitochondrial oxidase is an aa_3-type cytochrome *c* oxidase, where heme *a* is a six-coordinate heme and heme a_3 is the site where oxygen binds and reacts. Many bacterial oxidases are also aa_3-type cytochrome *c* oxidases, but there are members of this superfamily which contain *b*-type or *o*-type hemes [1,7–9]. Furthermore, not all members of this superfamily utilize a cytochrome *c* as a substrate. The *bo*-type oxidase from *Escherichia coli*, for example, is a ubiquinol-8 oxidase [10]. All the cytochrome *c* oxidases in this superfamily contain a fourth metal prosthetic group, Cu_A, which is absent in the quinol oxidases [1,11,12].

The sequences of bacterial operons encoding the subunits of the heme-copper oxidases have revealed genes homologous to the three mitochondrially encoded genes of the eukaryotic oxidases [1]. The largest of these subunits, subunit I, is very highly conserved, reflecting the fact that this subunit contains the ligands for the six-coordinate heme (i.e., heme *a*) and the binuclear center (i.e., heme a_3-Cu_B). Subunits II and III are less highly conserved. The residues implicated in both cytochrome *c* binding and Cu_A ligation are within subunit II of those members of the superfamily that utilize a *c*-type cytochrome as substrate [1,3,4]. Well characterized preparations of the aa_3-type cytochrome *c* oxidase from *Paracoccus denitrificans* demonstrate that subunits I and II together are sufficient for electron transport activity as well as for proton-pumping activity [13].

In this project, the aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* is being characterized along with a number of site-directed point mutants. The goal is to identify the structural or functional roles of critical amino acid residues and to use these data to construct a plausible and testable model of this enzyme. The targets for mutagenesis are provided by previous spectroscopic studies which implicate histidines as metal ligands, and by a knowledge of which residues are totally or very highly conserved in the many species from which subunit sequences are available. The project involves our research group at Urbana as well as the groups of Dr. S. Ferguson-Miller (Michigan State University), Dr. G. Babcock (Michigan State University) and Dr. J. Alben (Ohio State University).

Rb. sphaeroides contains three respiratory oxidases, of which two have been shown to be members of the heme-copper oxidase superfamily [14]. One of these oxidases is an aa_3-type cytochrome *c* oxidase. The enzyme has been purified by J. Hosler and S. Ferguson-Miller (unpublished data) as a three-subunit enzyme with high specific activity (> 1600 e^-/s) and efficient proton-pumping activity (0.85 H^+/e^-). The genes encoding each subunit have been cloned and sequenced [15]. The sequence of the *ctaD* gene, encoding subunit I, reveals that this subunit is 50% identical

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to subunit I of bovine cytochrome *c* oxidase [15]. This *ctaD* gene has been deleted from the bacterial genome, and it can be replaced by a plasmid-borne copy to fully restore the aa_3-type oxidase to the bacterial membrane. A number of site-directed mutations have been made by using this plasmid-based system [16]. The resulting mutant enzyme species can be analyzed by Fourier transform infrared (FTIR), resonance Raman, electron spin resonance and optical spectroscopic techniques, as well as by enzyme kinetics procedures. The FTIR technique probes the stretching frequency of CO bound to both heme a_3 and Cu_B [14,17], and this analysis can be performed using isolated bacterial membranes. Other evaluative techniques generally require purification of the mutant oxidases.

Results and Discussion

The studies to date have focussed on several regions within subunit I which appear to be functionally or structurally important. Fig. 1 is a two-dimensional model of subunit I of the aa_3-type cytochrome *c* oxidase from *Rb. sphaeroides*. The location of the twelve membrane-spanning helices in this model is supported by studies on the related *bo*-type oxidase from *E. coli* [18]. The results from mutating ten highly conserved residues, indicated in Fig. 1, are summarized below.

