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Catalytic intermediates of cytochrome *bd* terminal oxidase at steady-state: Ferryl and oxy-ferrous species dominate

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ABSTRACT

The cytochrome *bd* ubiquinol oxidase from *Escherichia coli* couples the exergonic two-electron oxidation of ubiquinol and four-electron reduction of O_2 to $2H_2O$ to proton motive force generation by transmembrane charge separation. The oxidase contains two *b*-type hemes (b_{558} and b_{595}) and one heme *d*, where O_2 is captured and converted to water through sequential formation of a few intermediates. The spectral features of the isolated cytochrome *bd* at steady-state have been examined by stopped-flow multiwavelength absorption spectroscopy. Under turnover conditions, sustained by O_2 and dithiothreitol (DTT)-reduced ubiquinone, the ferryl and oxy-ferrous species are the mostly populated catalytic intermediates, with a residual minor fraction of the enzyme containing ferric heme *d* and possibly one electron on heme b_{558}. These findings are unprecedented and differ from those obtained with mammalian cytochrome *c* oxidase, in which the oxygen intermediates were not found to be populated at detectable levels under similar conditions [M.G. Mason, P. Nicholls, C.E. Cooper, The steady-state mechanism of cytochrome *c* oxidase: redox interactions between metal centres, Biochem. J. 422 (2009) 237–246]. The data on cytochrome *bd* are consistent with the observation that the purified enzyme has the heme *d* mainly in stable oxy-ferrous and ferryl states. The results are here discussed in the light of previously proposed models of the catalytic cycle of cytochrome *bd*.

1. Introduction

Terminal oxidases in the prokaryotic and eukaryotic respiratory chain catalyze the four-electron reduction of molecular oxygen to water. The members of the two major families of terminal oxidases, heme-copper oxidases and cytochrome *bd* (cyt *bd*) oxidases couple this exergonic chemical reaction to the generation of proton motive force. Differently from heme-copper oxidases, cyt *bd* is not a proton pump and creates membrane potential *via* transmembrane charge separation [1–7]. Cyt *bd* is widely distributed in prokaryotes where, along with its bioenergetic function, it performs a number of vital physiological functions. Cyt *bd* helps bacteria to survive under low oxygen pressure and other "stress" conditions [8–11]. It protects oxygen-labile enzymes by acting as oxygen scavenger [12–19]. Cyt *bd* supports disulfide bond formation upon protein folding [20] and provides the oxidizing power required in the penultimate step of heme biosynthesis [21]. Relevant to microbial resistance, cyt *bd* also contributes to hydrogen peroxide detoxification [22] and enhances microbial tolerance to nitric oxide [23–28]. Evidence for a positive correlation between virulence of bacterial pathogens responsible for various diseases and the expression level of cyt *bd* has been accumulated [8–10,29–34].

Cyt *bd* is an integral membrane protein with two different subunits carrying three redox-active centers, two iron protoporphyrins IX (hemes b_{558} and b_{595}) and one iron chlorin (heme *d*) (reviewed in [35–41]). The X-ray structure of cyt *bd* is not yet available, however it is suggested that all the heme groups are located closer to the periplasmic side of the membrane [42,43]. The low-spin hexa-coordinate heme b_{558} seems to be directly involved in the oxidation of the respiratory substrate, ubiquinol or menaquinol [44–48]. The high-spin heme *d* is the site where the oxygen molecule binds tightly to be subsequently reduced to water. The high-spin penta-coordinate heme b_{595} and heme *d* probably form a di-heme oxygen-reducing site somewhat analogous to the heme-Cu active site in heme-copper oxidases [3,49–56].

Cytochromes *bd* and heme-copper oxidases are suggested to share the key intermediates of the catalytic cycle. Based on previous reports [4,57–59], the cyt *bd* catalytic cycle (Fig. 1) is postulated to proceed through the following steps: R→OXY→P→F→R. Differently from

Abbreviations: cyt *bd*, cytochrome *bd*; DTT, dithiothreitol; Q_1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; O, ferric heme *d*; OXY, oxy-ferrous heme *d*; P, peroxy heme *d*; F, ferryl heme *d*; R, ferrous uncomplexed heme *d*

