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Oxygenated complex of cytochrome *bd* from *Escherichia coli*: Stability and photolability

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Abstract **Cytochrome *bd* is one of the two terminal ubiquinol oxidases in the respiratory chain of *Escherichia coli* catalyzing reduction of O_2 to H_2O. The enzyme is expressed under low oxygen tension; due to high affinity for O_2 it is isolated mainly as a stable oxygenated complex. Direct measurement of O_2 binding to heme *d* in the one-electron reduced isolated enzyme gives $K_{d(O_2)}$ of ~280 nM. It is possible to photolyse the heme *d* oxy-complex by illumination of the enzyme for several minutes under microaerobic conditions; the light-induced difference absorption spectrum is virtually identical to the inverted spectrum of O_2 binding to heme *d*.**
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Keywords: Heme *d*; Oxygen affinity; Photolysis; Microaerobic condition; Respiratory chain

1. Introduction

Under different growth conditions, *Escherichia coli* cells produce two quinol oxidases cytochrome bo_3 and cytochrome *bd* [1,2]. Cytochrome bo_3 predominates under normal aerobic conditions, whereas cytochrome *bd* is expressed under low oxygen tension [1,2]. The *E. coli* cytochrome *bd* reveals two peculiar features related to its interaction with O_2. First, unlike cytochrome bo_3 and other heme-copper terminal oxidases, cytochrome *bd* is isolated predominantly as a stable oxygenated complex ($b_{558}^{3+}b_{595}^{3+}d^{2+}$-$O_2$) characterized by an absorption band near 650 nm [3–5]. This unusual feature may be attributed to high affinity of the enzyme for oxygen. The previous studies of the affinity of the *E. coli* cytochrome *bd* for O_2 were mainly limited to measurements of the value for 'operational oxygen affinity' ($K_{m(O_2)}$). The reported $K_{m(O_2)}$ values vary 1000-fold (from 3 nM to 2 μM) depending on the nature and concentration of the primary substrate, the experimental setup and other factors [6–10]. However, the use of the Michaelis–Menten constant (K_m) as a measure of the enzyme affinity for substrate, i.e. the assumption that $K_m = K_d$ may often be incorrect [11]. For example, the K_d and apparent K_m can differ more than 100-fold for the bo_3-type oxidase [12]. The only determination of the $K_{d(O_2)}$ value for the *bd* oxidase was made by Hill et al. [13]. In that work, $K_{d(O_2)} = 25$ nM was obtained indirectly using the values for forward and reverse rate constants of oxygen reaction from different experiments [13].

Peculiarly, the cytochrome *bd* oxy-complex has proved uniquely photostable. All the attempts to photolyse it under aerobic conditions turned out to be unsuccessful. O_2 photodissociation from heme *d* could not be detected even with UV-excitation and with ps time resolution [14]. It was concluded that, in the one-electron reduced cytochrome *bd*, the quantum yield of O_2 dissociation from heme *d* is zero [14]. This is in contrast to the CO adduct of heme *d* that can be easily photodissociated under the same conditions [14,15].

In this work, we have measured directly the $K_{d(O_2)}$ by titrating the deoxygenated one-electron reduced cytochrome *bd* with O_2. In addition, we were able to photolyse the oxy-complex of heme *d* by continuous illumination at low oxygen concentration.

2. Materials and methods

Cytochrome *bd* from *E. coli* strain GO105/pTK1 was isolated as described [16], with modifications reported in [17]. Its concentration was determined from the reduced *minus* 'air-oxidized' difference absorption spectra using $\Delta\varepsilon_{628-607}$ of 10.8 mM^{-1} cm^{-1} [18].

Absorption measurements were performed at room temperature as previously reported ([19] and references therein) using a diode array spectrophotometer made by Unisoku Instruments (Kyoto, Japan). The sample was placed in a rectangular gas-tight fluorescence-type cuvette (total volume of ~14.6 mL, sample volume of 0.35 mL, internal dimensions of 4 × 10 mm), equipped with the joints for attachment to a vacuum/gas line. The assays were performed in 100 mM Mops-KOH buffer (pH 7.0) containing 0.05% *N*-lauroyl-sarcosine.

