



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

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ABSTRACT

Cytochrome *bd* is a terminal quinol:O₂ oxidoreductase of respiratory chains of many bacteria. It contains three hemes, *b*₅₅₈, *b*₅₉₅, and *d*. The role of heme *b*₅₉₅ remains obscure. A CO photolysis/recombination study of the membranes of *Escherichia coli* containing either wild type cytochrome *bd* or inactive E445A mutant was performed using nanosecond absorption spectroscopy. We compared photoinduced changes of heme *d*–CO complex in one-electron-reduced, two-electron-reduced, and fully reduced states of cytochromes *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 *d* perturbs ferrous heme *b*₅₉₅ causing loss of an absorption band centered at 435 nm, thus supporting interactions between heme *b*₅₉₅ and heme *d* in the di-heme oxygen-reducing site, in agreement with previous works. Photolyzed CO recombines with the fully reduced enzyme monoexponentially with $\tau \sim 12 \mu\text{s}$, whereas recombination of CO with one-electron-reduced cytochrome *bd* shows three kinetic phases, with $\tau \sim 14 \text{ ns}$, $14 \mu\text{s}$, and $280 \mu\text{s}$. 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 *d*. The 14- μs component reflects bimolecular recombination of CO with heme *d* and electron backflow from heme *d* to hemes *b* in $\sim 4\%$ of the enzyme population. The final, 280- μs component, reflects return of the electron from hemes *b* to heme *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 *b*₅₉₅, and not that of heme *b*₅₅₈, controls the pathway(s) by which CO migrates between heme *d* and the medium.

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1. Introduction

Cytochrome *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 *bd* endows bacteria with a number of specific physiological functions. Cytochrome *bd* facilitates both pathogenic and commensal bacteria to colonize oxy-

gen-poor environments [11–14], serves as an oxygen scavenger and inhibits degradation of O₂-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 *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 *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 *b*₅₅₈, heme *b*₅₉₅, and heme *d*, with stoichiometry of 1:1:1 per enzyme molecule.

The roles of the three hemes in cytochrome *bd* are different. The low-spin hexacoordinate heme *b*₅₅₈ is the electron entry site; it directly accepts electrons from quinol [29,30]. The high-spin and likely pentacoordinate

Abbreviations: WT, wild type; R, fully reduced (three-electron-reduced) species ($b_{558}^2 b_{595}^2 d^{2+}$); MV¹, one-electron-reduced “mixed-valence” species ($b_{558}^2 b_{595}^+ d^{2+}$); MV², two-electron-reduced “mixed-valence” species ($b_{558}^+ b_{595}^+ d^{2+}$); fwhm, full-width at half-maximum; τ , time constant; $t_{1/e}$, reciprocal of rate constant

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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 *b*₅₉₅ apparently accepts electrons from heme *b*₅₅₈ 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 *b*₅₉₅ 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 *b*₅₉₅ can be observed [44]. It has also been proposed that heme *b*₅₉₅ 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 *b*₅₅₈ and heme *b*₅₉₅ 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 geminate 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 *b*₅₉₅ [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 *d*²⁺, 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 [47] and cannot be completely reduced even with excess dithionite [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 *bo*₃ 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 ethylenediaminetetraacetate, 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-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonate)/50 mM 2-(*N*-cyclohexylamino)-ethanesulfonate (pH 8.0), and 0.5 mM ethylenediaminetetraacetate 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, *b*₅₅₈²⁺*b*₅₉₅²⁺*d*²⁺–CO) and (b) dithionite-reduced mutant (E445A MV²–CO, *b*₅₅₈²⁺*b*₅₉₅³⁺*d*²⁺–CO) were obtained by bubbling the sample, prerduced with 50–100 mM sodium dithionite for 30 min, with 100% CO; and (c) one-electron-reduced wild type (WT MV¹–CO, *b*₅₅₈³⁺*b*₅₉₅³⁺*d*²⁺–CO) was prepared by purging the as isolated membrane-bound cytochrome *bd* (which is mainly a one-electron-reduced oxy species, *b*₅₅₈³⁺*b*₅₉₅³⁺*d*²⁺–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–“air-oxidized” difference absorption spectra using Δε_{628–607} of 10.8 mM^{–1} cm^{–1} [38] and from the (CO-bound/dithionite-reduced)–minus–(dithionite-reduced) difference spectra using Δε_{643–623} = 13.2 mM^{–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 Δε_{643–623} = 11.1 mM^{–1} cm^{–1} that corresponds to Δε_{628–670} of 25 mM^{–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], 5 ns fwhm) 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 $\tau \sim 12 \mu\text{s}$ 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 $\sim 8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, 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

