Cytochrome *bd* from *Azotobacter vinelandii*: Evidence for High-Affinity Oxygen Binding†

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ABSTRACT: Cytochrome *bd* from *Azotobacter vinelandii* is a respiratory quinol oxidase that is highly efficient in reducing intracellular oxygen concentration, thus enabling nitrogen fixation under ambient aerobic conditions. Equilibrium measurements of O_2 binding to ferrous heme *d* in the one-electron-reduced form of the *A. vinelandii* enzyme give $K_{d(O_2)} = 0.5\ \mu M$, close to the value for the *Escherichia coli* cytochrome *bd* (*ca.* $0.3\ \mu M$); thus, both enzymes have similar, high affinity for oxygen. The reaction of the *A. vinelandii* cytochrome *bd* in the one-electron-reduced and fully reduced states with O_2 is extremely fast approaching the diffusion-controlled limit in water. In the fully reduced state, the rate of O_2 binding depends linearly on the oxygen concentration consistently with a simple, single-step process. In contrast, in the one-electron-reduced state the rate of oxygen binding is hyperbolic, implying a more complex binding pattern. Two possible explanations for the saturation kinetics are considered: (A) There is a spectroscopically silent prebinding of oxygen to an unidentified low-affinity saturatable site followed by the oxygen transfer to heme *d*. (B) Oxygen binding to heme *d* requires an "activated" state of the enzyme in which an oxygen channel connecting heme *d* to the bulk is open. This channel is permanently open in the fully reduced enzyme (hence no saturation behavior) but flickers between the open and closed states in the one-electron-reduced enzyme.

Cytochrome *bd* is a respiratory quinol oxidase widely spread in bacteria which reduces O_2 to H_2O (*1*−*5*). It shows no apparent sequence homology to the heme-copper terminal oxidases (*6*, *7*) and has no copper (*8*). Cytochrome *bd* conserves energy by transmembrane charge separation (*9*, *10*) but, unlike heme-copper oxidases, it is not a proton pump (*11*).

The enzyme contains three hemes: a low-spin b_{558} and two high-spin b_{595} and *d*. Heme b_{558} is believed to be the electron entry site directly accepting electrons from mena- or ubiquinol (*12*, *13*). Heme *d* is the site where O_2 is first trapped and then reduced to water. The role of heme b_{595} is not fully understood, but there is evidence that it may, together with heme *d*, form a di-heme site for O_2 reduction (*10*, *14*−*19*). Although the X-ray structure of cytochrome *bd* is not available, conventional studies of the protein topology in the membrane suggest that all three hemes are located near the periplasmic side of the membrane (*20*, *21*).

Apart from its obvious role in energy conservation, cytochrome *bd* has been shown to carry out many other physiological functions in bacteria. The enzyme is preferentially expressed under unfavorable ('stressful') conditions (*1*), particularly those associated with inadequate oxygen supply. Cytochrome *bd* supports disulfide bond formation upon protein folding catalyzed by the DsbA−DsbB system (*22*). In *Azotobacter vinelandii*, cytochrome *bd* is required for the "respiratory protection" during aerotolerant N_2 fixation (*23*). Recent investigations show that expression of cytochrome *bd* is prerequisite for virulence of pathogenic microorganisms (*24*, *25*): first, the enzyme increases the capability to colonize O_2-poor environments during infection (*26*−*28*); second, the *bd*-type oxidases help pathogenic bacteria to overcome host immune response mediated by NO production. The unusually high rate of NO dissociation from cytochrome *bd* may explain in part fast recovery of the NO-inhibited bacterial respiration and enhanced bacterial tolerance to the nitrosative stress (*29*, *30*).

