Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1655 (2004) 263–273
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Review

Time-resolved optical absorption studies of cytochrome oxidase dynamics☆

Ólöf Einarsdóttir*, Istvan Szundi

Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064, USA

Received 24 April 2003; accepted 31 July 2003

Abstract

Time-resolved spectroscopic studies in our laboratory of bovine heart cytochrome *c* oxidase dynamics are summarized. Intramolecular electron transfer was investigated upon photolysis of CO from the mixed-valence enzyme, by pulse radiolysis, and upon light-induced electron injection into the cytochrome *c*/cytochrome oxidase complex from a novel photoactivatable dye. The reduction of dioxygen to water was monitored by a gated multichannel analyzer using the CO flow-flash method or a synthetic caged dioxygen carrier. The pH dependence of the intermediate spectra suggests a mechanism of dioxygen reduction more complex than the conventional unidirectional sequential scheme. A branched model is proposed, in which one branch produces the P form and the other branch the F form. The rate of exchange between the two branches is pH-dependent. A cross-linked histidine–phenol was synthesized and characterized to explore the role of the cross-linked His–Tyr cofactor in the function of the enzyme. Time-resolved optical absorption spectra, EPR and FTIR spectra of the compound generated after UV photolysis indicated the presence of a radical residing primarily on the phenoxyl ring. The relevance of these results to cytochrome oxidase function is discussed.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Flow-flash; Branched mechanism; Oxygen carrier; Tyrosyl radical

1. Introduction

The mechanism of electron and proton transfer in heme-copper oxidases continues to be a major challenge in the field of bioenergetics. The reduction of dioxygen to water has generally been studied by the flow-flash technique developed by Gibson and Greenwood [1,2], in which the reaction of the reduced enzyme with dioxygen is initiated upon photodissociation of carbon monoxide bound to heme a_3. Transient optical absorption flow-flash measurements have provided important information regarding the kinetics of the electron transfer [3–13], and time-resolved resonance Raman studies [14–24], including pioneering experiments by Babcock and his co-workers, have revealed the structures of some of the postulated intermediates. Transient optical absorption measurements, in combination with site-directed mutagenesis studies, have also recently provided important insights into the role of the two major proton transfer pathways, D- and the K-pathways [25–30], postulated based on crystallographic studies [31–33].

Despite significant advances, several questions remain unanswered. What are the details of the mechanism of intramolecular electron transfer in cytochrome *c* oxidase and the cytochrome *c*/cytochrome *c* oxidase complex? Does the sequential mechanism, conventionally proposed to describe the flow-flash reaction of the fully reduced enzyme with dioxygen, adequately describe the reaction? Why has the so-called P_R intermediate, proposed to be generated during the reduction of dioxygen to water, not been observed in time-resolved resonance Raman studies carried out by both Varotsis et al. [20] and Han et al. [34]? Does the cross-linked tyrosine play a role in the function of the enzyme? Does the CO flow-flash technique represent the physiological reaction of the heme-copper oxidases under turnover conditions or is this technique potentially compromised by the fate of the photodissociated CO? This review will focus on time-resolved optical absorption studies of

☆ This work was supported by the National Institutes of Health Grant GM 53788.

* Corresponding author. Tel.: +1-831-459-3155; fax: +1-831-459-2935.
E-mail address: olof@chemistry.ucsc.edu (O. Einarsdóttir).

0005-2728/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2003.07.011

cytochrome *c* oxidase that we have conducted in the last few years as they relate to these questions.

2. Intramolecular electron transfer in cytochrome oxidase

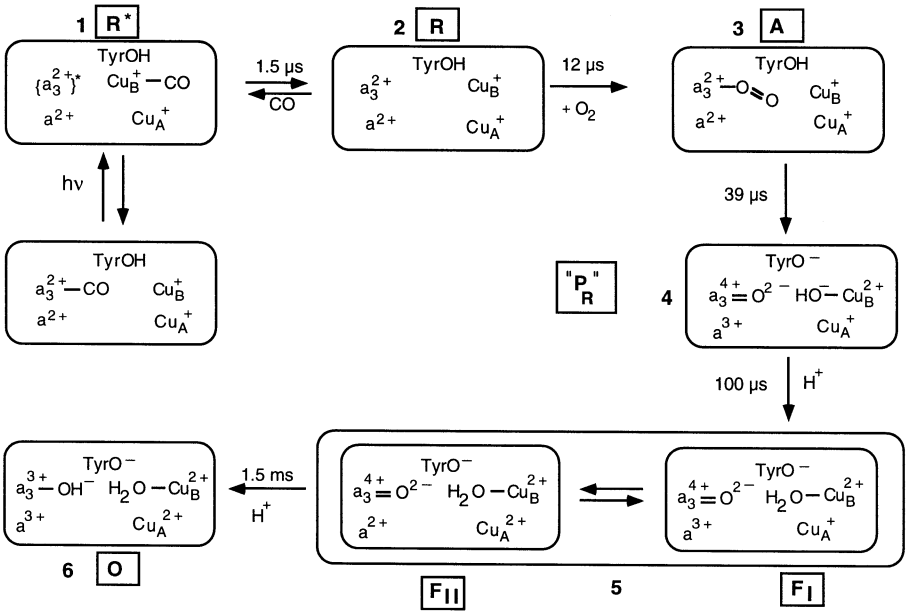
One way to study intramolecular electron transfer in heme-copper oxidases is to photodissociate CO bound to the mixed-valence enzyme and follow the reverse flow of electrons from the binuclear center to the low-potential redox centers, heme *a* and Cu_A [35,36]. Our laboratory has followed the optical absorption changes associated with this reaction and the photolysis of CO bound to the fully reduced enzyme using multichannel detection. This detection method has allowed us to collect high-resolution spectra in the Soret [37], visible [38,39] and near-infrared regions [39,40] on nanosecond to millisecond time scales. Singular value decomposition and global exponential fitting were used to analyze the time-resolved spectral changes. The data were fitted to a sequential mechanism with accompanying equilibria, and the absorption spectra of the intermediates were extracted. The spectral changes on ~ 5 and $\sim 50-100$ μs time scale were consistent with electron transfer from heme a_3 to heme *a* and between heme *a* and Cu_A, respectively. These results are in agreement with previous photolysis studies of the mixed-valence CO-bound enzyme [41–43]. Our kinetic modeling also allowed the extraction of the spectral contribution of Cu_A^{2+} in the bovine heart enzyme between 480 and 550 nm for the first time [39].

Alternatively, intramolecular electron transfer between Cu_A and heme *a* in cytochrome oxidase has been investigated by pulse radiolysis [44,45]. Kobayashi et al. [44] were the first to show by pulse radiolysis that Cu_A is the primary acceptor of electrons from cytochrome *c*, followed by electron transfer to heme *a*. Flow-flash studies on the cytochrome *c*/cytochrome *c* oxidase complex by Hill [46], laser-induced electron transfer experiments using photoactivatable compounds [47–52] and recent pulse radiolysis studies by us and our collaborators [45] have confirmed these conclusions. Our results indicated an equilibrium constant of 3.4 in the direction of reduced heme *a* and oxidized Cu_A, corresponding to a difference in reduction potential between heme *a* (Fe(III)/Fe(II)] and Cu_A [(Cu(II)/(I)] of +31 mV. This equilibrium constant is similar to an equilibrium constant of ~2 between heme *a* and Cu_A observed following photolysis of the mixed-valence CO complex [37] and during flow-flash studies of the fully reduced enzyme [10,53]. From the thermodynamic and activation parameters of the electron transfer reactions, and assuming that the reorganization energy of Cu_A in cytochrome oxidase is the same as that of the Cu_A center in purple azurin [54], we determined that heme *a* has a similar unusually small reorganization energy of 0.4 eV [45].

Our laboratory has also recently investigated electron transfer reactions in the electrostatic cytochrome *c*/cytochrome *c* oxidase complex using a novel photoactivatable dye. Laser photolysis of thiouredopyrenetrisulfonate (TUPS), covalently linked to a single cysteine on yeast *iso*-1-cytochrome *c*, generates a triplet state of the dye which donates an electron to cytochrome *c*, followed by electron transfer to cytochrome *c* oxidase [51,52]. The intramolecular electron transfer was monitored using single wavelength and multichannel detection. The results of the kinetic analysis of the time-resolved optical absorption data are consistent with Cu_A being the initial electron acceptor from cytochrome *c*, followed by subsequent electron transfer between Cu_A and heme *a*. The maximum efficiency of the reduced cytochrome *c* produced by a single laser pulse is significantly higher than that obtained previously with Ru-cytochrome *c* derivatives [48,49]. Future experiments with TUPS include direct labeling of specific cysteines on bacterial heme-copper oxidases. By varying the distance between the labeled cysteine and the TUPS dye and by introducing breaks into presumed electron transfer pathways by site-directed mutagenesis, we expect to provide detailed information about intramolecular electron transfer pathways in heme-copper oxidases.