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heme-copper oxidases, the fully oxidized species ($b_{558}^{3+}b_{595}^{3+}d^{3+}$—OH) has been proposed not to be an intermediate of the cyt *bd* catalytic cycle [59], although it can be generated *in vitro* [2,28,60]. The uncomplexed species with both one ($b_{558}^{3+}b_{595}^{3+}d^{2+}$) and three ($b_{558}^{2+}b_{595}^{2+}d^{2+}$) electrons can be obtained under anaerobic conditions. The one-electron-reduced **OXY** species ($b_{558}^{3+}b_{595}^{3+}d^{2+}$—$O_2$) predominates in the enzyme isolated under aerobic conditions, as the affinity of ferrous heme *d* for oxygen is high [61,62]. A very short-lived three-electron reduced **OXY** species ($b_{558}^{2+}b_{595}^{2+}d^{2+}$–$O_2$) was observed in flow-flash studies [2,4,62,63]. In these studies, sequential formation of a transient peroxy complex (**P**, $b_{558}^{2+}b_{595}^{3+}d^{3+}$—OOH) [4,5] and ferryl species (**F**, $b_{558}^{3+}b_{595}^{3+}d^{4+}=O^{2-}$) [2,4,5,63] was also documented. A noticeable fraction of **F** is also seen in the "as-prepared" enzyme, i.e., the enzyme obtained at the end of the purification procedure. **F** can also be produced *in vitro* by addition of excess hydrogen peroxide to cyt *bd* in the **O** or **OXY** state [2,24,64,65].

In the present work, we studied the steady-state behavior of isolated cyt *bd* from *Escherichia coli* at ambient temperature using stopped-flow spectrophotometry and found that, differently from mammalian cytochrome *c* oxidase [66], in cyt *bd* the **OXY** and **F** catalytic intermediates are mostly populated under steady-state conditions.

2. Materials and methods

2.1. Chemicals and enzyme purification

DTT, Q_1, dithionite and *N*-lauroyl-sarcosine were from Sigma-Aldrich. Recombinant cyt *bd* was expressed in the *E. coli* strain GO105/pTK1 devoid of cytochrome bo_3 and cyt *bd* oxidases according to Ref [67]. Enzyme purification was carried out following the protocol described in Refs [68,69]. As isolated, the enzyme contains ferric hemes b_{558} and b_{595}, and heme *d* as a mixture of O_2-bound ferrous (**OXY**), ferryl (**F**) and most likely ferric (**O**) states. Cyt *bd* concentration was determined from the fully reduced-*minus*-"as isolated" difference absorption spectrum using $\Delta\epsilon_{628-607}=10.8\ mM^{-1}\ cm^{-1}$ [51].

2.2. Stopped-flow spectroscopy

Stopped-flow experiments were carried out with an instrument (DX.17MV, Applied Photophysics, Leatherhead, UK), equipped with a photodiode array detector (light path = 1 cm). Assays were performed at 20 °C in 50 mM K/phosphate buffer pH 7.3, 0.05% N-lauroyl-sarcosine. Typically, 20 μM "as isolated" cyt *bd* was degassed, anaerobically reduced with 10 mM DTT and 0.1 ÷ 1.2 mM Q_1, and mixed in a 1:1 ratio with O_2-equilibrated buffer (containing ~1.35 mM O_2). Afterwards, the reaction was followed up to 500 s by collecting 500 absorption spectra according to a logarithmic time scale, with an acquisition time of 4 ms/spectrum and a wavelength resolution of ~2.1 nm. To prevent possible photochemical artefacts, UV light in the incident beam was filtered in these experiments.

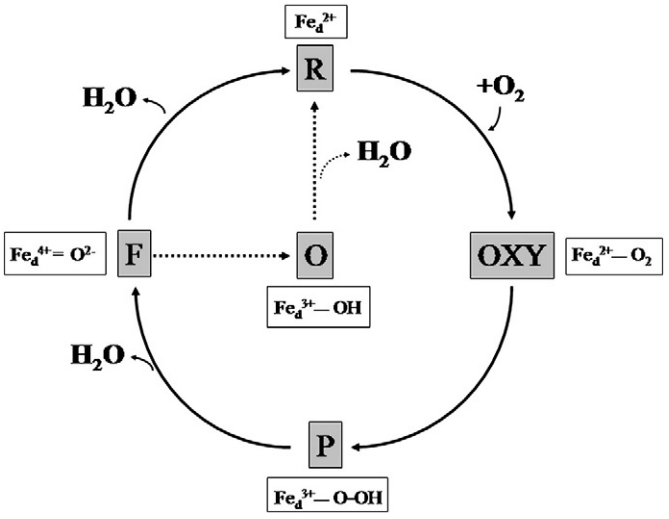

Fig. 1. Scheme of the catalytic cycle of cyt *bd* with the indication of the redox and the ligation state of heme *d* at the active site. Protonation and electronation reactions as well as hemes b_{558} and b_{595} are not shown for simplicity.

