Transmembrane Charge Separation during the Ferryl-oxo → Oxidized Transition in a Nonpumping Mutant of Cytochrome c Oxidase*

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The N139D mutant of cytochrome c oxidase from Rhodobacter sphaeroides retains full steady state oxidase activity but completely lacks proton translocation coupled to turnover in reconstituted liposomes (Pawate, A. S., Morgan, J., Namslauer, A., Mills, D., Brzezinski, P., Ferguson-Miller, S., and Gennis, R. B. (2002) Biochemistry 41, 13417–13423). Here, time-resolved electron transfer and vectorial charge translocation in the ferryl-oxo → oxidized transition (transfer of the 4th electron in the catalytic cycle) have been studied with the N139D mutant using ruthenium(II)-tris-bipyridyl complex as a photocative single-electron donor. With the wild type oxidase, the flash-induced generation of ΔGc in the ferryl-oxo → oxidized transition begins with rapid vectorial electron transfer from CuA to heme a (τ ~ 15 μs), followed by two protonic phases, referred to as the intermediate (0.4 ms) and slow electrogenic phases (1.5 ms). In the N139D mutant, only a single protonic phase (τ ~ 0.6 ms) is observed, which was associated with electron transfer from heme a to the heme a3/CuB site and decelerates ~4-fold in D2O. With the wild type oxidase, such a high H2O/D2O solvent isotope effect is characteristic of only the slow (1.5 ms) phase. Presumably, the 0.6-ms electrogenic phase in the N139D mutant reports proton transfer from the inner aqueous phase to Glu-286, replacing the “chemical” proton transferred from Glu-286 to the heme a3/CuB site. The transfer occurs through the D-channel, because it is observed also in the N139D/K362M double mutant in which the K-channel is blocked. It is concluded that the intermediate electrogenic phase observed in the wild type enzyme is missing in the N139D mutant and is because of translocation of the “pumped” proton from Glu-286 to the D-ring propionate of heme a3 or to release of this proton to the outer aqueous phase. Significantly, with the wild type oxidase, the protonic electrogenic phase associated with proton pumping (~0.4 ms) precedes the electrogenic phase associated with the oxygen chemistry (~1.5 ms).

Cytochrome c oxidase (COX)1 is the terminal enzyme of the aerobic respiratory chain in mitochondria and in many prokaryotes (1–4). The enzyme conducts electrons from cytochrome c to O2 and couples the energetically downhill electron transfer to the uphill electrogenic movement of protons across the membrane from the bacterial cytoplasm (or mitochondrial matrix) to the periplasm (or mitochondrial intermembrane space).

The transmembrane charge separation by COX involves two different mechanisms. Half of the free energy (corresponding to the transmembrane translocation of four elementary electric charges per dioxygen reduced) is conserved by a direct mechanism; the four electrons and four protons required to convert O2 to water come from opposite sides of the membrane. This direct mechanism was postulated by Mitchell (5, 6) in the original chemiosmotic hypothesis. Charge separation via this mechanism is an intrinsic part of the reaction chemistry and cannot be dissociated from it without re-engineering the proton and/or electron delivery pathways to the active site. Any mutation resulting in inhibition of this electrogenic function would inevitably inhibit electron transfer activity of the enzyme.

The other half of the free energy is conserved by virtue of “proton pumping” discovered by Wikström in 1977 (7) and resulting in the transmembrane electrogenic translocation of four more protons per catalytic cycle. This proton pumping occurs by an “indirect” coupling mechanism and is not an obligatory part of the reaction chemistry. In principle, residues constituting the proton pump may be located in a separate part of the protein from the catalytic center, and the coupling can occur by virtue of finely tuned mechano-electrical interactions mediated by minor conformational changes of the protein (cf. Refs. 8 and 9).

The indirect coupling of the proton pump has been demonstrated recently by the isolation of cytochrome oxidase mutants that have full catalytic electron transfer activity but that do not pump protons. This was first shown for the N131D mutant of the COX from Paracoccus denitrificans (10), which places an aspartic acid residue near the entrance of the D-channel. This mutant COX has wild type oxidase activity but does not pump protons (10), which is exactly the phenotype expected for cytochrome oxidase in which electron transfer is decoupled from proton translocation at the molecular level. The equivalent mutant of the COX from Rhodobacter sphaeroides, N139D,

1 The abbreviations used are: COX, cytochrome c oxidase; RuBpy, tris-bipyridyl complex of ruthenium(II); F.O.E-ferro-oxo, oxidized and “electronated” (single-electron reduced) states of cytochrome c oxidase, respectively; P- and N-phases, the positively and negatively charged aqueous phases separated by the coupling membrane.
reveals a similar phenotype, i.e. loss of proton pumping, whereas the electron transfer activity is not only maintained but is enhanced about 2-fold (11).

In this work the properties of the N139D mutant of the *R. sphaeroides* COX have been further examined by observing the time-resolved intraprotein electron transfer and electrogenic activity associated with the F → O transition, corresponding to the transfer of the fourth electron in the catalytic cycle. In the N139D mutant, the rapid initial electrogenic step (vectorial electron transfer Cu₄ → heme α; τ ≈ 15 μs) is followed by a single protonetric electrogenic phase with τ of ≈ 0.6 ms. The data strongly suggest that the 0.6-ms electrogenic phase is because of proton uptake through the D-channel, probably to re-protonate Glu-286, currently believed to be the internal donor of the "chemical" proton required at the active site for the catalytic chemistry of the *F* → *O* step (see Refs. 12–15). The N139D mutant is missing the "intermediate" electrogenic phase which thus can be tentatively identified as vectorial transfer of the "pumped" proton within the protein in the direction of the outer aqueous phase across part of the insulating barrier.