3. Flow-flash studies—sequential mechanism

The most common method used to follow the electron transfer in cytochrome *c* oxidase during the reduction of dioxygen to water is the CO flow-flash approach [1,2]. The results have ordinarily been analyzed using a unidirectional sequential mechanism (for reviews, see Refs. [55–57]). Scheme 1 shows such a scheme and the postulated intermediates. The unidirectional sequential scheme provides a simple mathematical solution to the kinetics and allows calculations of the intermediate spectra without assumptions. The reaction sequence and the number of postulated intermediates have primarily been based on transient optical measurements carried out at selected wavelengths [3,5–7]. Four lifetimes, ~ 10, 30–40, 80–100 μs and 1–2 ms, have been reported [5], and an additional lifetime of 1.5 μs, attributed to the conversion of R* to R (Scheme 1), has been observed based on our time-resolved optical absorption measurements [9,10]. Time-resolved resonance Raman experiments by Babcock, Rousseau and Kitagawa and coworkers have provided strong evidence for the formation of the heme a_3-dioxygen bound intermediate (compound A) on 10 μs time scale [14–16] and an oxoferryl state ($Fe_{a3}^{4+}=O^{2-}$) on late microsecond time scale [17–19]. The oxoferryl is converted to the oxidized enzyme on a millisecond time scale. However, the P intermediate (P_R), postulated to be generated on the 30–40 μs time scale based on transient optical absorption measurements [7,8,58], was not observed during the reduction of dioxygen to water by time-resolved resonance Raman [20,34]. There-



Scheme 1. Conventional sequential reaction mechanism for the reduction of dioxygen to water by cytochrome *c* oxidase [12]. The "P_R" intermediate has not been observed in time-resolved resonance Raman experiments.

fore, there appears to be an unresolved discrepancy between the time-resolved resonance Raman and optical absorption measurements.

4. The sequential intermediate spectra—P_M versus "P_R"

Our laboratory has used multichannel detection to investigate the reduction of dioxygen to water in the Soret region [10–12] and the visible region [9,11,12,59] on nanosecond to millisecond time scales. Fig. 1 shows typical time-resolved absorption difference spectra (post-minus pre-photolysis) in both spectral regions at pH 7.5. We have also monitored the reaction at nine selected wavelengths in the near-infrared region [53]. Singular value decomposition and global exponential fitting were used to analyze the time-resolved spectra. Five apparent lifetimes were observed at the three pH values studied: 1.5, 13, ~35, 80–240 µs and 1.1–2.4 ms, with the two slowest rates decreasing by a factor of 2–3 upon increase

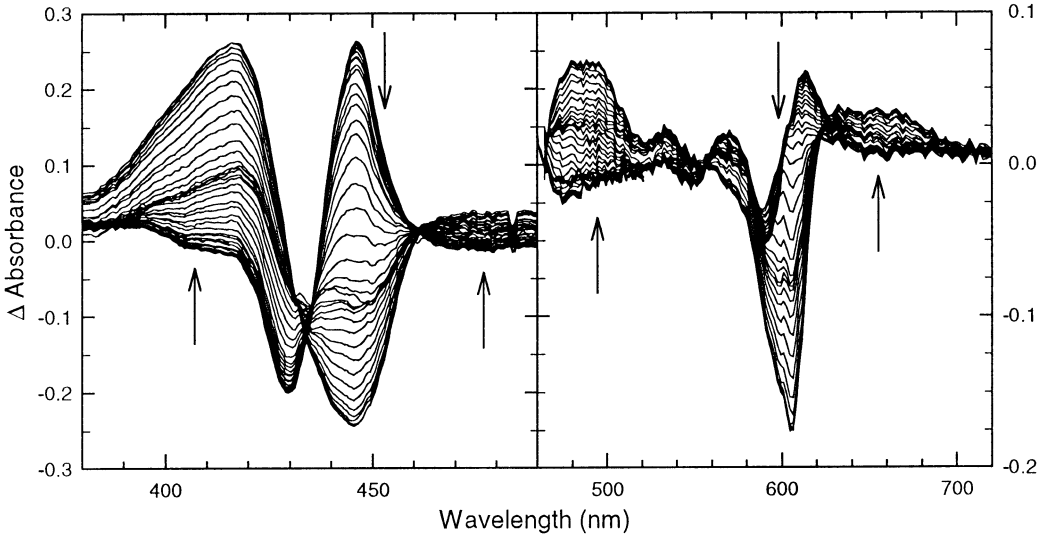


Fig. 1. The time-resolved optical absorption spectra (post-minus pre-photolysis) collected during the reaction of the fully reduced cytochrome oxidase with dioxygen at pH 7.5. The spectra were obtained at 44 and 42 delay times, equally spaced on a logarithmic time scale, between 50 ns and ~10 ms after photolysis of the fully reduced CO complex.

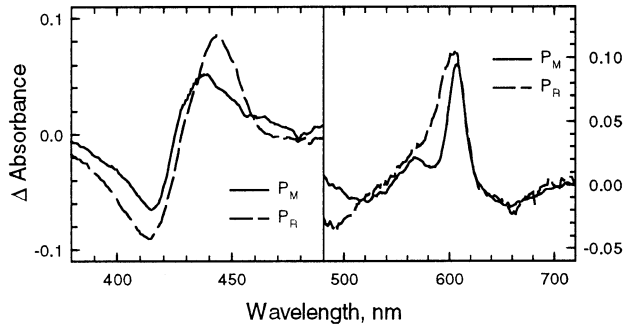


Fig. 2. The difference spectra of P_M (—) and intermediate 4 of the sequential scheme (P_R) (– – –) [11]. Both spectra are referenced against the spectrum of the oxidized enzyme. The spectra of P_M and P_R were extracted from the transient data on the reaction of the mixed-valence and fully reduced enzyme with dioxygen, respectively, using a sequential unidirectional mechanism. The oxidized spectrum of Cu_A was added to the spectrum of P_R in order to match the redox state of Cu_A in P_M.

in pH from pH 6.2 to 8.5 [12,59], in agreement with previous studies [60,61].

Our initial approach toward understanding the mechanism of the reduction of dioxygen to water was to fit the data to the conventional unidirectional sequential scheme (Scheme 1). The testing of the mechanism involves extracting the intermediate spectra and comparing them to model spectra. The latter are linear combinations of spectra of various derivatives of the enzymes, including that of the oxidized, reduced, mixed-valence and fully reduced CO-bound enzyme derivatives, the spectra of P and F [62], and the oxidized spectrum of Cu_A [63].

The early steps in the reaction of the fully reduced enzyme with dioxygen are believed to be analogous to the ones for the mixed-valence enzyme, and it was expected that the two reactions would generate comparable intermediate spectra. Our flow-flash studies on the mixed-valence enzyme [11] produced the four expected intermediates, R*, R, A and the P form (P_M). However, a comparison of the P_M intermediate and the so-called P_R intermediate showed unexpected spectral disagreement discussed below.

The first intermediate in the reaction of fully reduced enzyme with dioxygen, R*, (Scheme 1) represents CO bound to Cu_B following CO photolysis from heme a_3 [64,65]. The 1.5 μs process, which is also observed following photolysis of CO from the fully reduced and mixed-valence enzyme in the absence of O_2 [37,39,66], is attributed to a conformational change at heme a_3 [66,67]. The spectral change associated with this process occurs on the same time scale as the CO dissociation from Cu_B [64]. The extracted spectra of the reduced enzyme (intermediate 2) and oxidized enzyme (intermediate 6) are in good agreement with the model spectra [9,10]. While a model spectrum of compound A is not available, the extracted spectrum of compound A agrees well with spectra of compound A observed at low temperature [68–70]. Moreover, the extracted spectrum of compound A for the fully reduced enzyme (A_R) is identical to that extracted for the analogous intermediate formed during the reaction of the mixed-valence enzyme with dioxygen (A_M) [11].