2.3. Spectral deconvolution analysis

Spectral deconvolution analysis was carried out using the "left matrix division" operator implemented in the software MATLAB (The Mathworks, South Natick, MA). Given a set of reference optical components, this operator yields the linear combination of reference spectra best-fitting an experimental spectrum in the least squares sense. Spectral deconvolution analysis was carried out after subtracting the spectrum of the fully reduced enzyme from the experimental set of time-resolved spectra, acquired after stopped-flow mixing reduced cyt *bd* with O_2-equilibrated buffer. For data analysis, the following reference difference spectra were utilized: 1) "heme b_{558}"=[Fe_{b558}^{3+}]-*minus*-[Fe_{b558}^{2+}]; 2) "heme b_{595}"=[Fe_{b595}^{3+}]-*minus*-[Fe_{b595}^{2+}]; 3) **"O"**=[Fe_d^{3+}]-*minus*-[Fe_d^{2+}]; 4) **"F"**=[$Fe_d^{4+}=O^{2-}$]-*minus*=[Fe_d^{2+}]; 5) **"OXY"**=[Fe_d^{2+}–O_2]-*minus*-[Fe_d^{2+}]. Spectra 1), 2) and 3) were taken from [70] and spectrum 4) from [64]. Spectrum 5) was obtained from the absorption changes observed during deoxygenation of the "as isolated" enzyme upon O_2 removal after addition of catalytic amounts of glucose oxidase in the presence of excess glucose.

3. Results

Peculiarly, due to the high O_2-affinity of ferrous heme *d* [61,62], cyt *bd* is not isolated as fully oxidized, but rather in a heterogeneous form in which the hemes b_{558} and b_{595} are oxidized and heme *d* is a mixture of ferrous oxygenated (**OXY**), ferryl (**F**) and likely ferric (**O**) states. The "as isolated" enzyme can be easily converted into the fully reduced uncomplexed state after addition of an excess of reductants (dithionite or DTT plus Q_1). The absorption spectra of the "as isolated" and fully reduced cyt *bd* exhibit well distinct, characteristic optical features (Fig. 2, top panel). Of relevance for the present study, the O_2-reactive heme *d* absorbs in the visible region at wavelengths (>600 nm) where the optical contributions of the hemes b_{558} and b_{595} are negligible. This makes cyt *bd* an ideal system to investigate by absorption spectroscopy the redox and ligation state of the O_2-reactive heme *d* and measure the steady-state occupancy of the catalytic O_2 intermediates during turnover.

Upon rapidly mixing reduced cyt *bd* with O_2-equilibrated buffer in the presence of excess DTT and Q_1 (5 and 0.6 mM after mixing, respectively), the enzyme during turnover undergoes noticeable absorption changes in the visible region, involving both hemes *b* and heme *d* (Fig. 2, bottom panel). By inspection of the raw data collected up to 500 s after mixing, it appears that part of the reaction occurs during the instrumental dead-time (a few ms), followed by at least three major kinetic phases: an oxidation phase (<100 ms), a steady-state phase (up to ~30 s), where by definition only minor absorption changes are observable, and finally after O_2-exhaustion a reduction phase that eventually restores the fully reduced enzyme. To acquire information on the catalytic intermediates populated during turnover and their actual occupancy, time-resolved spectral data in Fig. 2 were converted into difference spectra with reference to the fully reduced enzyme, i.e., the most homogeneous form of the enzyme that can be obtained experimentally. Afterwards, the contribution of each optical species was deconvoluted using the reference spectra depicted in Fig. 3A. These reference spectra include the oxidized-*minus*-reduced spectrum of the hemes b_{558} and b_{595} and the difference spectra of ferric (**O**), ferryl (**F**) and ferrous oxygenated (**OXY**) heme *d* relative to