A peculiar feature of *bd*-type oxidases is that they are purified mainly as a stable oxygenated (oxy-) complex ($b_{558}^{3+}b_{595}^{3+}d^{2+}-O_2$) characterized by an absorption peak at 645−650 nm (*31*−33). Such a stability of the oxy-complex points to high affinity of heme d^{2+} for oxygen. The data on the apparent oxygen affinity of the *A. vinelandii bd*-type

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oxidase as inferred from the measurements of the Michaelis−Menten constant for oxygen, $K_{m(O_2)}$, are controversial. In some works the oxygen affinity was found to be much lower than that for the *Escherichia coli* enzyme ($K_{m(O_2)}$ values of 4.5 μM and 5 nM, respectively) (*34*, *35*), whereas rather close $K_{m(O_2)}$ values (4−6 μM vs 2 μM) have been reported by other authors (*36*, *37*). Biosynthesis of the *A. vinelandii* oxidase is elevated at high aeration (*38*, *39*), whereas the *E. coli* enzyme is expressed maximally under microaerobic conditions (*40*, *41*). The remarkable difference in the regulation of expression of these two enzymes was thought to be consistent with the strikingly different $K_{m(O_2)}$ values reported by D'mello et al. (*34*, *35*). Accordingly, the cytochromes *bd* from *E. coli* and *A. vinelandii* have been often referred in the literature as the high- and low-affinity oxidases, respectively (*42*−*44*), despite their similarity in amino acid sequence and protein topology (*45*−*47*), and at variance with the similar $K_{m(O_2)}$ values reported in refs *36* and *37*.

It has to be pointed out that the use of the Michaelis−Menten constant (K_m[1]) as a measure of the enzyme affinity for a substrate may often be misleading (i.e., the assumption that $K_m = K_d$ is not always correct (*48*)). For instance, in cytochrome bo_3, the K_d and the apparent K_m values differ by more than 100-fold (*49*).

In this work, we measured some basic thermodynamic and kinetic parameters for the reaction of cytochrome *bd* from *A. vinelandii* with oxygen and compared them to those of the *E. coli* enzyme.

MATERIALS AND METHODS

Cytochrome *bd* from *E. coli* strain GO105/pTK1 was isolated as described (*50*), with modifications reported in ref *10*, and its concentration was determined from the dithionite-reduced minus air-oxidized difference absorption spectrum using $\Delta\epsilon_{628-607}$ of 10.8 mM^{-1} cm^{-1} (*16*). Cytochrome *bd* from *A. vinelandii* strain MK8 was isolated as reported in ref *51*. The heme *d* concentration in the *A. vinelandii* enzyme was determined from the dithionite-reduced minus air-oxidized difference absorption spectrum using $\Delta\epsilon_{628-605}$ of 9.5 mM^{-1} cm^{-1}; the latter value was used instead of 12 mM^{-1} cm^{-1} reported earlier (*51*). With the new extinction coefficient, the normalized spectrum for the α-band of the *A. vinelandii* ferrous heme *d* is virtually identical to the corresponding spectrum of the *E. coli* enzyme (not shown).

Determination of $K_{d(O_2)}$ was performed essentially as described (*52*). The sample was placed in a gastight fluorescence-type cuvette (total volume, ~11.75 mL; sample volume, 0.5 mL; internal dimensions, 4 × 10 mm) equipped with joints for the attachment to the vacuum/gas line. Absorption spectra were collected using a visible-range spectrophotometer HR2000+ (Ocean Optics Inc., Dunedin, FL). Oxygen concentration in air and water at +21 °C and 1 atm was assumed to be 8.6 mM and 278 μM, respectively (*53*).

Time-resolved spectrophotometric measurements were performed using a home designed CCD-based instrument (*54*). A pulsed 150 W xenon arc lamp (Applied Photophysics, Leatherhead, Surrey, U.K.) was used as the probe light source. Light from the lamp was passed through fibers to a stopped-flow module (SFM-300, Bio-Logic, Grenoble, France) equipped with a fluorescence cuvette (TC-100/10F, optical path 1 cm) with the sample. The light was further directed at a compact imaging spectrograph (Triax-180, HORIBA Jobin Yvon, Edison, NJ), which delivers spectral imaging over a fast kinetic CCD matrix (DV420-UV-FK, Andor Technology, Belfast, Northern Ireland). The setup allows continuous recording of absorption changes as consecutive spectra with a delay of 1 μs between the spectra.