Fig. 2 (– – –) shows the extracted spectrum of intermediate 4 (Scheme 1), the so-called P_R, in the Soret and visible regions at pH 7.5. The spectrum is referenced against the oxidized enzyme and compared to the spectrum of P_M (—), the P intermediate formed at the binuclear center during the reaction of the mixed-valence enzyme with dioxygen. The binuclear center is the same in P_M and intermediate 4 (P_R), $Fe_{a3}^{4+}{=}O^{2-}Cu_B^{2+}{-}OH^-$, and heme *a* is oxidized in both. However, in P_M the fourth electron required to break the dioxygen bond has been proposed to arise from the cross-linked tyrosine, while heme *a* is the proposed electron donor in P_R [24]. It is clear that there are significant discrepancies between intermediate 4 and P_M in both regions (Fig. 2, [11,12]). While modeling the spectrum of intermediate 4 with a ~ 1:1 mixture of P and F, with heme *a* reduced in P and oxidized in F, resulted in good agreement in the Soret region, differences in bandwidth between intermediate 4 and the P/F mixture were still observed in the visible region [10]. Proshlyakov et al. [24] have reported a resonance Raman vibration due to P_M at 804/768 cm^{-1} ($^{16}O_2/^{18}O_2$), but failed to observe this frequency during the reduction of dioxygen to water by the fully reduced enzyme [20]. This frequency is identical to that observed for the 607 nm form generated upon addition of hydrogen peroxide to the oxidized enzyme under alkaline conditions (P_H) [22–24,71]. We have also recently shown

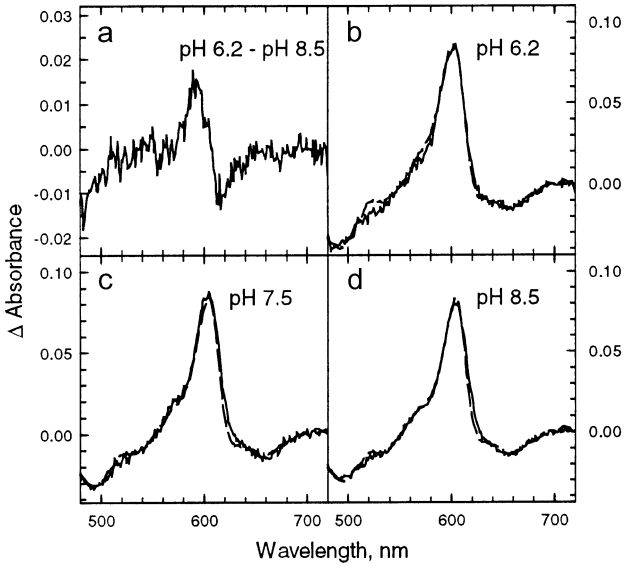


Fig. 3. (a) The difference between the spectrum of the sequential intermediate 4, the putative P_R at pH 6.2 and 8.5. (b–d) The visible experimental spectrum (—) of intermediate 4 (P_R) versus the oxidized enzyme at pH 6.2 (b), 7.5 (c) and 8.5 (d). The spectra were extracted from the time-resolved optical absorption visible data using a unidirectional sequential mechanism (Scheme 1). A linear combination of the spectrum of compound A and the bench-made spectra of P and F were used to model the data (– – –). The model spectrum of P_R contains 45% A + 5% P + 50% F at pH 6.2, 33% A + 34% P + 33% F at pH 7.5, and 30% A + 40% P + 30% F at pH 8.5 [12].

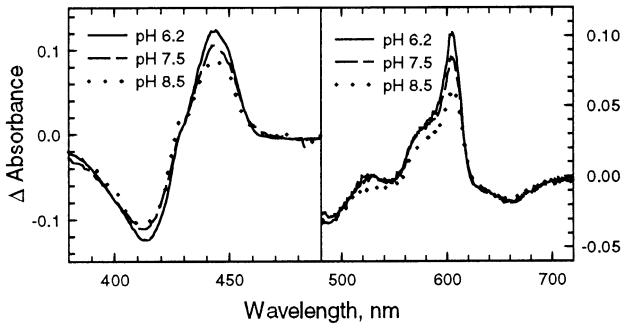


Fig. 4. The Soret and visible spectra of intermediate 5 (F_I/F_{II}) (Scheme 1), referenced against the oxidized enzyme, at three pHs (6.2, 7.5, and 8.5). The spectra were extracted from the time-resolved data using a unidirectional sequential mechanism.

that P_M, P_H and the P form generated upon exposing the oxidized enzyme to a mixture of CO and O_2 (P_{CO/O_2}) all have identical absorption spectra [11].

5. The pH dependence of the catalytic intermediates

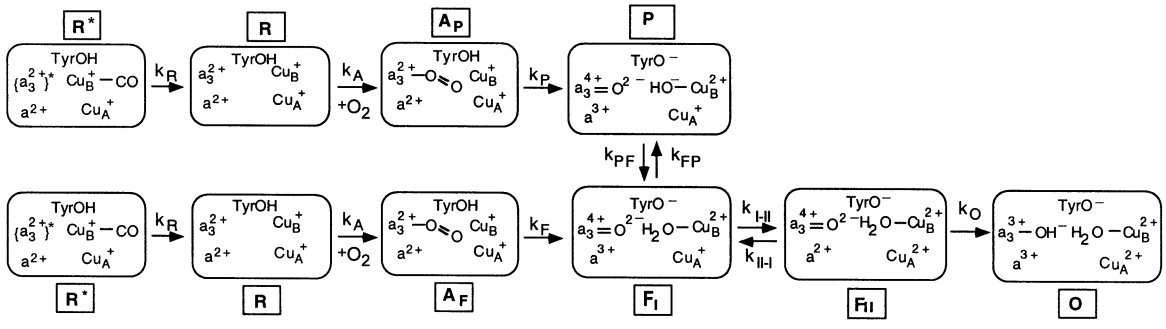
Recent time-resolved optical absorption measurements in our laboratory have shown that the spectrum of the sequential intermediate 4, the so-called P_R, changes as a function of pH [12]. This is demonstrated in Fig. 3a, which shows the difference between the spectrum of intermediate 4 at pH 6.2 and 8.5. The spectrum of intermediate 4 (P_R), was found to be best represented by a pH-dependent mixture of the spectrum of compound A and the bench-made spectra of P (or P_M) and F (Fig. 3b–d, [12]). While the contributions of A, P and F are approximately equal at pH 8.5, the contribution of P is only 5% at pH 6.2. The pH dependence of the P-to-F ratio is in line with previous steady-state measurements, which have shown that the addition of H_2O_2 favors the formation of P at high pH and F at low pH [62,72–77]. Furthermore, the bench-made P form has been shown to convert to the F form upon uptake of a proton with a pH-dependent rate [76].

Intermediate 5, which represents the F form and the equilibrium between heme *a* and Cu_A, is also pH-dependent [12]. It should be noted that F_I and F_{II} both represent the F form with respect to the binuclear site, while the redox states of heme *a* and Cu_A differ. In the unidirectional sequential scheme the two states, F_I and F_{II}, are not resolved, and the equilibrium is shifted toward oxidized Cu_A and reduced heme *a* (F_{II} in Scheme 1) at lower pH (Fig. 4). This is consistent with previous studies of Brzezinski et al., which have indicated that the electron transfer is controlled by proton transfer [26,78,79].

6. Branched mechanism

The significant difference between intermediate 4 (P_R) and P_M (Fig. 2, [11]) and the pH-dependent spectral composition of intermediate 4, which includes compound A, P and F (Fig. 3, [12]), cast doubt on the conventional unidirectional sequential mechanism. In order to account for these observations we have recently analyzed the reaction of the fully reduced cytochrome oxidase with dioxygen using a model consisting of branched pathways, with one branch producing the 607 nm P form and the other the 580 nm F form (Scheme 2) [13]. The P form in the branched scheme has the same structure as the so-called P_R form, but has the spectrum of P_M. The A_P (P-branch) and A_F (F-branch) intermediates are isospectral, but kinetically not equivalent. They decay with lifetimes of 20–40 and 80–100 μs, but which branch is slower cannot be determined at this point [13]. The majority of the molecules (60–85% depending on pH) go through the slow branch [13]. The two pathways are interconnected, with the rate of exchange being pH-dependent [13]. The branched scheme is mathematically underdetermined and testing the scheme involves a novel algebraic approach, which transforms the intermediates of the branched scheme into intermediates comparable to those derived on the basis of a sequential model. The approach is described in detail in our recently published paper [13].

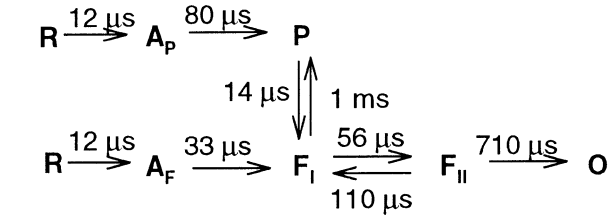
The branched model reproduces the experimental data well. Fig. 5 shows reproduced data at pH 6.2 using the branched scheme and the listed microscopic lifetimes assuming that 75% of the molecules go through the slow branch. Fig. 6(a and b) shows the spectra of intermediates 4 (P_R) and 5 extracted from the experimental flow-flash data at pH 6.2 on the basis of a unidirectional sequential scheme



Scheme 2. Branched mechanism used to analyze the time-resolved flow-flash pH-dependent data.