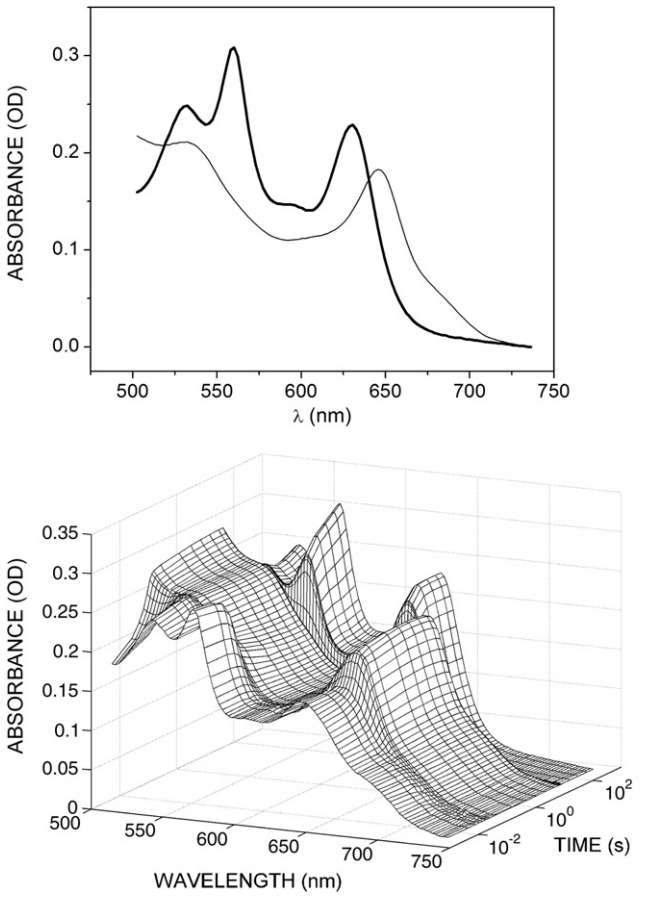

Fig. 2. *Top:* Absorption spectra of 10 μM cyt *bd*, "as isolated" (thin line) and in the fully reduced state (thick line). *Bottom:* Time-resolved absorption spectra collected up to 500 s after stopped-flow mixing O_2-equilibrated buffer with cyt *bd*, pre-reduced by DTT and Q_1. Concentrations after mixing: 10 μM enzyme; 5 mM DTT; 600 μM Q_1. T = 20 °C.

