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The Oxygen Affinity of Cytochrome *bo'* in *Escherichia coli* Determined by the Deoxygenation of Oxyleghemoglobin and Oxymyoglobin: K_m Values for Oxygen Are in the Submicromolar Range

RITA D'MELLO,[1] SUSAN HILL,[2] AND ROBERT K. POOLE[1*]

Division of Life Sciences, King's College London, London W8 7AH,[1] and Nitrogen Fixation Laboratory, AFRC IPSR, University of Sussex, Brighton, BN1 9RQ,[2] United Kingdom

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Apparent oxygen affinities for *Escherichia coli* cells and membranes containing a terminal oxidase with only one type of ligand-binding heme, cytochrome *o'*, were measured with oxyleghemoglobin and oxymyoglobin as sensitive oxygen reporters. Two K_m values (0.15 to 0.35 μM and 0.016 to 0.085 μM) were detected, well below values determined for the purified oxidase by insensitive electrode methods.

Escherichia coli contains two terminal quinol oxidases (21, 22, 28). One of these, cytochrome *bo'*, contains the ligand-binding heme O and a second low-spin heme of either the B or O type (29). This oxidase has also been called cytochrome $b_{562}o$ (16), bo_3, and oo_3 (29), but the *bo'* nomenclature is in accordance with IUB recommendations (25). Cytochrome *bo'* is a member of the heme-copper superfamily of terminal oxidases (7) and is a proton pump (28). The alternative oxidase, cytochrome *bd*, is structurally unrelated to this family (13) and appears not to pump protons (28). Cytochome *bo'* is synthesized under conditions of high aeration (8, 15), whereas maximal synthesis of cytochrome *bd* occurs microaerobically (12). Apparently consistent with these patterns of regulation are results obtained from studies of whole cells (30) and purified oxidases (16) suggesting that cytochrome *bd* has a high oxygen affinity, whereas cytochrome *bo'* has a much lower affinity. However, these measurements, and a recent claim that *E. coli* cytochrome *bd* has a surprisingly low affinity (17), have relied upon polarographic methods in which the sensitivity at low oxygen tensions is limited by the unstirred layer at the electrode membrane (18). Recognizing the physiological significance of the disparate oxygen affinities yet the absence of reliable data on their values, we have measured the K_m values for oxygen using a strain containing only cytochrome *bo'* by a method that allows highly sensitive and continuous monitoring of oxygen consumption (4).

E. coli UNF3502 (14) was constructed by transduction of UNF3501 (*trp his rpsE*) with bacteriophage P1 grown on GO103, which carries a kanamycin resistance cassette close to the partially deleted *cydAB* locus. Strain AN2342 is regarded as wild type (27), has a different genetic background, and was used to assess the generality of the data obtained with UNF3502. Cells were grown at 37°C, with shaking (200 rpm), in five 2-liter baffled flasks (Belco Biotechnology, Vineland, N.J.), each containing 400 ml of Luria-Bertani medium (LB) (19) supplemented with 0.2% (wt/vol) glucose and (for UNF3502) kanamycin (50 μg/ml). Cultures were grown to mid-exponential phase, harvested, and washed, and a portion of the cell paste (approximately 1 g) was resuspended in twice its volume of 25 mM phosphate buffer (pH 7.5 ml). The suspension was kept at 4°C and used for K_m determinations within 24 h. Remaining cells were suspended in 50 ml of 50 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] buffer (pH 6.5), containing 8 mM magnesium acetate and a few grains of DNase (Sigma), and stored at −70°C for membrane preparation.

