Heme–heme and heme–ligand interactions in the di-heme oxygen-reducing site of cytochrome \textit{bd} from \textit{Escherichia coli} revealed by nanosecond absorption spectroscopy

Fabrice Rappaport \textsuperscript{a}, Jie Zhang \textsuperscript{b}, Marten H. Vos \textsuperscript{c,d}, Robert B. Gennis \textsuperscript{b}, Vitaliy B. Borisov \textsuperscript{e,*}

\textsuperscript{a} Institut de Biologie Physico-Chimique, Unité Mixte de Recherche 7141 CNRS, Université Paris 6, 13 Rue Pierre et Marie Curie, 75005 Paris, France
\textsuperscript{b} Department of Biochemistry, University of Illinois, 600 South Mathews Street, Urbana, IL 61801, USA
\textsuperscript{c} Laboratoire d’Optique et Biosciences, CNRS, Ecole Polytechnique, France
\textsuperscript{d} INSERM U696, F-91128 Palaiseau, France
\textsuperscript{e} Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Leninskie Gory, Moscow 119991, Russian Federation

\section*{Abstract}

Cytochrome \textit{bd} is a terminal quinol:O\textsubscript{2} oxidoreductase of respiratory chains of many bacteria. It contains three hemes, \textit{b}_{\textit{558}}, \textit{b}_{\textit{595}}, and \textit{d}. The role of heme \textit{b}_{\textit{558}} remains obscure. A CO photolysis/recombination study of the membranes of \textit{Escherichia coli} containing either wild type cytochrome \textit{bd} or inactive E445A mutant was performed using nanosecond absorption spectroscopy. We compared photodissociated changes of heme \textit{d}–CO complex in one-electron-reduced, two-electron-reduced, and fully reduced states of cytochromes \textit{bd}. The line shape of spectra of photodissociation of one-electron-reduced and two-electron-reduced enzymes is strikingly different from that of the fully reduced enzyme. The difference demonstrates that in the fully reduced enzyme photolysis of CO from heme \textit{d} perturbs ferrous heme \textit{b}_{\textit{595}} causing loss of an absorption band centered at 435 nm, thus supporting interactions between heme \textit{b}_{\textit{595}} and heme \textit{d} in the di-heme oxygen-reducing site, in agreement with previous works. Photolyzed CO recombines with the fully reduced enzyme monoelectronically with \(\tau\sim 12\) μs, whereas recombination of CO with one-electron-reduced cytochrome \textit{bd} shows three kinetic phases, with \(\tau\sim 14\) ns, 14 μs, and 280 μs. The spectra of the absorption changes associated with these components are different in line shape. The 14 ns phase, absent in the fully reduced enzyme, reflects geminate recombination of CO with part of heme \textit{d}. The 14-μs component reflects bimolecular recombination of CO with heme \textit{d} and electron backflow from heme \textit{d} to hemes \textit{b} in ~4% of the enzyme population. The final, 280-μs component, reflects return of the electron from hemes \textit{b} to heme \textit{d} and bimolecular recombination of CO in that population. The fact that even in the two-electron-reduced enzyme, a nanosecond geminate recombination is observed, suggests that namely the redox state of heme \textit{b}_{\textit{595}}, and not that of heme \textit{b}_{\textit{558}}, controls the pathway(s) by which CO migrates between heme \textit{d} and the medium.

© 2010 Elsevier B.V. All rights reserved.

\section*{1. Introduction}

Cytochrome \textit{bd} is a terminal oxidase of aerobic respiratory chains of many bacteria [1–3]. It catalyzes electron transfer from quinol to molecular oxygen (to produce water) [4,5] and couples this exergonic reaction to the generation of a membrane potential [6–10].

Apart from energy conservation, cytochrome \textit{bd} endows bacteria with a number of specific physiological functions. Cytochrome \textit{bd} facilitates both pathogenic and commensal bacteria to colonize oxygen-poor environments [11–14], serves as an oxygen scavenger and inhibits degradation of O\textsubscript{2}-sensitive enzymes [15], increases virulence and survival in host mammalian cells [16,17] of pathogens, enhances bacterial tolerance to nitrosative stress [18–23], supports disulfide bond formation upon protein folding [24], and may contribute to mechanisms of detoxification of hydrogen peroxide in the bacterial cell [25].