**EXPERIMENTAL PROCEDURES**

All chemicals used were reagent or analytical grade. Dodecyl-β-o-maltoside was obtained from Anatrace. Ni²⁺-nitrotriacetic acid-agarose was obtained from Qiagen. D₂O (98%) was purchased in St. Petersburg (Russia) from a local source. Experiments with D₂O were done at the same pH meter reading (pH₀) as in H₂O.

Wild type and mutant cytochrome *c* oxidase, modified by a six-histidine affinity tag, was purified from *R. sphaeroides* by chromatography on a nickel column as described previously (16). Purified cytochrome *c* oxidase was reconstituted in phospholipid (sphingolipid) vesicles by a cholate dialysis method (17).

**Photoexcitation of RuBpy—** Ru(II)-tris-bipyridyl (RuBpy) was photoexcited by 9-ns light pulses (λ = 532 nm) from a neodymium YAG laser (Spectra Physics, model Lab-170-10) operated in a double frequency mode. The light pulse energy was 100–150 mJ for the electromagnetic measurements and about 300 mJ for absorption measurements.

**Time-resolved Absorption Measurements—** Absorption transients were made by using a thermostatted (21 °C, if not stated otherwise) 4 × 4-mm rectangular quartz cell (Starna Cells) with four optically transparent windows. The total volume of the sample was 250 μl. The monitoring light from a 70-watt lamp was passed through a grating monochromator (Jobin-Yvon) and projected onto the cell in the sample holder at a right angle relative to the excitation laser beam (10 mm in diameter), passing through the entire sample volume (vertically downward). After the sample, the monitoring light was passed to the photomultiplier via a second grating monochromator and a dark-blue glass filter (OD540/OD450 > 7.5). The photomultiplier signal was digitalized using a PC-installed Gage 8012 card (2,000,000 points, 12-bit, time resolution of 0.1 μs). An RC filter was placed before the analog-to-digital converter to improve the signal-to-noise ratio. 25–30 3–5-s spaced traces were averaged for each absorption trace.

**Time-resolved Measurements of Membrane Potential Generation—** Time-resolved electrometric measurements of membrane potential generation by liposome-reconstituted COX were carried out as described previously (18, 19) using RuBpy as a photoactivated single-electron donor for cytochrome oxidase Cu₄ (20). The signal was digitized with the aid of the same PC-installed Gage 8012 card as used for the absorption measurements. The kinetic traces were resolved into individual exponents using the program Discrete (21) and the Micr Olympic 7 software package (Micr Olympic Software).

**RESULTS**

The *F* state of the oxidase was generated as described previously (18, 22) by treating the oxidized solubilized or liposome-bound enzyme with H₂O₂. With the wild type oxidase from *R. sphaeroides*, the conversion to the *F* state was found to be complete upon the addition of 2 mM H₂O₂ at pH 8. This conclusion is significant for interpretation of the electrometric data; therefore, it is worthwhile to delineate in some detail the procedure used to evaluate the yield of the *F* state.

For instance, if the wild type cytochrome oxidase concentra-

**Fig. 1.** The kinetics of heme oxidoreduction in the *F* → *O* transition in purified wild type (*WT*) and N139D cytochrome oxidase from *R. sphaeroides*. The final sample contained COX (20 μM) in 5 mM Tris acetate and 0.05–0.1% dodecyl maltoside 40 μM RuBpy, 10 mM aniline, and 2 mM H₂O₂. The two absorption curves are given normalized by the amplitude of heme a photoreduction. The inset shows initial parts of the same traces, but normalized by the amplitude of heme a reoxidation, and superimposed on the y axis by the maximal reduction of heme a.

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tion is determined by applying extinction coefficients as published by Rich and co-workers (23, 24) (e.g. Δε₁₁₄₄₄ = 141 mm⁻¹ cm⁻¹ or Δε₄₄₄₄₄₄₁₄₁ = 209.6 mm⁻¹ cm⁻¹ for the reduced minus oxidized spectrum), then the absorption changes in the Soret band of the wild type *R. sphaeroides* oxidase after 2 mM peroxide addition correspond to Δε₅₂₁₅₅₂₂₂₂ of 50.5 mm⁻¹ cm⁻¹ given by Rich and co-workers (23) as 100% yield of the ferryl-oxo state in the wild type COX from *R. sphaeroides*. Similar Δε₅₂₁₅₅₂₂₂₂.min values (49–54 mm⁻¹ cm⁻¹) were obtained for the peroxide-induced Soret band changes using extinction coefficients for the reduced minus oxidized spectrum of COX, bovine, or *R. sphaeroides* as reported by other laboratories (25–27).

Alternatively, by using extinction coefficients as given by Rich and co-workers (24, 28) for the visible part of the *R. sphaeroides* COX reduced minus oxidized difference spectra (e.g. Δε₆₃₆₆₃₆₂₁ = 25.7 mm⁻¹ cm⁻¹), a value of Δε₆₃₆₆₃₆₂₁ ~5 mm⁻¹ cm⁻¹ was obtained for 2 mM H₂O₂-induced absorption changes in the visible part, which also indicates complete formation of the ferryl-oxo state of the wild type cytochrome oxidase from *R. sphaeroides* (cf. Refs. 29 and 30).

