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Rapid report

Interaction between the formyl group of heme *a* and arginine 54 in cytochrome aa_3 from *Paracoccus denitrificans*

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Abstract

The optical spectrum of heme *a* is red-shifted in aa_3-type cytochrome *c* oxidases compared to isolated low-spin heme A model compounds. Early spectroscopic studies indicated that this may be due to hydrogen-bonding of the formyl group of heme *a* to an amino acid in the close vicinity. Here we show that most of the optical spectral shift of native heme *a* is due to a hydrogen-bonding interaction between the formyl group and arginine-54 in subunit I of cytochrome aa_3 from *Paracoccus denitrificans*, and that a smaller part is due to an electrostatic interaction between the D ring propionate of heme *a* and arginine-474.

Keywords: Cytochrome *c* oxidase; heme *a*

Cytochrome *c* oxidases of mitochondrial or bacterial origin catalyze the respiratory reduction of O_2 to water at the bimetallic heme iron (a_3)–copper (Cu_B) center in subunit I of these membrane-bound enzymes. This reaction is coupled to proton translocation across the mitochondrial or bacterial membrane [1]. Subunit II contains the binuclear Cu_A center, which is the primary acceptor of electrons from cytochrome *c*, and donates electrons to heme *a* in subunit I, from which they are transferred to the O_2 reduction site. The iron of heme *a* of cytochrome *c* oxidases is low spin with two histidine residues as axial ligands. In cytochrome *c* oxidase, from *Paracoccus denitrificans* these ligand residues are His-94 and His-413, in transmembrane helices 2 and 10 of subunit I, respectively [2].

Heme A is unique to the respiratory chain. It differs from protoheme (heme B) in having hydroxyethylfarnesyl and formyl side chains in place of vinyl and methyl groups in positions 2 and 8 of the porphyrin ring, respectively [3]. Synthesis of heme A from protoheme occurs in two steps with heme O as an intermediate [4]. The formyl group is strongly electron-withdrawing, and this alters redox and ligand binding reactions, interactions of the porphyrin π system with the protein, as well as the optical absorption spectrum [3].

Spectroscopic studies showed that the optical spectrum of both ferric and ferrous heme *a* are considerably red shifted in mitochondrial cytochrome *c* oxidase, as compared to isolated heme A model com-

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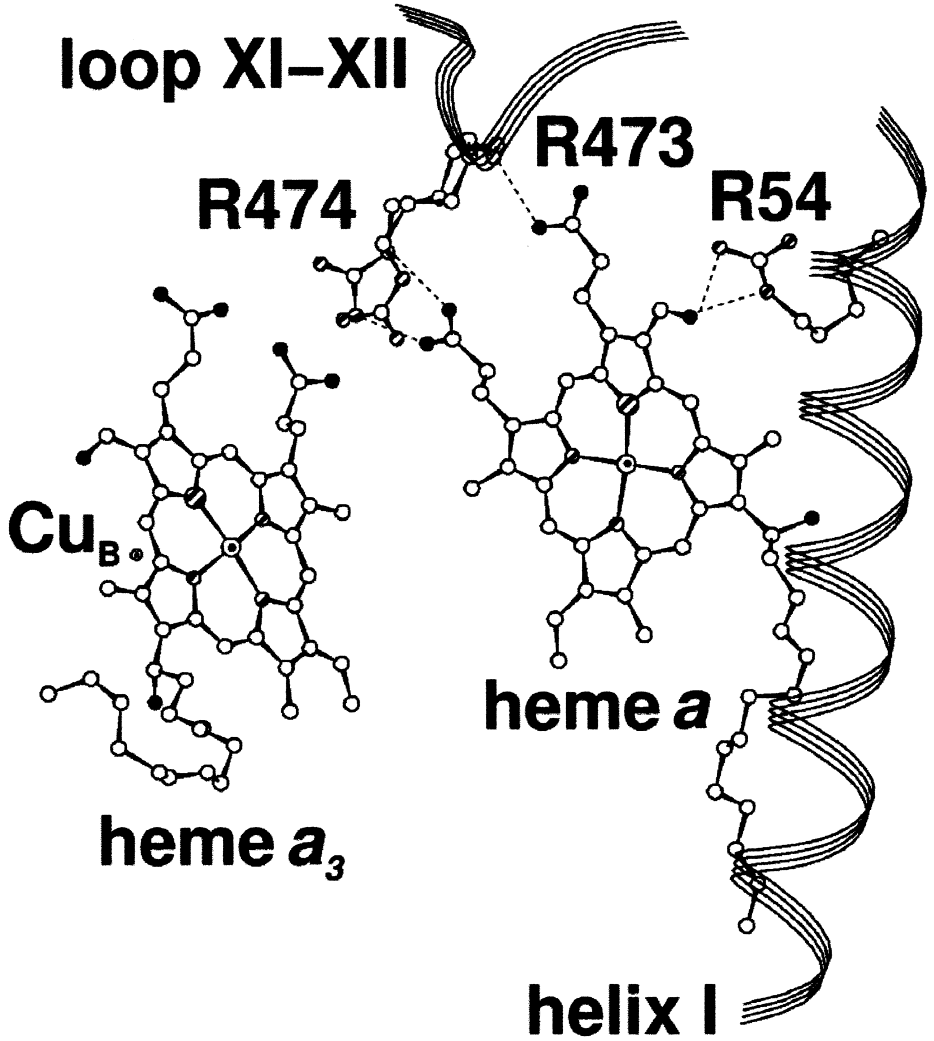