In the flow−flash experiments, cytochrome *bd* in the mixed-valence (one-electron-reduced, **MV**) or fully reduced (**R**) state was prepared in a gastight cuvette. Deoxygenated **MV** enzyme was obtained by equilibration with argon gas using the vacuum/gas line. To obtain the **R** enzyme, the deoxygenated **MV** enzyme was further reduced under anaerobic conditions with 2.5 mM sodium ascorbate and 5 μM TMPD. Then the enzyme in either the **MV** or **R** state was equilibrated with 1% CO/99% argon mixture. After formation of the cytochrome *bd*−CO complex, the sample was transferred into the stopped-flow module in an argon-flushed gastight syringe (Hamilton, Reno, NV), and then mixed with O_2-enriched water. Desired concentrations of O_2 were achieved by varying the volume ratio of anaerobic (0 μM O_2), air-saturated (278 μM O_2), and oxygen-saturated (1.32 mM O_2) aliquots of water. CO photolysis was initiated by a laser flash (Brilliant B, Quantel, Les Ulis, France; frequency-doubled YAG, 532 nm, pulse energy, 120 mJ).

The spectroelectrochemical redox titrations of cytochrome *bd* were carried out in an optically transparent, thin layer electrode (OTTLE) cell as described (*55*). Potentials within a range of −100 to +450 mV *versus* NHE were set with 20 mV steps during both oxidative and reductive titration using a potentiostat (PAR 263A, Princeton Applied Research, Oak Ridge, TN). At each potential step, the onset of equilibrium on the working electrode was determined. Two layers of gold minigrid (300 lpi, each layer with a 70% transmittance, Buckbee-Mears Europe GmbH, Germany) served as the working electrode. A platinum wire immersed in 3 M KCl and a saturated Ag/AgCl half-cell served as the counter and reference electrodes, respectively. Hexaammineruthenium (E_m = +50 mV), pentaaminepyridineruthenium (E_m = +250 mV), and ferrocenyl ethanol (E_m = +430 mV), all at 200 μM, were present as redox mediators. All redox potentials quoted refer to NHE.

Instrumental software for experimental setups was developed and written by N. Belevich (Helsinki, Finland). All measurements were performed at +21 °C. MATLAB (The Mathworks, South Natick, MA) was used for data analysis.

RESULTS

Equilibrium Oxygen Binding. Cytochrome *bd* from *A. vinelandii* is isolated predominantly as a stable oxygenated complex of the **MV** state of the enzyme ($b_{558}^{3+}b_{595}^{3+}d^{2+}$−$O_2$), similarly to the *E. coli* enzyme (*52*). The oxygenated complex can be converted reversibly into the one-electron-reduced, oxygen-free form by purging the sample repeatedly with argon. Removal of oxygen from heme *d* can be followed as a blue shift of its α absorption band from 645 to 630 nm.

[1] Abbreviations: E_m, midpoint redox potential; $K_{d(O_2)}$, apparent dissociation constant for the heme d^{2+}−O_2 complex defined as k_{off}/k_{on}; K_m, Michaelis−Menten constant; **R**, fully reduced enzyme; **MV**, "mixed valence" (one-electron-reduced) enzyme; τ, time constant, reciprocal of rate constant; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine; NHE, normal hydrogen electrode.

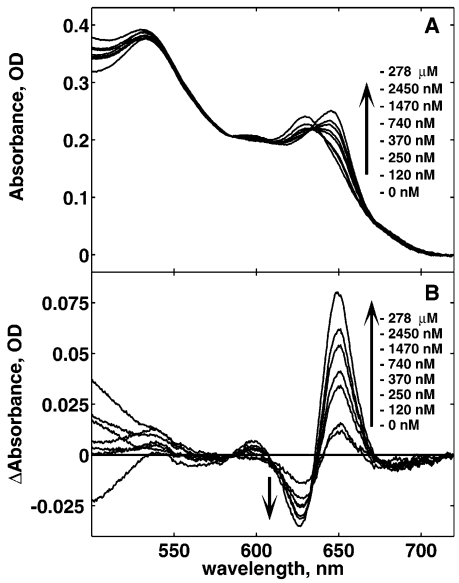


FIGURE 1: Equilibrium oxygen binding to *A. vinelandii* cytochrome *bd* in the **MV** state. (A) Absolute absorption spectra. (B) Differences between the spectra after the addition of O_2 and the anaerobic spectrum. The arrows indicate increase in [O_2]. Conditions: cytochrome *bd*, 12.7 μM; 0.05% *n*-dodecyl-β-D-maltoside, 65 mM Mops−KOH, 35 mM potassium phosphate, pH 7.0; 1 cm optical path. The enzyme was made anaerobic by equilibration with argon and then was titrated with O_2.