As prepared, aerobic cytochrome *bd* is mainly in a form with O_2 bound to ferrous heme *d*. To determine $K_{d(O_2)}$, a one-electron reduced oxygen-free form of the enzyme was prepared first. This was done by purging the sample repeatedly with argon; using the vacuum/gas line, the atmosphere in the cuvette was replaced with oxygen-free argon, and the cuvette was then gently rocked to equilibrate the sample with the new gas phase. After several cycles, removal of oxygen from heme *d* could be observed by a shift of heme *d* absorbance from 645 to 629 nm. Titration of anaerobic one-electron reduced enzyme was then performed with increasing amounts of air added as a gas or air-equilibrated water, assuming that oxygen concentrations in air and in water at 21 °C and 1 atm are 8.6 mM and 278 μM, respectively [20]. A typical experiment was performed as follows. An aliquot of air was added to the bottom of the cuvette (liquid phase) by a gas-tight microsyringe with a long needle against argon flux. Then the cuvette was shaken until the equilibrium between the liquid and gas phases was established judged by the intensity of 648 nm band, i.e, oxygen was distributed between the two phases according to partition

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coefficient of ~31. As the O_2 concentration and the volume of the gas phase in the cuvette were much larger than those of the liquid phase, the gas phase over the sample served as oxygen buffer to maintain the concentration of O_2 in the liquid phase.

The $K_{d(O_2)}$ value was determined according to the simple reaction scheme:

$$d^{2+} + O_2 \Longleftrightarrow d^{2+} - O_2$$

The model leads to the following equation:

$$[d^{2+} - O_2] = \frac{[O_2]}{(K_d + [O_2])},$$

where $[d^{2+}-O_2]$ is a fraction of the heme *d* oxy-complex, and $[O_2]$ is concentration of free (unbound) oxygen dissolved in the sample solution.

Prior to the photolysis experiments, concentration of free O_2 dissolved in the sample solution was reduced to ~800 nM. The sample was photolysed with the focused beam from a 150-W xenon lamp and absorption spectra were collected during the illumination in a diode array kinetic spectrophotometer (Unisoku Instruments, Kyoto, Japan).

MATLAB (The Mathworks, South Natick, MA) was used for data procession and presentation.

3. Results

Oxygenated cytochrome *bd* can be converted into the single-electron reduced oxygen-free species by sequential exchange the head space of the air-equilibrated sample, with argon atmosphere. Deoxygenation can be seen as shift of the heme *d* α band maximum from 648 to 626 nm, in agreement with an earlier report [3]. The heme *d* oxy-complex can be regenerated by successive addition of aliquots of oxygen to the deoxygenated enzyme (Fig. 1) and the [oxy-complex] vs $[O_2]$ dependence obtained can be used to determine directly the $K_{d(O_2)}$ for the heme $d^{2+}-O_2$ complex. Data in Fig. 1B show that increase in the O_2 concentration added to the anaerobic one-electron reduced cytochrome *bd* is accompanied by increase in magnitude of the oxygen-induced difference absorption spectra. To determine $K_{d(O_2)}$, the O_2 induced absorption changes (a maximum at 648 nm *minus* a minimum at 626 nm) were plotted as a function of the O_2 concentration and the data were fitted according to the equation (see Section 2) yielding the $K_{d(O_2)}$ value of ~280 nM (Fig. 2).

In the course of continuous illumination of oxygenated cytochrome *bd* with white light under microaerobic conditions ($[O_2] \sim 800$ nM), absorbance at 648 nm decreases, whereas absorbance at 626 nm increases (Fig. 3A). The spectrum of these changes is virtually identical to the inverted difference spectrum of oxygen binding to heme *d* (Fig.3B). Therefore, we can conclude that the light-induced absorption spectrum corresponds to photodissociation of O_2 from heme *d*. The photostationary state is achieved within 3 min after the onset of illumination. The 3-min illumination induces only minor, if any, reduction of heme(s) *b* as evidenced by the absence of significant absorption changes around 560 nm (Fig. 3B). The light-induced absorption changes were reversed following equilibration between the liquid and gas phases of the sample upon shaking in the dark. The control incubation of the same sample for 3 min but without illumination does not result in any significant spectral changes (not shown).