In contrast, the reaction of the N139D mutant oxidase with 2 mM H₂O₂ resulted in only ~70% yield of the ferryl-oxo state as determined from Δε₅₂₁₅₅₂₂₂₂. Notably, incomplete reaction with hydrogen peroxide has been observed routinely with several other mutants of the *R. sphaeroides* oxidase with replacements in the D-channel such as D132N, E286Q, E286D, and E286D/D132N. Incomplete reaction with H₂O₂ was also noted by Rich and co-workers (23) as 100% yield of the ferryl-oxo state of the oxidase from *R. sphaeroides* COX reduced minus oxidized difference spectra (e.g. Δε₆₃₆₆₃₆₂₁ = 25.7 mm⁻¹ cm⁻¹), a value of Δε₆₃₆₆₃₆₂₁ ~5 mm⁻¹ cm⁻¹ was obtained for 2 mM H₂O₂-induced absorption changes in the visible part, which also indicates complete formation of the ferryl-oxo state of the wild type cytochrome oxidase from *R. sphaeroides* (cf. Refs. 29 and 30).

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25.7 mM H₂O₂ addition correspond to Δε₅₂₁₅₅₂₂₂₂ of 50.5 mm⁻¹ cm⁻¹ given by Rich and co-workers (23)

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Fig. 1 shows the kinetics of photoreduction of heme a and its subsequent reoxidation in the *F* → *O* transition of the wild type and N139D oxidases. Although the rate of heme a reduction is the same for the wild type and mutant enzymes (not shown), the reoxidation kinetics (i.e. electron transfer from heme a to ferryl-oxo heme a₉) differ significantly.

With the wild type enzyme, reoxidation of the photoreduced heme a is complete, as indicated by the absorption changes at 444 nm. The A₄₄₄₄ trace ends up slightly below the initial level.

2 S. Siletsky, T. Vygodina, and C. Pecoraro, unpublished observations.
because of the smaller absorption contribution at this wavelength from ferric heme a₃ (final state) compared with the initial ferryl-oxo state of heme a₃. The reoxidation of heme a in the wild type enzyme is fitted best by a biexponential curve with a major phase (70%) with τ of 0.7 ms and a minor phase (30%) with τ of 3.5 ms (Table I). A single exponent fit of the same trace gives τ of 1.2 ms, which compares well with the value of τ ∼1.4 ms reported previously (31).

The reoxidation of heme a in the N139D mutant oxidase differs from that observed with the wild type oxidase in two respects. First, ~30% of the flash-reduced heme a in the mutant oxidase is not reoxidized on the time scale observed. This correlates with the observation that the yield of the ferryl-oxo heme a₃ species is only about 70% with the N139D mutant oxidase. Presumably, in the 30% of the enzyme population in which heme a₃ is not converted to the ferryl-oxo state, the photoreduced heme a remains reduced over the time course of the experiment. Second, in the population of the N139D mutant oxidase in which heme a is reoxidized, the kinetics of the reoxidation is different from that observed with the wild type enzyme (Table I). The N139D reoxidation trace is triphasic with two major components of about equal magnitude (40% each) and τ values of 180 μs and 1.8 ms, followed by a slow residual oxidation phase (τ ∼8 ms, ∼20%), well outside the turnover time range of the enzyme.

Fig. 2 shows the electrogenic response coupled to the F → O transition in N139D oxidase, along with the responses observed with both the fully active wild type oxidase and the “dead” E286Q mutant. The traces are normalized by the amplitude of the initial rapid phase, i.e. by the amount of heme a flash-reduced in each of the samples. With the wild type oxidase, the response includes the fast phase (vectorial electron transfer from CuA to heme a) and a slower part resulting from the vectorial intra-protein transfer of protons coupled to the reoxidation of heme a by the binuclear site. This slower cyanide-sensitive part of the response is known to consist of two exponential components, denoted as the intermediate and slow phases (18, 22).

Characteristics of the electrogenic phases are summarized in Table II. The data for bovine COX are also included for comparison. The wild type trace (Fig. 2) is composed of three phases as follows: rapid (τ ∼15 μs), intermediate (τ ∼0.5 ms), and slow (τ ∼1.5 ms) with the relative amplitudes of 1:0.7:1.7, respectively (or 30, 20, and 50% of the total response), in agreement with the data reported previously for COX from R. sphaeroides (22) and for the P. denitrificans oxidase (32, 33). In contrast, the photoelectric trace observed with the N139D mutant, shows only two phases with τ values of about 15 μs and 0.6 ms. The 15-μs phase is obviously the same rapid phase as observed with the wild type oxidase and is assumed to be due to electron transfer from CuA to heme a. The 0.6-ms phase of the N139D mutant is eliminated by performing the experiment in the presence of cyanide (Fig. 2, inset), indicating that, as expected, this phase is linked to electron transfer from heme a to the binuclear site.

Previous work with the bovine oxidase has shown that the electrogenic proton translocation in the F → O transition (τ ∼4.5 ms) lags behind heme a reoxidation (τ ∼1.5 ms) (34, 35). Fig. 3A shows that with the wild type R. sphaeroides oxidase, there is a closer match between the optically monitored oxidation kinetics of heme a and the electrogenic proton translocation. Nevertheless, although each of the two traces deconvolutes into two exponentials (Tables I and II), attempts to fit the two curves by the same set of two exponentials gave poor fits.