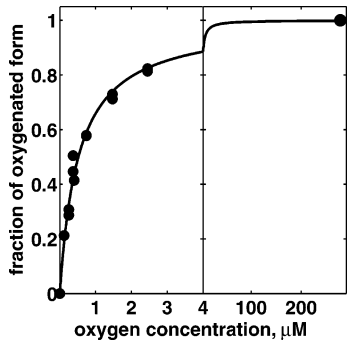


FIGURE 2: Oxygen concentration dependence of the absorption changes of the *A. vinelandii* cytochrome *bd*. The ΔA increase at 650 minus 626 nm was measured and plotted *versus* [O_2]. The experimental data (circles) were fitted as described in ref *52* giving the $K_{d(O_2)}$ value of 0.52 μM. Conditions, as for Figure 1.

Conversely, titration of the anaerobic one-electron-reduced enzyme with oxygen regenerates the oxygenated form of heme *d* (Figure 1). Figure 1B shows that addition of O_2 aliquots to the anaerobic **MV** cytochrome *bd* from *A. vinelandii* induces stepwise increase in the magnitude of the difference absorption spectra (a peak at 650 nm, a trough at 626 nm) corresponding to a red shift of the α-band of the ferrous heme *d*. The magnitude of the O_2-induced absorption changes ($\Delta A_{650-626}$) plotted as a function of the O_2 concentration shows a saturation curve (Figure 2) with the $K_{d(O_2)} = 0.5$ μM for the heme *d* oxy-complex.

It is noted that apart from the red shift of the 630 nm band of ferrous heme *d*, the oxygen binding to heme d^{2+} correlates with a weak but well-discernible absorption increase around 595 nm which may resemble the maximum of ferrous heme b_{595} (Figure 1A,B). However, no evidence for a concomitant increase of absorption at 560 nm diagnostic of heme b_{595} reduction is observed (similar findings have been made with cytochrome *bd* from *E. coli*, V.B.B., unpublished results). Hence, the minute 595 nm maximum is unlikely to indicate reduction of heme b_{595} but most probably is a spectral feature associated with oxygen binding to heme *d*.

Kinetics of Oxygen Binding. The flow−flash method was used to determine the rate of O_2 binding to the **MV** and **R** forms of the *A. vinelandii* cytochrome *bd*. In this approach, the CO-bound enzyme was photolyzed in the presence of oxygen and the flash-induced spectral changes associated with the reaction of O_2 with the enzyme were monitored with a microsecond time resolution.

Figure 3A shows typical time courses for the reaction of oxygen with the **MV** enzyme following CO photolysis at different O_2 concentrations. The reaction follows apparent monoexponential kinetics. The kinetic spectrum of the process derived from the global data fitting (Figure 3B) is identical to the static difference spectrum characteristic of the heme *d* oxy-complex formation (*cf.* Figure 1B). The rate of the reaction increases with O_2 concentration, but the increase is obviously nonlinear and reveals a saturation behavior. The dependence of the oxygen binding rate on oxygen concentration can be fitted by a hyperbolic function (Figure 3C). The finding may implicate that the binding of O_2 to the **MV** cytochrome *bd* is not an elementary single-step process and includes at least one additional intermediate step.

The reaction of oxygen with the **R** form of cytochrome *bd* does not stop at the stage of the oxy-complex formation as in the case of the **MV** enzyme, but proceeds further yielding the ferryl species, as reported earlier for the *E. coli* enzyme (*9*, *56*). In this work, our interest in the reaction of O_2 with the enzyme in the **R** state has been confined to the very first phase, *i.e.*, to the formation of the $d^{2+}-O_2$ adduct. The kinetic spectrum of this phase (not shown) is virtually identical to that observed for the **MV** enzyme shown in Figure 3B. The rate of the oxy-complex formation in the case of the **R** state depends linearly on the O_2 concentration (Figure 3C, squares) without any evidence for saturation behavior as observed for the **MV** cytochrome *bd* (Figure 3C, circles) and gives a second-order rate constant k_{on} of 1.96×10^9 M^{-1} s^{-1}. Notably, this rate is very close to the limit controlled by oxygen diffusion in water (6.5×10^9 M^{-1} s^{-1}).