4. Discussion

The *E. coli* cytochrome *bd* is isolated as a mixture of oxygenated (at least 70%), ferryl-oxo (10–20%) and ferric (5–10%) forms. A possibility to generate a stable oxy-complex by reversible binding of oxygen to the one-electron reduced enzyme [3] is a unique feature of cytochrome *bd* oxidase and can be used for direct measuring its $K_{d(O_2)}$. This is not the case for any of the heme-copper oxidases, such as mitochondrial cytochrome *c* oxidase, where $K_{d(O_2)}$ can only be indirectly determined [21,22]. The $K_{d(O_2)}$ value obtained in this study by titration of the anaerobic one-electron reduced *E. coli* cyto-

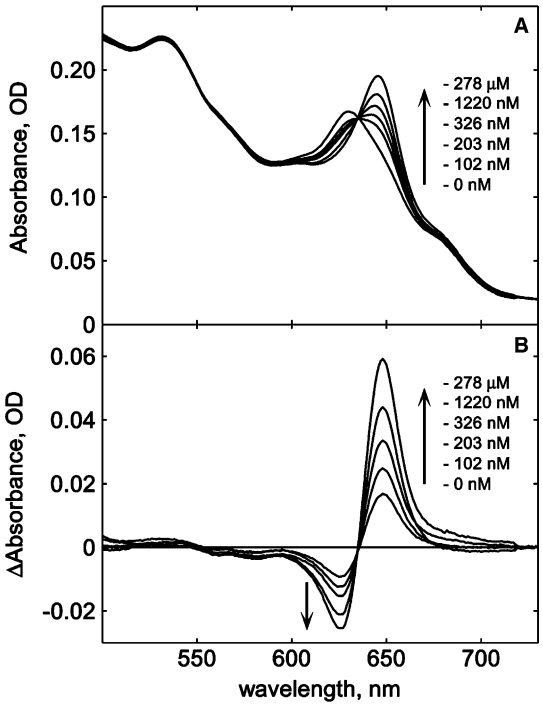

Fig. 1. Oxygen binding to the one-electron reduced cytochrome *bd*. The enzyme (23.8 μM) made anaerobic by argon-equilibration was titrated with O_2. (A) Absolute absorption spectra. The arrow shows the direction of optical changes corresponding to increase in $[O_2]$. (B) Difference between the spectra after addition of O_2 and the anaerobic spectrum. The arrows show that ΔA_{648} increases and ΔA_{626} decreases as $[O_2]$ increases.

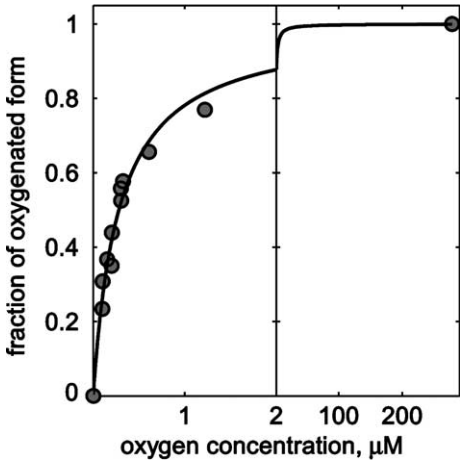

Fig. 2. Oxygen concentration dependence of the O_2-induced spectral changes. Absorbance increase at 648 *minus* 626 nm was measured and plotted versus $[O_2]$-equilibrated. The experimental data (filled circles) were fitted (solid line) according to the equation (see Section 2).

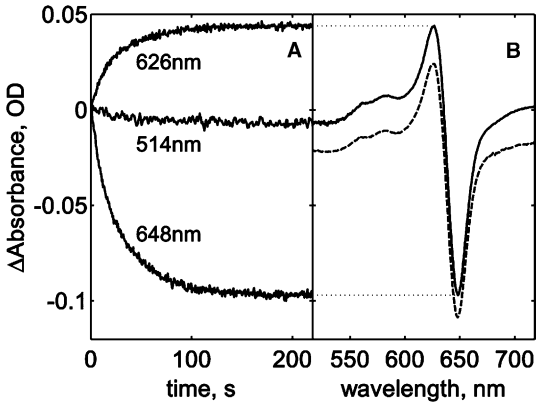

Fig. 3. Absorption changes induced by oxygen photodissociation from the one-electron reduced cytochrome *bd*. (A) Kinetics of absorption changes at 648, 514 and 626 nm during illumination. (B) Photodissociation spectrum (solid line): 3 min after the onset of illumination *minus* before the photolysis. Inverted spectrum of O_2 binding (dashed line) has been normalized to the amplitude of photodissociation spectrum.

chrome *bd* with O_2 appeared to be ~280 nM (Fig. 2). Thus, the oxygen affinity of the *E. coli* cytochrome *bd* is about 3-times higher than that of sperm whale myoglobin (~880 nM), another hemoprotein forming a stable oxy-complex [23], and two to three orders of magnitude higher than that of cytochrome *c* oxidase (40–280 μM) [21,22]. The *E. coli* cytochrome *bd* is actually a high O_2 affinity oxidase.