For the N139D mutant, the kinetics of heme a reoxidation and of the protonic phase of voltage generation are also not coincident, which is evident even by visual inspection (Fig. 3B).

The kinetics of voltage generation was also monitored in the presence of D₂O in place of H₂O. The rapid phase of the response is not affected in agreement with the observations on P. denitrificans oxidase (32). In contrast, the cyanide-sensitive part of the response manifests a significant solvent isotope effect with both wild type and N139D forms of the oxidase. With the wild type oxidase (Fig. 4A and Table III), the effect is mainly associated with the slow electrogenic phase (3.5–4-fold deceleration in the presence of D₂O), whereas the intermediate phase slows down by only ∼1.4-fold. This different sensitivity to D₂O/H₂O replacement provides an important independent experimental basis to distinguish the intermediate and slow phases of the voltage generation in the F → O transition. With the N139D mutant, the cyanide-sensitive phase is decelerated by about 3.5-fold (Fig. 4B and Table III), similar to the H₂O/D₂O kinetic isotope effect observed for the slow, but not the intermediate, phase in the wild type oxidase. Hence, the residual protonic phase observed in the N139D mutant is proposed to correspond to the slow phase of the wild type oxidase.

In order to determine whether the 0.6-ms electrogenic phase in the N139D mutant requires the K-channel, the double mu-
tant N139D/K362M was constructed and examined. This double mutant is inactive in steady state turnover as a result of the K362M mutation but has normal absorption characteristics and readily forms the F state with H2O2 with about the same yield of 60–70% as the N139D mutant (not shown). As shown in the inset of Fig. 5, the protonic phase observed with the N139D mutant oxidase is retained in the double mutant (N139D/K362M). It is concluded that the 0.6-ms electrogenic phase in the N139D mutant does not involve proton translocation through the K-channel.

At the same time, there are minor but noticeable changes in the charge translocation kinetics in the 50–1000-s time window resulting from the presence of the K362M mutation in the N139D background (Fig. 5, main panel). Compared with the N139D mutant, the K362M/N139D double mutant exhibits a lag prior to the onset of the electrogenic proton transfer, and the protonic phase itself becomes slightly faster. The modulation of the kinetics of electrogenic proton transfer during the F3O transition can also be observed in comparing the electrogenic traces of the single K362M mutant with that of the wild type oxidase (22). This effect may indicate some cross-talk between the D- and K-channels during the F3O transition.

DISCUSSION

One clear conclusion from this work is that the N139D mutant of the R. sphaeroides oxidase, although not pumping protons (11), generates an electric potential difference across the membrane. The time-resolved studies of charge translocation coupled to the F3O transition with the N139D mutant reveal two electrogenic phases with τ values of about 15 μs and 0.6 ms (at 22 °C, pH 8) that are likely to represent the steps of vectorial electron and proton transfer, respectively, from the opposite sides of the membrane to the oxygen-reducing site buried within the enzyme (Fig. 6A). Hence, the N139D COX from R. sphaeroides may exemplify an oxidase operating by the original redox-loop principle of ΔμH+ generation postulated by Mitchell (5).

TABLE II

*Photoelectric responses of the wild type and N139D mutant forms of cytochrome c oxidase*

In all samples, the amplitude of the rapid electrogenic phase was taken as 1.0.

| Phase       | Bovinea | R. sphaeroides, wild type | R. sphaeroides, N139D |
|-------------|---------|--------------------------|----------------------|
|             | τ       | τ                        | Amplitude            | Amplitude observed | Amplitude correctedb |
| Rapid       | 45 μs   | 15 μs                    | 1.0                  | 1.0                | 1.0                  |
| Intermediate| 1.2 ms  | 0.4 ms                   | 0.7                  | 1.7                | 1.3                  |
| Slow        | 4.5 ms  | 1.5 ms                   | 2.4                  | 0.9                | 1.3                  |
| Sum         |         |                          | 3.4                  | 1.9                | 2.3                  |

a Data from Refs. 18 and 19.
b Corrected for 30% of the enzyme in which flash-reduced heme a is not reoxidized (see the text).

![Fig. 3. Comparison of heme a reoxidation kinetics with the kinetics of electrogenic protonic phases in the F → O transition. A, wild type (WT) COX. Although the two curves match each other visually, the matching is apparent. The kinetics of heme a reoxidation deconvolutes into two exponents with τ and contributions of 0.7 (70%) and 3.5 ms (30%), whereas the best fit for electrogenic proton transfer reveals two exponents with τ values of 0.4 (30%) and 1.5 ms (70%). See the text for more details. B, N139D. Mismatch between the kinetics of the two curves is evident visually.](http://www.jbc.org/)

![Fig. 4. Effect of H2O substitution by D2O on the kinetics the electrogenic phases in the wild type (A) and N139D mutant COX (B). The experiments have been carried with the same samples of collodion film-adhered liposomes with wild type (WT) or N139D COX, first recording the response in H2O and then replacing the buffer by that made in D2O. The responses are given normalized by the amplitude of the rapid electrogenic phase.](http://www.jbc.org/)

![TABLE III

*Hydrogen isotope sensitivity of the F → O transition*

| Protonic electrogenic phase | Wild type | N139D |
|----------------------------|-----------|-------|
|                            | H2O       | D2O   | H2O   | D2O   | ms    | ms    |
| Intermediate              | 0.53      | 0.77  | 0.62  | 2.44  |       |       |
| Slow                      | 1.46      | 4.9   | 0.62  | 2.44  |       |       |

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**Origin of the Protonic Electrogenic Phase in the N139D Mutant**

Several arguments suggest that the 0.6-ms protonic phase in the N139D mutant corresponds to the slow, rather than inter-
enzyme. Rather the 0.6-ms phase is a “truncated” counterpart of the slow electrogenic phase observed with the wild type oxidase. It is concluded that the N139D mutant is specifically missing the intermediate electrogenic phase.