Cytochrome \textit{bd} is not a member of the well-known family of heme–copper oxidases. Neither of its two subunits (CydA and CydB) shows sequence homology to any subunit of heme–copper family members [26,27]. In contrast to heme–copper oxidases, cytochrome \textit{bd} is not a proton pump and does not contain copper in the active site [5,28]. It contains only hemes as redox cofactors, which are heme \textit{b}_{\textit{558}}, heme \textit{b}_{\textit{595}}, and heme \textit{d}, with stoichiometry of 1:1:1 per enzyme molecule.

The roles of the three hemes in cytochrome \textit{bd} are different. The low-spin hexacoordinate heme \textit{b}_{\textit{558}} is the electron entry site; it directly accepts electrons from quinol [29,30]. The high-spin and likely pentacoordinate
heme d is the site where binding, activation, and further reduction of O₂ by four electrons to H₂O occurs. This chlorin cofactor is likely responsible for the remarkably high affinity of the enzyme for oxygen leading to formation of a stable oxygenated complex [31,32]. The high-spin pentacoordinate heme b₉₉₉₅ apparently accepts electrons from heme b₅₅₈ to deliver them to heme d [33,34], but the issue as to whether this is its only role remains unanswered. A number of observations indicate that heme b₉₉₉₅ and heme d can form a common di-heme site for the oxygen reduction [8,9,35–43]. Nevertheless, no significant redox interactions between hemes d and b₉₉₉₅ can be observed [44]. It has also been proposed that heme b₉₉₉₅ may serve as a second oxygen-binding site [45,46].

It was shown that the interaction of heme d with ligands differs in the fully reduced (R) enzyme (all the three hemes are reduced) and the one-electron-reduced “mixed-valence” (MV¹) enzyme (heme d is reduced, heme b₅₅₈ and heme b₉₉₉₅ are oxidized). In particular, it was found that:

(i) In the MV¹ CO-bound isolated WT cytochrome bd from Escherichia coli, upon photodissociation of CO from heme d, a significant part of photodissociated CO (~50-70%) does not leave the protein but recombines with heme d within a few hundred ps. In contrast, for the enzyme in the R state under the same conditions, no such heme d-CO germinade recombination is observed [39,41]. In addition, this ultrafast spectroscopy study also showed that the spectra of CO dissociation from the R and MV¹ forms of the WT isolated cytochrome bd on a picosecond time scale are different in line shape, pointing to the interaction between the close-lying hemes d and b₉₉₉₅ [39,41]. The possible presence of later processes before bimolecular CO recombination has not been investigated so far.

(ii) The apparent rate constants for thermal (spontaneous) dissociation of NO and CO from the protein are much higher for the R cytochrome bd from E. coli than in case of the enzyme in the MV¹ state [19].

(iii) In the reaction of the R cytochrome bd from Azotobacter vinelandii with oxygen, the rate of O₂ binding depends linearly on the oxygen concentration up to the air level. On the contrary, when the enzyme is in the MV¹ state, the rate of O₂ binding is hyperbolic, thus revealing a saturation behavior. It was proposed that in case of the MV¹ cytochrome bd, the enzyme in equilibrium exists in the two different conformations, but only one of which can bind oxygen. When in the “closed” conformation, cytochrome bd provides no access for O₂ to heme d⁵⁺, whereas in the “open” conformation, oxygen binds easily. The R enzyme is always in the open conformation [32]. Thus, the redox state of one or both of hemes b modulates ligand binding properties to heme d.

In the present work, we performed a systematic nanosecond study of the E. coli membranes containing cytochrome bd by varying the number of electrons in the bd oxidase. We used both the WT cytochrome bd and the E445A mutant of subunit I (CydA) that is catalytically inactive [8] and cannot be completely reduced even with excess dithionate [8]. This unique property of the mutant allowed us to generate not only the R and MV¹ redox states but also the two-electron-reduced (MV²) state of cytochrome bd which is impossible to generate in the WT and has remained uncharacterized in the previous transient absorption spectroscopy studies. Here we were able to compare in detail the photoinduced absorption changes in various redox states of the enzyme on time scales that were not investigated previously and obtain new information about the heme–heme and heme–CO interactions.