Fig. 1. The hydrogen-bonding interactions between the formyl group of heme *a* and Arg-54 in helix 1 of subunit I in the aa_3-type cytochrome *c* oxidase from *P. denitrificans*. Arginines 473 and 474 in the loop between helices 11 and 12 interact with the propionates of heme *a* through the backbone nitrogen of Arg-473 and through the side chain nitrogens of Arg-474. The protein backbone is marked with ribbons. Atoms are marked as spheres: carbons (white), oxygens (black), nitrogens (hatched) and metals (cocentric circles). Dotted lines show the possible interactions between amino acids and heme *a*.

pounds [5]. In the α-band, the absorption peak of ferrous heme *a* is shifted ca. 17 nm to the red when compared to isolated bis-imidazole heme A [6]. In resonance Raman spectra, the stretching frequency of the formyl carbonyl of heme *a* is shifted to a lower wavenumber [6]. Based on these observations, it was suggested that there is a hydrogen-bonding interaction between the formyl oxygen of heme *a* and a proton donor from the protein, perhaps the hydroxyl group of a tyrosine residue [6]. In the crystal structure model of the aa_3-type cytochrome *c* oxidase from *P. denitrificans*, it was proposed that the formyl group of heme *a* forms a hydrogen bond to the side chain of Arg-54 in helix 1 of subunit I [2] (Fig. 1).

The hydrogen bond to the formyl group was proposed to be involved in proton translocation of the mitochondrial enzyme [6]. Since there is good reason to assume that the proton translocation mechanism is the same in all heme–copper oxidases, and when it was discovered that cytochrome bo_3 from *Escherichia coli* functions as a proton pump, this mechanism was rendered unlikely [7]. Arg-54 has also been suggested to be involved, via its interaction with the formyl