Because the N139D mutant oxidase does not pump protons and, concurrently, the intermediate electrogenic phase is absent, it is proposed that the intermediate phase in the wild type enzyme is associated only with the transfer of the pumped proton, and the electrogenic movements of the “chemical proton” take place within the slow phase. Hence, electrogenic transfer of the pumped proton precedes the uptake of the proton consumed at the oxygen-reducing site to form water.

**Relating the Electric Data to Proton Pumping Models**

There is now general belief that both the chemical and pumped protons in the $F \rightarrow O$ transition are delivered from the inner aqueous phase via the D-channel to Glu-286 located near the buried active site of the enzyme. Glu-286 serves as a branch point for subsequent proton translocation along pathways resulting in either pumping (via an “exit channel”) or chemistry (catalyzed at the heme-copper site). A gating “toggle switch” has been postulated to direct protons from Glu-286 either to the heme $a_3$-Cu$_b$ center or to the heme $a_3$ D-ring propionate, depending on the specific step in the catalytic cycle (11). A number of specific mechanisms for such a gating switch at Glu-286 are conceivable (e.g. Refs. 12–15 and 36–38).

Proton transfer from Glu-286 to the D-ring propionate of heme $a_3$ and from there to the bulk outer aqueous phase is speculated to be a likely path for the pumped proton (e.g. Refs. 12–14 and 36–40) (Fig. 6). The major proton transfer steps involved in the eventual release of the pumped proton to the $P$-phase (Glu-286 $\rightarrow$ heme $a_3$ propionate $\rightarrow$ . . . $\rightarrow$ outer phase) are directed “across” the membrane and must be electrogenic. It is noted that the voltage generation concurrent with the last steps of this process, i.e. “propionate $\rightarrow$ . . . $\rightarrow$ outer phase” (denoted by a question mark in Fig. 6B), may be less significant because of the short transfer distance and the apparent high dielectric constant of this region of the protein.

Alternatively, the proton can be transferred from Glu-286 to the heme $a_3$-Cu$_b$ center and be consumed there in water formation (12, 13, 15, 37, 38). The transfer of the chemical proton from Glu-286 to the heme $a_3$-Cu$_b$ center is not expected to be significantly electrogenic because the direction of charge movement is essentially parallel to the plane of the membrane. Finally, each time Glu-286 donates a proton, either to heme $a_3$ propionate or to the binuclear site, the deprotonated Glu-286 must be reprotonated from the N-aqueous phase via the D-channel.

Within this framework, a detailed sequence of steps can be proposed as comprising the $F \rightarrow O$ transition, both for the nonpumping N139D mutant (Fig. 6A) and for the pumping oxidase (wild type or bovine) (Fig. 6B).

**N139D Mutant**—Step 1 consists of electrogenic electron transfer from Cu$_b$ to heme $a$. This is the 15-$\mu$s rapid electrogenic phase. For step 2, there occurs essentially nonelectrogenic electron transfer along the membrane from heme $a$ to heme $a_3^{4+} = O^{2-}$. Step 3 corresponds essentially to nonelectrogenic proton transfer along the membrane from Glu-286 to heme $a_3$-bound oxygen, resulting in the formation of the heme $a_3^{3-} - OH^-$ complex. Step 4 involves electrogenic reprotonation of Glu-286 from the N-aqueous phase via Asp-132 at the entrance of the D-channel. This is the 0.6-ms electrogenic phase.

The amplitude ratio of the two major electrogenic steps 1 and 4 associated with Cu$_b$ $\rightarrow$ heme $a$ electron transfer (step 1) and proton uptake from the N-phase to the binuclear site (step 4) is $\sim 1:1.3$. This value is significantly different from the ratio of
Fig. 6. Electrogenic steps in the F → O transition of cytochrome c oxidase. A, the nonpumping mutant with N139D substitution in subunit I. B, the proton pumping oxidase. Major electrogenic steps are denoted by thick arrows. Green arrows, electron transfer; dark red arrows, chemical proton transfer; bright red arrows, proton pumping. See text for further details. The structure of the R. sphaeroides wild type cytochrome c oxidase was obtained from Protein Data Bank (36). The picture was prepared with the aid of Visual Molecular Dynamics software from Amersham Biosciences (50).

−1.07 assigned to the analogous electrogenic phases during single electron photoreduction of P. denitrificans oxidase (32) but is in qualitative agreement with the physical location of the hemes closer to the P-side of the membrane in the observed structures of cytochrome oxidase.

Wild Type Cytochrome Oxidase—Steps 1 and 2 are the same as for N139D mutant above.

