Effects of Mutation of the Conserved Lysine-362 in Cytochrome *c* Oxidase from *Rhodobacter sphaeroides*†

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ABSTRACT: We describe the effects of a mutation, K362M, of the conserved lysine in cytochrome *c* oxidase from *Rhodobacter sphaeroides*, a residue located in a putative proton channel that may convey substrate protons to the binuclear center. Spectra of the "as prepared", ferricyanide-oxidized, and dithionite-reduced forms of the mutant protein confirm that the redox centers remain intact. Ligand binding kinetics of the ferricyanide-oxidized enzyme and of the dithionite-reducible fraction are similar to those of the wild type, indicating that the K channel is not the major route for CO, cyanide, formate, or peroxide entry into the structure. Protonation of the lysine residue is not redox-linked to heme *a* or Cu_B as judged from the essentially unaltered midpoint potentials of these centers in the cyanide-ligated enzyme. A difficulty in electron transfer from heme *a* to the binuclear center is indicated by the slow and only partial reduction of heme a_3 by dithionite or ferrocytochrome *c* and by the presence of some reduced heme *a* in the as prepared mutant enzyme and under steady-state conditions. Further characterization of the K362M enzyme in the steady state shows that up to one electron, but not two, can enter the binuclear center easily. It is this inability to form the two-electron-reduced, oxygen-reactive R state that prevents activity. A model is proposed where the K channel serves as a dielectric well of high dielectric strength and low proton conductivity, rather than as a pathway for proton entry to the binuclear center. The function of this structure would be to decrease the cost of introducing a transiently uncompensated charge into the binuclear center prior to formation of a stable, charge-compensated R-state.

The crystal structures of cytochrome oxidase from *Paracoccus denitrificans* (Iwata et al., 1995) and bovine heart (Tsukihara et al., 1996) have been solved to 2.8 Å resolution. Possible routes for proton uptake and sites of charge-linked protonation can be identified in subunit I. The most obvious candidates for stable sites of charge-linked protonation include the histidine ligands to the metal centers, the heme propionates and nearby residues, and the conserved protonable residues lysine-362, glutamate-286, and tyrosine-288 (all numbering refers to the *Rhodobacter sphaeroides* sequence). Some of these residues have been suggested previously to be involved in proton transfer from site-directed mutagenesis (Hosler et al., 1993; Thomas et al., 1993). The residue K362 is located in a hydrophobic environment between transmembrane helices VI and VIII, approximately 15 Å below the binuclear center, and forms part of a proposed proton pathway (pore B). A second possible proton channel (pore A), lined by residues on helices II, III, IV, and VI, leads to E286, a residue that is close to heme *a* and the binuclear center metals. Since pore B appears to lead to the binuclear center, a model has been proposed (Iwata et al., 1995) in which this pathway serves to transfer substrate protons (H^+_S),[1] whereas A is a channel for translocated protons (H^+_T), and where H^+_T rather than H^+_S are loaded during the initial reduction of the binuclear center (O → R).

However, this concept of distinct proton channels has been questioned recently. It has been shown in K362 mutants that both electron transfer and proton uptake are normal in experiments where the fully reduced enzyme is reoxidized with oxygen using the flow-flash method (Svensson et al., 1995; Brzezinski & Ädelroth, 1997). Furthermore, in *Rb. sphaeroides* oxidase the electrogenic reactions associated with the F to O transition are not impaired in the K362M mutant (Konstantinov et al., 1997). Since transfer of both H^+_T and H^+_S is required for these reactions, it has been proposed that the two channels may be functionally associated with different steps in the catalytic cycle rather than being designed to convey exclusively H^+_T or H^+_S (Konstantinov et al., 1997). Despite relatively normal reactivity in the above experiments, mutants of K362 in *Escherichia coli* or *Rh. sphaeroides* are completely inactive catalytically, although the redox centers appear to be spectrally (resonance Raman and FTIR) normal (Thomas et al., 1993; Fetter et al., 1995; Hosler et al., 1996). Some indication of why the mutant may be inactive comes from the observation that reduction of heme a_3 is impaired in the K362M and K362A enzymes from *Rh. sphaeroides* (Hosler et al., 1996). Overall, these findings suggest that it may be necessary to reconsider either the assignment of the channels or the proposed stages of H^+_T and H^+_S uptake during the catalytic cycle.

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[1] Abbreviations: O, R, P, and F, *o*xidized, two-electron *r*educed, *p*eroxy, and *f*erryl intermediates of cytochrome oxidase; H^+_S, substrate protons; H^+_T, pumped protons; CO, carbon monoxide; FTIR, Fourier transform infrared; NTA, nitrilotriacetic acid.

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Table 1: Extinction Coefficients for Reduced, Oxidized, and Various Ligand-Bound States of Cytochrome Oxidase Used in This Work[a]

	wavelengths used (nm)	extinction coefficient (mM^{-1} cm^{-1})
absolute oxidized	425	160
reduced − oxidized	446 minus 416	209.6
	446 minus 462	140.6
	606 minus 630	28.9
P − oxidized	439 minus 414	50.7
	607 minus 630	12[b]
F − oxidized	436 minus 414	50.5
(oxidized + CN^-) − oxidized	433 minus 412	58
(reduced + CO) − reduced	430 minus 446	123[c]
(reduced + CN) − reduced	593 minus 614	22

[a] Data are taken from Rich and Moody (1997) unless otherwise indicated and were used in the present work for the *Rh. sphaeroides* enzyme after adjustment of wavelengths where necessary. [b] From Mitchell et al. (1992). [c] Calculated for the *Rh. sphaeroides* oxidase based on $\Delta\epsilon_{\text{reduced-oxidized, 446-462nm}} = 140.6\ mM^{-1}\ cm^{-1}$.

This paper focuses on a mutation of the conserved lysine, K362M, in cytochrome *c* oxidase from *Rh. sphaeroides*. Here we ask the question of how the mutation K362M disturbs individual electron and proton transfer reactions in the enzyme. Since charge changes in the binuclear center are strictly balanced by protonation changes (Mitchell & Rich, 1994) and at least one internal electron transfer step is kinetically coupled to protonation changes of a group in the vicinity of the binuclear center (Ädelroth et al., 1996), the roles for specific residues in internal protonation changes are clearly important. We have applied a set of basic, quantitative tests (including redox properties, ligand reactivity, and steady-state behavior) with the view of evaluating the role of the conserved lysine-362 in such redox-linked protonation processes.