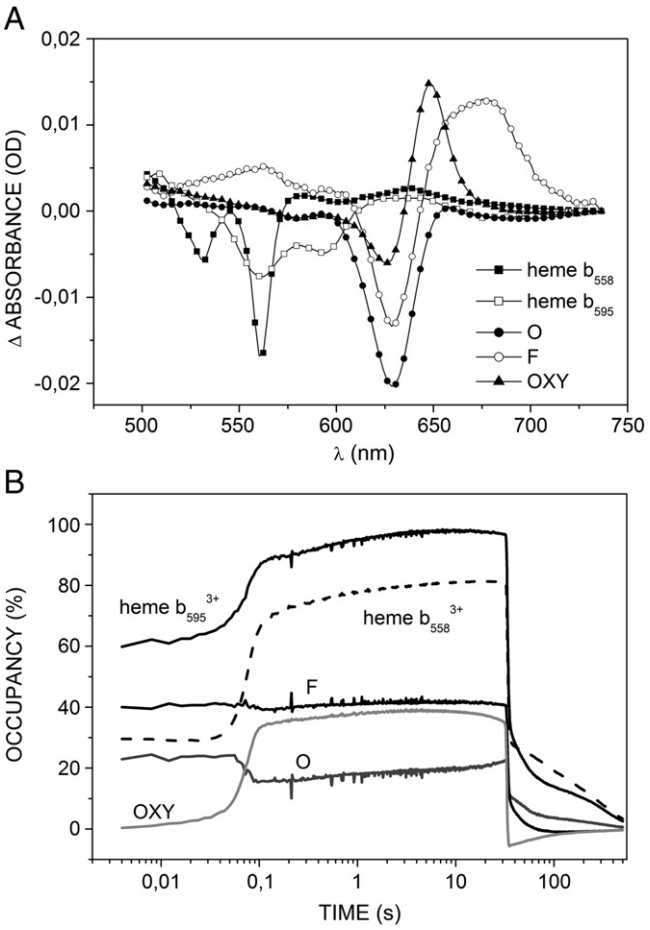

Fig. 3. A: Optical components (normalized to 1 μM enzyme): "heme b_{558}"=[Fe_{b558}^{3+}]-*minus*-[Fe_{b558}^{2+}]; "heme b_{595}"=[Fe_{b595}^{3+}]-*minus*-[Fe_{b595}^{2+}]; "**O**"=[Fe_d^{3+}]-*minus*-[Fe_d^{2+}]; "**F**"=[Fe_d^{4+}=O^{2-}]-*minus*-[Fe_d^{2+}]; "**OXY**"=[Fe_d^{2+}—O_2]-*minus*-[Fe_d^{2+}]. B: Percent occupancy of the optical species depicted in *A*, as obtained from the analysis of the spectra in the bottom panel of Fig. 2.

the reduced uncomplexed heme. It is worth to stress that the **F** species is the only one contributing at λ >670 nm and contribution appears important in amplitude. This makes detection of such a catalytic intermediate relatively straightforward, at least in comparison with the well known aa_3-type heme-copper respiratory oxidases in which, due to optical overlap between heme *a* and heme a_3, detection of the **F** intermediate under steady-state conditions is definitely more challenging.

Fig. 3B shows the output of the deconvolution analysis carried out on the data in Fig. 2 (bottom panel). According to this analysis, in the steady state phase (100 ms–30 s), hemes b_{558} and b_{595} are mostly oxidized (>70% and >90%, respectively), whereas up to 80% of heme *d* is almost equally distributed between the **F** and **OXY** states, with a residual ~ 20% ferric heme *d* (**O**). Both the pre-steady state phase and enzyme reduction following the steady-state phase proceed according to complex kinetic profiles. Both hemes b_{558} and b_{595} are oxidized to a significant extent within the stopped-flow dead-time and then adjust to their steady-state redox level within 100 ms. As expected, when O_2 vanishes (at ~30s), the steady-state phase ends and the enzyme eventually starts to be reduced, but full reduction is only achieved within 500 s, because there is a minor fraction of hemes b_{558} and b_{595} (~30% each) that is reduced only slowly. Despite kinetic complexity, it is important to notice that at steady-state the large majority of the enzyme exhibits heme *d* in the **F** and **OXY** state and this represents the main result obtained in the present study.

Since detection of these intermediates under steady-state is unprecedented, as a control we attempted to systematically fit spectral data with all heme *d* spectral components, but one. As shown in Fig. 4A, the steady-state spectrum collected at ~10 s (dotted) is nicely fitted if the complete set of reference spectra is linearly combined. The steady-state spectrum, however, cannot be adequately fitted when the reference spectrum of either **F** or **OXY** is omitted (Fig. 4B and C, respectively), and lower quality fits are obtained if the reference spectrum of the **O** species is excluded from the analysis (Fig. 4D). These results strongly suggest that all these intermediates are populated at a significant extent when the enzyme is in turnover with an excess of reductants and O_2.

The effect of Q_1 concentration on the steady-state occupancy of each intermediate was assayed by carrying out the described experiment at [Q_1] = 0.05, 0.15, 0.3 and 0.6 mM, keeping DTT concentration constant and equal to 5 mM. Fig. 5 shows the time course relative to each optical component, as obtained from spectral deconvolution of the data collected under different "reductive pressure," i.e., at different Q_1 concentrations. It may be appreciated that, as Q_1 concentration lowers, a faster pre-steady state oxidation of hemes b_{558} and b_{595} and a longer steady-state phase are observed; at the lowest Q_1 concentration assayed (0.05 mM), the steady-state phase was so long that exceeded the time window of observation (500 s). In contrast, no major changes were observed in terms of steady-state occupancy of each optical component by changing [Q_1]; only heme b_{558} modestly increased its steady-state oxidation level upon lowering Q_1 concentration (Fig. 5).