For step 3, there may occur proton transfer from the Arg-481/heme a$_3$ D-ring propionate clusters to an acceptor at or near the outer surface (P-side of the membrane). As pointed out previously (see Refs. 15, 38, and 41 but compare with Ref. 14), the carboxylate group of the propionate forms a strong salt bridge with the guanidinium group of Arg-481 and, in this state, cannot accept an additional H$^+$ from Glu-286 without first deprotonating. Proton transfer from the Arg-481/D-propionate cluster may proceed by a nonelectrogenic H$^+$ shift to a nearby group, e.g. His-334 (15, 38). Alternatively, the H$^+$ transfer in step 3 might proceed to a more distant acceptor or even to the outside bulk solution, in which case the charge transfer would be electrogenic and contribute to the 0.4-ms intermediate electrogeneric phase (cf. Fig. 3B in Ref. 35).

Step 4 involves electrogenic H$^+$ transfer from Glu-286 to the D-propionate of heme a$_3$. This step may be responsible for the major part of the 0.4-ms intermediate electrogeneric phase. Two alternative hypotheses have been put forth to explain why the proton goes from Glu-286 to the propionate (the pumped proton pathway), rather than to the binuclear site (chemical proton pathway): (i) The affinity of the propionate for protons in this state is higher than the affinity of the heme a$_3$-bound oxene (14). (ii) H$^+$ transfer to the propionate is less favorable thermodynamically but is faster than delivery of the proton to the binuclear site because of the more loose structure of the water chain forming the latter pathway (15, 38). Notably, according to Ref. 13, it is proton transfer from Glu-286 to the D-propionate of heme a$_3$ that gates electron transfer from heme a to heme a$_3$. Hence, according to this scheme, steps 3 and 4 will precede step 2.

For step 5, there occurs electrogenic reprotonation of Glu-286 from the N-aqueous phase via the D-channel to replace the proton transferred from Glu-286 to D-propionate in the previous proton-pumping step 4.

Step 6 consists of essentially nonelectrogenic transfer of the chemical proton from Glu-286 to heme a$_3$-bound oxygen formed the heme a$_3$-OH$^-$ complex.

Step 7 involves electrogenic expulsion of the pumped proton transiently reposed on His-334 (15) or on the D-propionate of heme a$_3$ (14) to the P-aqueous phase. This step may be missing because of the more loose structure of the water chain forming the latter pathway (15, 38). Notably, according to Ref. 13, it is proton transfer from Glu-286 to the D-propionate of heme a$_3$ that gates electron transfer from heme a to heme a$_3$. Hence, according to this scheme, steps 3 and 4 will precede step 2.

For step 8, there occurs electrogenic reprotonation of Glu-286 via the D-channel replaces the proton transferred from Glu-286 to the binuclear site in the preceding step 6.

Note that the slow electrogenic phase (−1.5 ms) in the F → O transition involves up to four reaction steps (steps 5–8) but appears kinetically as a single process (18, 22, 33). Similarly, the intermediate electrogenic phase integrates steps 2–4 in a single transient.

Identification of the Electrogenic Protonic Phases

The Intermediate Electrogenic Phase—The intermediate phase of the electrogeneric response coupled to F → O transition has been originally assigned to the uptake of the chemical proton from the N-side of the membrane (18). However, an alternative interpretation, namely proton release from the H$^+$-preloaded binuclear center to the P-side, was also pointed out (18) and discussed in detail (35). According to the present data, the intermediate electrogeneric phase, missing in the N139D mutant, is not likely to be associated with the uptake and delivery of the chemical proton to the heme a$_3$/Cu$_{II}$ center, because the latter process must still occur in the N139D mutant. Alternatively, the intermediate phase in the wild type enzyme may be related to the electrogeneric ejection of the pumped proton (see Fig. 3B in Ref. 35 and Fig. 6B in this work). The elimination of proton pumping in the N139D mutant correlates with the elimination of this electrogeneric phase.

The Slow Electrogeneric Phase—The major part of the slow electrogeneric phase in the wild type oxidase is likely to be due to the two consecutive reprotonations of Glu-286 from the N-aqueous phase following the donations of the pumped and chemical protons by this group to the D-ring propionate of heme a$_3$ and to the binuclear site, respectively. Furthermore, release of the pumped proton from some intermediate site “above the hemes” to the P-phase, electrostatically driven by the uptake of the chemical proton, as proposed by most of the current pumping models, may also be part of the slow electrogeneric phase (Fig. 6B). However, the electrogeneric contribution of
this step is likely to be small, as discussed above. In the N139D mutant, there is a single reprotonation of Glu-286 following the donation of the chemical proton by Glu-286 to the binuclear site. This reprotonation step is presumed to account for the entire 0.6-ms protonic phase in the mutant and would represent part of what constitutes the slow electrogenic phase in the $F \rightarrow O$ transition of the wild type oxidase.

The Electrogenic Response of the $F \rightarrow O$ Transition in the Nonpumping N139D Mutant as a Tool to Calibrate the Charge Translocations in the Pumping Oxidases

It is reasonable to assume that the electrogenicity of the $F \rightarrow O$ step in the N139D mutant is because of translocation of one elementary charge across the membrane, the electron and proton coming from opposite sides of the membrane to the ferryl-oxo heme $a_0$ (Fig. 6A) (10, 11). In such a case, the electrogenic response of the nonpumping N139D mutant can be used to calibrate the magnitude of charge translocation in the $F \rightarrow O$ transition of the proton pumping oxidases. In Table II, the amplitudes of the photoelectric responses observed for the $F \rightarrow O$ transition with the wild type, N139D mutant, and mitochondrial oxidases are compared. The responses have been normalized by the magnitude of the rapid electrogenic phase that has been assigned a value of 1 unit in each case, instead of indicating the percentage contribution of each electrogenic phase to the overall response in a particular sample, as done earlier (18, 22, 32, 33).