MATERIALS AND METHODS

Growth of Organism and Preparation of Enzyme. Wild-type and mutant forms of cytochrome *c* oxidase from *Rhodobacter sphaeroides* were derived from strains where a histidine tag is attached to the C-terminus of subunit I to allow rapid purification by Ni^{2+}−NTA affinity chromatography (Mitchell & Gennis, 1995). Growth of cells and preparation of the purified enzyme were carried out as described previously (Mitchell & Gennis, 1995). After elution of the oxidase from the Ni^{2+}−NTA column (Mitchell & Gennis, 1995), the buffer was exchanged by gel-filtration chromatography to 50 mM Tricine, pH 8.5, and 0.1% (w/v) lauryl maltoside. Glycerol (5% w/v) was added prior to storage at −70 °C.

The optical spectra of wild-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* are very similar to those of the bovine enzyme (Hosler et al., 1992). Extinction coefficients for the latter (Rich & Moody, 1997) were used to quantitate the levels of reduced, oxidized, and various ligand-bound states of the wild-type *Rh. sphaeroides* oxidase (Table 1). The same extinction coefficients were used for the mutant K362M form after adjustment of wavelengths where necessary, as indicated in the text.

Optical Spectroscopy and Kinetic Measurements. Optical spectra and multiwavelength kinetics were monitored at room temperature in the same sample using a single-beam instrument built in-house. Photolysis of the ferrous CO and cyanide compounds of the oxidase was carried out with a frequency-doubled Nd/YAG laser (Spectron Laser Systems, Rugby, U.K.). This provided a 10 ns pulse of light at 532 nm with an energy in excess of 100 mJ/pulse. Kinetic spectra were constructed from averaged transients taken cyclically at individual wavelengths.

Electron Paramagnetic Resonance Spectroscopy. Continuous-wave EPR spectra were recorded on a Jeol RE1X spectrometer fitted with an Oxford Instruments cryostat. Low-spin signals (222−414 mT scan) were recorded at 12 K, microwave power 4 mW, modulation amplitude 1.25 mT, and microwave frequency 9.055 GHz. Conditions of measurement for high-spin cytochromes (71−167 mT scan) were as follows: temperature 7 K, microwave power 10 mW, modulation amplitude 1.6 mT, microwave frequency 9.055 GHz.

Redox Titrations. Conventional anaerobic redox titrations (Dutton & Wilson, 1974) of cyanide-ligated oxidase (approximately 2 μM) were carried out under an argon atmosphere in a medium containing 50 mM potassium phosphate, pH 7.0, 2 mM EDTA, 1.5 mM potassium cyanide, and 0.1% (w/v) lauryl maltoside. Redox potentials were measured in the stirred sample using a glassy-carbon electrode and a Ag/AgCl reference electrode. Redox mediators were as follows ($E_m{}^7$ in millivolts, concentration in micromolar): phenazine methosulfate (+80, 8), 2,6-dichlorophenolindophenol (+220, 15), 2,3,5,6-tetramethylphenylenediamine (+275, 50), ferricyanide (+440, 50), 1,2-naphtho-4-sulfonate (+215, 5), 1,2-naphthoquinone (+143, 5), 1,4-benzoquinone (+293, 50), 2,6-dimethyl-1,4-benzoquinone (+168, 40), methylhydroquinone (+224, 40), and cytochrome *c* (+255; 3). The potential was varied with additions of sodium ascorbate (20 mM stock) or potassium permanganate (10 mM stock). After stabilization of the potential, stirring was stopped and the spectrum between 390 and 490 nm was recorded. In order to minimize interference from redox dyes or baseline drifts, titration curves were derived from triple wavelength measurements at 445 nm − (435 nm + 455 nm)/2. The data represent points taken in both oxidative and reductive directions.

RESULTS

(1) Analysis of the "As Prepared" State. As a starting point for characterization of the K362M mutant, we used optical and EPR spectroscopy to define the state of the enzyme in the as prepared form. The optical spectra of wild-type and mutant oxidase, as prepared, are shown in Figure 1. The wild-type enzyme is essentially fully oxidized and in the fast form, as indicated by a symmetrical Soret peak at 425 nm, an α-band near 600 nm (Figure 1A), and a high Soret: α-band ratio of 6.7 in the absolute oxidized spectra [cf. Rich and Moody (1997)]. Only a very small ferricyanide-oxidizable component can be discerned (see Figures 1A and 2C). In contrast, as prepared K362M protein shows a prominent shoulder on the red side of the Soret peak whose position is shifted to 423 nm. The α-band is located at 606 nm and has an increased intensity relative to the Soret peak (Soret: α-band ratio of 3.3). These features revert to a more typically ferric spectrum on incubation with 100 μM ferricyanide, although the Soret peak remains at the lower wavelength of 423 nm (Figure 1B). The Soret peak and

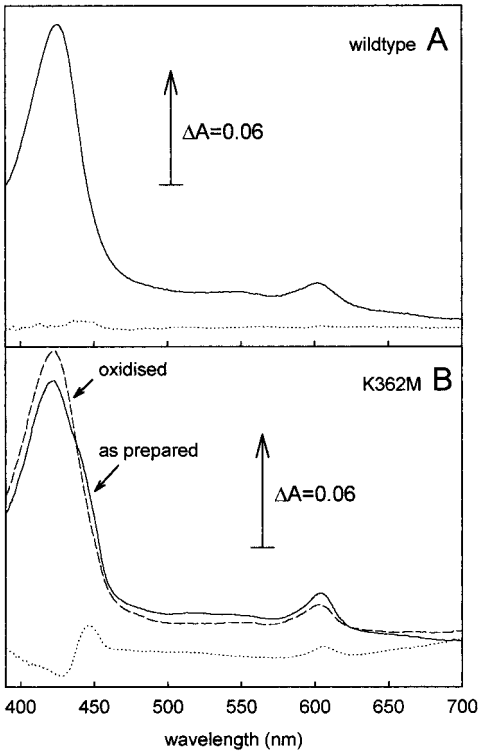

FIGURE 1: Optical spectra of as prepared and oxidized wild-type and K362M mutant cytochrome c oxidase. Oxidase samples were diluted to 0.6 μM in 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA and 0.1% (w/v) lauryl maltoside. Ferricyanide (100 μM) was used as an oxidant, and oxidation was judged to be complete when no further spectral changes could be detected. (A) Wild-type as prepared absolute spectrum (solid line), as prepared minus oxidized difference spectrum (dotted line); (B) K362M as prepared (solid line) and oxidized (broken line) absolute spectra, as prepared minus oxidized difference spectrum (dotted line, manually displaced along *y*-axis).