2. Materials and methods

2.1. Chemicals

Carbon monoxide was from Air Liquide; sodium dithionite was from Merck. Other basic chemicals and biochemicals were from Sigma-Aldrich, Merck, and Fluka.

2.2. Strains and plasmids

E. coli strain GO105 (cyd AB:kan, cyo, recA) devoid of cytochrome bo₃ and cytochrome bd quinol oxidases [48] was used as the host strain for expressing both the wild type and E445A mutant cytochrome bd from a plasmid. In both cases, plasmid pTK1 containing the whole operon encoding cytochrome bd and the ampicillin resistance gene was introduced into the strain [47].

2.3. Cell growth and membrane preparation

The WT cells of E. coli were grown aerobically as reported in [39]; the E445A mutant cells were grown anaerobically as described in Zhang et al. [47]. To obtain the E. coli membranes, both the WT and E445A mutant cells, washed twice with 5 mM sodium phosphate (pH 7.5), 0.17 M NaCl, and a few grains of solid 4-(2-aminoethyl)-benzenesulfonyl fluoride, were suspended in 20 mM Tris(hydroxymethyl)-aminomethan/HCl (pH 8.3), 0.5 mM ethylenediaminetetra-acetate, 5 mM MgSO₄, 15 mM benzamidine, 1 mM DL-dithiothreitol, 0.5 mg/L leupeptin, and a few grains of solid deoxyribonuclease I and 4-(2-aminoethyl)-benzenesulfonyl fluoride; then the suspension was passed twice by 30-ml portions through a French press. Intact and partially broken cells were removed by centrifugation at 17,600 × g for 5 min at 4 °C. The membranes were pelleted (125,000 × g, 4 h, 4 °C), frozen in liquid nitrogen, and stored at −80 °C.

2.4. Sample preparation

All measurements were performed in 50 mM N-(2-hydroxethyl)piperazine-N’-(2-ethanesulfonate)/50 mM 2-(N-cyclohexylamino)-ethanesulfonate (pH 8.0), and 0.5 mM ethylenediaminetetraacetae in a homemade optical cell of 2.5 mm pathway at room temperature. Cytochrome bd concentrations in the WT and E445A mutant membranes in the samples were 7.3 μM and 1 μM, respectively. The optical cell was first purged with argon, and the sample was flowed into the cell under argon pressure. Experiments were carried out with the three stable states of carbon monoxide-bound cytochromes bd: (a) dithionite-reduced wild type (WT R-CO, b₉₉₆₉₇₅₅₆ₕ₅₆ₕ–CO) and (b) dithionite-reduced mutant (E445A MV²-CO, b₉₉₆₉₇₅₅₆ₕ₅₆ₕ–d²–CO) were obtained by bubbling the sample, prereduced with 50–100 mM sodium dithionite for 30 min, with 100% CO; and (c) one-electron-reduced wild type (WT MV¹–CO, b₉₉₆₉₇₅₅₆ₕ₅₆ₕ–d²–CO) was prepared by purging the as isolated membrane-bound cytochrome bd (which is mainly a one-electron-reduced oxy species, b₉₉₆₉₇₅₅₆ₕ₅₆ₕ–d²–O₂) with argon gas and then by replacing argon with 100% CO. To check the redox and ligation status of cytochrome bd, static absorption spectra of the samples were recorded before and after the measurements with the use of a dual-pathway spectrophotometer described in Joliot et al. [49].

2.5. Enzyme concentration

The cytochrome bd content in the E. coli membranes was judged from the heme d concentration. In the WT membranes, the heme d concentration was determined from the dithionite-reduced-minus-“air-oxidized” difference absorption spectra using ΔA₂₆₂₈–₆₇₆ of 10.8 M⁻¹ cm⁻¹ [38] and from the (CO-bound/dithionite-reduced) minus-dithionite-reduced difference spectra using ΔA₆₄₃–₆₂₃ = 13.2 M⁻¹ cm⁻¹ [10]. In the E445A mutant membranes, the heme d concentration was determined from the (CO-bound/dithionite-reduced) minus-dithionite-reduced difference spectra using ΔA₆₄₃–₆₂₃ = 11.1 M⁻¹ cm⁻¹ that corresponds to ΔA₂₆₂₈–₆₇₆ of 25 M⁻¹ cm⁻¹ for the dithionite-reduced absolute absorption spectra of the isolated enzyme [8].
2.6. Nanosecond spectroscopy