Thermodynamic Characteristics of the Hemes in Cytochromes bd from A. vinelandii and E. coli. Figure 4 shows the redox titration curves for the three hemes (*d*, b_{595}, and b_{558}) in cytochromes *bd* from both *A. vinelandii* and *E.coli*. The experiments were performed in a spectroelectrochemical cell under the same conditions. Both reductive and oxidative titrations were carried out. In each case, the experimental points fit reasonably well the Nernst $n = 1$ curves. The results of redox titrations are summarized in Table 1. In general, the E_m values of the hemes determined in this work are within the range of the values reported earlier for the *A. vinelandii* and *E. coli* cytochromes *bd* (*57*−*60*). However, whereas a very good agreement is observed between the E_m values of the low-spin heme b_{558} in the two enzymes, the midpoint redox potentials of the both high-spin hemes are 50−80 mV higher in the *A. vinelandii* cytochrome *bd*, as compared to the *E. coli* enzyme.

DISCUSSION

The ability of a one-electron-reduced cytochrome *bd* to form reversibly a stable myoglobin-type oxy-complex, with

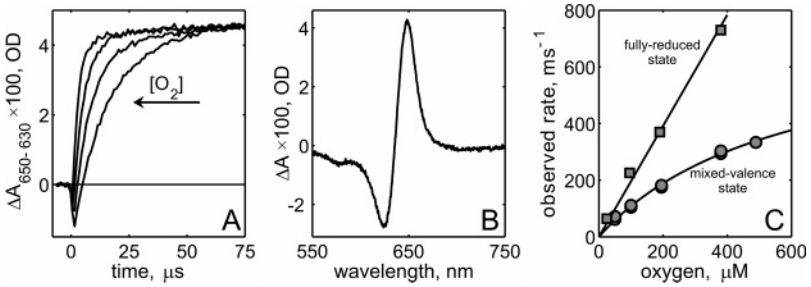


FIGURE 3: The kinetics of oxygen binding to *A. vinelandii* cytochrome *bd*. (A) Time courses of the O_2 binding at 650 minus 630 nm at 50, 100, 195, and 380 μM O_2 (concentrations after mixing, the arrow indicates the direction of $[O_2]$ increase) to the **MV** enzyme. (B) The kinetic spectrum of the reaction obtained by global analysis of the **MV** enzyme data. (C) The O_2 concentration dependence for the observed rate of the reaction (squares, the **R** enzyme; circles, the **MV** enzyme). Lines are the best fits to the data (linear, the **R** enzyme; hyperbolic, the **MV** enzyme). Each data point is an average of four to five independent measurements. Concentration of the enzyme after mixing is 11 μM; buffer, the same as for Figure 1. For other conditions, see Materials and Methods.