(—) and the derived equivalent intermediate spectra generated based on the branched scheme (– – –). It is clear (Fig. 5) that the branched model reproduces the sequential spectrum of intermediate 4 much better than the model P_M spectrum (Fig. 2) using only known and spectrally well-defined forms as intermediates. The branched model also resolves the discrepancy between the time-resolved resonance Raman and optical absorption measurements regarding P. The time-profiles based on the branched scheme indicate that very little P is present, particularly at pH 6.2 (Fig. 6c), which explains why Babcock's and Rousseau's groups have been unable to detect the resonance Raman frequency attributed to this form. If the enzyme followed a sequential mechanism, significant amounts of P would be expected and the resonance Raman frequency should be easily detected (Fig. 6d).

The microscopic rate for the P-to-F transition in Scheme 2 is highly pH-dependent, increasing sharply at low pH [13]. Fabian and Palmer [76] have recently shown that P under non-catalytic conditions is converted to F upon proton uptake. The branched model (Scheme 2) kinetically separates the F_I and F_{II} forms, which are unresolved in the conventional sequential model (Scheme 1). The microscopic rate constants for the reversible electron transfer step



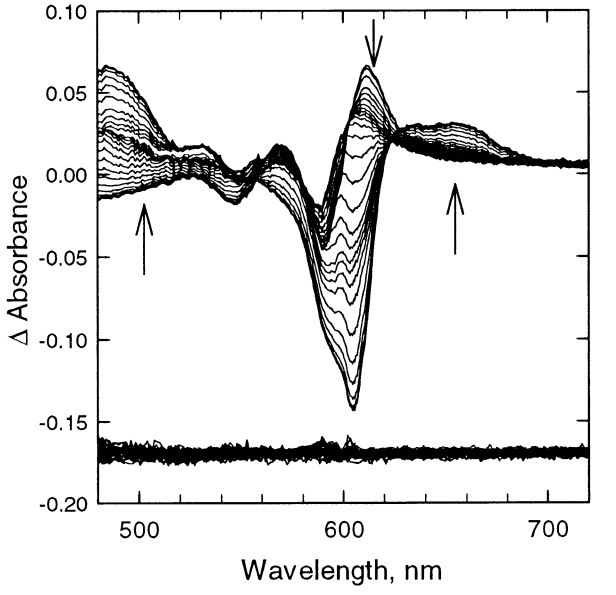


Fig. 5. The reproduced data at pH 6.2 using the listed microscopic lifetimes. The residuals represent the difference between the experimental data and the reproduced data generated using the branched scheme. The reproduced data correspond to 75% of the molecules going through the P-branch.

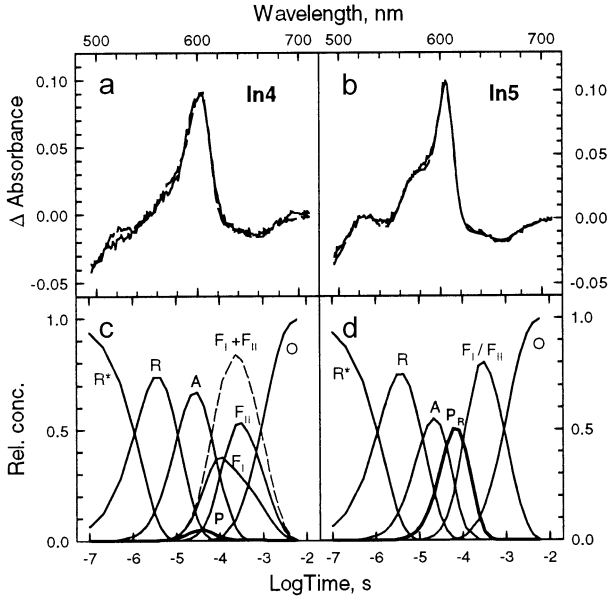


Fig. 6. (a and b). The spectra of intermediate 4 (P_R) and intermediate 5 (F_I/F_{II}) extracted from the experimental data at pH 6.2 on the basis of a unidirectional sequential scheme (—) and the calculated equivalent intermediate spectra (– – –) generated based on the slow-P/fast F branched scheme (the reproduced spectra correspond to 80% of the molecules going through the P-branch). (c) The time-dependent concentration profiles of the intermediates in the branched scheme at pH 6.2. The isospectral intermediates, R*, R, and (A_P and A_F), in the two branches (Scheme 2) are combined. (d) The time-dependent concentration profiles of the intermediates of a unidirectional sequential scheme at pH 6.2.

reproduce the 50 µs apparent electron transfer rate between heme *a* and Cu_A reported previously upon photolysis of the two- and three-electron-reduced CO-bound enzyme [39,41–43].

The two branches may arise from different ligand conformers or different conformations of protonated amino acid(s) in response to ligand state change. Multiple ligand conformers have been observed in the FTIR spectra of CO-bound heme-copper oxidases [80–84], and the binding of CO to Cu_B following CO photolysis has been found to be associated with a change in the environment around glutamic acid 286 (E286, *Rhodobacter sphaeroides* numbering) [85–88]. This change has been interpreted in terms of hydrogen-bonded connectivity between E286 and Cu_B [85] and a conformational isomerization of the side chain of E286 [89–91]. Thus the two different pathways in the branched model may represent E286 in different conformations [85,89–91], with different abilities to participate in protonation or deprotonation events at the binuclear center.

7. Proton-transfer reactions on the surface of bovine heart cytochrome *c* oxidase

We recently investigated the proton-transfer reactions on the surface of bovine heart cytochrome *c* oxidase in collab-

oration with Professor Gutman's laboratory at Tel Aviv University [92]. The approach involved a laser-induced proton-pulse technique in which the protonation state of pyranine in the bulk phase and that of a fluorescein indicator specifically attached to the native Cys(III-115) residue of subunit III of cytochrome oxidase were monitored simultaneously. The kinetic data were analyzed by numerical integration of coupled nonlinear differential rate equations, which provided the rate constants of protonation of all the reacting groups, their pK values and the rates of proton exchange among them. The kinetic analysis showed that the carboxylates on the surface of the protein act as a proton-collecting antenna, which is able to rapidly transfer protons to nearby histidine moieties on the surface. These results are in agreement with previous proton-pulse results on the *R. sphaeroides* cytochrome *c* oxidase [93]. These properties may enable cytochrome oxidase to carry out its redox-linked proton translocation. Molecular modeling of the fluorescein-binding site allowed the calculation of the possible orientations of the fluorescein molecule [92]. Three functional groups were found to shuttle protons between the bulk and the dye, and the structure of the most stable configuration of the fluorescein was found to be consistent with the conclusions of the kinetic analysis. Therefore, the kinetic measurements provide a basis for determining local protein conformations, which may be relevant to the function of the enzyme.

8. What is the role of the cross-linked tyrosine in the reaction mechanism?

The discovery of a tyrosine-histidine cross-link at the active site of cytochrome oxidase prompted Proshlyakov et al. [24] to propose a mechanism in which tyrosine functions as an electron and proton donor to the dioxygen bound at heme a_3, thus facilitating the breaking of the dioxygen bond. Babcock also pointed out potential parallels between the O—O bond cleavage by cytochrome oxidase and the O—O formation during O_2 evolution in photosynthesis, both possibly involving a tyrosyl radical [24]. The P_M intermediate in the mixed-valence cytochrome oxidase was proposed to be $Fe_{a3}^{4+}{=}O^{2-}Cu_B^{2+}{-}OH^-$ with a tyrosyl radical. However, the expected EPR signals from the tyrosyl radical and the oxidized copper have not been observed in the P_M form of the enzyme. Babcock attributed this to a possible spin coupling between the two radicals mediated by the cross-linked histidine [24]. Babcock et al., as well as other groups [94–96], have provided support for the existence of the tyrosyl radical, but direct evidence has been lacking.

If the cross-linked tyrosine plays a role in the breaking of the O—O bond by facilitating proton transfer, one might expect the redox properties and the pK_a of the tyrosine to be changed as a result of the cross-link. McCauley et al. [97] have indeed shown that the oxidation potential of a cross-linked 2-imidazol-1-yl-4-methylphenol is increased by $\sim$66 mV compared to *p*-cresol. The acid dissociation constant of the phenol in the cross-linked compound also decreased by more than 1.5 orders of magnitude compared to *p*-cresol. This observation supports the proposal that the cross-link facilitates proton delivery to the active site during dioxygen reduction.