We determined the apparent affinity for oxygen uptake by intact cells and membranes as previously described (10). In brief, oxygenated soybean leghemoglobin or myoglobin was diluted to 10 to 15 μM in phosphate buffer (25 mM, pH 7.0) containing 1 mM EDTA, previously sparged with 1% oxygen in argon. Globin solutions were kept under argon plus 1% oxygen and used within 6 h. Deoxygenation of the globins by respiration was monitored by multiwavelength spectrophotometry (6). A special glass cuvette (10) was filled (1.3 ml) with a solution of either oxyleghemoglobin (~15 μM) or oxymyoglobin (~10 μM) and sealed with a finely perforated stopper allowing injection of substrate (1 M succinate; 10 μl). The stability of the oxygenated globin was checked by monitoring ΔA for 5 to 10 min. After addition of bacteria or membranes (5 to 50 μl), deoxygenation was continuously monitored by plotting the change in absorbance between 575 and 560 nm. The ΔA traces were digitized with a scanner by using Apple Scan and Flexitrace software. Rates of oxygen consumption and the average concentrations of free dissolved oxygen were calculated as described elsewhere (10) by using Microsoft EXCEL software. Graphs were drawn by using Cricket Graph, and lines were fitted by using the linear regression feature of that software. The values of K' (k [oxygen dissociation rate]/k' [association rate]) used in these calculations were 786×10^{-9} M (oxymyoglobin) and 50×10^{-9} M (oxyleghemoglobin) (2). Plots of V (velocity) versus V/S (Eadie-Hofstee) were used to calculate K_m values. At least five separate determinations were carried out for each sample. Analyses of variance were done with Minitab software.

Determination of the oxygen affinities of cytochrome *bo'* in intact cells. Figure 1 shows the deoxygenation kinetics of oxymyoglobin (a) and oxyleghemoglobin (b) by intact *E. coli* UNF3502. Absorption at wavelengths appropriate for the oxy and deoxy forms was recorded continuously; the individual datum points in Fig. 1a and b were those extracted from these

* Corresponding author. Mailing address: Division of Life Sciences, King's College London, Kensington Campus, Campden Hill Road, London W8 7AH, United Kingdom. Phone: (0)71 333 4275. Fax: (0)71 333 4500.