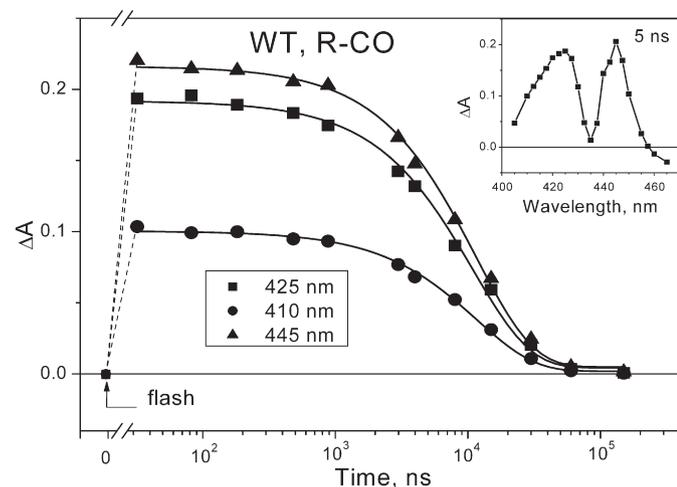


Fig. 1. Absorption changes measured after photodissociation of CO from the *E. coli* membranes containing the WT cytochrome *bd* in the R-CO state. *Main panel:* Kinetics of absorption changes at selected wavelengths during CO recombination. The kinetic data points (symbols) are shown with their best fits to single exponentials (solid lines) yielding $\tau \sim 12 \mu\text{s}$. Arrow indicates the moment of laser flash. *Inset:* Transient absorption spectrum at a delay time of 5 ns. For conditions, see Materials and methods.

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 multiphasic. It can be fitted with three exponential phases with time constants of $\sim 14 \text{ ns}$, $14 \mu\text{s}$ (10–15 μs in different experiments), and $280 \mu\text{s}$ (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 homogeneous 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 $\sim 3.8\%$ of heme *d*, with the electron simultaneously returning from heme *b*₅₉₅ and heme *b*₅₅₈ in the proportion of $\sim 70\%/30\%$, 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 have 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 $\sim 14 \text{ ns}$ and $42 \mu\text{s}$ (38–42 μs in different experiments). Using three exponentials