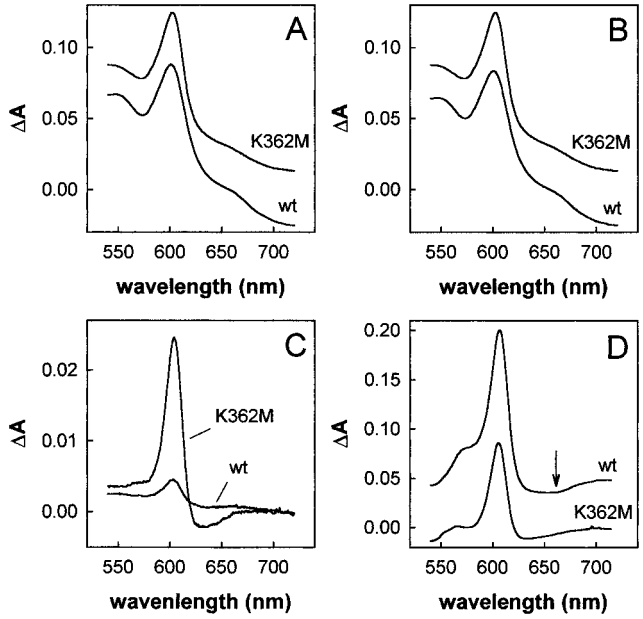

FIGURE 2: Spectra of oxidized and reduced cytochrome oxidase in the visible region. In order to obtain high-quality spectra in the visible region, oxidase samples were used in isolation buffer [50 mM Tricine, 0.1% (w/v) lauryl maltoside, and 5% (w/v) glycerol, pH 8.5] without prior dilution. Oxidase concentrations were 5.5 μM for wild type and 4.5 μM for mutant enzyme. Ferricyanide (100 μM) was used as an oxidant, and samples were reduced with dithionite (15 min incubation). (A) As prepared, absolute spectrum; (B) ferricyanide-oxidized absolute spectrum; (C) as prepared minus ferricyanide-oxidized; (D) dithionite-reduced minus ferricyanide-oxidized. The arrow indicates the trough caused by the bleaching of the 655 nm charge transfer band of ferric heme a_3.

trough positions (428 and 446 nm, respectively) and a comparatively intense signal at 606 nm in the as prepared minus ferricyanide-oxidized difference spectrum (ratio of signal intensities Soret:α = 6.0 in the difference spectrum; Figure 1B, also see Figure 2C) are characteristic of heme *a* (Liao & Palmer, 1996) and indicate the presence of some reduced heme *a* in the as prepared sample. On the basis of a quantitation of the total concentration of the K362M mutant from the Soret absorbance in the absolute, fully oxidized spectrum using ϵ = 160 mM^{-1} cm^{-1} (423 nm) (Rich & Moody, 1997) and quantitation of heme *a* using extinction coefficients given in Liao and Palmer (1996), approximately 30% of heme *a* is reduced in this as prepared sample. The ferricyanide-treated material might still not be fully oxidized but contain a small amount (up to 20%) of another species, as indicated by the less than quantitative ligand reactivity of the ferricyanide-treated enzyme, as described below. This unreactive species has properties similar to P in that the α-peak at 604 nm is still somewhat red-shifted compared with the oxidized wild-type enzyme, and the Soret:α-band ratio of 6.5 is still slightly lower than the expected value of 6.7 (Rich & Moody, 1997).

Fast, oxidized cytochrome oxidase shows a charge-transfer band near 655 nm ascribed to high-spin ferric heme a_3 (Beinert et al., 1976). This charge transfer band is clearly seen in the as prepared or ferricyanide-treated wild-type enzyme (Figure 2A,B) but is weaker in the K362M mutant (Figure 2A,B). Unlike the wild-type enzyme, the band is not bleached by dithionite in the K362M mutant over 15 min of incubation with reductant at pH 8.5; i.e., mostly heme *a*, but not a_3, reduction occurs over this time (absence of the 655 nm trough in the K362M trace, indicated by an arrow in Figure 2D). Figure 2C shows the as prepared minus ferricyanide-oxidized difference spectra in the visible region. The sizes of the wild-type and K362M signals in these spectra confirm that as prepared wild-type enzyme is essentially ferric (96%), whereas heme *a* is around 30% reduced in the present K362M mutant preparation. In addition, the lack of a 655 nm trough in the as prepared minus oxidized difference spectrum in Figure 2C indicates that in the K362M mutant it is heme *a*, not heme a_3, which is ferricyanide-oxidizable in the as prepared sample.

A similar conclusion can be reached from EPR spectroscopy. The as prepared K362M enzyme has similar features as the wild-type enzyme, although the g = 2.8 signal due to low-spin heme *a* (Hosler et al., 1992) is smaller than in wild-type enzyme, compatible with a proportion of ferrous heme *a* in the mutant (not shown).

(2) Ligand Reactivity of the Ferricyanide-Oxidized Enzyme. The reactions with formate, cyanide, peroxide, or CO were used to characterize the oxidase in the ferricyanide-oxidized state. Incubation of the ferricyanide-oxidized K362M mutant enzyme with formate (25 mM) and cyanide (10 mM) at pH 7.0 gave normal binding spectra corresponding to 70−80% formation of the formate- or cyanide-ligated compound (not shown). The kinetics of cyanide binding to the reactive fraction, monitored at 433 minus 412 nm, were monophasic and similar to those of the wild-type enzyme (wild type 0.025 s^{-1}; K362M 0.055 s^{-1} at 10 mM HCN and pH 7).

