Site-Directed Mutagenesis of Residues Lining a Putative Proton Transfer Pathway in Cytochrome *c* Oxidase from *Rhodobacter sphaeroides*†

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ABSTRACT: Several putative proton transfer pathways have been identified in the recent crystal structures of the cytochrome oxidases from *Paracoccus denitrificans* [Iwata et al. (1995) *Nature 376*, 660−669] and bovine [Tsukihara (1996) *Science 272*, 1138−1144]. A series of residues along one face of the amphiphilic transmembrane helix IV lie in one of these proton transfer pathways. The possible role of these residues in proton transfer was examined by site-directed mutagenesis. The three conserved residues of helix IV that have been implicated in the putative proton transfer pathway (Ser-201, Asn-207, and Thr-211) were individually changed to alanine. The mutants were purified, analyzed for steady-state turnover rate and proton pumping efficiency, and structurally probed with resonance Raman spectroscopy and FTIR difference spectroscopy. The mutation of Ser-201 to alanine decreased the enzyme turnover rate by half, and was therefore further characterized using EPR spectroscopy and rapid kinetic methods. The results demonstrate that none of these hydrophilic residues are essential for proton pumping or oxygen reduction activities, and suggest a model of redundant or flexible proton transfer pathways. Whereas previously reported mutants at the start of this putative channel (e.g., Asp-132-Asn) dramatically influence both enzyme turnover and coupling to proton pumping, the current work shows that this is not the case for all residues observed in this channel.

Cytochrome *c* oxidase is an integral membrane metalloprotein that couples the four-electron reduction of dioxygen to water to the translocation of protons across a lipid bilayer. The proton gradient created by this catalysis is used in various energy-requiring processes, including the synthesis of adenosine 5′-triphosphate (ATP).[1] The molecular mechanism by which the enzyme translocates protons has been the subject of much study and speculation (Gelles et al., 1987; Iwata et al., 1995; Musser & Chan, 1995; Rousseau et al., 1993; Wikström et al., 1994; Woodruff, 1993). However, the recently determined high-resolution structures of cytochrome oxidase from *Paracoccus denitrificans* (Iwata et al., 1995) and bovine (Tsukihara et al., 1996) will without doubt greatly facilitate the elucidation of this mechanism. In this paper, site-directed mutagenesis of the *Rhodobacter sphaeroides* oxidase is used to examine the functional importance of several residues highlighted by the structure of the very closely related oxidase from *P. denitrificans*. Two putative proton transfer pathways have been identified in the crystal structure of the bacterial oxidases: one is speculated to be for protons consumed in water formation and another for protons translocated across the membrane (Iwata et al., 1995). The latter pathway was found to begin at Asp-132 (*R. sphaeroides* numbering), proceed by a series of hydrophilic residues and solvent molecules to Glu-286, and end in a cluster of carboxylic acid residues at the periplasmic surface. Three conserved hydrophilic residues originating from helix IV are present in this proton transfer pathway: (bottom to top) Thr-211, Asn-207, and Ser-201. These correspond to *Paracoccus*: T203, N199, S193; and bovine: T167, N163, and S157. According to Iwata et al. (1995), Thr-211 and Asn-207 form a gate on the cytoplasmic side of the proton transfer pathway in conjunction with Asp-132, whereas Ser-201 is positioned further up the pathway, lining a solvent-filled cavity with other hydrophilic residues and leading, via hydrogen bonding interactions, to Glu-286. Site-directed mutagenesis studies in *R. sphaeroides* have previously demonstrated the importance of Asp-132 and Glu-286 residues in maintaining enzyme activity (Fetter et al., 1995; Hosler et al., 1993). In the bovine oxidase structure (Tsukihara et al., 1996), Ser-201 is also noted to be within a possible proton transfer pathway,

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; PCR, polymerase chain reaction; EPR, electron paramagnetic resonance; FTIR, Fourier-transform infrared; Ser, serine; Asn, asparagine; Thr, threonine; CO, carbon monoxide; C-terminus, carboxy-terminus; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid]; Tris, tris(hydroxymethyl)aminomethane; Cu_A, copper A; Cu_B, copper B.

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although Asn-207 and Thr-211 are not mentioned. The importance of Asp-132 has been best documented in studies of mutants of the equivalent residue in the *E. coli* cytochrome bo_3 ubiquinol oxidase (Garcia-Horsman et al., 1995; Thomas et al., 1993). Hence, there is little doubt that the putative channels observed in the X-ray structures are functionally important.