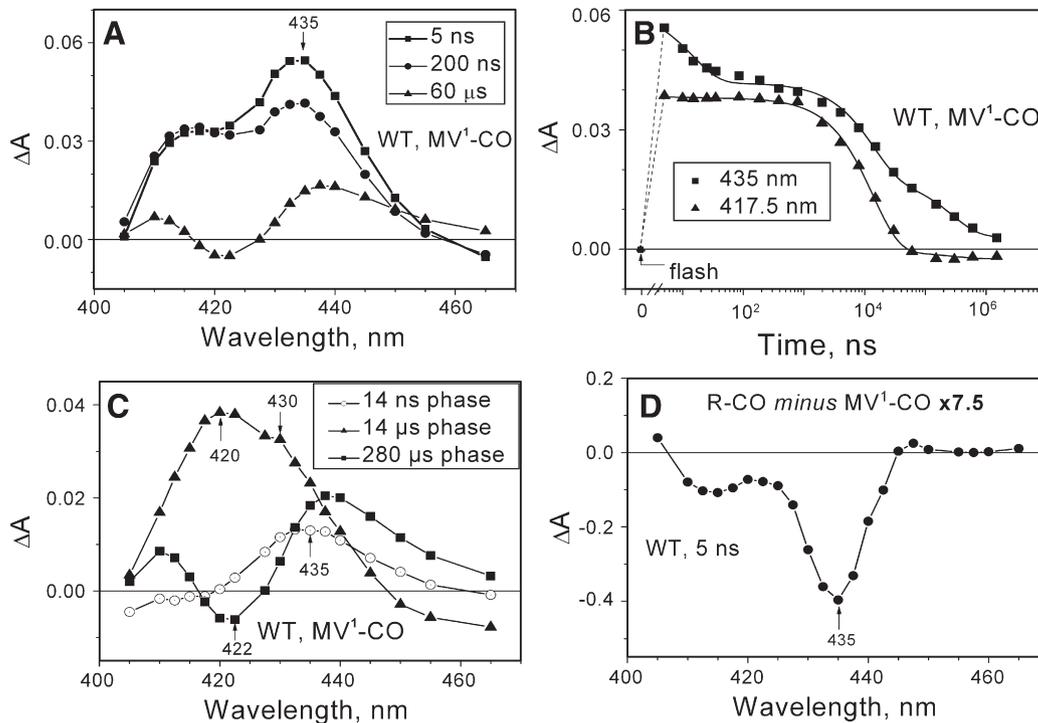


Fig. 2. Absorption changes measured after photodissociation of CO from the *E. coli* membranes containing the WT cytochrome *bd* in the MV¹-CO state. (A) Transient absorption spectra at delay times of 5 ns, 200 ns, and 60 μ s. (B) Kinetics of absorption changes at selected wavelengths during CO recombination. The kinetic data points (symbols) are shown with their best fits (solid lines). The kinetics at 435 nm is fitted to the sum of three exponentials yielding $\tau \sim 14$ ns, 14 μ s, and 280 μ s. 417.5 nm has been selected as the wavelength isosbestic for the 14 ns and 280 μ s components; therefore, for that kinetics, a one-exponential fit with $\tau \sim 14$ μ s is sufficient. Arrow indicates the moment of laser flash. (C) Spectra of the absorption changes associated with the 14-ns, 14- μ s, and 280- μ s 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- μ s phase is the spectrum measured 60 μ s after the flash. The amplitude of the latter has been divided by $e^{-60/280}$ to correct for the decay at 60 μ s. The spectrum of the 14- μ s phase is calculated as the difference between the transient spectrum measured at a delay time of 200 ns and the spectrum of the 280- μ s 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- μ s component observed in the MV¹ state is absent. This implies that, under these conditions, back electron transfer from heme *d* to heme *b*₅₉₅ requires heme *b*₅₅₈ 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 heme *b*₅₉₅ 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 *b*₅₉₅.
- (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,