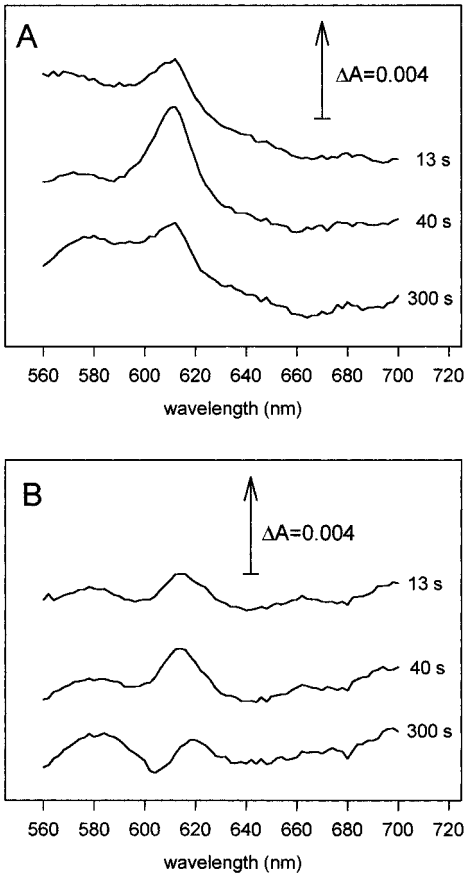

FIGURE 3: Reaction of ferric wild-type and K362M mutant oxidase with hydrogen peroxide. Hydrogen peroxide (2 mM) was added to oxidase samples (0.6 μM) in 100 mM potassium phosphate buffer, pH 8.0, containing 0.1% (w/v) lauryl maltoside. Samples had been pretreated with 100 μM ferricyanide. The H_2O_2-reacted minus oxidized spectra are at 13, 40, and 300 s after addition of H_2O_2, as indicated. (A) Wild type; (B) K362M mutant.

Oxidized heme−copper oxidases react with hydrogen peroxide to transiently form compound P followed by conversion to F, the kinetics of the reaction depending on pH and H_2O_2 concentration (Wrigglesworth, 1984; Fabian & Palmer, 1995; Brittain et al., 1996). In the experiment of Figure 3 we have chosen conditions that allow a clear separation of the two phases, i.e., alkaline pH and high (millimolar) peroxide concentrations that maximize transient occupancy of the P state. Spectra in the visible region (Figure 3) show that both wild-type and K362M mutant proteins form the P (absorbing at 608 nm) and F (band near 580 nm) states. In K362M this reaction is overlaid by some heme degradation or oxidation indicated by distortion of the P-spectrum by a small trough at 600−605 nm (Figure 3B), which may account for as much as 15% of the total enzyme concentration (based on degradation of oxidized heme *a* with $\epsilon \approx 24$ mM^{-1} cm^{-1}; Rich & Moody, 1997). Peroxide reactivities of near 100% for the wild type and 70−75% for the K362M mutant were estimated from the binding spectrum in the Soret region (not shown), where the P and F states have similar spectra and extinction coefficients (50.5 mM^{-1} cm^{-1}, peak to trough; Rich & Moody, 1997), although the band position of compound F is slightly shifted in the K362M mutant (trough at 414 nm, peak at 435 nm) compared to the wild type (trough at 413 nm, peak at 437 nm). The kinetics of the reaction with H_2O_2 at pH 8, monitored at the respective Soret peak minus trough wavelengths, can be overlaid for the two samples (biphasic reaction with rate constants at 4 mM H_2O_2 of 0.6 s^{-1} and 0.05 s^{-1}, not shown).

Table 2: Summary of the Extent of Enzyme Reactivity with Ligands in Various Redox States

	extent of reaction[a] (rate)	
	wild-type	K362M
composition of as prepared enzyme		
oxidized	≥96%	
heme *a* reduced	≤4%	≤30%
other		7−17% (P?)
reactivity of ferricyanide-treated enzyme with		
cyanide (10 mM)	102% (0.025 s^{-1})	70−80% (0.055 s^{-1})
formate (25 mM)	99%	70−80%
H_2O_2 (4 mM)	94%	70−75%
CO (1 mM)	92%	50−60%
reactivity of dithionite-reduced enzyme with		
CO (1 mM)	100% (52 s^{-1})	30−50% (48 s^{-1})
cyanide (10 mM)	86% (10 s^{-1})	25−30% (12 s^{-1})

[a] The extent of ligand reactivity was calculated from the difference spectra compared to the signal intensity expected for 100% reactivity, based on the extinction coefficients in Table 1.

Bubbling carbon monoxide through a solution of fully oxidized beef heart oxidase results in two-electron reduction of the binuclear center (Bickar et al., 1984). Under aerobic conditions, this mixed-valence species reacts with oxygen to form the P state. The conversion is nearly quantitative in the wild type. The P state of the K362M mutant can also be prepared using the CO method, however, only at a yield of about 60%, slightly less than in the reaction with H_2O_2 .

These ligand reactivities of the ferricyanide-oxidized enzyme are summarised in Table 2.

(3) Behavior of the Reduced Enzyme. Preparation of the wild-type oxidase results in the fast form of the enzyme with characteristic fast reduction of heme a_3 by dithionite and fast cyanide binding kinetics to the ferric enzyme [$k_{on} = 2.5$ M^{-1} s^{-1} at pH 7, compared to 1−2 M^{-1} s^{-1} in bovine oxidase; cf. Moody (1996)]. In contrast, dithionite reduction of the K362M mutant is slow and yields only a partially reduced product. In order to quantitate the amounts of reduction of the individual hemes we have used the (wild-type) reduced minus oxidized spectra of hemes *a* and a_3, which were determined for the *Rh. sphaeroides* enzyme (not shown) as previously described for the bovine oxidase (Liao & Palmer, 1996): Reconstitution of the reduced minus oxidized spectrum of the K362M mutant using these individual heme spectra shows that incubation with dithionite at pH 7 for 20−30 min results in about 30−40% heme a_3 reduction, increasing to 60−70% after several hours before degradation sets in (not shown).