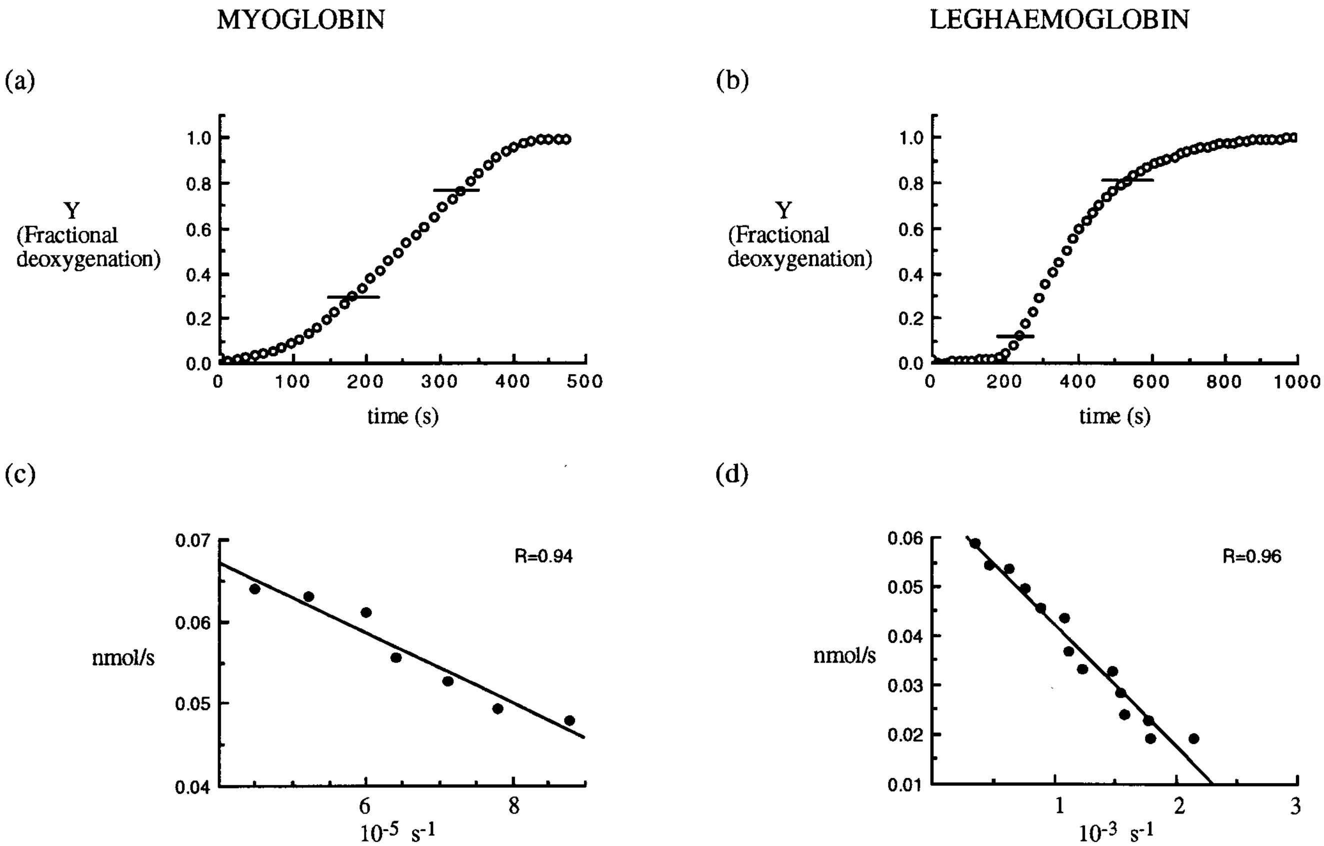

FIG. 1. Determination of oxygen affinities of cytochrome *bo′* in a cytochrome *d*-deficient strain (UNF3502) of *E. coli* by the deoxygenation kinetics of (a) oxymyoglobin and (b) oxyleghemoglobin. The horizontal lines define, for each carrier, the fractional oxygenation equivalent to the working range used (see text); only datum points between the lines were used to derive data for the Eadie-Hofstee plots (panels c and d). The K_m values calculated from the slopes were 0.43 μM for oxymyoglobin (c) and 0.026 μM for oxyleghemoglobin (d).

traces and used in the calculations. The oxygenated globins provide dispersed free oxygen at concentrations defined by the kinetic constants of their oxygenation and deoxygenation, and their optical spectra indicate the average oxygen concentration (2, 4). Oxyleghemoglobin and oxymyoglobin allow oxygen consumption rates to be measured when the free oxygen concentrations are in the ranges of 0.003 to 0.3 μM and 0.1 to 10 μM, respectively (5). Only data that fell in these ranges were examined. Controls (10) ensured that the absorbance changes accurately reflected deoxygenation kinetics. Experiments using intact cells with oxymyoglobin and oxyleghemoglobin each revealed a single component in Eadie-Hofstee plots (Fig. 1c and d). The mean K_m values from several such experiments were 0.35 and 0.025 μM for oxymyoglobin and oxyleghemoglobin, respectively (Table 1). The V_{max} values for the two components were similar. Comparable results were obtained with cells of a wild-type strain grown so as to maximize the contribution of cytochrome *o* and minimize the contribution of cytochrome *bd* (see below). Again, one component was found with each globin (Table 1).

Determination of the oxygen affinities of cytochrome *bo′* in membranes. The two K_ms found in experiments with intact cells, particularly of UNF3502 (which lacks cytochrome *d*), were unexpected. To test whether one component might be

TABLE 1. Oxygen affinities of cytochrome *o* in *E. coli* cells and membranes[a]

Strain and phenotype	Preparation	Myoglobin		Leghemoglobin	
		K_m (μM)	V_{max} (nmol/s/mg)	K_m (μM)	V_{max} (nmol/s/mg)
AN2342 (Cyo$^+$ Cyd$^+$)	Cells	0.16 (0.51)	1.17 (0.16)	0.085 (0.0029)	1.77 (0.19)
	Membranes	0.15 (0.055)	1.33 (0.73)	0.016 (0.0036)	5.62 (1.6)
UNF3502 (Cyo$^+$ Cyd$^-$)	Cells	0.35 (0.19)	1.21 (0.47)	0.025 (0.0038)	1.41 (0.31)
	Membranes	0.20 (0.067)	0.15 (0.059)	0.046 (0.014)	0.10 (0.043)