4. Discussion

The data clearly show that in the Q_1H_2/O_2 oxidoreductase reaction catalyzed by *E. coli* cyt *bd* oxidase under near-physiological

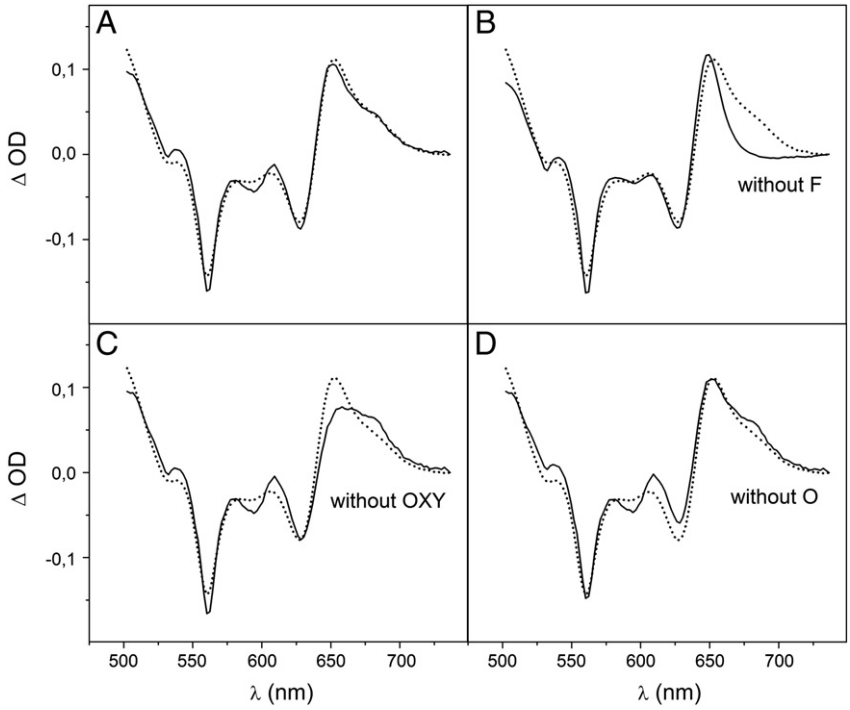

Fig. 4. Best fit of the steady-state spectrum (dotted) acquired at 10 s after mixing reduced cyt *bd* with O_2-equilibrated buffer (experimental conditions as in Fig. 2). Fitting was carried out by using the whole optical components set (panel A) or the same set without the reference spectrum of **F** (panel B), **OXY** (panel C) or **O** (panel D) species.

conditions, the **OXY** and **F** species are the most populated steady-state turnover intermediates (~40% each). The results are in agreement with catalytic cycle models proposed earlier [4,57–59] (see also Fig. 1). The data also validate the intermediates observed by investigating the reaction of the fully reduced cyt *bd* with oxygen in flow-flash single turnover experiments, i.e. under somewhat artificial, non-physiological conditions [2,4,5,63]. Moreover, detection of the **OXY** and **F** intermediates at steady-state is consistent with the observation that, in the 'as-prepared' isolated enzyme or in cyt *bd*-containing native bacterial membranes, most of heme *d* exhibits the spectral features characteristic of the **OXY** and **F** states.

Overall, the data are compatible with the proposal by Yang et al. [59] that the intermediates populated in the cyt *bd* catalytic cycle are characterized by an odd number of electrons relative to the fully ferric form (**O**, $b_{558}^{3+}\, b_{595}^{3+}\, d^{3+}$—OH), i.e., that the reaction cycle runs through the three-electron reduced species (**R**$^{\mathbf{3}}$, $b_{558}^{2+}\, b_{595}^{2+}\, d^{2+}$), the single-electron reduced O_2-bound species (**OXY**, $b_{558}^{3+}\, b_{595}^{3+}\, d^{2+}$—$O_2$) and a one-electron deficient ferryl intermediate (**F,** $b_{558}^{3+}\, b_{595}^{3+}\, d^{4+}=O^{2-}$). The steady-state **OXY** and **F** species observed in our experiments most likely are the above-mentioned intermediates, and it is not surprising that, based on its high reactivity with O_2, **R**$^{\mathbf{3}}$ is not populated at detectable levels in steady-state.