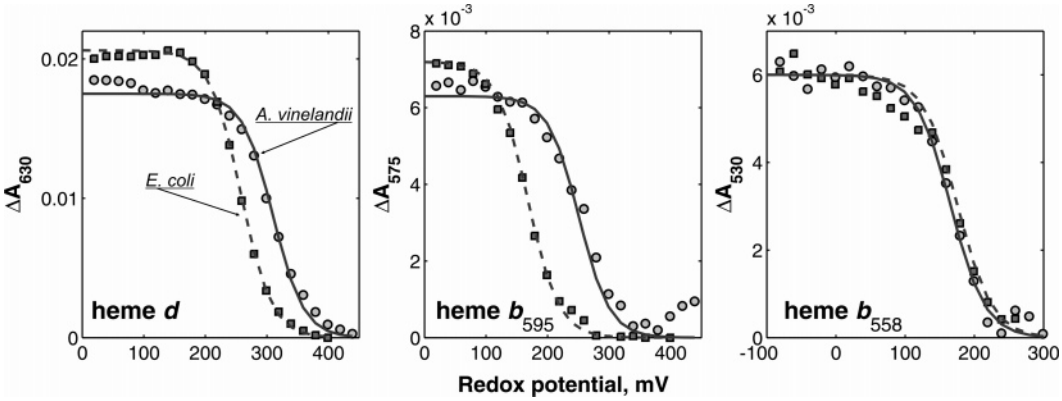


FIGURE 4: Spectroelectrochemical redox titrations of *A. vinelandii* (circles) and *E. coli* (squares) cytochromes *bd*. The titration profiles for the hemes *d*, b_{595}, and b_{558} are shown at the wavelengths where the spectral contributions of each heme have maxima (630, 575, and 530 nm, respectively). Each data point is the average of the oxidative and reductive titrations at matching potentials. Solid lines are one-electron Nernstian curves. The data points of the *E. coli* cytochrome *bd* (200 μM) are normalized by the enzyme concentration to those of the *A. vinelandii* cytochrome *bd* (150 μM). The measurements were performed in 0.05% *n*-dodecyl-β-D-maltoside, 200 mM potassium phosphate buffer, pH 7.0. For other conditions, see Materials and Methods.

Table 1: Apparent Redox Midpoint Potentials of the Heme Groups of Cytochrome *bd*

	E_m, mV (*vs* NHE)[a]	
heme	*E. coli*	*A. vinelandii*
b_{558}	+176	+166
b_{595}	+168	+251
d	+258	+310

[a] The standard deviation for the E_m values reported was ±5 mV.

oxygen bound to heme d^{2+} (*31*), is unique among the respiratory oxidases. This feature renders it possible to measure directly the oxygen affinity of cytochrome *bd* in the **MV** state, whereas in the oxidases of the heme-copper family, such as mitochondrial cytochrome *c* oxidase, $K_{d(O_2)}$ can only be determined indirectly (*61*, *62*).

In this work, the $K_{d(O_2)}$ value has been determined for the *A. vinelandii* cytochrome *bd* by direct titration of the anaerobic **MV** form of the enzyme with oxygen (Figure 2). The obtained value of ~0.5 μM is but slightly higher than K_d (ca. 0.3 μM) measured earlier by the same method for the *bd*-type quinol oxidase from *E. coli* (*52*). In other words, the *bd*-type oxidases from the two bacteria have similar and quite high affinity for oxygen. This finding is in apparent disagreement with the earlier conclusion of D'Mello and coauthors (*34*, *35*) who reported the catalytic Michaelis–Menten constant, $K_{m(O_2)}$, for cytochrome *bd* from *A. vinelandii* to be ~1000 times higher than that for cytochrome *bd* from *E. coli* (~5 μM and ~5 nM, respectively). On the basis of those results (*34*, *35*), cytochromes *bd* from *E. coli* and *A. vinelandii* have been often considered in the literature to exemplify the "high-" and "low-affinity" *bd*-type quinol oxidases. Such a conclusion is not supported by our data. At the same time, our data are consistent with the $K_{m(O_2)}$ values reported by other groups where no discrepancy between the *A. vinelandii* and *E. coli* enzymes were found (*36*, *37*). As suggested by Junemann and coauthors (*37*), the low level of ubiquinol and the resultant limitation of electron-transfer rate would result in lower apparent $K_{m(O_2)}$, explaining the extremely low $K_{m(O_2)}$ values measured in the *E. coli* cytochrome *bd* in ref *35*. It is clear that both types of cytochrome *bd* oxidases should be considered as high O_2 affinity oxidases, in contrast with the earlier suggestions (*34*, *35*, *42*).

A. vinelandii is an obligate aerobe, but at the same time survival of the bacterium depends on its ability to fix nitrogen. The latter is not an easy task under aerobic conditions as nitrogenase is highly susceptible to the oxidative damage (*63*). To cope with this problem, an efficient "respiratory protection" is required for cell survival. The highly active *bd*-type oxidase of *A. vinelandii* is believed to play an essential role in oxygen depletion (*23*). This is fully consistent with the high O_2 affinity of the *A. vinelandii* cytochrome *bd* measured in this work. Increased expression of cytochrome *bd* in *A. vinelandii* in response to the increase

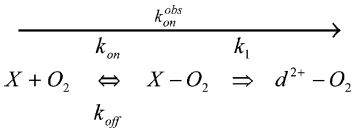
of oxygen supply (*38*, *39*) matches perfectly the oxygen-protective function of the oxidase and can hardly be used as an argument against the high affinity of the *A. vinelandii* cytochrome *bd* for oxygen.