Another question concerns the possible spin coupling between Cu_B^{2+} and the tyrosyl radical and whether the proposed radical resides on tyrosine or the histidine. Our laboratory and collaborators have recently synthesized and characterized a histidine–phenol cross-linked compound, a chemical analogue of the active site of cytochrome *c* oxidase [98]. In agreement with the results of McCauley et al. [97], the pK_a of the cross-linked tyrosine was found to be lowered (8.34) compared to that of tyrosine (10.1) and *p*-cresol (10.2). Room-temperature time-resolved optical absorption spectra and low-temperature FTIR and EPR spectra recorded following exposure of the complex to UV-light confirmed the presence of a radical residing primarily on the phenoxyl ring, with a small delocalization of spin density onto the imidazole. Efforts to synthesize a His–phenol cross-link incorporated into a Cu-containing ligand system are underway. The detection (or lack of detection) of a UV-generated tyrosyl radical in this ligand system by time-resolved optical absorption, EPR and FTIR spectroscopies should provide important insight into the functional role of the cross-linked histidine–tyrosine cofactor in cytochrome oxidase.

9. The reduction of dioxygen to water studied by photolysis of caged dioxygen carriers

While the flow–flow flash technique has provided an excellent means to study the reduction of dioxygen to water by cytochrome oxidase, in some instances this technique may not represent the physiological reaction under turnover conditions. Time-resolved infrared experiments have shown that CO binds to Cu_B^+ both after photolysis of CO from heme a_3 [64,65] and on its route into the enzyme [66,67]. It has been proposed that oxygen likewise binds to Cu_B^+ prior to binding to heme a_3 [7,99,100]. While CO dissociates from Cu_B^+ on a 1.5 μs time scale in the bovine enzyme [64], in cytochrome bo_3 from *E. coli*, the rate constant has been reported to be much slower or $\sim$500 s^{-1} [101], thus imposing severe limitations on the CO flow-flash kinetic studies.

An alternative to the CO flow-flash method involves the production of dioxygen in situ by photodissociating synthetic caged dioxygen carriers. Our laboratory has synthesized a (μ-peroxo)(μ-hydroxo)bis[bis(bipyridyl)cobalt(III)] complex, which is stable under anaerobic conditions at pH 7, and releases dioxygen on nanosecond time scales upon irradiation with 355 nm light with a quantum yield of 0.04 [102]. We have shown that oxyhemoglobin is formed from deoxyhemoglobin upon photodissociation of this complex

[102] and have recently used this complex to investigate the reduction of dioxygen to water by cytochrome *c* oxidase [103]. The reaction between the reduced enzyme and the photoproduced oxygen was monitored in the visible region using single wavelength detection and a gated diode array spectrometer. Fig. 7 shows the absorbance changes at 604 nm following photolysis of the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2)]^{3+}$ complex alone, the reduced unliganded enzyme alone, and the reduced enzyme in the presence of the complex [103]. It is clear that the photolysis does not induce photochemistry in the enzyme, and after the initial photolysis, the cobalt complex and its photoproduct do not undergo further spectral changes at 604 nm (Fig. 7). Although we were not able to resolve all the apparent lifetimes observed in the CO flow-flash experiment, our results indicated that ~ 90 μM oxygen could be generated upon a single laser pulse at 355 nm [103]. It seems likely that the O_2 quantum yield could be improved by using a wavelength closer to 300 nm, for example 308 nm illumination from an eximer laser. Ludovici et al. [104] recently investigated the photooxidation reaction of cytochrome bo_3 by FTIR after the photochemical release of dioxygen from the nitrate salt of the (μ-peroxo)(μ-hydroxo)bis[bis(bipyridyl)cobalt (III)] complex, and reported a significantly higher quantum yield at 308 nm.

The generation of dioxygen by photolysis of the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2)]^{3+}$ complex circumvents the rate limitation imposed by stopped-flow methods. It also has the potential to provide valuable information regarding the mechanisms of other enzymes in which dioxygen is a substrate.

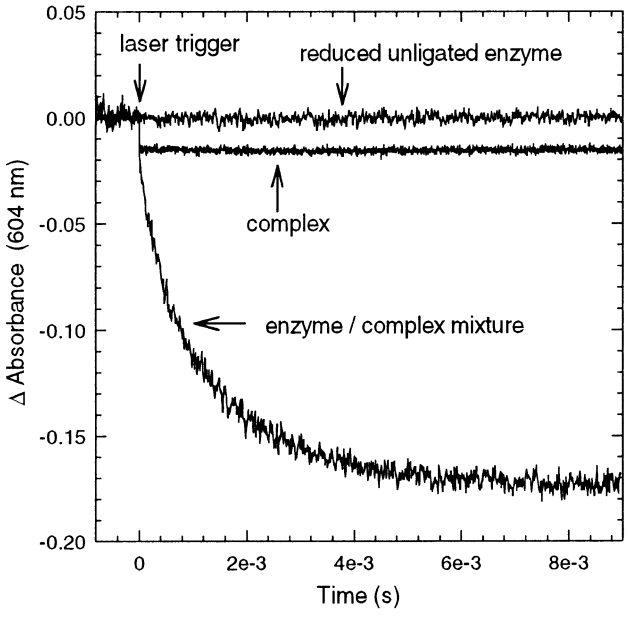


Fig. 7. The time dependence of the absorption changes recorded at 604 nm following photolysis of the $[(\mu\text{-}O_2)(\mu\text{-}OH)(Co(bpy)_2)_2)]^{3+}$ complex alone, the reduced enzyme alone and the mixture of the enzyme and the complex [103].

10. Conclusions and future directions

Considerable progress has been made toward establishing the kinetic mechanism of the reduction of dioxygen to water by cytochrome *c* oxidase, and a lot of the credit for this progress goes to Jerry Babcock and his coworkers. As suggested by Proshlyakov et al. [24], the formation of the P intermediate in the mixed-valence enzyme (P_M) is likely to involve a transient formation of an oxoferryl form and a tyrosyl radical. However, this intermediate may not be observed during the reduction of dioxygen to water by the fully reduced enzyme due to rapid electron transfer from heme *a* to the tyrosine radical to form tyrosinate. Our time-resolved optical absorption spectra, EPR and FTIR spectra, recorded after UV photolysis of a cross-linked His–phenol chemical analog of the active site of cytochrome *c* oxidase, indicate the presence of a radical that resides primarily on the tyrosine. Future spectroscopic studies on the His–phenol cross-link incorporated into a Cu-containing ligand system are expected to give information regarding the structural and functional role of the cross–linked His–Tyr cofactor in heme-copper oxidases.

Our flow-flash studies suggest that the mechanism of the dioxygen reduction by cytochrome *c* oxidase is more complex than the conventional unidirectional sequential pathway [13]. The pH-dependent spectral composition of intermediate 4, including compound A, P and F, is consistent with a branched model, in which P is formed in one branch and F in the other, with a pH-dependent exchange between the two. Further support for the branched scheme comes from preliminary results on the effect of Zn^{2+} on the reduction of dioxygen to water, which cannot be explained in terms of the conventional unidirectional sequential mechanism.

How important is it to know the detailed mechanism in order to understand the function of the enzyme, and does it matter whether the mechanism is sequential or branched? A kinetic mechanism is in fact essential if we are to fully understand the sequence of events on a molecular level. Every time-dependent observation, including transient absorbance, time-resolved resonance Raman, time-resolved IR or step-can FTIR, requires a kinetic scheme in order to assign the spectral changes to a particular molecular event or an intermediate form. It should be emphasized that the results of the global exponential fit, namely the apparent rates and the corresponding spectral changes or the so-called kinetic spectra (b-spectra), do not provide a complete kinetic analysis, but instead are mathematical abstractions that do not have a physical meaning. The effect of pH and temperature on the apparent rates and b-spectra also cannot be correlated to specific molecular events. Only the microscopic rates and intermediate spectra, which are obtained from the apparent values via a kinetic scheme, can be assigned to actual physical events and intermediates that occur during enzyme function. The sequential model, which has been generally accepted because of its simplicity, assigns each apparent rate to a microscopic rate constant that reflects a

single molecular event, while in the branched model an apparent rate can be a combination of two or more microscopic rates. The same applies to the spectral changes. Thus, interpretation of the same experimental results may lead to very different conclusions depending on the model. Unfortunately, there is no model-independent kinetic analysis and the only way a particular model can be considered to be better than a previous model is if it provides molecular interpretations that are more consistent with available experimental results. Further spectroscopic studies are expected to give more insight into the details of the branched model, and our novel algebraic procedure used to test the branched scheme can now be extended to other biological systems that have more complex mechanisms than a sequential unidirectional scheme.