The photoinduced absorption changes in the membranes were measured with a home-built nanosecond spectrophotometer described in Beal et al. [50]. The flash (excitation at 640 nm, near the α band of heme d [44, 51, 52]) was provided by a Nd:Yag pumped dye laser. The absorption changes were probed at discrete wavelengths and delay times after the exciting flash by flashes provided by an optical parametric oscillator pumped by the third harmonic of a Nd:Yag (5 ns fwhm).

2.7. Data analysis

Origin 7 (OriginLab Corporation) was used for data manipulation and presentation.

3. Results

3.1. Recombination of CO with the WT cytochrome bd in the R state

CO recombines with the WT cytochrome bd in the R state monoexponentially with τ ∼ 12 µs at 100% CO, as evidenced from the kinetics of flash-induced absorption changes at selected wavelengths (Fig. 1, main panel). This value corresponds to a second-order rate constant of CO recombination to heme d of ∼ 8 × 10^7 M⁻¹ s⁻¹, in line with the previously reported values for the isolated enzyme from E. coli [53] and A. vinelandii [40, 54]. A difference transient absorption spectrum recorded 5 ns after the flash has a reversed “W” shape (Fig. 1, inset). Such peculiar W-shape is consistently observed as well in static Soret difference absorption spectra of CO binding to the enzyme in the R state [38, 40, 52, 55, 56].

We have previously shown that CO does not recombine with the isolated fully reduced enzyme in the time scale up to 300 ps [39]. Recent experiments (M.H.V. and V.B.B., unpublished results) have extended this range up to 4 ns. Altogether, our experiments show that only bimolecular recombination occurs after dissociation of the heme d–CO bond in the R-CO state.

3.2. Recombination of CO with the WT cytochrome bd in the MV¹ state

Flash-induced absorption changes of the WT cytochrome bd in the MV¹–CO state were monitored under the same conditions. Fig. 2A shows the transient spectra at delay times of 5 ns, 200 ns, and 60 µs. It can be seen that the 5-ns spectrum of the WT MV¹–CO enzyme (Fig. 2A) is clearly different in line shape from its counterpart of the WT R-CO enzyme (Fig. 1, inset). The difference between the 5-ns R-CO and MV¹–CO transient spectra is mainly a bleaching at 435 nm (Fig. 2D), in agreement with earlier measurements on the picoseconds time scale [41]. As shown in Fig. 2B, the spectral evolution in the WT MV¹ enzyme is multihasic. It can be fitted with three exponential phases with time constants of ∼ 14 ns, 14 µs (10–15 µs in different experiments), and 280 µs (140–290 µs in different experiments). This is in contrast to a single (12 µs) phase observed for the WT R enzyme (Fig. 1). The spectra of the absorption changes associated with the three components are different in line shape. The spectrum of the 14 ns component, absent in the WT R cytochrome bd, reflects the decay of an induced absorption with a maximum at 435 nm (Fig. 2C), which we assign to geminate recombination of CO with part of WT MV¹ enzyme. The spectral characteristics of this component are similar to that of the ~100-ps phase [39] also attributed to geminate recombination. The spectrum of the absorption changes associated with the 14-µs component shows a maximum at 420 nm with a shoulder around 430 nm (Fig. 2C). This component is not homogenous and likely reflects at least two different processes—bimolecular recombination of CO with heme d on the microsecond time scale and the photolysis-induced electron transfer (backflow) from heme d to hemes b in a small fraction of the enzyme molecules (see Discussion). The spectrum of the absorption changes associated with the 280 µs component has a maximum at 438 nm and a minimum at 422 nm (Fig. 2C) and may be attributed to reversal of the electron backflow and bimolecular recombination in this enzyme fraction. According to modeling (not shown), the 280 µs component reflects re-reduction of ∼ 3.8% of heme d with the electron simultaneously returning from heme b₄₅ and heme b₃₂ in the proportion of ~70%/30%, respectively. Such relative contributions of the hemes b to the reversed electron transfer are consistent with those observed recently with the isolated enzyme at 1% CO [10].