Kinetics of CO or cyanide recombination to the reduced oxidase are monophasic in the wild-type enzyme with rate constants of 52 s^{-1} at 1 mM CO and 10 s^{-1} at 10 mM HCN, respectively (both pH 7). These values compare to rate constants for CO recombination (1 mM) of around 70 s^{-1} for bovine (Gibson & Greenwood, 1963) and yeast oxidase (Brown et al., 1994) and 50 s^{-1} for the *E. coli* enzyme (Mitchell et al., 1995) and about 2 s^{-1} for 10 mM cyanide binding in the bovine and *E. coli* enzymes [cf. Hill and Marmor (1991) and Mitchell et al. (1995)]. Biphasic kinetics and deviation from the characteristic rate constant indicate protein heterogeneity such as may be induced by partial denaturation or lack of copper in the binuclear center (Mitchell et al., 1995). Therefore, binding of CO [where

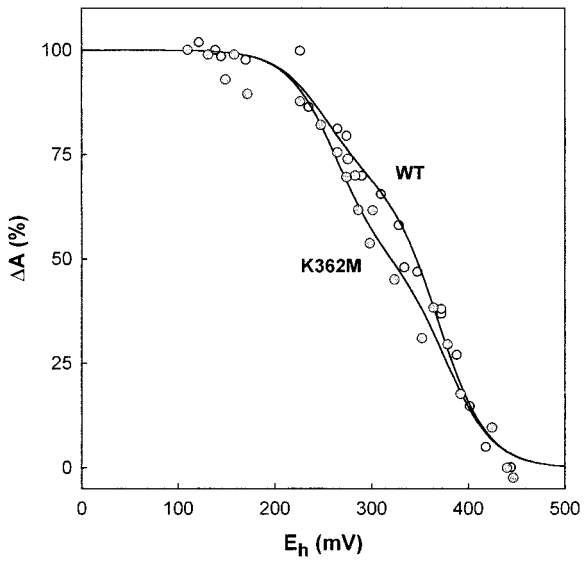

FIGURE 4: Redox titration at pH 7 of cyanide-ligated wild-type and K362M mutant oxidase. Redox titrations of approximately 2 μM oxidase in the presence of 1.5 mM cyanide were performed as described in Materials and Methods. The experimental data represent points taken in both oxidative and reductive directions and are overlaid with calculated traces based on a model of interaction between heme a_3 and Cu_B (Moody & Rich, 1990). The following parameters were used: for wild type, $E_{m,a}(Cu_{B,ox}) = 360$ mV, $E_{m,a}(Cu_{B,red}) = 280$ mV, and $E_{m,CuB}(a_{ox}) = 340$ mV. For K362M, $E_{m,a}(Cu_{B,ox}) = 360$ mV, $E_{m,a}(Cu_{B,red}) = 285$ mV, and $E_{m,CuB}(a_{ox}) = 360$ mV.

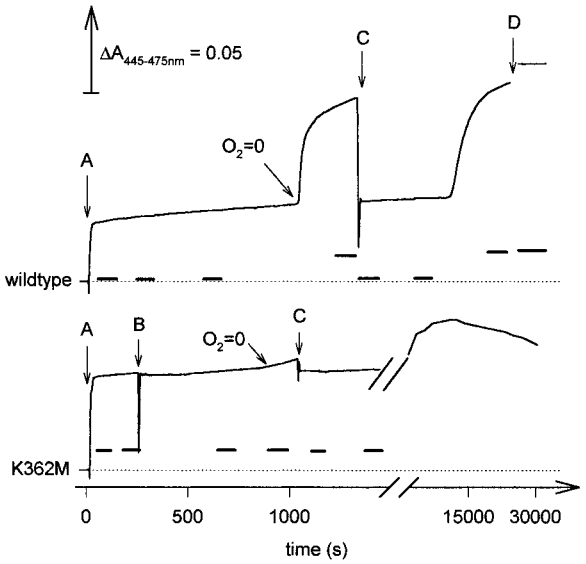

FIGURE 5: Steady-state behavior of wild-type and K362M mutant oxidase. Levels of steady-state heme reduction and intermediates were monitored at 445 minus 475 nm in samples containing 0.6 μM oxidase in 100 mM potassium phosphate and 0.1% (w/v) lauryl maltoside, pH 7.5. Before the reaction, samples were oxidized with 100 μM ferricyanide. The following additions were made: (A) 0.6 μM cytochrome *c*, 10 mM ascorbate, and 10 μM TMPD; (B) 10 mM glucose, 5 units/mL glucose oxidase, and 50 units/mL catalase (K362M only), (C) oxygen-pulse (stirring); (D) dithionite (wild type only). The traces still contain spectral contributions of ferrocytochrome *c*. The cytochrome *c* absorbance was separately derived from spectra taken during the course of the real-time experiment and is indicated by bars. The dotted reference lines indicate the $\Delta A = 0$ point for each experiment. Note that the break in the time axis only applies to the K362M mutant, as indicated, and not to the wild-type sample.

no proton is involved (Mitchell & Rich, 1994)] and cyanide [where one proton is bound simultaneously (Mitchell & Rich, 1994)] to the reduced oxidase was used to monitor whether the binuclear center reacts normally. In the K362M mutant, both CO (1 mM, at pH 7) and cyanide (10 mM, at pH 7) gave binding spectra and kinetics similar to those of the wild-type enzyme (Table 2). Quantitation of the ligand-reactive proportion of the mutant oxidase using the extinction coefficients given in Materials and Methods showed that although reduction of heme a_3 is only partial, the proportion of enzyme that does become fully ferrous appears to bind both ligands quantitatively and with normal rate constants (not shown).

(4) Effects of Mutation on Redox Potentials. A redox titration of the cyanide-ligated enzyme at pH 7 shows behavior very similar to that of the beef heart enzyme (Figure 4). Simulations of the experimental data were carried out as described in Moody and Rich (1990), assuming a model of anticooperative interaction between heme *a* and Cu_B but not taking into account the weak interaction with Cu_A. The data are best simulated with midpoint potentials of 360 and 340 mV for heme *a* and Cu_B, respectively, in wild-type enzyme (-80 mV redox interaction) and 360 mV for both heme *a* and Cu_B with a redox interaction of -75 mV in the K362M mutant (all ± 10 mV).