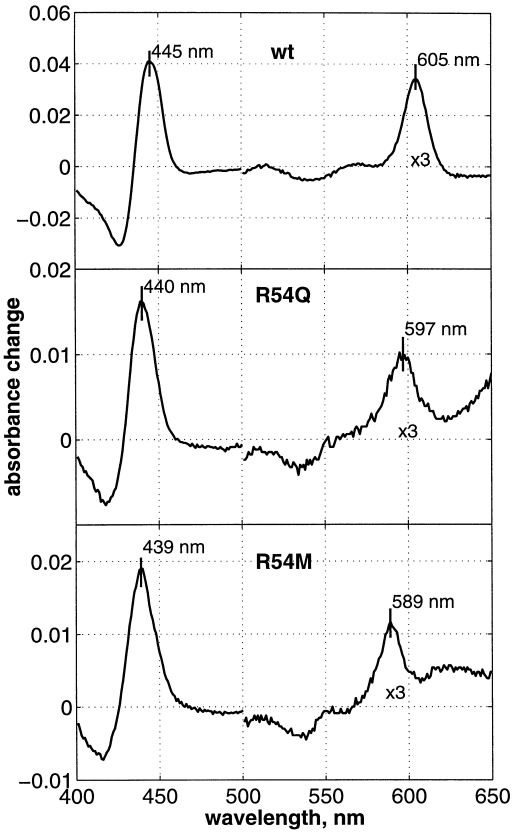

Fig. 2. The reduced minus oxidized difference spectra of heme *a* from wild-type enzyme (top panel) and two mutant enzymes Arg-54-Gln (R54Q, central panel), and Arg-54-Met (R54M, bottom panel). The spectrum of oxidized cyanide-inhibited enzyme was subtracted from spectra of the same enzyme after reduction with ascorbate and TMPD. The α-region is expanded 3-fold. The measurements were done in 50 mM Bis-Tris propane buffer, pH 7.5, 0.02% dodecyl maltoside, and 0.5 mM EGTA, supplemented with 5 mM KCN, 2.5 mM ascorbate and 56 μM TMPD. The enzyme concentrations used were 0.7 μM for wild-type (wt) and 0.5 μM for the R54Q and R54M mutant enzymes.

group, in the mechanism of the Ca^{2+}-induced spectral shift of heme *a* [8].

To change the strength of the hydrogen bond between the Arg-54 and the formyl group of heme *a*, and to find out whether modification of this arginine affects the optical spectrum of heme *a*, this residue was substituted with glutamine and methionine. In the isolated R54Q mutant enzyme the reduced *minus* oxidized absorption spectrum of heme *a* is blue-shifted by 8 nm from the 605 nm peak of wild-type to 597 nm in the mutant (Fig. 2). In the R54M mutant enzyme the blue-shift is even more extensive; the absorption maximum is at 589 nm, a shift of 16 nm (Fig. 2). The oxygen consumption activity of the R54Q mutant enzyme was about one fourth of the wild-type activity, while mutation of Arg-54 to methionine caused almost complete loss of activity.

As shown by Callahan and Babcock [5], hydrogen-bonding to the formyl group is stronger in the ferrous than in the ferric heme *a*, indicating that this effect would tend to increase the midpoint redox potential (E_m). Hence, weakening or abolishing hydrogen-bonding in the present mutants would be expected to lower the E_m of heme *a*, relative to the wild-type enzyme. The decreased turnover of the mutant enzymes could be due to such an effect, which is supported by our finding that heme *a* was reduced to an extent of 23 and 18% in the R54Q and R54M mutants during steady-state respiration, whereas it was reduced to 40% in the wild-type enzyme (not shown). On the other hand, there were no indications of major structural pertubations of heme a_3 in the mutant enzymes. Pyridine hemochrome spectra of both mutant enzymes indicate a small amount of heme O. However, heme O did not appear in reduced *minus* oxidized difference spectra of the enzymes. The presence of heme O seems to be a general feature of the enzyme expression system used [8]. The proton translocation activity of the R54Q mutant was as in wild-type enzyme, but could not be measured in the R54M mutant due to the very low enzyme activity.

Our data suggest that there is still hydrogen-bonding to the formyl group in the R54Q mutant enzyme, but that the bond is weakened due to an increased bond length and/or a less favorable bond angle, either of which would reduce the conjugation effect on the porphyrin ring. In the R54M mutant, the absence of hydrogen-bonding shifts the absorption peak of heme *a* almost, but not quite, to the position for isolated heme A. Therefore, hydrogen-bonding to the formyl group of heme *a* is the major, but not the only, reason for the red shift in the spectrum.

The loop between transmembrane helices 11 and 12 in subunit I of the *P. denitrificans* enzyme contains two conserved arginines, 473 and 474, which interact with the ring D propionates of the two heme groups by charge interactions as well as hydrogen bonds [2,9–11]. Arg-474 is closer to the D-propionate of heme *a*, whereas Arg-473 is closer to the D-propionate of heme a_3. We have earlier mutagen-

ized Arg-474 to an asparagine, and observed a blue shift of 2–3 nm in the optical absorption spectrum of ferrous heme *a* [8]. This interaction is hence probably responsible for the remainder of the red shift of the absorption spectrum of heme *a*, compared to isolated heme A.

We conclude that the observed red shift of heme *a* in cytochome *c* oxidase, compared to isolated model heme A compounds, is mainly due to a specific hydrogen-bonding interaction between the heme *a* formyl group and Arg-54, and partially to an electrostatic effect exerted via the interaction between the D-propionate of the heme and Arg-474.

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