We determined redox midpoint potentials of the hemes in the two enzymes by spectroelectrochemical titrations under the *identical conditions*. Indeed, although E_m values of the hemes *d*, b_{595}, and b_{558} of cytochromes *bd* from *E. coli* and *A. vinelandii* have been measured earlier (*57*−*60*), the reported values scatter considerably and seem to depend on the specific experimental conditions (particularly, on the detergent used). Thus the fact that the heme midpoint potentials have never been measured in the two bacteria under the identical conditions greatly limits validity of the comparison. In this work we find that whereas the E_m values for the low-spin input heme b_{558} are very close in the two cytochromes *bd*, the midpoint potentials of the both high-spin hemes b_{595} and *d* are considerably higher in the *A. vinelandii* enzyme as compared to the *E. coli* enzyme (Figure 4, Table 1).

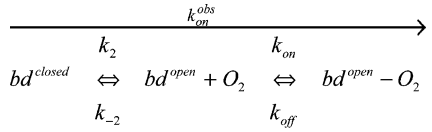
The k_{on} value for oxygen binding with the *A. vinelandii* enzyme in the **R** state reported here (1.96×10^9 M^{-1} s^{-1}) is about 1 order of magnitude higher than that estimated from $K_{m(O_2)}$ and V_{max} (*37*). At the same time, the O_2 binding rates for the **R** enzymes from *A. vinelandii* (this work) and *E. coli* (1.9×10^9 M^{-1} s^{-1} (*56*)) are essentially the same. It must be emphasized that the latter constant actually approaches the diffusion-controlled limit for oxygen in water. This circumstance suggests that the site in cytochrome *bd*, where O_2 is trapped and reduced, may be located nearby or even at the surface of the protein molecule. Another possibility is that, like in cytochromes aa_3 and ba_3 (*64*, *65*), cytochrome *bd* possesses a special channel delivering O_2 to its active site. Such channel is thought to endow cytochrome oxidase with a high rate of O_2 binding ($\sim 10^8$ M^{-1} s^{-1} (*61*, *62*)). Cytochrome oxidase binds oxygen at least 10-fold faster than most of the other hemoproteins (*66*), but still about 10-fold slower than cytochrome *bd*.

It should also be taken into account that actually the experimental system is not a homogeneous aqueous solution but is rather heterogeneous. The enzyme molecules are surrounded by detergent micelles, and the equilibrium concentration of oxygen in the hydrophobic phase of the micelles is envisaged to be much higher than in the aqueous phase (0.25×10^{-4} and 12×10^{-4} mole fractions at 101.325 kPa partial pressure of oxygen for water and *n*-octanol, respectively (*67*)). Therefore, the apparent second-order rate constants for oxygen binding to heme d^{2+} in the single- or few-turnover experiments may need to be corrected properly. Two factors may be of importance in this correction. On the one hand, it seems unlikely that the entire enzyme is surrounded with the molecules of detergent, but rather only the hydrophobic membrane spanning parts—leaving the hydrophilic part of the enzyme's surface exposed to the aqueous phase. However, it is reasonable to assume that similarly to heme-copper oxidases (*64*, *68*−*70*), the oxygen channel of cytochrome *bd* would open into the hydrophobic phase of the phospholipid bilayer surrounding the membrane spanning part of the enzyme. In the few-turnover experiments, the increase in local oxygen concentration within the surfactant layer may be offset by the higher viscosity of the layer and, hence, lower O_2 diffusion rates. Thus, we cannot estimate the exact correction factor. Probably, it should be less than the difference of the oxygen concentrations in water and *n*-octanol (*i.e.*, <48-fold), but higher than 1.

Scheme 1



Scheme 2



It is of interest that the concentration dependences of the observed rates of oxygen binding to heme d^{2+} are clearly different in the **MV** and **R** states of *A. vinelandii* cytochrome *bd*. In the **R** state, the dependence is linear (Figure 3C, squares) and can be described by a simple single-step reaction of oxygen binding with $k_{on} = 1.96 \times 10^9$ M^{-1} s^{-1}.