(5) Steady State. Low levels of reductant (cytochrome *c* plus ascorbate and TMPD) were used to assess the behavior of the oxidase in the aerobic steady state. In the wild-type oxidase, a comparatively fast (seconds) approach to the steady state was observed following addition of substrate to the ferricyanide-oxidized enzyme (Figure 5). The difference spectrum under turnover conditions with maxima at 608 and 444 nm and a trough at 421 nm can be interpreted as showing partial heme *a* reduction together with the presence of the oxygen intermediates P (as indicated by the position of the α-peak) and F (featured by a prominent band near 580 nm) (Figure 6B). At the substrate levels used (in particular limiting TMPD), cytochrome *c* is approximately 90% oxidized in the steady state (see Figure 5). Due to this low concentration of reduced cytochrome *c*, the observed turnover is extremely low, in the region of 2 electrons s^{-1}. This also means that the enzyme is also mostly oxidized (Figure 6A,B). Anaerobiosis is marked by a sharp increase in absorbance as both heme *a* and the binuclear center become fully reduced (Figure 5). Simultaneously, reduction of cytochrome *c* is observed. An oxygen pulse returns the system to the previous steady state.

In the K362M mutant, addition of substrate to the ferricyanide-oxidized enzyme also led to a rapid approach to a steady state (Figure 5). However, the spectrum of this state corresponded to nearly pure heme *a* reduction with no indication of oxygen intermediates (Figure 6A,C). The cytochrome *c* remains 90% reduced. Since the activity of the mutant protein is close to zero, a glucose/glucose oxidase/catalase system was used to induce anaerobiosis. On oxygen depletion, a clear anaerobic transition is observed (Figure 5), although heme reduction in the mutant protein proceeds at a much lower rate than in the wild type. An oxygen pulse given to the anaerobic, partly reduced sample returns it to the previous steady-state where heme *a* is reduced and the binuclear center is essentially oxidized (Figure 5). The partly reduced minus after O_2 pulse difference spectrum shows a clear Soret maximum at 445 nm with only a weak and broad band near 600 nm. This is compatible with some heme a_3

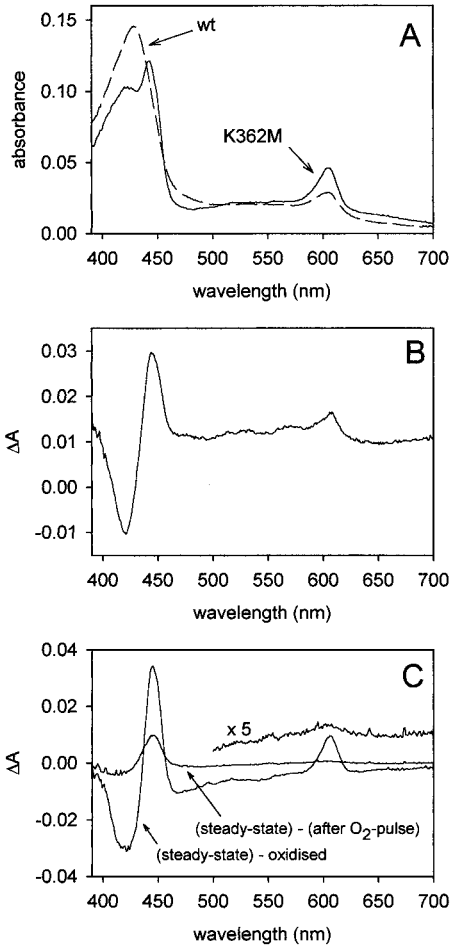

FIGURE 6: Steady-state spectra of wild-type and K362M mutant oxidase. Spectra were recorded in the same samples during the real-time experiment presented in Figure 5. For clarity, the spectral contributions of cytochrome *c* (ferric and ferrous) have been subtracted. (A) Absolute steady-state spectra of wild-type and K362M enzymes; (B) steady-state minus ferricyanide-oxidized difference spectrum of wild-type enzyme; (C) steady-state minus ferricyanide oxidized and anaerobic minus (after O_2 pulse) difference spectra of the K362M mutant.

reduction. Further reduction of heme a_3 at pH 7.5 is very slow and after several hours reaches about 60−70% before enzyme degradation sets in.

In order to further assess the reduction state of the binuclear center in the aerobic steady state, the enzyme was reacted with cyanide or formate in the presence of low levels of substrate (cytochrome *c* plus ascorbate and TMPD). The cyanide-binding spectrum of K362M mutant oxidase in the steady state is that of ferric heme a_3 (not shown) and shows essentially quantitative reaction. No extra reduction of heme *a* is observed, consistent with the above conclusion that this is already fully ferrous in the presence of substrate. Whereas cyanide-binding to ferric heme a_3 does not require cupric Cu_B [cf. Yoshikawa et al. (1995)], formate will only bind when both metals in the binuclear center are oxidized (Kojima & Palmer, 1983). As described above, under the conditions used turnover is very low in the wild-type enzyme, which is therefore still mostly oxidized. Thus, the spectrum of interaction of the wild-type enzyme with formate in the aerobic steady state, shown in trace C of Figure 7, can be interpreted as an overlay of quantitative formate binding to ferric heme a_3 (peak/trough at 414 and 430 nm, trace D) and reduction of heme *a* (peaks at 446 and 605 nm) in the

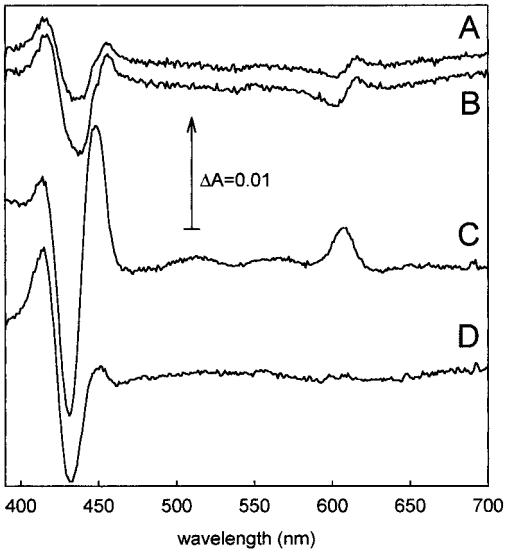

FIGURE 7: Formate binding to wild-type and K362M mutant oxidase in the aerobic steady state. Samples were suspended in 50 mM potassium phosphate, 2 mM EDTA, and 0.1% (w/v) lauryl maltoside, pH 7.0, to give a final concentration of 0.6 μM. Cytochrome *c* (0.6 μM, plus 10 mM sodium ascorbate and 5 μM TMPD) was used as a substrate. Potassium formate (25 mM) was added after a stable steady-state level of heme reduction had been reached. The formate/steady-state minus steady-state difference spectra shown are (A) K362M, 1 min; (B) K362M, 10 min after formate addition; (C) wild type (oxidized), 10 min after formate addition. Trace D shows the formate minus oxidized difference spectrum of wild-type enzyme.