3.3. Recombination of CO with the E445A mutant cytochrome bd in the MV² state

Flash-induced absorption changes of the E445A mutant cytochrome bd in the MV²–CO state (Fig. 3) are generally similar to those observed with the WT MV¹ cytochrome bd but also markedly different from the WT R cytochrome bd. Fig. 3A shows the corresponding transient spectra at 5 ns, 200 ns, and 1.5 ms. The 5-ns spectrum of the E445A MV²–CO enzyme (Fig. 3A) resembles that of the WT MV¹–CO enzyme (Fig. 2A). It is worth mentioning that the 5 ns difference spectra for WT MV¹–CO and E445A MV²–CO ha comparable amplitudes (within a factor of 2), though the concentrations of WT and E445A differ considerably (7.3 µM vs. 1.0 µM). A few explanations of such difference which do not exclude each other can be considered. First, in MV¹–CO only part of heme d is reduced (initially as oxy ferrous heme d complex), whereas in MV²–CO all heme d population is in the ferrous state. Therefore, after replacement of O₂ with CO, the actual heme d–CO concentration in MV¹–CO should be substantially lower than 7.3 µM because neither ferryl nor oxidized heme d species being also present in the as-prepared MV¹ state can react with CO. Second, MV¹–CO and MV²–CO may differ in a fraction that undergoes geminate recombination of CO and heme d at earlier (subnanosecond) times. If in the former case the enzyme population involved in subnanosecond recombination is larger, the amplitude at a delay time of 5 ns will become smaller. Third, the quantum yield of photodissociation of CO from heme d in E445A MV²–CO may be larger than that in WT MV¹–CO.

The kinetics of CO recombination at selected wavelengths for the MV²–CO state (Fig. 3B, symbols) can be reasonably fitted with two exponentials (Fig. 3B, solid lines) with time constants of ∼ 14 ns and 42 µs (38–42 µs in different experiments). Using three exponentials...
measured at delay times of 5 ns and 200 ns. The spectrum of the 280-μs component; therefore, for that kinetics, a one-exponential fit with τ ~ 14 μs is sufficient. Arrow indicates the moment of laser flash. (C) Spectra of the absorption changes associated with the 14-ns, 14-μs, and 280-μs components. The spectrum of the 14-ns phase is calculated as the difference between the transient spectra measured at delay times of 5 ns and 200 ns. The spectrum of the 280-μs phase is the spectrum measured 60 μs after the flash. The amplitude of the latter has been divided by e^{-60/280} to correct for the decay at 60 μs. The spectrum of the 14-μs phase is calculated as the difference between the transient spectrum measured at a delay time of 200 ns and the spectrum of the 280-μs phase. (D) Difference between R-CO and MV1-CO transient spectra at a delay time of 5 ns. The spectra are normalized at 445 nm (spectrum MV1-CO multiplied by 7.5) as the spectral properties at this wavelength are independent of the oxidation state of the b-hemes [41].

does not improve fit significantly. These changes can mainly reflect geminate and bimolecular recombination of CO with heme d on the nanosecond and microsecond time scales, respectively. Remarkably, the 280-μs component observed in the MV1 state is absent. This implies that, under these conditions, back electron transfer from heme d to heme b595 requires heme b595 to be in the oxidized state.

The spectrum of the bimolecular recombination component differs somewhat from the nanosecond component. This may be due to interaction of dissociated CO with the hemes while it is sequestered close to the active site.

CO recombination to the E445A MV2 enzyme (Fig. 3B) appeared to be about 3-fold slower than that to the WT MV1 cytochrome bd (Fig. 2B). Two possible explanations can be suggested. First, the mutation could affect an access channel for ligand transfer between the bulk phase and heme d. The existence of such channel(s) in cytochrome bd has been proposed earlier [19,32,39]. Second, the mutation could decrease the affinity of ferrous heme d for CO.