To examine the possible role of the hydrophilic face of helix IV, Ser-201, Asn-207, and Thr-211 were individually changed to alanine, and the mutants were fully characterized. The results clearly demonstrate that these residues are nonessential for enzyme activity when mutated individually, although mutation of Ser-201 does affect enzyme turnover rate. A model involving multiple proton transfer pathways is suggested.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Mutants were constructed using a single-step PCR method. Single-stranded oligonucleotide mutagenic primers were synthesized at the University of Illinois Biotechnology Center, Urbana, IL, for each of the mutants. Ser-201-Ala: 5′-GGT CG**T GAT CA**T GTT GAT CGC GCC GAG GAT CGC GGA GGC-3′; Asn-207-Ala: GGT CG**T GAT CA**T GGC GAT CGC GC-3′; Thr-211-Ala: 5′-CAA CA**T GAT CA**C GGC CTT CC-3′. Each of the primers overlapped a unique *Bcl*I restriction site (in boldface) in the *Bam*HI−*Sal*I restriction fragment of the *ctaD* gene, which codes for subunit I of cytochrome aa_3 from *R. sphaeroides* (Shapleigh & Gennis, 1992). The *Bam*HI-*Sal*I restriction fragment was cloned into the commercial phagemid pT7T3 19U (Pharmacia) for use as a template for PCR reactions. A universal forward or reverse primer was used in conjunction with the mutagenic primers for amplification. Vent polymerase (New England Biolabs) was used for PCR reactions. PCR products were digested with *Bcl*I and other appropriate restriction enzymes for cloning into pJS3, a plasmid containing the wild-type *ctaD* gene (Shapleigh & Gennis, 1992). Due to the *dam*-methylation of the *Bcl*I restriction site in common *E. coli* strains, a *dam*- strain, GM2199, was used for all cloning steps involving *Bcl*I restriction digests. Mutations were verified by DNA sequencing.

Protein Purification. The mutants were grown and purified as described previously (Hosler et al., 1992). In the case of Ser-201-Ala, additional enzyme was purified by affinity chromatography. This was facilitated by constructing a genetic fusion of six histidines to the C-terminus of subunit I, allowing the tagged enzyme to be purified using nickel(II) nitrilotriacetic acid−agarose, as previously described (Mitchell & Gennis, 1995). Such a modification has been shown to have no detectable effects on the structure or function of the enzyme (Mitchell & Gennis, 1995).

Activity Assays. Activity was monitored spectrophotometrically by following oxidation of ferrocytochrome *c* at 550 nm. Reaction conditions were as follows: 50 mM phosphate, pH 6.5, 0.02% lauryl maltoside, 20−30 μM ferrocytochrome *c*. Proton pumping assays were performed as previously described (Hosler et al., 1992).

FTIR Spectroscopy. FTIR samples were prepared as described previously (Shapleigh et al., 1992a). Glycerol-dehydrated samples were pressed between two CaF_2 windows to a thickness of 25 μm. FTIR absorption spectra were obtained using a Mattson Sirius 100 FTIR interferometer at a resolution of 0.5 cm^{-1} using a liquid nitrogen cooled indium−antimonide detector. Spectra were obtained at 10−30 K. Spectra are presented as light-*minus*-dark difference spectra. Photodissociation of CO was achieved by using continuous radiation from a focused 500 W tungsten bulb. Light and dark spectra are averages of 512 scans.

Resonance Raman Spectroscopy. Resonance Raman spectra were obtained using a Spex 1401 spectrometer equipped with a photon-counting photomultiplier detector. Excitation at 441.6 nm was provided by pumping Stilbene 420 (Coherent 599 dye laser) with multiline ultraviolet light from an argon ion laser (2 W). Cytochrome *c* oxidase samples (30 μM in 100 mM KH_2PO_4, pH 7.5/0.1% lauryl maltoside) were reduced with sodium dithionite under N_2 flow and placed in capillary tubes for spectra acquisition. The spectra were taken with 14.3 mW of power on the sample, and the average of 8 scans is shown. The temperature was maintained between 1 and 7 °C with a stream of cold N_2 gas.

Internal Electron Transfer Kinetics. Enzyme purified by the above affinity methods was used. Mixed-valence oxidase was prepared as described previously (Brzezinski & Malmström, 1987), in 0.1 M HEPES, pH 7.2. The excitation laser and observation equipment have been described in detail earlier (Hallén & Brzezinski, 1994). The analysis of kinetic traces was performed by the same methods as used by Hallén et al. (1994).

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, 10 K; microwave power, 2 mW; microwave frequency, 9.45 GHz; modulation amplitude, 2 mT; time constant, 200 ms; recording time, 100 s. Samples were 60−80 μM in 50 mM Tris, pH 8.0, 0.1% lauryl maltoside.