By contrast, in the **MV** state of cytochrome *bd* from *A. vinelandii* the O_2 concentration dependence of the observed oxygen binding rates is hyperbolic (Figure 3C, circles). The finding may indicate that either (i) the reaction of O_2 with the **MV** enzyme includes more than one step, so that the simple model in Scheme 1 is not sufficient to describe the process, or (ii) the reaction pathway for oxygen binding to heme d^{2+} is controlled by the redox state of the heme(s) *b*.

Two models (see Schemes 1 and 2) can be considered. The first model implies that there is a spectrally invisible prebinding of oxygen to a saturatable site X (other than heme d^{2+}) from which the molecule of oxygen is further transferred to heme d^{2+}. Since the putative O_2 binding to the X-site is spectroscopically silent, the assignment of X to any of the hemes is unlikely. Besides in the **MV** enzyme, both hemes b_{595} and b_{558} are in the oxidized state and thus are not likely to participate in oxygen binding. Rather, a gas molecule accommodating cavity near heme *d* would be kept in mind (see, *e.g.*, ref *70*).

According to Scheme 1, the observed kinetics will be nearly monoexponential. k_{on}^{obs}, given by

$$k_{on}^{obs} \sim k_1 \frac{[O_2]}{K_{d(X)} + [O_2]} \quad (1)$$

is the observed rate of O_2 binding to heme d^{2+}; $K_{d(X)}$ is the equilibrium dissociation constant for O_2 binding to the site X.

The fit of the $[O_2]$ vs k_{on}^{obs} data according to eq 1 for the **MV** enzyme (Figure 3C) gives the value of k_{on}^{obs} at the maximum $[O_2]$ that corresponds to rate constant $k_1 = 7.2 \times 10^5$ s^{-1}, with the observed equilibrium dissociation constant $K_{d(X)} = 5.5 \times 10^{-4}$ M and the initial slope corresponding to the bimolecular rate constant of 1.3×10^9 M^{-1} s^{-1}. The latter value defines the model-free, upper limit for the true oxygen biding rate constant (k_{on}). The k_{on} value however is significantly lower than the bimolecular rate constant for O_2 binding to heme *d* measured for the **R** state of the enzyme (1.96×10^9 M^{-1} s^{-1}). Therefore we assume that this model is not very likely, although cannot be discarded.

In the second model, there are two states of the **MV** cytochrome *bd* in equilibrium, only one of which can bind O_2 (Scheme 2).

When in the "closed" conformation, cytochrome *bd* provides no access for oxygen to heme d^{2+}, whereas in the "open" conformation, oxygen binds easily. The fully reduced enzyme is always in the open configuration. The fraction of the closed conformation in the **MV** enzyme (or K_2 in Scheme 2) can be defined by the ratio $k_{on}^{MV}/k_{on}^{R} = 0.67$ (or $K_2 = 1.5$). Fitting the kinetic curves (Figure 3A) within a framework of Scheme 2 at several O_2 concentrations gives $k_2 = 5.1 \times 10^5\ s^{-1}$. Although Scheme 2 predicts biexponential behavior, under the conditions of the present experiments, the solution is indistinguishable from the monoexponential approximation (not shown).

In the **R** state of cytochrome *bd*, heme *d* is always directly accessible to O_2 and the conformation is presumed to be permanently "open", whereas in the **MV** state (heme b_{595} oxidized) it undergoes an "open−closed" transition. The estimated time of channel opening and closing would be 2 μs ($1/k_2$) and 3 μs (K_2/k_2), respectively. There are two observations in the literature that support the above hypothesis. (i) Flash-photolysis of the CO-complex of the **R** cytochrome *bd* from *E. coli* results in complete photodissociation of the CO molecule into the bulk aqueous phase. If the experiment is repeated with the **MV−CO** complex, a significant part of CO flashed off heme d^{2+} (up to 70%) gets trapped inside the protein and undergoes geminate recombination with heme d^{2+} on a sub-nanosecond time scale (*17*). (ii) The apparent *off* rate constants for spontaneous dissociation of the ligands such as CO or NO from heme d^{2+} are markedly slower for the **MV** state of the *E. coli* cytochrome *bd*, as compared to the **R** state of the enzyme (*30*). We propose that in cytochrome *bd*, the redox state of the *b*-type hemes, presumably that of heme b_{595}, controls the pathway for ligand transfer between heme *d* and the bulk phase (*17*, *30*).

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