Acknowledgements

This paper is dedicated to the memory of Jerry Babcock with gratitude for all his support throughout the years.

References

[1] Q.H. Gibson, C. Greenwood, Reactions of cytochrome oxidase with oxygen and carbon monoxide, Biochem. J. 86 (1963) 541–554.
[2] C. Greenwood, Q.H. Gibson, The reaction of reduced cytochrome *c* oxidase with oxygen, J. Biol. Chem. 242 (1967) 1782–1787.
[3] B.C. Hill, C. Greenwood, The reaction of fully reduced cytochrome *c* oxidase with oxygen studied by flow-flash spectrophotometry at room temperature, Biochem. J. 218 (1984) 913–921.
[4] Y. Orii, Intermediates in the reaction of reduced cytochrome oxidase with dioxygen, Ann. N.Y. Acad. Sci. 550 (1988) 105–117.
[5] M. Oliveberg, P. Brzezinski, B.G. Malmström, The effect of pH and temperature on the reaction of fully reduced and mixed-valence cytochrome *c* oxidase with dioxygen, Biochim. Biophys. Acta 977 (1989) 322–328.
[6] B.C. Hill, Modeling the sequence of electron transfer reactions in the single turnover of reduced, mammalian cytochrome *c* oxidase with oxygen, J. Biol. Chem. 269 (1994) 2419–2425.
[7] M.I. Verkhovsky, J.E. Morgan, M. Wikström, Oxygen binding and activation: early steps in the reaction of oxygen with cytochrome *c* oxidase, Biochemistry 33 (1994) 3079–3086.
[8] J.E. Morgan, M.I. Verkhovsky, M. Wikström, Observation and assignment of peroxy and ferryl intermediates in the reduction of dioxygen to water by cytochrome *c* oxidase, Biochemistry 35 (1996) 12235–12240.
[9] A. Sucheta, K.E. Georgiadis, Ó. Einarsdóttir, Mechanism of cytochrome *c* oxidase-catalyzed reduction of dioxygen to water. Evidence for peroxy and ferryl intermediates at room temperature, Biochemistry 36 (1997) 554–565.
[10] A. Sucheta, I. Szundi, Ó. Einarsdóttir, Intermediates in the reaction of fully reduced cytochrome *c* oxidase with dioxygen, Biochemistry 37 (1998) 17905–17914.
[11] Ó. Einarsdóttir, I. Szundi, N. Van Eps, A.S. Sucheta, P_M and P_R forms of cytochrome *c* oxidase have different spectral properties, J. Inorg. Biochem. 91 (2002) 87–93.
[12] N. Van Eps, I. Szundi, Ó. Einarsdóttir, pH dependence of the reduction of dioxygen to water by cytochrome *c* oxidase: 1. The P_R state is a pH-dependent mixture of three intermediates, A, P, and F, Biochemistry 42 (2003) 5065–5073.
[13] I. Szundi, N. Van Eps, Ó. Einarsdóttir, pH dependence of the reduction of dioxygen to water by cytochrome *c* oxidase: 2. Branched electron transfer pathways linked by proton transfer, Biochemistry 42 (2003) 5074–5090.
[14] C. Varotsis, W.H. Woodruff, G.T. Babcock, Direct detection of a dioxygen adduct of cytochrome a_3 in the mixed-valence cytochrome oxidase/dioxygen reaction, J. Biol. Chem. 265 (1990) 11131–11136.
[15] T. Ogura, S. Takahashi, K. Shinzawa-Itoh, S. Yoshikawa, T. Kitagawa, Observation of the $Fe^{II}-O_2$ stretching Raman band for cytochrome oxidase compound A at ambient temperature, J. Am. Chem. Soc. 112 (1990) 5630–5631.
[16] S. Han, Y.-C. Ching, D.L. Rousseau, Primary intermediate in the reaction of oxygen with fully reduced cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 2491–2495.
[17] C. Varotsis, G.T. Babcock, Appearance of the $\upsilon(Fe^{IV}=O)$ vibration from a ferryl-oxo intermediate in the cytochrome oxidase/dioxygen reaction, Biochemistry 29 (1990) 7357–7362.
[18] S. Han, Y.-C. Ching, D.L. Rousseau, Ferryl and hydroxy intermediates in the reaction of oxygen with reduced cytochrome *c* oxidase, Nature 348 (1990) 89–90.
[19] T. Ogura, S. Takahashi, K. Shinzawa-Itoh, S. Yoshikawa, T. Kitagawa, Observation of the $Fe^{4+}=O$ stretching Raman band for cytochrome oxidase compound B at ambient temperature, J. Biol. Chem. 265 (1990) 14721–14723.
[20] C. Varotsis, Y. Zhang, E.H. Appelman, G.T. Babcock, Resolution of the reaction sequence during the reduction of O_2 by cytochrome oxidase, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 237–241.
[21] T. Ogura, S. Takahashi, S. Hirota, K. Shinzawa-Itoh, S. Yoshikawa, E.H. Appelman, T. Kitagawa, Time-resolved resonance Raman elucidation of the pathway for dioxygen reduction by cytochrome *c* oxidase, J. Am. Chem. Soc. 115 (1993) 8527–8536.
[22] D.A. Proshlyakov, T. Ogura, K. Shinzawa-Itoh, S. Yoshikawa, E.H. Appelman, T. Kitagawa, Selective resonance Raman observation of the "607 nm" form generated in the reaction of oxidized cytochrome *c* oxidase with hydrogen peroxide, J. Biol. Chem. 269 (1994) 29385–29388.
[23] D.A. Proshlyakov, T. Ogura, K. Shinzawa-Itoh, S. Yoshikawa, T. Kitagawa, Microcirculating system for simultaneous determination of Raman and absorption spectra of enzymatic reaction intermediates and its application to the reaction of cytochrome *c* oxidase with hydrogen peroxide, Biochemistry 35 (1996) 76–82.
[24] D.A. Proshlyakov, M.A. Pressler, G.T. Babcock, Dioxygen activation and bond cleavage by mixed-valence cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 8020–8025.
[25] A.A. Konstantinov, S. Siletsky, D. Mitchell, A. Kaulen, R.B. Gennis, The roles of the two proton input channels in cytochrome *c* oxidase from *Rhodobacter sphaeroides* probed by the effects of site-directed mutations on time-resolved electrogenic intraprotein proton transfer, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 9085–9090.
[26] P. Ädelroth, M.S. Ek, D.M. Mitchell, R.B. Gennis, P. Brzezinski, Glutamate 286 in cytochrome aa_3 from *Rhodobacter sphaeroides* is involved in proton uptake during the reaction of the fully-reduced enzyme with dioxygen, Biochemistry 36 (1997) 13824–13829.
[27] P. Brzezinski, P. Ädelroth, Pathways of proton transfer in cytochrome *c* oxidase, J. Bioenerg. Biomembranes 30 (1998) 99–107.
[28] T.V. Vygodina, C. Pecoraro, D. Mitchell, R. Gennis, A.A. Konstantinov, Mechanism of inhibition of electron transfer by amino acid replacement K362M in a proton channel of *Rhodobacter sphaeroides* cytochrome *c* oxidase, Biochemistry 37 (1998) 3053–3061.
[29] D. Zaslavsky, R.B. Gennis, Substitution of lysine-362 in a putative proton-conducting channel in the cytochrome *c* oxidase from *Rhodobacter sphaeroides* blocks turnover with O_2 but not with H_2O_2, Biochemistry 37 (1998) 3062–3067.
[30] M. Bränden, H. Sigurdson, A. Namslauer, R.B. Gennis, P. Ädelroth, P. Brzezinski, On the role of the K-proton transfer pathway: cyto-

chrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 5013–5018.
[31] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*, Nature 376 (1995) 660–669.
[32] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å, Science 272 (1996) 1136–1144.
[33] C. Ostermeier, A. Harrenga, U. Ermler, H. Michel, Structure at 2.7 Å resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody FV fragment, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10547–10553.
[34] S. Han, S. Takahashi, D.L. Rousseau, Time dependence of the catalytic intermediates in cytochrome *c* oxidase, J. Biol. Chem. 275 (2000) 1910–1919.
[35] R. Boelens, R. Wever, Electron-transfer processes in carboxy-cytochrome *c* oxidase after photodissociation of cytochrome a_3^{2+}-CO, Biochim. Biophys. Acta 547 (1979) 296–310.
[36] R. Boelens, R. Wever, B.F. Van Gelder, Electron transfer after flash photolysis of mixed-valence carboxy cytochrome *c* oxidase, Biochim. Biophys. Acta 682 (1982) 264–272.
[37] K.E. Georgiadis, N.-I. Jhon, Ó. Einarsdóttir, Time-resolved optical absorption studies of intramolecular electron transfer in cytochrome *c* oxidase, Biochemistry 33 (1994) 9245–9256.
[38] Ó. Einarsdóttir, T.D. Dawes, K.E. Georgiadis, New transients in the electron-transfer dynamics of photolyzed mixed-valence CO-cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 6934–6937.
[39] Ó. Einarsdóttir, K.E. Georgiadis, A. Sucheta, Intramolecular electron transfer and conformational changes in cytochrome *c* oxidase, Biochemistry 34 (1995) 496–508.
[40] Ó. Einarsdóttir, K.E. Georgiadis, T.D. Dawes, Evidence for a band III analogue in the near-infrared absorption spectra of cytochrome *c* oxidase, Biochem. Biophys. Res. Commun. 184 (1992) 1035–1041.
[41] J.E. Morgan, P.M. Li, D.-J. Jang, M.A. El-Sayed, S.I. Chan, Electron transfer between cytochrome *a* and copper A in cytochrome *c* oxidase: a perturbed equilibrium study, Biochemistry 28 (1989) 6975–6983.
[42] M. Oliveberg, B.G. Malmström, Internal electron transfer in cytochrome *c* oxidase: evidence for a rapid equilibrium between cytochrome *a* and the bimetallic site, Biochemistry 30 (1991) 7053–7057.
[43] M.I. Verkhovsky, J.E. Morgan, M. Wikström, Intramolecular electron transfer in cytochrome *c* oxidase: a cascade of equilibria, Biochemistry 31 (1992) 11860–11863.
[44] K. Kobayashi, H. Une, K. Hayashi, Electron transfer in cytochrome oxidase after pulse radiolysis, J. Biol. Chem. 264 (1989) 7976–7980.
[45] O. Farver, Ó. Einarsdóttir, I. Pecht, Electron transfer rates and equilibrium within cytochrome *c* oxidase, Eur. J. Biochem. 267 (1999) 950–954.
[46] B.C. Hill, The reaction of the electrostatic cytochrome *c*-cytochrome oxidase complex with oxygen, J. Biol. Chem. 266 (1991) 2219–2226.
[47] T. Nilsson, Photoinduced electron transfer from tris(2,2′-bipyridyl)-ruthenium to cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 6497–6501.
[48] L.P. Pan, S. Hibdon, R.-Q. Liu, B. Durham, F. Millet, Intracomplex electron transfer between ruthenium-cytochrome *c* derivatives and cytochrome *c* oxidase, Biochemistry 32 (1993) 8492–8498.
[49] L.M. Geren, J.R. Beasley, B.R. Fine, A.J. Saunders, S. Hibdon, G.J. Pielak, B. Durham, F. Millet, Design of a ruthenium-cytochrome *c* derivative to measure electron transfer to the initial acceptor in cytochrome oxidase, J. Biol. Chem. 270 (1995) 2466–2472.
[50] P. Brzezinski, M.T. Wilson, Photochemical electron injection into redox-active proteins, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 6176–6179.
[51] A.B. Kotlyar, M. Hazani, N. Borovok, I. Szundi, Ó. Einarsdóttir, Photoinduced electron transfer between cytochrome *c* oxidase and TUPS-modified cytochrome *c*, Eur. J. Biochem. 267 (2000) 5805–5809.
[52] I. Szundi, J. Cappuccio, N. Borovok, A.B. Kotlar, Ó. Einarsdóttir, Photoinduced electron transfer in the cytochrome *c*/cytochrome *c* oxidase complex using thiouredopyrenetrisulfonate-labeled cytochrome *c*. Optical multichannel detection, Biochemistry 40 (2001) 2186–2193.
[53] I. Szundi, G.-L. Liao, Ó. Einarsdóttir, Near-infrared time-resolved optical absorption studies of the reaction of fully reduced cytochrome *c* oxidase with dioxygen, Biochemistry 40 (2000) 2332–2339.
[54] O. Farver, Y. Lu, M.C. Ang, I. Pecht, Enhanced rate of intramolecular electron transfer in an engineered purple Cu_A azurin, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 899–902.
[55] G.T. Babcock, M. Wikström, Oxygen activation and the conservation of energy in cell respiration, Nature 356 (1992) 301–309.
[56] S. Ferguson-Miller, G.T. Babcock, Heme/copper terminal oxidases, Chem. Rev. 96 (1996) 2889–2907.
[57] D. Zaslavsky, R.B. Gennis, Proton pumping by cytochrome oxidase: progress, problems and postulates, Biochim. Biophys. Acta 1458 (2000) 164–179.
[58] J.E. Morgan, M.I. Verkhovsky, G. Palmer, M. Wikström, Role of the P_R intermediate in the reaction of cytochrome *c* oxidase with O_2, Biochemistry 40 (2001) 6882–6892.
[59] S. Paula, A. Sucheta, I. Szundi, Ó. Einarsdóttir, Proton and electron transfer during the reduction of molecular oxygen by fully reduced cytochrome *c* oxidase: a flow-flash investigation using optical multichannel detection, Biochemistry 38 (1999) 3025–3033.
[60] M. Oliveberg, S. Hallén, T. Nilsson, Uptake and release of protons during the reaction between cytochrome *c* oxidase and molecular oxygen: a flow-flash investigation, Biochemistry 30 (1991) 436–440.
[61] S. Hallén, T. Nilsson, Proton transfer during the reaction between fully reduced cytochrome *c* oxidase and dioxygen: pH and deuterium isotope effects, Biochemistry 31 (1992) 11853–11859.
[62] M. Fabian, G. Palmer, The interaction of cytochrome oxidase with hydrogen peroxide: the relationship of compounds P and F, Biochemistry 34 (1995) 13802–13810.
[63] C.E. Slutter, D. Sanders, P. Wittung, B.G. Malmström, R. Aasa, J.H. Richards, H.B. Gray, J.A. Fee, Water-soluble recombinant Cu_A-domain of the cytochrome ba_3 from subunit II from *Thermus thermophilus*, Biochemistry 35 (1996) 3387–3395.
[64] R.B. Dyer, Ó. Einarsdóttir, P.M. Killough, J.J. López-Garriga, W.H. Woodruff, Transient binding of photodissociated CO to Cu_B^+ of eukaryotic cytochrome oxidase at ambient temperature. Direct evidence from time-resolved infrared spectroscopy, J. Am. Chem. Soc. 111 (1989) 7657–7659.
[65] R.B. Dyer, K.A. Petersen, P.O. Stoutland, W.H. Woodruff, Ultrafast photoinduced ligand transfer in carbonmonoxy cytochrome *c* oxidase. Observation by picosecond infrared spectroscopy, J. Am. Chem. Soc. 113 (1991) 6276–6277.
[66] Ó. Einarsdóttir, R.B. Dyer, D.D. Lemon, P.M. Killough, S.M. Hubig, S.J. Atherton, J.J. López-Garriga, G. Palmer, W.H. Woodruff, Photodissociation and recombination of carbonmonoxy cytochrome oxidase: dynamics from picoseconds to kiloseconds, Biochemistry 32 (1993) 12013–12024.
[67] W.H. Woodruff, Ó. Einarsdóttir, R.B. Dyer, K.A. Bagley, G. Palmer, S.J. Atherton, R.A. Goldbeck, T.D. Dawes, D.S. Kliger, Nature and functional implications of the cytochrome a_3 transients after photodissociation of CO-cytochrome oxidase, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 2588–2592.
[68] B. Chance, C. Saronio, J.S. Leigh Jr., Functional intermediates in the reaction of membrane-bound cytochrome oxidase with oxygen, J. Biol. Chem. 250 (1975) 9226–9237.
[69] M.G. Clore, L.-E. Andréasson, B.G. Karlsson, R. Aasa, B. Malm-