Proton Pumping. Measurement of proton pumping was performed by the cytochrome *c* pulse method after reconstitution of purified enzyme into asolectin vesicles as described by Fetter et al. (1995). Vesicles were diluted into 2.5 mL of 50 μM $NaHCO_3$, 45 mM KCl, and 44 mM sucrose with 50 μM phenol red at pH 7.4, to give 43 nM of wild type and 50 nM S201A mutant *R. sphaeroides* cytochrome *c* oxidase, and equilibrated with the addition of 4 μM valinomycin. Changes in extravesicular pH upon addition of 0.95 nmol of ferrocytochrome *c* were measured in an Aminco DW2A spectrophotometer using the absorption at 556.8 minus 504.7 nm. The total alkalinization caused by the same amount of cytochrome *c* was measured after addition of 4 μM CCCP. Additions of 1 nmol of HCl were used to calculate the H^+/e^- ratios.

RESULTS

The mutants Ser-201-Ala, Asn-207-Ala, and Thr-211-Ala are each functional, demonstrating nearly normal turnover rates and normal efficiency of proton translocation. Ser-201-Ala, however, has a turnover rate of approximately 50% of wild type (~550 s^{-1}).

The effects of each mutation on the structure of the binuclear center and on the structural environment of the hemes were examined using Fourier-transform infrared absorbance difference spectroscopy and resonance Raman spectroscopy. In the FTIR analysis, the CO adduct of fully

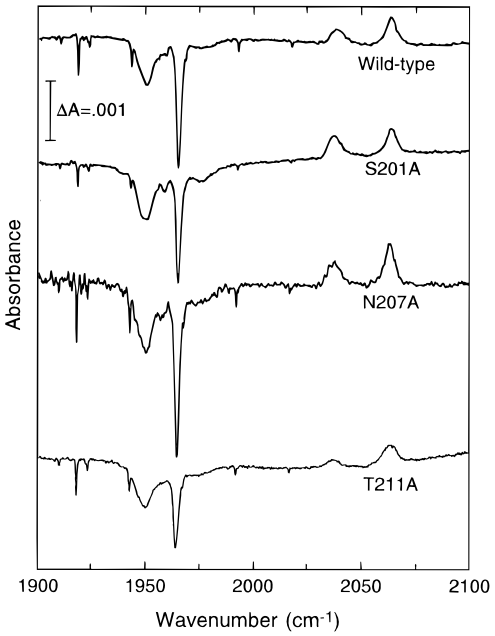

FIGURE 1: Low-temperature (10−30 K) FTIR light-*minus*-dark difference spectra of membranes containing the wild type aa_3-type oxidase and the Ser-201-Ala, Asn-207-Ala, and Thr-211-Ala mutants in the fully-reduced CO-complexed form.

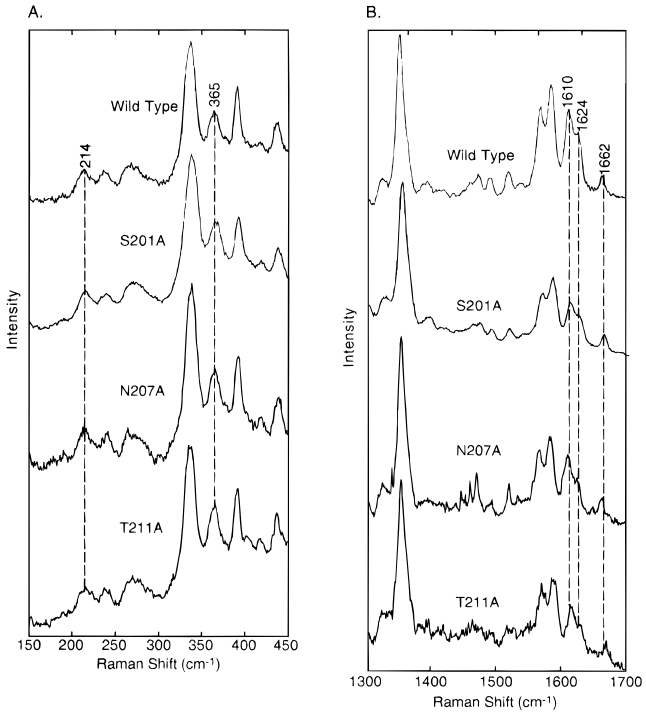

FIGURE 2: Resonance Raman spectra of the purified aa_3-type oxidase from *R. sphaeroides*. The wild type spectrum is shown along with those from three mutants, Ser-201-Ala (S201A), Asn-207-Ala (N207A), and Thr-211-Ala (T211A). Panel A: Low-frequency region. The indicated bands at 214 and 365 cm^{-1} are associated with the Fe−histidine bond of heme a_3 and the ring bending mode, respectively. Panel B: High-frequency region. The indicated bands correspond to the heme *a* formyl stretch (1610 cm^{-1}), the heme *a* vinyl stretch (1624 cm^{-1}), and the heme a_3 formyl stretch (1662 cm^{-1}).