Because of the large discrepancy among the $K_{m(O_2)}$ values reported for the *E. coli* cytochrome *bd* [6–10], it is still an open question whether this oxidase is designed to trap O_2 kinetically by reducing it to ferryl-oxo species ($K_{d(O_2)} > K_{m(O_2)}$) like cytochrome *c* oxidase and cytochrome bo_3 [12,22], or for thermodynamic trapping of oxygen ($K_{d(O_2)} < K_{m(O_2)}$) as proposed in [13]. Conceivably, both factors may contribute to the high efficiency of cytochrome *bd* reaction with oxygen. Additional work is necessary to elucidate this issue in sufficient detail.

The $K_{d(O_2)}$ value measured in this work (280 nM) is about 10-fold higher than previously reported (25 nM) [13]. The latter was calculated from the experimentally obtained forward (k_1) and reversed (k_{-1}) rate constants ($K_d = k_{-1}/k_1$), the value used for k_{-1} was obtained from a ligand-exchange (O_2/CO) experiment but the data were not shown [13]. We do not have a reasonable explanation for this discrepancy. Obviously, further work on the k_{-1} determination has to be performed under different experimental conditions and, if the dissociation rate of the heme *d* oxy-complex is determined by measuring its reactivity with CO, it is important to assure that exchange of the ligands is complete.

Although O_2 bound to the 'as isolated' cytochrome *bd* can be reversibly removed by purging the sample with argon, attempts to photolyse the heme d^{2+}–O_2 bond in the mixed-valence enzyme under aerobic conditions were not successful. The conclusion was made that O_2 cannot be photodissociated from the mixed-valence cytochrome *bd* at all, and that the quantum yield for heme *d*–O_2 photodissociation is zero [14]. This is in contrast to the studies of O_2 photodissociation from myoglobin and some synthetic oxy-hemes where the light-induced release of O_2 was observed [24–27]. In this work we found that, under microaerobic conditions ($[O_2] \sim 800$ nM), illumination of the oxy-complex of cytochrome *bd* induces the ΔA_{648} decrease and the ΔA_{626} increase (Fig. 3A). The photoinduced difference absorption spectrum is virtually identical with the inverted difference spectrum of O_2 binding to heme *d* (Fig. 3B).Therefore it appears to be that, under microaerobic conditions it is possible to photolyse the heme *d* oxy-complex with continuous illumination.

The finding is in contradiction with the data in [14]. At the moment, there is no obvious clear explanation for the discrepancy. An important detail is that the photolysis could have been observed in this work at low but not at high oxygen concentration; notably, the experiments in [14] were carried out at high concentrations of O_2. It would be of great interest to repeat the ultrafast experiments on *d*–O_2 photodissociation [14] at microaerobic conditions. The faster rate of oxygen recombination is unlikely to account for the effect of high O_2 concentration, since the rate of geminate recombination that might have been observed in the ultrafast measurements [14] (cf. experiments on CO photodissociation) should not depend on ambient O_2 concentration. Rather, the oxygen concentration effect implies an existence of a cavity near heme *d* that serves as a second low-affinity oxygen-binding site. During the photolysis, the cavity provides the space for the initial transient accomodation of the O_2 photodissociated from heme *d*; an evidence for such a transient intraprotein binding site has been reported in [28]. The low-affinity site may be expected to be empty under our microaerobic conditions thus allowing for oxygen photodissociation, whereas at high O_2, the cavity will be occupied by dioxygen locking the photoexcited O_2 at heme *d*. A similar model has been invoked recently to explain the decreased yield of NO photodissociation from heme a_3 in cytochrome *c* oxidase [29]. It is noted that, the low-affinity O_2 binding site can not be a b_{595} heme, as this would be clearly resolved by absorption spectra.

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