The $F \rightarrow O$ step in bovine oxidase (5 units) is more electrogenic by $-2.7$ units than the overall photoelectric response of the N139D mutant (2.3 units), corrected for the 30% of the enzyme in which heme $a$ is not oxidized. If the overall electrogenic response of the mutant $R. sphaeroides$ oxidase ($-2.3$ units) corresponds to transfer of one elementary charge across the membrane, the difference between the bovine oxidase and the N139D mutant of $R. sphaeroides$ oxidase is equivalent to $-1$ proton pumped across the membrane by the mitochondrial oxidase. This value is in agreement with the original models of proton pumping by cytochrome oxidase (6, 42, 43) as well as the predictions of the recent schemes postulating translocation of 1 proton coming from opposite sides of the membrane to the protonic phase in the mutant and would represent the percentage contribution of each electrogenic phase to the overall response in a particular sample, as done earlier (18, 22, 32, 33).

However, the same comparison of the N139D mutant with the wild type $R. sphaeroides$ oxidase indicates that the additional charge translocation because of proton pumping (1.1 unit) can account in this case for translocation of only $-0.5$ elementary charges across the membrane. This apparent shortfall is obviously related to a lower protonic/electronic electrogenic phase amplitude ratio observed in the $R. sphaeroides$ oxidase discussed below.

The Difference between the Electrogenic Patterns in the $F \rightarrow O$ Transitions of the Bacterial and Bovine Oxidase

The mitochondrial and wild type $R. sphaeroides$ cytochrome $c$ oxidases reveal similar sets of three electrogenic phases (rapid, intermediate, and slow) in the $F \rightarrow O$ transition. However, the amplitude ratio of those phases is known to differ for the mitochondrial oxidase (20:20:60% (18)) and bacterial enzyme from $R. sphaeroides$ (22) or $P. denitrificans$ (32, 33) (30:20:50%), i.e., a relocation of about 10% of the total electrogenic response from the slow to the rapid phase is observed in the bacterial enzyme relative to the bovine oxidase. Because of this shift, the amplitude of the overall response, normalized to the amplitude of the rapid phase taken as 1 unit, is significantly larger for the mitochondrial oxidase (1 + 1 + 3 = 5 units) than for the bacterial oxidases (1 + 0.7 + 1.7 = 3.4 units) (Table II).

It is argued sometimes that the true amplitude ratio of the electrogenic phases in the bacterial oxidases may be the same as for the mitochondrial enzyme but that it is distorted because of incomplete yield of the $F$-state in the bacterial oxidases treated with $H_2O_2$. As described under “Results,” no evidence for incomplete reactivity of the oxidized wild type oxidase from $R. sphaeroides$ with $H_2O_2$ was found. Furthermore, reoxidation of the photoreduced heme a in the flash-induced $F \rightarrow O$ transition in the wild type $R. sphaeroides$ oxidase is complete on a turnover time scale, and its magnitude does not differ significantly from that observed in the $F \rightarrow O$ step for bovine oxidase (not shown).

Therefore, certain differences in the electrogenic mechanisms between the bacterial and mitochondrial enzyme cannot be excluded. There can be a minor electrogenic partial proton transfer step (~10% of the overall electrogenic response of the wild type enzyme) that is included in the slow phase in the case of the mitochondrial oxidase but takes place within the rapid phase in the bacterial enzyme. For example, perhaps the pumped proton shifts position coincident with the fast phase instead of the slow phase in the bacterial enzyme but not in the bovine enzyme. There are many possibilities that do not alter the fundamental mechanism of the proton pump but which could lead to the different ratio of electrogenic phase amplitudes observed.

Summary

The two major conclusions from this work are as follows. 1) For the $F \rightarrow O$ transition of cytochrome oxidase initiated by photoreduction of the $H_2O_2$-generated $F$ state, the electrogenic proton movements associated specifically with the proton pump precede the proton movements driven by the oxygen chemistry at the active site, namely vectorial proton transfer from Glu-286 to a site in the exit pathway of the pump is postulated to precede electrogenic proton uptake from the $N$-aqueous phase. 2) Proton translocation from the bulk aqueous $N$-phase to Glu-286 through the D-channel is about 1.3 times more electrogenic than the vectorial electron transfer from $Cu_A$ to heme $a$.