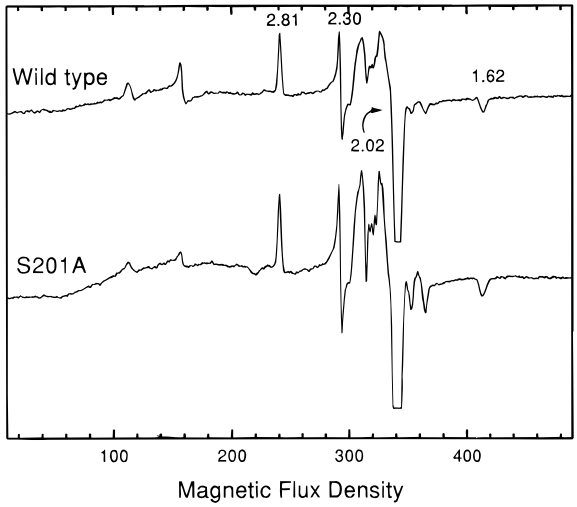

FIGURE 3: Electron paramagnetic resonance spectra of purified wild type cytochrome oxidase and Ser-201-Ala mutant. See Materials and Methods for conditions. *g*-values are indicated.

reduced cytochrome oxidase was examined. In this method, the vibrational frequency of CO, complexed to Fe_{a3}, is first monitored at 10−30 K. Upon photolysis, which yields a stable Cu_B−CO complex, the vibrational frequency of CO is once again observed. The resulting light-*minus*-dark FTIR difference spectrum has been used successfully as a sensitive probe of the structure of the binuclear center (Alben et al., 1981; Hosler et al., 1993). Figure 1 shows the FTIR difference spectra of each of the helix IV mutants compared to wild type. It is clear that no significant deviations in the structure of the binuclear center are present in these mutants.

Resonance Raman spectroscopy of fully reduced cytochrome oxidase, with excitation in the Soret region (441.6 nm), has been used as a sensitive probe of the environment surrounding the two heme A porphyrin rings (Babcock et al., 1981; Babcock & Salmeen, 1979; Ogura et al., 1983; Woodruff et al., 1981, 1982). Several distinct porphyrin vibrational modes, assigned to heme *a* and heme a_3, have been used previously to interpret effects of site-directed mutants on the structure of the enzyme (Hosler et al., 1993; Shapleigh et al., 1992b). The resonance Raman spectra of the helix-4 mutants compared to wild type (Figure 2), in agreement with the FTIR results, indicate that none of the mutations have a substantial effect on the environment of the porphyrin rings or the Fe−His bond of heme a_3.

The Ser-201-Ala mutant, because of its significant decrease in turnover rate, was further characterized by EPR and rapid kinetic methods. EPR spectra of wild type and Ser-201-Ala, shown in Figure 3, indicate that the Ser-201-Ala mutant is essentially the same as wild type, and has normal heme *a* and Cu_A environments as well as a coupled binuclear center.

Flash photolysis of CO from mixed-valence cytochrome oxidase has been used previously to follow internal electron transfers and CO recombination on a microsecond to second time scale (Ädelroth et al., 1995; Hallén et al., 1994). The mixed-valence enzyme is in a two-electron-reduced state with heme a_3 and Cu_B of the binuclear center reduced and CO bound to heme a_3 as the distal axial ligand, causing an apparent increase in the reduction potential of heme a_3. Upon photolysis of the Fe−CO bond, the electron is no longer stabilized on heme a_3 and will equilibrate with heme *a* and Cu_A, i.e., be transferred backward (opposite to the physiological direction) through the enzyme. This is observed as three phases in the absorbance changes following CO dissociation, where the first and second phases have been interpreted as representing electron transfer from heme a_3 to heme *a* (time constant 3 μs) and then from heme *a* to

Table 1: Wild Type and Mutant Cytochrome Oxidase Turnover Rate and Proton Pumping Efficiency

mutant	turnover rate (electrons/s)[a]	H^+/e^- ratio
wild type	1250	0.4−0.8
Ser-201-Ala	550	0.4−0.7
Asn-207-Ala	1200	0.5−0.6
Thr-211-Ala	1100	0.5−0.6

[a] Relative error: ∼10%.