At the same time, the analysis shows that in addition to **OXY** and **F**, enzyme species with ferric heme *d* (**O**) are also populated up to ~20% at steady-state (Figs. 3B and 5). At first glance, this finding may appear inconsistent with the proposal that the fully oxidized enzyme is not involved in the cyt *bd* catalytic cycle under physiological conditions [59]. However, analysis of the redox state of hemes *b* shows that at steady-state around 20% cyt *bd* contains reduced heme b_{558} (Figs. 3B and 5). In principle, this fraction of the enzyme may contain heme *d* in any of the states detected at steady-state (**OXY**, **F** or **O**). On the other hand, enzyme species with **F** or **OXY** heme *d* and reduced heme b_{558} have a little chance to accumulate in turnover. Like in heme-copper oxidases, the **F** intermediate of cytochrome *bd* most likely has a very high redox potential (~ +1 V), therefore the **F** species with an electron located on heme b_{558} is expected to be very unstable and quickly convert into all-ferric form of the enzyme, provided that protons are available:

$$b_{558}^{2+}\, b_{595}^{3+}\, d^{4+}=O^{2-}(\mathbf{F})\rightarrow b_{558}^{3+}\, b_{595}^{3+}\, d^{3+}\text{–OH}(\mathbf{O})$$

Similarly, the **OXY** species with an electron located on heme b_{558} would also be unstable, yielding the **P** state:

$$b_{558}^{2+}\, b_{595}^{3+}\, d^{2+}\text{–}O_2(\mathbf{OXY})\rightarrow b_{558}^{3+}\, b_{595}^{3+}\, d^{3+}\text{–OOH}(\mathbf{P})$$

Hence we conclude that the species with reduced heme b_{558} observed in turnover has the following structure: $b_{558}^{2+}\, b_{595}^{3+}\, d^{3+}$—OH (herein referred to as '**O**$^{\mathbf{1}}$'). Based on this finding, we propose a modified scheme of the catalytic cycle of cyt *bd*, that includes such a species (Fig. 6). Why in the presence of excess O_2 does **O**$^{\mathbf{1}}$ accumulate at a detectable level (~20%) rather than being quickly converted into the one-electron **OXY** species?

$$\left(b_{558}^{2+}\, b_{595}^{3+}\, d^{3+}\text{–OH} + O_2\rightarrow b_{558}^{3+}\, b_{595}^{3+}\, d^{2+}\text{–}O_2 + H_2O\right)$$

A possible explanation is that the electron transfer from heme b_{558} to ferric heme *d* may be rate-limited due to reorganization of the heme *d* coordination sphere. The reorganization may comprise conversion of the heme-bound hydroxyl group into water or, alternatively, binding/replacement of an endogenous *protein* ligand at heme *d*. The latter scenario would be consistent with the proposal that, upon enzyme reduction, the heme *d* iron binds an endogenous π-acceptor protein ligand [71]. Further work is needed to clarify this issue.

In this study, we also tried to estimate a possible contribution of the **P** species to the overall population of the steady-state catalytic intermediates. As a model we attempted to use the kinetic spectrum of **P** formation as obtained earlier by flow-flash [4]. The analysis