now inhibited enzyme. In contrast to this behavior of the wild-type enzyme, the K362M reaction with formate, as estimated from the Soret absorbance (414 minus 430 nm, $\Delta\epsilon = 34$ mM^{-1} cm^{-1}; Rich & Moody, 1997) does not proceed beyond about 30% (traces A and B, Figure 7). In addition, the spectrum shows some unusual features, a Soret band at 456 nm and a red shift in the α-peak region (602−615 nm).

DISCUSSION

State of the Mutant Enzyme in the "As Prepared" and Ferricyanide-Oxidized Forms. As shown in Figures 1 and 2, the K362M mutant sample that we describe here contains up to 30% ferrous heme *a* in the as prepared state that can be reoxidized with ferricyanide. This treatment produces an enzyme whose spectral features are close to those of the (fast) wild-type oxidase and that shows about 80% extent of reactivity toward cyanide, peroxide, or formate with kinetics close to the wild-type behavior (Table 2). A minor heterogeneity problem is posed by the presence of up to about 20% of a non-ferricyanide-oxidizable species. On the basis of its Soret and visible band features, this fraction appears similar to a P state, although this is not consistent with the behavior of the major fraction of this mutant. At present it is not clear whether this compound can be reduced with dithionite since reduction of the mutant enzyme at pH 7 could not be completed before some degradation was observed. It should be noted that the above composition of the as prepared K362M enzyme cannot be an absolute quantitation but that it is expected to vary between different preparations. Below we concentrate on the properties of the major ferricyanide-oxidizable fraction.

The EPR and optical spectra of the ferricyanide-treated K362M mutant in various ligation states show features

essentially identical to the wild-type form, indicating that the redox centers, in particular the binuclear center, are intact in the mutant. This is consistent with previous findings from FTIR and resonance Raman spectroscopy of the *Rh. sphaeroides* (Hosler et al., 1996) and *E. coli* (Thomas et al., 1993) enzymes, which indicated no major structural changes in the redox centers of the K362M mutant, except for attenuated $Fe-N_{His}$ stretching and porphyrin bending modes (Hosler et al., 1996). A structurally intact binuclear center in the major fraction of the mutant is also suggested by the similarities to the wild-type enzyme of the extents and features of ligand binding spectra and their kinetics, both for the ferricyanide-oxidized form and for that fraction which could be reduced with sodium dithionite (see below).

Reduction of the Binuclear Center. Whereas heme *a* reduction by dithionite or ferrocytochrome *c* is rapid and quantitative, heme a_3 can only be reduced slowly and partially. The presence of some reduced heme *a* in as prepared mutant enzyme (Figure 1) and, quantitatively, in the steady state (see below) also points to a block preventing electron transfer from heme *a* to the binuclear center. Addition of CO or cyanide to dithionite- reduced enzyme yields less than 50% of the respective adduct compared to the wild-type enzyme (Table 2), although, once reduced, the spectral features and ligand binding kinetics of the mutant enzyme are as in the wild type. Inhibition of heme a_3 reduction and lower than wild type ($\leq$ 60%) amounts of CO binding have previously been noted by Hosler et al. (1996).

Redox Titration. The redox behavior at pH 7 of heme *a* in cyanide-ligated K362M oxidase was unaltered, within error limits, from that of the wild-type enzyme, indicating that the K362M mutation essentially does not affect the redox properties of heme *a* and Cu_B. Most importantly, the data on the mutant enzyme still showed a distorted two-wave shape of the heme *a* titration that can be attributed to a heme *a*/Cu_B redox interaction and that could be simulated using parameters close to the wild-type values (Figure 4). It has been proposed (Moody & Rich, 1990) that the anticooperative interaction between these two redox centers is mediated *via* a common protonable group(s). Our present results indicate that the residue K362 is unlikely to be one of these groups. However, although K362 is unlikely to be redox-linked to heme *a* and Cu_B, the question remains open as to whether it is redox-linked to heme a_3.

Nature of the Steady State. As shown by the optical spectra in Figure 6, the steady state of the K362M mutant oxidase is characterised by full heme *a* reduction with no indication of any alteration in the content of oxygen intermediates. Since two-electron reduction of the binuclear center is extremely difficult in the K362M enzyme, as discussed above, while the E_m of Cu_B appears to be unaltered, we further probed the redox state of the binuclear center in the presence of respiratory substrate and oxygen by assessing its reactivity with the ligands cyanide and formate. The size of the cyanide-binding spectrum of the K362M mutant in the aerobic steady state confirmed that heme a_3 had remained largely oxidized, although this result does not provide information about the redox state of Cu_B since this does not significantly affect the cyanide binding spectrum of ferric heme a_3 (Yoshikawa et al., 1995). However, the low extent of formate binding (approximately 30%, in comparison to 70−80% for the ferricyanide-oxidized form) in the presence of substrate and oxygen suggests that the binuclear center was not fully oxidized. We interpret these results as showing that in the aerobic steady state the binuclear center of the K362M enzyme was at least partially in a one-electron-reduced state.

Unusual spectral features are observed following addition of formate (Figure 7). It is unlikely that these are due to spectral shifts in the mutant enzyme, since all other spectral features, in particular the formate binding spectrum to oxidized enzyme, are close to those seen in the wild type. It cannot be excluded that formate binds to the mutant enzyme in an unusual manner in the presence of substrate. However, it has previously been noted (Moody & Rich, 1990) that the position of the α-peak of reduced heme *a* in bovine heart oxidase undergoes slight shifts depending on the redox state of Cu_B, a red shift being associated with oxidation of Cu_B. Thus, we interpret the red shift in the formate binding spectrum in the presence of respiratory substrate as suggesting formate-induced reoxidation of the one-electron-reduced binuclear center (Cu_B^{1+} a_3^{3+}) to the fully oxidized, formate-ligated species.