4. Discussion

Earlier, recombination of CO with the dithionite-reduced E. coli membranes containing the WT cytochrome bd was studied on the micro/millisecond time scale at the 532-nm excitation [57]. These membranes were treated with detergent. Treatment of membrane-bound cytochrome bd with detergent can markedly attenuate scattering in the near UV typical of the native membranes that allowed resolving flash-induced absorption changes in the Soret. However, treatment of cytochrome bd with detergent can lead to appearance of a denatured fraction of heme b reacting with CO [2,56]. Such a heme b–CO complex can be easily photolyzed at the 532 nm excitation that resulted in additional slower phases of CO recombination with heme b [40,54,57], significantly complicating interpretation of the data [57]. In the present experiments, native membranes of E. coli, devoid of such an undesired reaction, were used. The use of a specific setup in the photolysis experiments allows us to monitor absorption changes with a very high resolution even in the near UV region, starting from the time of 5 ns (see Materials and methods). Upon selective excitation of the α band of heme d (at 640 nm), CO is photolyzed only from heme d.

In the present study, we showed that at the 640 nm excitation the flash-induced absorption changes for the E. coli membranes containing the WT cytochrome bd in the R-CO and MV1-CO states under the same conditions differ in (i) line shape of the transient spectra, (ii) number of recombination phases, and (iii) amplitude of the response.

(i) As previously observed for the picosecond CO photodissociation spectra of the isolated WT enzyme [41], the transient spectra at 5 ns of the WT membranes containing cytochrome bd in the R and MV1 states are clearly different in line shape. The WT R spectrum has a reversed W-shape with a minimum at 435 nm (Fig. 1, inset), whereas the main feature of that of the WT MV1 is the maximum at 435 nm (Fig. 2A). The difference between the normalized R and MV1 spectra is the bleaching at 435 nm (Fig. 2D) [41]. An explanation suggested in [39,41] for the picosecond spectra is that, in the R enzyme, photolysis of CO from heme d perturbs ferrous ferrous heme b595 causing loss of an absorption band centered at 435 nm. The data of nanosecond spectroscopy support this conclusion and further substantiate the assignment of the bleaching at 435 nm (Fig. 2D) to the interaction between heme d and heme b595.

(ii) One of the main results of this work is that the dynamics of the flash-induced absorption changes for WT cytochrome bd is strikingly different in the R-CO and MV1-CO states. In R-CO,
there is a single phase of bimolecular recombination with $\tau \sim 12 \mu s$ at 1 atm CO (Fig. 1). In contrast, in MV$^1$-CO, there are three phases of recombination with time constants of $\sim 14$ ns, $14 \mu s$, and $280 \mu s$ (Fig. 2B) plus a picosecond phase of geminate recombination ($\tau \sim 70$–200 ps) observed earlier [39]. Thus, there are totally four phases in MV$^1$-CO.

The line shape of the spectra associated with the three phases of recombination in MV$^1$-CO is different. The spectrum of the 14 ns component, absent in the R enzyme, is the induced absorption with a $\sim 70$–200 ps component, showing a maximum at 438 nm and a shoulder at 455 nm. This similarity allows us to suggest that the 14-ns component, absent in the R enzyme, is the induced absorption with a maximum at 435 nm and a shoulder at 455 nm. This is in agreement with the spectrum of the R-CO state and the E445A mutant cytochrome $bd$ in the MV$^1$-CO state (Fig. 3A).

In this view, the 14-ns component consists of at least two different processes: (a) bimolecular recombination of CO with the remaining ferrous unligated heme $d$ on the microsecond time scale and (b) the electron backflow from heme $d$ to hemes $b$ in $\sim 3.8\%$ of the enzyme molecules (see Fig. 4B).

The conclusion that the 14-ns component includes recombination of CO with heme $d$ that is bimolecular rather than geminate is based on the data of Junemann et al. [58] who first observed such recombination with the $A. vinelandii$ cytochrome $bd$ in the MV$^1$ state monitoring the heme $d$ $\alpha$-band. They showed that the rate of recombination increases linearly with the CO concentration, hence the recombination is indeed bimolecular. Their observation that the second-order rate constant of recombination for the MV$^1$ enzyme ($1 \times 10^8$ M$^{-1}$ s$^{-1}$) is only slightly slower than that for the R enzyme ($1.5 \times 10^8$ M$^{-1}$ s$^{-1}$) [58] is also in agreement with our data.