(1) Four helices (II, VI, VII, X) provide the metal ligands

An alignment of the sequences of subunit I from over 25 different organisms reveals only six totally conserved histidine residues [1,16]. Spectroscopic data indicate that heme *a* is ligated to two histidines [19–21], heme a_3 is ligated to one histidine [22,23] and Cu_B is ligated to at least two, or possibly three, histidines [24,25]. Not surprisingly, amino acid substitutions for any of the six conserved histidines results in inactive oxidase [16]. Spectroscopic analyses of several mutants clearly identify His-102 (helix II) and His-421 (helix X) as the heme *a* ligands [16]. Hence, helix II and helix X must be adjacent since heme *a* is ligated between them. The mutations of the remaining four conserved histidines all perturb the heme a_3-Cu_B binuclear center and have relatively little or no influence on spectroscopic properties of heme *a*. The data so far are insufficient to uniquely assign which histidine is the

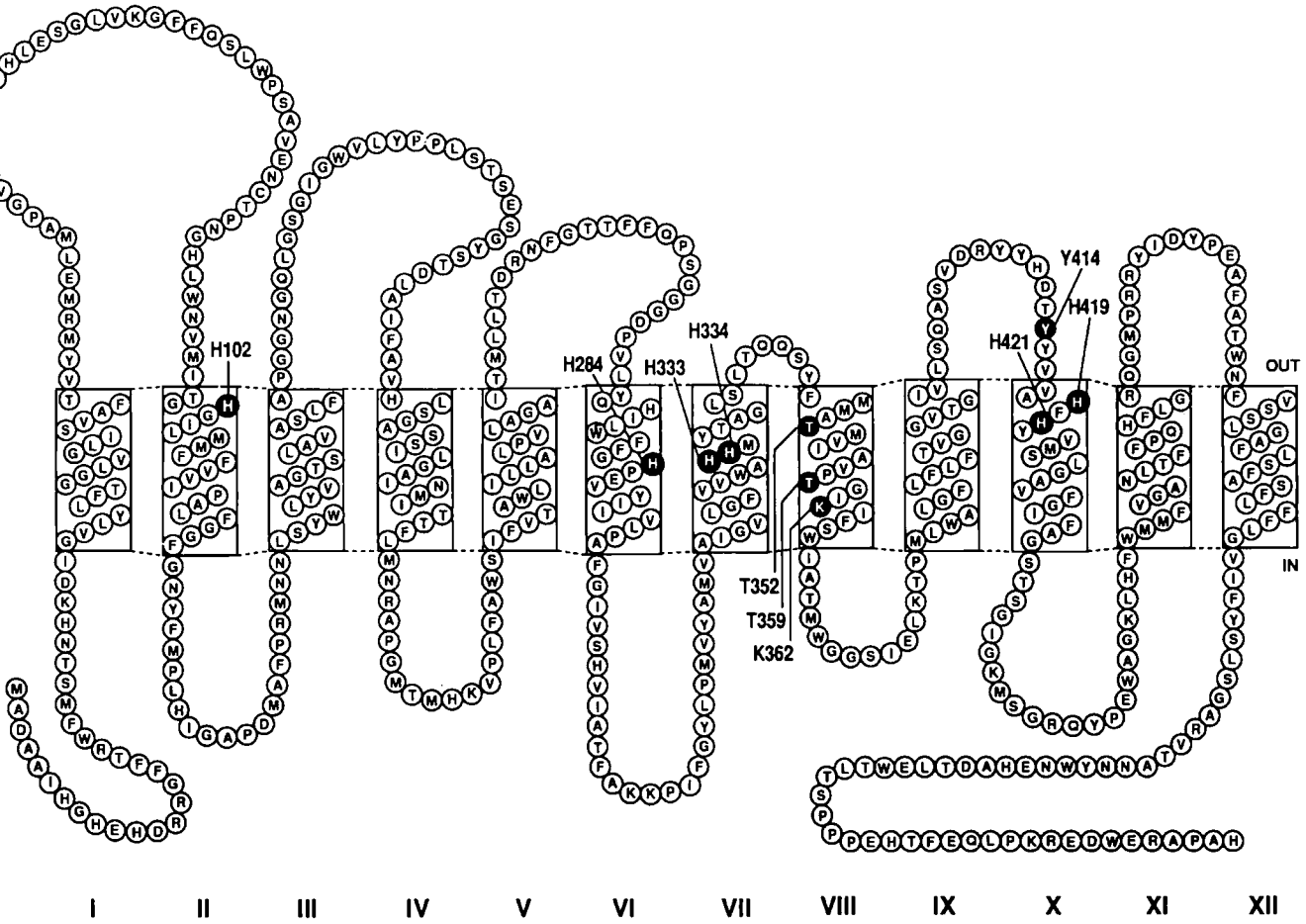

Fig. 1. A two-dimensional model of subunit I of the aa_3-type cytochrome *c* oxidase from *Rb. sphaeroides*. Twelve membrane spanning α-helical segments are indicated. The ten residues discussed in the text are highlighted.

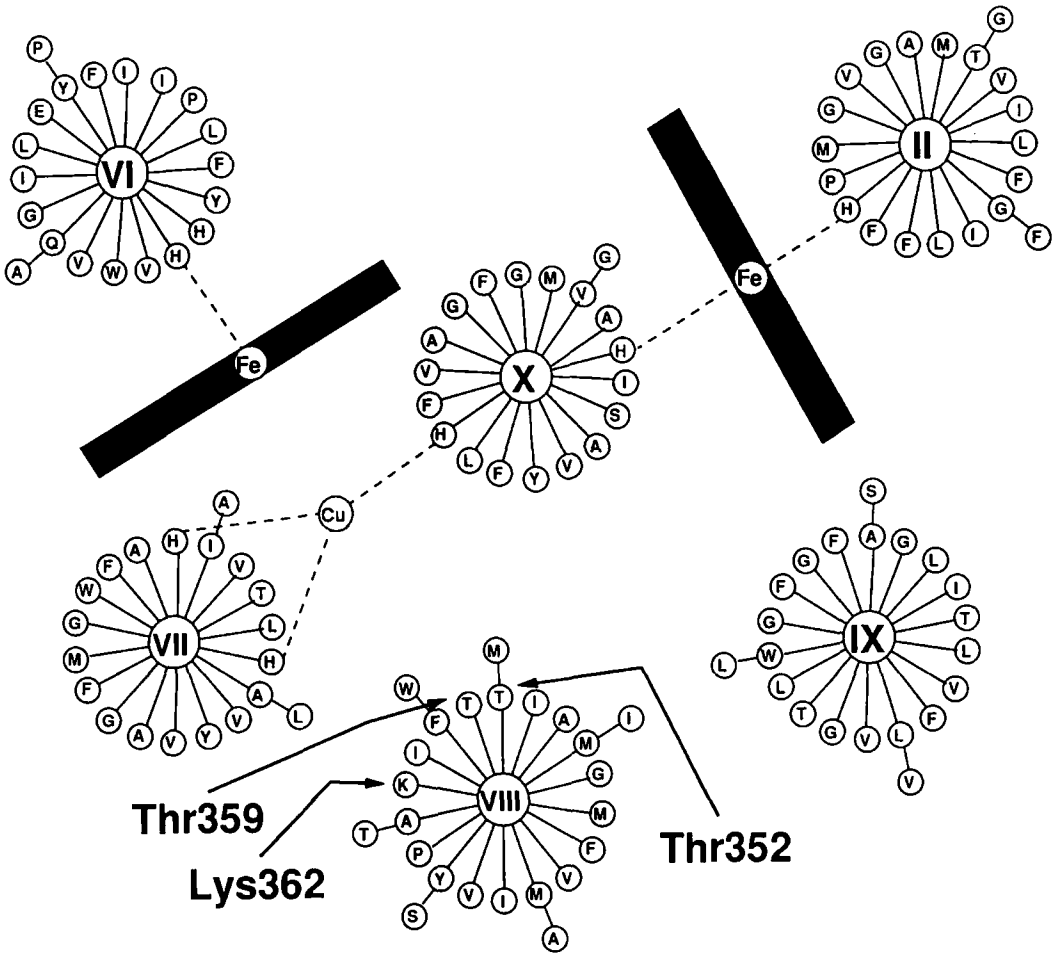

Fig. 2. A view from the periplasmic surface of a model in which heme *a*, heme a_3, and Cu_B are placed in relationship to six of the twelve membrane-spanning helices of subunit I. Each helix is indicated as a helical wheel. In this model, His-284 is proposed as the proximal ligand of heme a_3. Tyr-414, located between helix IX and helix X (Fig. 1) is not shown in this diagram. Data in support of this model are discussed in the text.