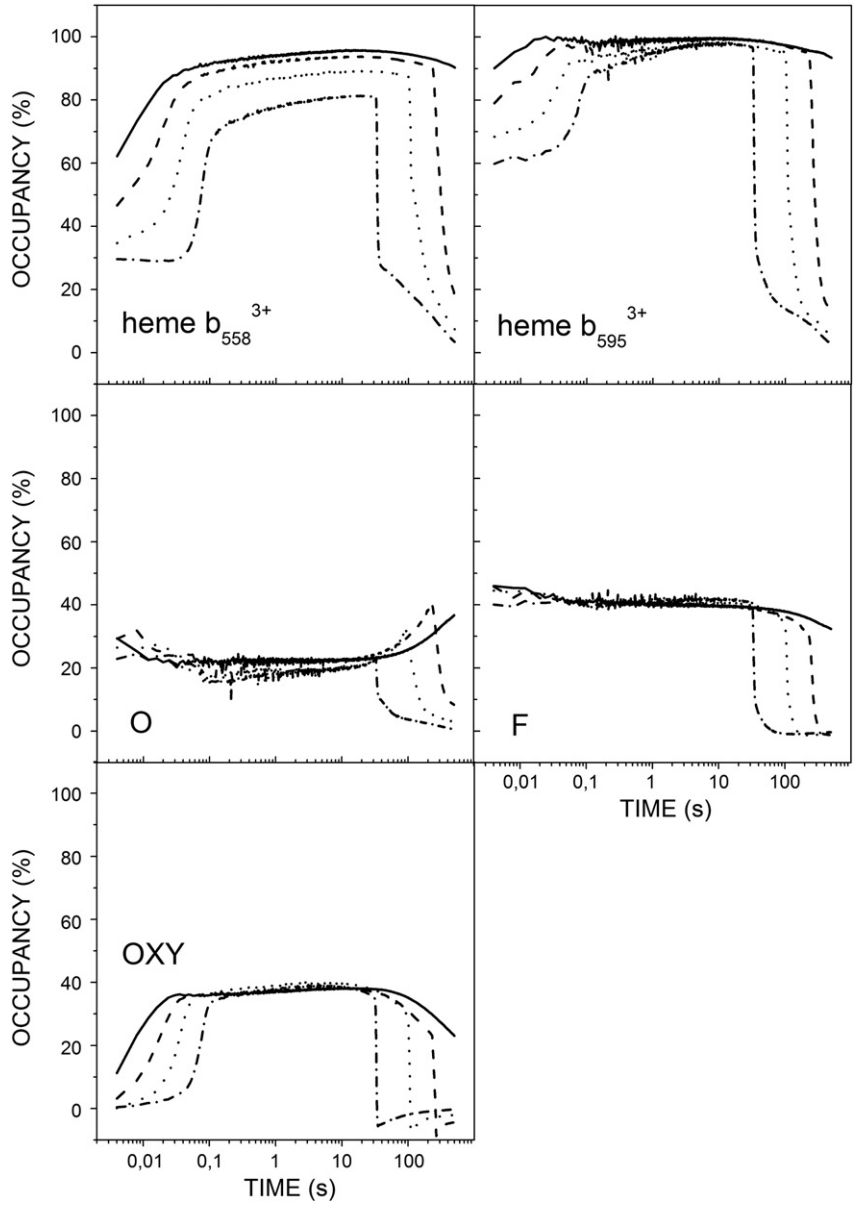

Fig. 5. Percent occupancy of each optical component as measured with cyt *bd* in turnover at increasing [Q_1]. Concentrations after mixing: 10 μM enzyme; 5 mM DTT; 50 μM (solid), 150 μM (dashed), 300 μM (dotted) or 600 μM (dash-dotted) Q_1.

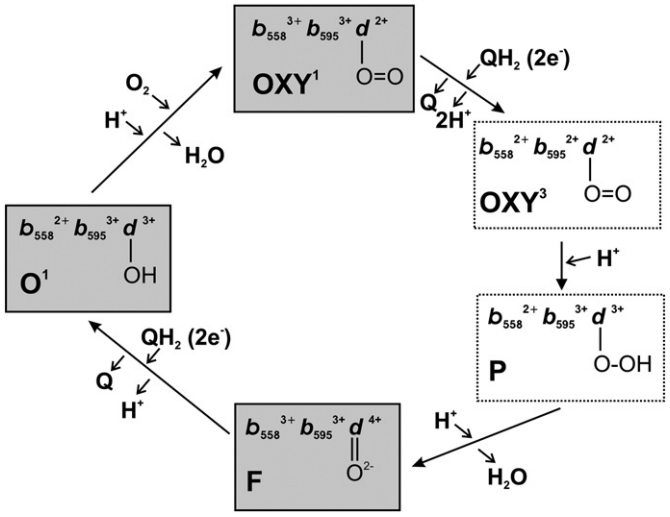

Fig. 6. Plausible scheme of the cyt *bd* catalytic cycle based on the results of the present study.

suggests that the **P** species likely does not contribute significantly at steady-state (data not shown). This is not surprising because **P** quickly decays to the **F** intermediate, without requiring electron supply from quinol (Fig. 6).

Finally, it is worth to notice that the results herein achieved with cyt *bd* clearly differ from those obtained earlier under similar conditions with mammalian cytochrome *c* oxidase, using cytochrome *c* as the electron donor [66]. In that study it was shown that the steady-state occupancy of the oxygen intermediates, particularly of the two different ferryl forms (called **P** and **F**), is very low (<10%, [66]). This may be due to differences in both the nature of the utilized respiratory substrates (cytochrome *c versus* ubiquinol) and/or intrinsic structural-functional properties of the two enzymes.

5. Conclusions

We have shown that, when cyt *bd* from *E. coli* is in turnover with ubiquinol and O_2, the enzyme intermediates detected at steady-state are the **OXY** and **F** species (~40% each) with oxidized hemes *b* and, to

a lesser extent (~20%), a species with ferric heme *d* and possibly one electron on heme b_{558} ($\mathbf{O^1}$).

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