Some further indication that heme a_3 remains ferric in the one-electron-reduced binuclear center comes from the unaltered E_m of Cu_B (see above) and the ligand (CO) reactivity of dithionite-reduced K362M enzyme. The amount of CO adduct formed in dithionite-treated enzyme approximately equals the observed reduction level of heme a_3 as estimated from the optical spectra. Since binding of CO requires a two-electron-reduced binuclear center (Lindsay et al., 1975; Wilson & Miyata, 1977), Cu_B must also be reduced in those enzymes with a reduced heme a_3. Likewise, preliminary EPR spectra (unpublished results) of anaerobic K362M oxidase in the presence of reductant show a transient increase in a $g \approx 6$ high-spin heme signal without any simultaneous appearance of a $g \approx 2$ signal, which has been described for Cu_B (Reinhammar et al., 1980; Karlsson & Andréasson, 1981). These findings are consistent with a reduction of the binuclear center where a first reducing equivalent resides primarily on Cu_B, producing a species that can bind cyanide but not formate or CO and that has an EPR-detectable form of heme a_3 which is no longer spin-coupled to Cu_B. Further reduction of heme a_3 is much slower and produces the form that can bind CO, pointing to a lower operative midpoint potential of this heme group.

Inhibitory Action of the Mutation. From the above it can be concluded that possibly one electron, but not two, can enter the binuclear center easily. Hence the inhibitory action of the mutation is its hindrance of formation of the doubly-reduced binuclear center (the R state), which is the intermediate required for productive reaction with oxygen to produce the P intermediate [cf. Babcock and Wikström (1992)]. In the light of this conclusion, the finding needs comment that the P form can still be produced at wild-type rates either by reaction with CO in the presence of oxygen (where around 60% P form is attained) or by direct reaction with hydrogen peroxide (which gives a yield of 70−75%; see Table 2).

Critical to this discussion is the point that the stable form of the R state needs two protonations to balance the charges of the two electrons (Mitchell & Rich, 1994). In the natural reaction cycle there must presumably be an unstable transient state, which we shall call R*, in which the two electrons have entered the binuclear center, before full charge compensation by protonation has occurred. It is argued below

that it is a difficulty of formation of R* that prevents catalytic turnover of the K362M mutant enzyme.

The mechanism of the CO reaction is as yet not fully understood. However, the equation of CO oxidation

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$$

suggests that the required protons are taken up into the structure as part of the reacting water. In the case of reaction with hydrogen peroxide, the required protons are supplied by the hydrogen peroxide itself. Hence, in both cases, it seems likely that the pathway to reaction product does not require the transient formation of R*, so that the reactions can proceed as in the wild-type form.

It has been shown by Svensson et al. (1995) that the fully reduced form of cytochrome *bo* with a mutation at this lysine residue is able to react with oxygen through to the F form at rates similar to those of the wild-type form. Again, this result may in part be explicable by consideration of associated protonations. In this case, prolonged incubation of the enzyme with reductant is required to produce the reduced state and to preload the binuclear center with its two redox-linked protons (Mitchell & Rich, 1994). Hence, again a forbidden R* transient state is not involved in reoxidation to the P state. This argument may also be used to explain the apparently normal further conversion of P to F in this mutant and the finding that the F to O conversion is also apparently normal in analogous mutants of cytochrome oxidase of *Rh. sphaeroides* ((Brzezinski & Ädelroth, 1997; Konstantinov et al., 1997). In these steps, an unstable state must occur on transient electron transfer to the P or the F state before charge compensation by protonation has occurred. However, in this case the electron density is not restricted to the two metal centers of the binuclear center and so may not be so affected by mutation in the K362 position.

Mechanistic Considerations. Hence, it seems that formation of the doubly reduced (and doubly protonated) R state can occur with prolonged incubation and that it is the rate of formation of this state which is slow in the mutant. The most obvious rationale is that the lysine is part of an obligatory H^+_S channel that allows proton transfer into the binuclear center, as described in Iwata et al. (1995). However, this cannot explain why oxidation from the fully reduced state to F (Svensson et al., 1995) or to O (Brzezinski & Ädelroth, 1997) is relatively normal, nor the normal electrogenic behavior in the F → O transition (Konstantinov et al., 1997), since the full sequence of these reactions requires uptake of both H^+_S and H^+_T. Furthermore, in the context of our glutamate trap model of coupling, we might have expected that the enzyme could proceed from the oxidized state into the P form without a requirement for H^+_S (Rich, 1995, 1996).

Another line of data of relevance is our finding that the binuclear center ligands formate, cyanide, and carbon monoxide bind with normal kinetics to this mutant enzyme. If the lysine residue were part of a hydrophilic channel that could conduct protons and polar groups to the binuclear center, then significant effects on some of the ligand reactivities might have been expected.

The above results, although emphasizing the importance of the pore B structure, cast doubts on a simple static role as a pathway for transfer of H^+_S (or ligands generally) into the binuclear center throughout the reaction cycle. This led Konstantinov et al. (1997) to suggest that the proton channels in the oxidase structure may not conduct exclusively H^+_S or H^+_T but may change their roles at different stages of the catalytic cycle. In this instance, pore B would act as a proton channel only for the H^+_S required for conversion of oxidized enzyme to the P stage, and the H^+_S required at subsequent steps would be provided by a different route. An alternative possibility is that it may represent a feature that we will call a dielectric well, a structure that might be contrasted with the classical Mitchellian proton well (Mitchell, 1968) in having a high dielectric strength but a low proton conductance. Its function would be to provide a pathway of dielectric relaxation for transient introduction of a (noncompensated) charge into the binuclear center, perhaps by hydrogen-bond network rearrangements between the charge and the N-phase solution. Such a pathway would decrease the energy cost of the unstable and energetically costly uncompensated R* intermediate. Hence, the inhibitory effect of the mutation is caused not by destabilizing the R state (which has two electrons and two charge-compensating protons) but instead by increasing the energy of the R* intermediate, which is an essential step in the natural catalytic cycle. This idea can be tested further by examining effects of other mutation positions and by direct electrostatic calculations on the available structural coordinates and offers an alternative working model to one that proposes a change of function of structural elements in different steps of the catalytic cycle.

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