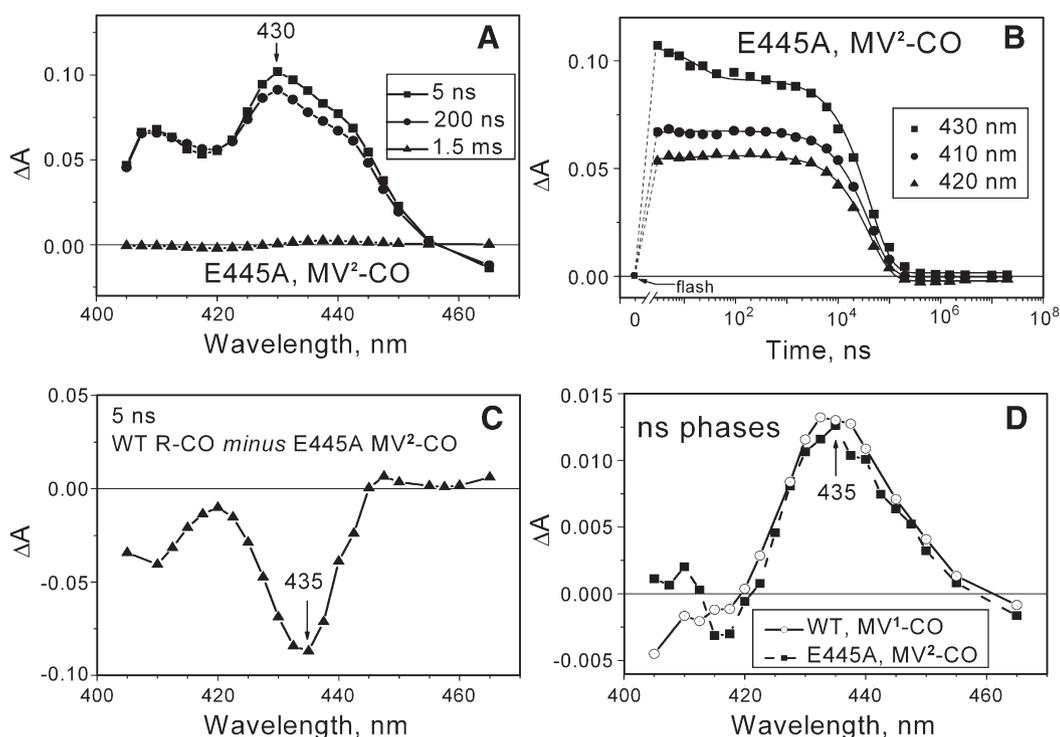


Fig. 3. Absorption changes measured after photodissociation of CO from the *E. coli* membranes containing the E445A mutant cytochrome *bd* in the MV²-CO state. (A) Transient absorption spectra at delay times of 5 ns, 200 ns, and 1.5 ms. (B) Kinetics of absorption changes at selected wavelengths during CO recombination. The kinetic data points (symbols) are shown with their reasonable fits to the sum of two exponentials (solid lines) yielding $\tau \sim 14$ ns and 42 μ s. Approximation with three exponentials does not improve the fit significantly. Arrow indicates the moment of laser flash. (C) Difference between transient absorption spectra of the WT cytochrome *bd* in the R-CO state and the E445A mutant cytochrome *bd* in the MV²-CO state at a delay time of 5 ns (normalized at 445 nm). (D) Comparison of the spectra of the absorption changes associated with the nanosecond components for the WT cytochrome *bd* in the MV¹-CO state and the E445A mutant cytochrome *bd* in the MV²-CO state (normalized on the maximum). The latter spectrum is a difference between the MV²-CO E445A transient spectra at delay times of 5 ns and 200 ns.

there is a single phase of bimolecular recombination with $\tau \sim 12$ μ s at 1 atm CO (Fig. 1). In contrast, in MV¹-CO, there are three phases of recombination with time constants of ~ 14 ns, 14 μ s, and 280 μ 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 MV¹-CO.

The line shape of the spectra associated with the three phases of recombination in MV¹-CO is different. The spectrum of the 14 ns component, absent in the R enzyme, is the induced absorption with a maximum at 435 nm (Fig. 2C), very similar to the ps MV¹-CO spectrum [41]. This similarity allows us to suggest that the 14-ns component reflects a second phase of geminate recombination of CO with heme *d* in part of MV¹, following the ~ 100 -ps phase [39]. The presence of at least two phases of CO geminate recombination indicates the presence of several distinct configurations of nonbound CO within the protein moiety. As discussed previously [39], the absence of geminate recombination in the fully reduced enzyme implies that the redox state of heme *b*₅₉₅ controls the pathways of unbound CO. Our present finding of a second geminate rebinding phase also only in MV complexes indicates that all geminate rebinding to heme *d* is influenced by heme *b*₅₉₅, suggesting that the nanosecond rebinding occurs via the conformation from which picosecond rebinding occurs (Fig. 4A), in agreement with the proposed proximity of hemes *d* and *b*₅₉₅.

Based on transmembrane electron transfer kinetics observed in previous electrogenicity studies (Fig. 7, trace 3 in Ref. [7]), and of our modeling of the 280- μ s phase, we propose that the 14 μ s and 280 μ s heme *d*-CO recombination phases also reflect backflow (14- μ s phase) and reverse backflow (280- μ s phase) between heme *d* and the *b* hemes. Fig. 4B depicts a scheme encompassing the ensemble of competing reactions on the microsecond time scale that summarizes our interpretation of the data, as discussed below.