Another factor that contributes to this phase is electron redistribution from heme $d$ to hemes $b$ in $\sim 3.8\%$ of the MV$^1$ cytochrome $bd$ induced by photolysis. This is in agreement with the spectrum of the next, 280-µs component, showing a maximum at 438 nm and a minimum at 422 nm (Fig. 2C), which reflects return of the electron from the hemes $b$ to heme $d$. It is worth mentioning that, even under optimum conditions, the amplitude of the electron backflow is quite small due to the large redox potential difference between $d$ and $b$ hemes. Furthermore, since there is a competition between CO recombination and backflow [7], the backflow could previously be detected by micro/millisecond absorption spectroscopy only at low CO concentrations [7,58]. At 1% CO, $\sim 11\%$ of heme $d$ is oxidized following CO photolysis and the electron simultaneously moves to hemes $b_{558}$ and $b_{595}$ [10] and then returns back as CO recombines. At 100% CO, no internal electron redistribution in the MV$^1$ cytochrome $bd$ has been detected by absorption spectroscopy hitherto. The reverse electron flow was found to be associated with the generation of
membrane potential [7]. The finding that the signal-to-noise ratio of
electrometric traces is superior to that of absorbance traces allowed us
to observe electrometric backlight transients even at high CO
concentrations [7]. At 100% CO, the electron backlight is present but
its amplitude decreases to be of about one-fourth of that at 1% CO (see
Fig. 7 of [7]), i.e., around 3% of heme d can be oxidized following CO
photolysis provided this value is 11% at 1% CO. The electrometric
backlight response decays with time constant of about 360 μs [7] that
is in rough agreement with the 280-μs phase observed in this work.
Owing to the fact that extinction of hemes b in the Soret is much larger
than that of heme d [44] and due to a very high sensitivity of the
technique used, we are now able to observe spectrophotometrically
flash-induced internal electron redistribution and its relaxation even
at 100% CO.

It should be noted that the 280-μs component is not observed in the
MV²-CO enzyme (Fig. 3). This agrees with our conclusion about the
origin of the microsecond phases in the MV¹-CO cytochrome bd
because flash-induced electron redistribution between hemes d and
b₃₅₈ is not possible in the MV²-CO state (both hemes are reduced in
this state). Electron backlight from heme d to heme b₃₅₈ in the E445A
MV²-CO enzyme is apparently also negligible under these conditions.
As such, electron transfer does occur to a significant extent starting
from the MV¹-CO state, this finding implies that the redox state of
heme b₃₅₈ influences the redox potential difference between hemes d
and b₉₅₅. This reasoning is consistent with the report of negative
redox interactions between hemes b₃₅₈ and b₉₅₅ [44]. The fact that the
difference between the normalized spectra of the 14-μs phase for
MV¹-CO and the 42-μs phase for MV²-CO (not shown) is similar to the
reversed spectrum of the 280-μs component further supports such
conclusion.

It has to be noted that the lack of heme b₉₅₅ reduction in photolyzed MV²-CO state seems to contrast with a report of Belevich et al. [8] where such reduction was observed. The experimental conditions of that work were different: it was carried out with the isolated detergent-solubilized enzyme, at 1% CO, the kinetics of the electron transfer was not resolved [8]. Two possible explanations of such apparent discrepancy can be suggested. First, due to the mutation, the rate of electron transfer between the hemes may become slower in the E445A enzyme as compared to the WT enzyme. Since, as mentioned above, there must be a competition between CO recombination and backlight, at 100% CO used in this study, the photolysis-induced electron transfer from heme d to heme b₃₅₈ may be indeed negligible by that reason. Second, the midpoint potential values for WT cytochrome bd from E. coli can significantly depend on the nature of membrane environment such as detergent used for the enzyme solubilization [59]. The latter has never been tested with the E445A mutant cytochrome bd. It is possible that in the mutant cytochrome bd of the bacterial membranes (this work) ΔE_m between heme d and heme b₉₅₅ is larger (i.e., E_m of b₉₅₅ is lower) than that in the isolated detergent-solubilized enzyme [8] that in the former case would make the electron backlight thermodynamically unfavorable to occur. The combination of these two reasons cannot also be excluded. (iii) R-CO and MV²-CO WT cytochromes bd differ in the yield of the observed photolysis of CO from ferrous heme d at 5 ns. Although recorded under the same enzyme concentration and other experimental conditions, a normalization factor of ~7.5 is required for the 5 ns WT MV¹-CO transient spectrum to match that of R-CO, provided the extinction coefficients of R-CO and
MV¹-CO at 445 nm are virtually identical [41]. Since in the “as prepared” state of WT cytochrome bd used to generate MV¹-CO, only about 70% of heme d is in the oxy form (MV¹-O²), the actual factor is ~5.2. This means that in case of WT MV¹
cytochrome bd, no more than ~20% of ferrous heme d can be observed as recombining with CO through a bimolecular mechanism on the microsecond time scale. This is in full agreement with the picosecond studies wherein at least half of the photolyzed WT MV¹-CO purified enzyme, subnanosecond geminate recombination of CO, and heme d occurs [39], and the quantum yield of photodissociation of CO from heme d was found to be ~3-fold diminished in the presence of oxidized hemes b [41].