ligand to heme a_3. One reasonable model, illustrated in Fig. 2, places His-284 in helix VI as the proximal ligand of heme a_3, which leaves His-333, His-334 (helix VII) and His-419 (helix X) as potential Cu_B ligands.

In any reasonable model, including the one shown (Fig. 2), the binuclear center must be located at about the same level as heme *a*, near the periplasmic (positive) side of the membrane. This model is consistent with rapid electron transfer observed between heme *a* and the heme a_3-Cu_B binuclear center [26]. The model also provides a potential mechanism for allosteric interactions [27] between heme *a* and the binuclear center via residues in helix X (see Fig. 2).

(2) The interhelical loop between helix IX and X may be in contact with heme a

It has been suggested previously that Tyr-414 might be hydrogen bonded to the formyl group of heme *a* [28]. This residue has been changed to a phenylalanine to test this hypothesis (unpublished data). The resulting mutant has substantial cytochrome *c* oxidase activity, and the resonance Raman spectrum indicates no perturbation of the heme *a* formyl group. However, the α-band of heme *a* is shifted from 607 nm to 611 nm. These data indicate that although Tyr-414 is unlikely to be a hydrogen bond donor to the heme *a* formyl group, it is very likely to be located close to heme *a*. This is consistent with the location of heme *a* near the periplasmic surface, and also suggests that helix IX should be located in the model (Fig. 2) to allow this residue within the interhelical loop to helix X to be close to heme *a*. This region of the polypeptide may provide a 'cap' over heme *a*, and possibly over the binuclear center as well.

(3) Helix VIII may be part of the proton-conducting channel

During oxidase turnover, protons must move from the cytoplasm of the bacterium to the site where oxygen is reduced to water [1,4]. In addition, protons (1 H^+/e^-) are pumped across the membrane during enzyme turnover [29]. These proton movements suggest

that the oxidase must contain at least one proton-conducting channel to facilitate these proton movements. Since subunits I and II appear to be sufficient for the proton pumping activity of cytochrome *c* oxidase [13], it is likely that at least some of the components of a proton-conducting channel are within the twelve transmembrane spans of subunit I. Subunit II has only two putative membrane spanning helices [1,3].

It is reasonable to assume that a proton-conducting channel within the oxidase would contain polar residues located within the putative membrane spanning helices, as is the case in bacteriorhodopsin [30,31]. Several very highly conserved polar residues (Thr-352, Thr-359, Lys-362) are located along one side of helix VIII. Substitutions for each of these three residues suggest that they may be important. The analysis is not yet complete, but it is clear, for example, that Lys-362-Met is enzymatically inactive. Optical spectroscopy and FTIR of bound CO indicate that both heme *a* and the heme a_3-Cu_B binuclear center are unperturbed by this mutation. In contrast, Thr-352-Ala, located three helical turns above Lys-362, results in substantial perturbation of the binuclear center by FTIR analysis, probably due to the loss of Cu_B.

These data support placing helix VIII in the model shown in Fig. 2 such that the polar face leads up to the binuclear center, potentially involved in conveying protons from the cytoplasm during enzyme turnover.

The model shown (Fig. 2), though obviously highly speculative, at least provides a rationale for the few data obtained so far. This model is a useful visual aid, and will doubtless be greatly refined or even drastically altered as more data are accumulated. Clearly the analysis of mutants will never result in the equivalent of a high resolution structure. However, there is reason to be optimistic that insights into both the structure and the functional mechanism of the oxidase can be gained by the application of molecular genetics techniques in combination with the powerful biophysical methods available for the analysis of this fascinating enzyme.

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