ström, Characterization of the low-temperature intermediates of the reaction of fully reduced soluble cytochrome oxidase with oxygen by electron-paramagnetic-resonance and optical spectroscopy, Biochem. J. 185 (1980) 139–154.
[70] M.G. Clore, L.-E. Andréasson, B.G. Karlsson, R. Aasa, B. Malmström, Characterization of intermediates in the reaction of mixed-valence-state soluble cytochrome oxidase with oxygen at low temperature by optical and electron-paramagnetic-resonance spectroscopy, Biochem. J. 185 (1980) 155–167.
[71] D.A. Proshlyakov, T. Ogura, K. Shinzawa-Itoh, S. Yoshikawa, T. Kitagawa, Resonance Raman/absorption characterization of the oxo intermediates of cytochrome *c* oxidase generated in its reaction with hydrogen peroxide: pH and H_2O_2 concentration dependence, Biochemistry 96 (1996) 8580–8586.
[72] J.M. Wrigglesworth, Formation and reduction of a 'peroxy' intermediate of cytochrome *c* oxidase by hydrogen peroxide, Biochem. J. 217 (1984) 715–719.
[73] L. Weng, G.M. Baker, Reaction of hydrogen peroxide with the rapid form of resting cytochrome oxidase, Biochemistry 30 (1991) 5727–5733.
[74] T. Brittain, R.H. Little, C. Greenwood, N.J. Watmough, The reaction of *Escherichia coli* cytochrome *bo* with H_2O_2: evidence for the formation of an oxyferryl species by two distinct routes, FEBS Lett. 399 (1996) 21–25.
[75] S. Jünemann, P. Heathcote, P.R. Rich, The reactions of hydrogen peroxide with bovine cytochrome *c* oxidase, Biochim. Biophys. Acta 1456 (2000) 56–66.
[76] M. Fabian, G. Palmer, Proton involvement in the transition from the "peroxy" to the ferryl intermediate of cytochrome *c* oxidase, Biochemistry 40 (2001) 1867–1874.
[77] C. Pecoraro, R.B. Gennis, T.V. Vygodina, A.A. Konstantinov, Role of the K-channel in the pH-dependence of the reaction of cytochrome *c* oxidase with hydrogen peroxide, Biochemistry 40 (2001) 9695–9708.
[78] M. Karpefors, P. Ädelroth, Y. Zhen, S. Ferguson-Miller, P. Brzezinski, Proton uptake controls electron transfer in cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 13606–13611.
[79] P. Ädelroth, M. Karpefors, G. Gilderson, F.L. Tomson, R.B. Gennis, P. Brzezinski, Proton transfer from glutamate 286 determines the transition rates between oxygen intermediates in cytochrome oxidase, Biochim. Biophys. Acta 1459 (2000) 533–539.
[80] J.O. Alben, P.P. Moh, F.G. Fiamingo, R.A. Altschuld, Cytochrome oxidase (a_3) heme and copper observed by low-temperature Fourier transform infrared spectroscopy of the CO complex, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 234–237.
[81] Ó. Einarsdóttir, M.G. Choc, S. Weldon, W.S. Caughey, The site and mechanism of dioxygen reduction in bovine heart cytochrome *c* oxidase, J. Biol. Chem. 263 (1988) 13641–13654.
[82] Ó. Einarsdóttir, P.M. Killough, J.A. Fee, W.H. Woodruff, An infrared study of the binding and photodissociation of carbon monoxide in cytochrome ba_3 from *Thermus thermophilus*, J. Biol. Chem. 264 (1989) 2405–2408.
[83] J. Wang, S. Takahashi, J.P. Hosler, D.M. Mitchell, S. Ferguson-Miller, R.B. Gennis, D.L. Rousseau, Two conformations of the catalytic site in the aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*, Biochemistry 34 (1995) 9819–9825.
[84] D.M. Mitchell, J.D. Müller, R.B. Gennis, G.U. Nienhaus, FTIR study of conformational substates in the CO adduct of cytochrome *c* oxidase from *Rhodobacter sphaeroides*, Biochemistry 35 (1996) 16782–16788.
[85] A. Puustinen, J.A. Bailey, R.B. Dyer, S.L. Mecklenburg, M. Wikström, W.H. Woodruff, Fourier transform infrared evidence for connectivity between Cu_B and glutamic acid 286 in cytochrome bo_3 from *Esherichia coli*, Biochemistry 36 (1997) 13195–13200.
[86] P.R. Rich, J. Breton, FTIR studies of the CO and cyanide adducts of fully reduced bovine cytochrome *c* oxidase, Biochemistry 40 (2001) 6441–6449.
[87] D. Heitbrink, H. Sigurdson, C. Bolwien, P. Brzezinski, J. Heberle, Transient binding of CO to Cu_B in cytochrome *c* oxidase is dynamically linked to structural changes around a carboxyl group: a time-resolved step-scan Fourier transform infrared investigation, Biophys. J. 82 (2002) 1–10.
[88] J.A. Bailey, F.L. Tomson, S.L. Mecklenburg, G.M. MacDonald, A. Katsonouri, A. Puustinen, R.B. Gennis, W.H. Woodruff, R.B. Dyer, Time-resolved step-scan Fourier transform infrared spectroscopy of the CO adducts of bovine cytochrome *c* oxidase and of cytochrome bo_3 from *Eschericia coli*, Biochemistry 41 (2002) 2675–2683.
[89] I. Hofacker, K. Schulten, Oxygen and proton pathways in cytochrome *c* oxidase, Proteins: Struct., Funct., Genet. 30 (1998) 100–107.
[90] R. Pomès, G. Hummer, M. Wikström, Structure and dynamics of a proton shuttle in cytochrome *c* oxidase, Biochim. Biophys. Acta 1365 (1998) 255–260.
[91] M. Swensson-Ek, J. Abramson, G. Larsson, S. Törnroth, P. Brzezinski, S. Iwata, The X-ray crystal structures of wild-type and EQ(I-286) mutant cytochrome *c* oxidases from *Rhodobacter sphaeroides*, J. Mol. Biol. 321 (2002) 329–339.
[92] Y. Marantz, Ó. Einarsdóttir, E. Nachliel, M. Gutman, Proton-collecting properties of bovine heart cytochrome *c* oxidase: kinetic and electrostatic analysis, Biochemistry 40 (2001) 15086–15097.
[93] Y. Marantz, E. Nachliel, A. Aagaard, P. Brzezinski, M. Gutman, The proton collecting function of the inner surface of cytochrome *c* oxidase from *Rhodobacter sphaeroides*, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 8590–8595.
[94] Y.R. Chen, B.E. Sturgeon, M.R. Gunther, R.P. Mason, Electron spin resonance investigation of the cyanyl and azidyl radical formation by cytochrome *c* oxidase, J. Biol. Chem. 274 (1999) 3308–3314.
[95] F. MacMillan, A. Kannt, J. Behr, T. Prisner, H. Michel, Direct evidence for a tyrosine radical in the reaction of cytochrome *c* oxidase with hydrogen peroxide, Biochemistry 38 (1999) 9179–9184.
[96] D.A. Proshlyakov, M.A. Pressler, C. DeMaso, J.F. Leykam, D.L. DeWitt, G.T. Babcock, Oxygen activation and reduction in respiration: involvement of redox-active tyrosine 244, Science 290 (2000) 1588–1591.
[97] K.M. McCauley, J. Vrtis, J. Dupont, W.A. Van der Donk, Insights into the functional role of the tyrosine-histidine linkage in cytochrome *c* oxidase, J. Am. Chem. Soc. 122 (2000) 2403–2404.
[98] J.A. Cappuccio, I. Ayala, G. Elliott, I. Szundi, J. Lewis, J.P. Konopelski, B.A. Barry, Ó. Einarsdóttir, Modeling the active site of cytochrome oxidase: synthesis and characterization of a cross-linked histidine–phenol, J. Am. Chem. Soc. 124 (2002) 1750–1760.
[99] R.S. Blackmore, C. Greenwood, Q.H. Gibson, Studies of the primary oxygen intermediate in the reaction of fully reduced cytochrome oxidase, J. Biol. Chem. 266 (1991) 19245–19249.
[100] J.A. Bailey, C.A. James, W.H. Woodruff, Flow-flash kinetics of O_2 binding to cytochrome *c* oxidase at elevated [O_2]: observations using high-pressure stopped-flow for gaseous reactants, Biochem. Biophys. Res. Commun. 220 (1996) 1055–1060.
[101] W.H. Woodruff, R.B. Dyer, Ó. Einarsdóttir, in: R.J.H. Clark, R.E. Hester (Eds.), Biological Spectroscopy, Part B. Wiley, Chichester, England, 1993, pp. 189–233.
[102] R. MacArthur, A. Sucheta, F.S. Chong, Ó. Einarsdóttir, Photodissociation of a (μ-peroxo)(μ-hydroxo)bis[bis(bipyridyl)cobalt(III)] complex: a tool to study fast biological reactions involving O_2, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 8105–8109.
[103] N. Van Eps, I. Szundi, Ó. Einarsdóttir, A new approach to study fast biological reactions involving dioxygen: the reaction of fully reduced cytochrome *c* oxidase with O_2, Biochemistry 39 (2000) 14576–14582.
[104] C. Ludovici, R. Fröhlich, K. Vogtt, B. Mamat, M. Lübben, Caged O_2. Reaction of cytochrome bo_3 oxidase with photochemically released dioxygen from a cobalt peroxo complex, Eur. J. Biochem. 269 (2002) 2630–2637.