Table 2: Internal Electron Transfer and CO Recombination Kinetics for Wild Type and Ser-201-Ala Mutant Enzymes, Measured in a Buffer Containing 0.1 M HEPES−KOH pH 7.2, with 0.1% Dodecyl Maltoside[a]

	wild type	S201A
(A) Mixed-Valence Enzyme		
(1) cyt a_3 to cyt a (1°)		
obsd[b] time constant (μs)	2.7	2.6
extent of cyt a reduced (%)	45	47
(2) cyt a to Cu_A		
obsd[b] time constant (μs)	40	70
extent of Cu_A reduced (%)	<10	<10
(3) CO recombination		
time constant[c] (ms)	60	130
(4) cyt a_3 to cyt a (2°)		
time constant[d] (ms)	1.6	1.3
(B) Fully Reduced Enzyme		
(1) CO-recombination time constant (ms)	20	23

[a] The enzyme and CO concentrations were 2 μM and 1 mM, respectively. [b] The observed time constant is the inverse of the sum of the forward and backward rate constants. [c] The value is for the major phase of the reaction. In both the wild type and Ser-201-Ala mutant enzymes, there is also a small slower phase of CO recombination whose origin is not fully understood at this moment. [d] This value was obtained at pH 8.8 instead of pH 7.2, because of the larger extent of the reaction at higher pH.

Cu_A (time constant 35 μs), respectively. A slower third phase (time constant around 1 ms at neutral pH) is associated with a second phase of electron transfer from heme a_3 to heme a which is slowed down due to an accompanying proton release. The results from Ser-201-Ala are shown in Table 2. Only one of the rate constants, the recombination rate of CO with heme a_3 in the mixed-valence state, was significantly altered, by approximately 2-fold.

DISCUSSION

The above results clearly suggest that the hydrophilic residues Ser-201, Asn-207, and Thr-211 are not essential for either enzyme turnover or efficient proton translocation of the *R. sphaeroides* oxidase. The X-ray structure of the *Paracoccus* oxidase (Iwata et al., 1995) shows that these three residues are in the same putative proton-conducting channel as are Asp-132, Asn-125, Asn-123, and Glu-286. Previous results from site-directed mutagenesis in *E. coli* (cytochrome bo_3) (Garcia-Horsman et al., 1995; Thomas et al., 1993) and in *R. sphaeroides* (Fetter et al., 1995; Mitchell et al., 1995) have demonstrated that Asp-132, Asn-125, Asn-123, and Glu-286 are functionally important. Clearly, the residues examined in the current work, which are located further up the channel, are not individually as critical for function.

There are several scenarios where removal of an individual residue would not necessarily be expected to affect proton transfer rate or efficiency. Possibly, these residues play roles that can be compensated in the mutants by adjustments of other residues or by internal water molecules (Baciou & Michel, 1995). Alternatively, the proton transfer pathway could be redundant in places, and be connected *via* several independent "proton wires". Since the Ser-201-Ala mutant has only 50% of the steady-state activity of wild type, but is unaltered in its structure according to spectroscopic evidence, it is possible that this mutation affects maximal turnover rates by altering the rate of a limiting proton transfer step. It should be noted that the assay of proton pumping used in these studies measures only efficiency, not rate.

A proton pump is distinguished from a proton channel by its ability to regulate the direction and rate of proton flow through the enzyme through the use of chemical energy. Conceivably, a single proton gate (amino acid residue or residues) is sufficient to regulate the direction of proton flow. Much of the proton transfer pathway through the enzyme could be nonspecific; i.e., multiple alternate pathways for certain segments could be present. It seems that the proton transfer pathway formed by the hydrophilic edge of helix IV is such a pathway. As noted (Tsukihara et al., 1996), many of the hydrophilic residues within this putative channel (hydrogen bond network) are often replaced by alanine or glycine in the oxidases from other species, possibly leaving cavities in which water molecules might be present to facilitate proton movement. A similar conclusion was reached in site-directed mutagenesis and second-site revertant studies of the photosynthetic reaction center from *Rhodobacter sphaeroides* in which residues in a proton transfer pathway were altered and effects on electron and proton uptake observed (Baciou & Michel, 1995; Hanson et al., 1993). Individual residues may not be essential either because alternate routes exist around the side chain or because the solvent within the channel rearranges in the mutant to provide a suitable pathway. Future work will examine the effects of multiple mutations on the function of this channel and will be directed to determine the minimum required set of residues for efficient proton transfer.

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