In all earlier studies where ligand reaction pattern was compared for WT cytochrome bd in R and MV¹ states and the differences were observed [19,32,39,41], the redox state of which of the two b hemes modulates ligand binding/dissociation properties of the heme d active site, was not established. This could be heme b₉₅₅, or heme b₃₅₈, or both. In this work, we used the E445A mutant cytochrome bd in which heme b₉₅₅ remains in the ferric state even in the presence of a strong reductant. This unique possibility to have two-electron-reduced mixed-valence enzyme (MV²) was used to answer this question. First, we found that the line shape of the photodissociation spectrum (at a delay time of 5 ns) of the E445A mutant in the MV²-CO state (Fig. 3A) is similar to that of the WT cytochrome bd in the MV¹-CO state (Fig. 2A) but strikingly different from that of the WT enzyme in the R state (Fig. 1, inset). In contrast to the latter spectrum, it does not display the sharp bleaching feature at 435 nm as clearly demonstrates the difference between the 5 ns transient spectra of the WT R and E445A MV² cytochromes bd (Fig. 3C). This difference is also reminiscent of that between the picosecond transient spectra of the

---

**Fig. 4.** (A) Minimal scheme of geminate recombination phases of CO starting from the MV¹-CO and R-CO states. Two different configurations of dissociated CO in the protein (dred…,..., CO_i = I, II) are required to explain the two geminate recombination phases. The ratio of forward and backward rates from these configurations is roughly estimated from the amplitudes of the phases; the sums of the rates correspond to the experimentally observed rates. The state (dred CO) denotes a state where CO has escaped from the protein. In this minimal scheme, if heme b₃₅₈ is reduced before dissociation of the heme d-CO bond, geminate recombination from the (dred…,..., CO_i) state does not compete efficiently with population of the (dred…,..., CO_i) state. (B) Minimal scheme of bimolecular CO recombination and electron transfer starting from the MV¹-CO. The first dissociation step comprises all steps in A. The ratio of forward and backward rates from the CO dissociated dred is roughly estimated from the amplitudes of the phases; the sums of the rates correspond to the experimentally observed rates.
R and MV¹ purified enzymes under isotropic conditions [41]. Hence, we can conclude that dissociation of CO from heme d perturbs the Soret band of heme b₅₅₅ but not that of heme b₁₅₅₅. The data thus further support interactions between high-spin protoporphyrin b₂₅₉₅ and chlorin d in the di-heme oxygen-reducing site, in agreement with previous works [8,9,36–43].

Second, in the case of E445A MV²-CO, apart from a microsecond phase of bimolecular recombination, there is also an additional phase of CO recombination on the nanosecond time scale with 7 ± 14 ns (Fig. 3), absent in the WT R cytochrome bd. The fact that the spectrum of the 14 ns component of E445A MV²-CO is very similar in line shape to that of WT MV²–CO (Fig. 3D) supports the conclusion that also in the oxidized di-heme and heme-copper oxygen-reducing centers of terminal oxidases: different reaction pathways and end-products. J. Inorg. Biochem. 103 (2009) 1185–1187.