REFERENCES

1. Babcock, G. T., and Wikstrom, M. (1992) Nature 356, 301–309
2. Ferguson-Miller, S., and Babcock, G. T. (1996) Chem. Rev. 97, 2989–2908
3. Ludwig, B., Bender, E., Arnold, S., Huttemann, M., Lee, I., and Radkenbach, B. (2001) ChemBioChem, 2, 392–403
4. Brazeinski, P. (2004) Trends Biochem. Sci. 29, 380–387
5. Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, pp. 1–111, Glynn Research Ltd., Bodmin
6. Mitchell, P. (1988) Ann. N. Y. Acad. Sci. 550, 185–198
7. Wikstrom, M. (1977) Nature 266, 271–273
8. Xavier, A. V. (2002) FEBS Lett. 532, 261–266, and references therein
9. Xavier, A. V. (2004) Biochim. Biophys. Acta 1655, 23–30, and references therein
10. Fütten, U., Hoffmeier, K., Harrenga, A., Kannt, A., Michel, H., Bamberg, E., Richter, O. M. H., and Ludwig, B. (2000) Biochemistry 39, 6767–6762
11. Pawate, A. S., Morgan, J., Namslander, A., Mills, D., Brezinskii, P., Ferguson-Miller, S., and Gennis, R. B. (2002) Biochemistry 41, 13417–13423
12. Namslander, A., Pawate, A., Gennis, R., and Brezinskii, P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15543–15547, and references therein
13. Wikstrom, M., Verkhovsky, M. I., and Hummer, G. (2003) Biochim. Biophys. Acta 1604, 61–65, and references therein
14. Siegbahn, P., and Blomberg, M. R. A. (2004) Biochim. Biophys. Acta 1655, 45–50, and references therein
15. Popovce, D. M., and Starchevski, A. A. (2004) FEBS Lett. 566, 126–130, and references therein
16. Mitchell, D. M., and Gennis, R. B. (1993) FEBS Lett. 368, 148–150
17. Hinkle, P. C. (1979) Methods Enzymol. 55, 748–751
18. Zaslavsky, D., Kaulen, A., Smirnova, I. A., Vygudin, T. V., and Konstantinov, A. A. (1993) FEBS Lett. 336, 389–393
19. Siletsky, S., Kaulen, A. D., and Konstantinov, A. A. (1999) Biochemistry 38, 4655–4661
20. Nilsson, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6497–6501
21. Provencher, S. W. (1976) Biophys. J. 16, 27–41
22. Konstantinov, A. A., Siletsky, S. A., Mitchell, D., Kaulen, A. D., and Gennis, R. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9085–9090
23. Junemann, S., Meunier, B., Gennis, R. B., and Rich, P. (1997) *Biochemistry* **36**, 14456–14464
24. Rich, P., and Moody, A. J. (1997) in *Bioenergetics* (Graber, P., and Milazzo, G., eds) Vol. 4, pp. 419–456, Birkhauser Boston, Inc., Cambridge, MA
25. Vanneste, W. H. (1966) *Biochemistry* **5**, 838–848
26. Hosler, J. P., Fetter, J., Tecklenberg, M. M. J., Espe, M., Lerma, C., and Ferguson-Miller, S. (1992) *J. Biol. Chem.* **267**, 24264–24272
27. Liao, G.-L., and Palmer, G. (1996) *Biochim. Biophys. Acta* **1274**, 109–111
28. Iwaki, M., Breton, J., and Rich, P. R. (2002) *Biochim. Biophys. Acta* **1555**, 116–121
29. Fabian, M., and Palmer, G. (2001) *Biochemistry* **40**, 1867–1874
30. Wikstrom, M., and Morgan, J. E. (1994) *J. Biol. Chem.* **269**, 10266–10273
31. Zaslavsky, D., Sadoski, R. C., Wang, K., Durham, B., Gennis, R. B., and Millett, F. (1998) *Biochemistry* **37**, 14910–14916
32. Ruitenberg, M., Kannt, A., Bamberg, E., Ludwig, B., Michel, H., and Fendler, K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4632–4636
33. Ruitenberg, M., Kannt, A., Bamberg, E., Fendler, K., and Michel, H. (2002) *Nature* **417**, 99–102
34. Siletsky, S. A. (1998) *Studies on Electrogenic Electron and Proton Transfer in Cytochrome Oxidase*, Ph.D. thesis, Moscow State University, Moscow
35. Konstantinov, A. (1998) *J. Bioenerg. Biomembr.* **30**, 121–130
36. Svensson-Ek, M., Abramson, J., Larsson, G., Tornroth, S., Brezezinski, P., and Iwata, S. (2002) *J. Mol. Biol.* **321**, 329–339
37. Zheng, X., Medvedev, D. M., Swanson, J., and Stuchebryukhov, A. A. (2003) *Biochim. Biophys. Acta* **1557**, 99–107
38. Popovic, D. M., and Stuchebryukhov, A. A. (2004) *J. Am. Chem. Soc.* **126**, 1868–1871
39. Puustinen, A., and Wikstrom, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 35–37
40. Michel, H. (1999) *Biochemistry* **38**, 15129–15140
41. Tsukihara, T., Shimokata, K., Katayama, Y., Shimada, H., Muramato, K., Aoyama, H., Mochizuki, M., Shinzawa-Itoh, K., Yamashita, E., Yao, M., Ishimura, Y., and Yoshikawa, S. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15304–15309
42. Konstantinov, A. A. (1977) *Dokl. Akad. Nauk SSSR* **237**, 713–716
43. Artzathanov, V. Y., Konstantinov, A. A., and Skulachev, V. P. (1978) *FEBS Lett.* **87**, 180–185
44. Bloch, D., Belevich, I., Jasaitis, A., Bihaacka, C., Puustinen, A., Verkhovsky, M. I., and Wikstrom, M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 529–533
45. Wikstrom, M. (1989) *Nature* **338**, 776–778
46. Morgan, J. E., Verkhovsky, M. I., and Wikstrom, M. (1994) *J. Bioenerg. Biomembr.* **26**, 599–608
47. Wikstrom, M. (2000) *Biochim. Biophys. Acta* **1458**, 188–198
48. Rich, P. R. (1995) *Aust. J. Plant Physiol.* **22**, 479–486
49. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature* **376**, 660–660
50. Humphrey, W., Dalke, A., and Schulten, K. (1996) *J. Mol. Graphics* **14**, 33–38
Transmembrane Charge Separation during the Ferryl-oxo → Oxidized Transition in a Nonpumping Mutant of Cytochrome c Oxidase
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