In this view, the 14- μ s 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- μ s 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 MV¹ state monitoring the heme *d* α -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 MV¹ enzyme (1×10^8 M⁻¹ s⁻¹) is only slightly slower than that for the R enzyme (1.5×10^8 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 MV¹ cytochrome *bd* induced by photolysis. This is in agreement with the spectrum of the next, 280- μ s 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 MV¹ cytochrome *bd* has been detected by absorption spectroscopy hitherto. The reverse electron flow was found to be associated with the generation of

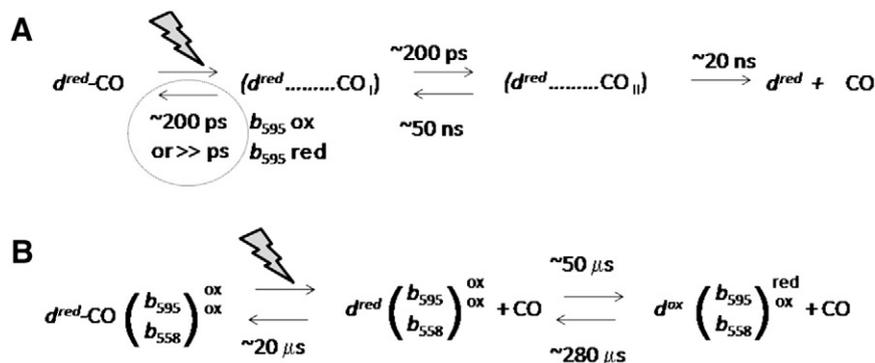


Fig. 4. (A) Minimal scheme of geminate recombination phases of CO starting from the MV¹-CO and R-CO states. Two different configurations of dissociated CO in the protein ($d^{red}\text{.....CO}_i$, $i = I, II$) are required to explain the two geminate recombination phases. The ratio of forward and backward 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 ($d^{red} + \text{CO}$) denotes a state where CO has escaped from the protein. In this minimal scheme, if heme b_{595} is reduced before dissociation of the heme d -CO bond, geminate recombination from the ($d^{red}\text{.....CO}_I$) state does not compete efficiently with population of the ($d^{red}\text{.....CO}_{II}$) 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 d^{red} is roughly estimated from the amplitudes of the phases; the sums of the rates correspond to the experimentally observed rates.

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 backflow transients even at high CO concentrations [7]. At 100% CO, the electron backflow 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 backflow 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 b_{558} is not possible in the MV²-CO state (both hemes are reduced in this state). Electron backflow from heme d to heme b_{595} 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 b_{558} influences the redox potential difference between hemes d and b_{595} . This reasoning is consistent with the report of negative redox interactions between hemes b_{558} and b_{595} [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 b_{595} 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 backflow, at 100% CO used in this study, the photolysis-induced electron transfer from heme d to heme b_{595} 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 b_{595} is larger (i.e., E_m of b_{595} is lower) than that in the isolated detergent-solubilized enzyme [8] that in the former case would make the electron backflow thermodynamically unfavorable to occur. The combination of these two reasons cannot also be excluded.

- (iii) R-CO and MV¹-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 $\sim 20\%$ 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 b_{595} , or heme b_{558} , or both. In this work, we used the E445A mutant cytochrome bd in which heme b_{595} 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

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 *b*₅₉₅ but not that of heme *b*₅₅₈. The data thus further support interactions between high-spin protoporphyrin *b*₅₉₅ 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 $\tau \sim 14$ 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 MV¹-CO (Fig. 3D) supports the conclusion that also in E445A MV²-CO cytochrome *bd* geminate recombination of CO with heme *d* on the nanosecond time scale occurs. Thus, the redox state of heme *b*₅₉₅ controls the pathway(s) by which CO migrates between heme *d* and the medium. In light of this finding, we can also suggest that the same holds true for O₂ [32] and NO [19].

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