[a] *E. coli* AN2342 is a wild-type strain and was grown to logarithmic phase to minimize the contribution of cytochrome *bd* (see text). UNF3502 is Δ*cydAB*. In all cases, succinate was used as the substrate and the values presented are means of four to six determinations with standard deviations in parentheses. A one-way analysis of variance for the six data sets obtained with cells and membranes using oxymyoglobin gave a *P* value of 0.52, indicating no statistically significant difference between the data sets. However, the K_m values measured with the two globins were clearly and markedly different; for example, a one-way analysis of variance for the whole-cell data obtained with oxymyoglobin and oxyleghemoglobin gave a *P* value of 0.002, indicating a statistically significant difference between the data sets.

due to the soluble flavohemoglobin (HMP) of *E. coli* (31), which has oxidase activity (23), the experiments were repeated with everted (20) membrane vesicles prepared as described elsewhere (1), in which the site of oxygen reduction is presumed to be on the outer face of the membrane, directly accessible to the oxygen-reporting globin. Thawed cells were broken by one passage through a French press (Aminco, Silver Spring, Md.) at 69 MPa. Centrifugation at 27,000 $\times$ *g* for 15 min removed cell debris; membrane vesicles were extracted from the supernatant (containing the soluble HMP) by centrifugation at 100,000 $\times$ *g* for 90 min. The membrane pellet was resuspended in the same buffer (4 ml, but lacking DNase), and the suspension was maintained at 4°C and used within 24 h without freezing. For UNF3502, the mean K_m values for membranes were 0.2 μM with oxymyoglobin and 0.046 μM with oxyleghemoglobin (Table 1). Thus, neither component can be attributed to HMP. The use of membranes from strain AN2342 confirmed the presence of two components (Table 1).

Spectral characterization of the strains used. The two K_ms with UNF3502 (Δ*cydAB*) might arise from the residual presence of cytochrome *bd*, for example, by a contribution from the uncharacterized products of the *appC* and *appB* genes, predicted to encode a homolog of cytochrome *bd* (9) and recently renamed *cyxA* and *cyxB* (3). Therefore, cells were examined spectrally for the presence of cytochrome *bd*. Reduced + CO minus reduced difference spectra recorded at room temperature for intact UNF3502 cells revealed symmetrical Soret bands at 430 nm (trough) and 416 nm (peak) attributed to cytochrome *o* and its CO adduct, respectively (results not shown). The α and β regions showed the undulations reported previously (25). No cytochrome *bd* or other CO-binding heme protein was detectable. In contrast, the corresponding spectrum of logarithmic-phase cells of AN2342 showed an additional, minor shoulder at 444 nm due to cytochrome b_{595} (21) of the cytochrome *bd* complex and signals in the 622- to 643-nm region due to the reaction of CO with cytochrome *d* (24).

Low-temperature photodissociation spectroscopy (26) confirmed the absence of cytochrome *bd* in UNF3502: a suspension of cells supplemented with 30% (vol/vol) ethylene glycol was reduced with succinate, and the CO adduct(s) of terminal oxidase(s) was prepared by bubbling the cell suspension with CO. The suspension was frozen and scanned in the temperature-controlled sample compartment of a dual-wavelength scanning spectrophotometer (26). The spectrum of the reduced CO-ligated sample was recorded and used as a baseline for the photodissociation spectrum, i.e., the postphotolysis-minus-prephotolysis difference spectrum. At -130°C, the spectrum of UNF3502 revealed no spectral signals other than those anticipated for cytochrome *o* (26). The Soret region showed symmetrical features at 414 nm (trough) and 432 nm (peak). The presence of the cytochrome *bd* complex would result in a red shift of the latter feature due to photodissociation of CO-ligated cytochrome b_{595}, as clearly demonstrated with *Azotobacter vinelandii* (11). Photodissociation spectra recorded anoxically at -100, -80, and -60°C all showed these features. Although the presence of cytochrome b_{595} can be detected in such spectra, cytochrome *d* itself cannot, since at temperatures above -130°C, geminate recombination of CO occurs too rapidly to be detected by conventional wavelength scanning spectrophotometry (21, 24). However, when oxygen is introduced into the sample before freezing, photolysis yields the characteristic oxy form of cytochrome *d* (24) with a clear absorption peak at about 650 nm. Nevertheless, photodissociation spectra scanned to 780 nm in the presence of oxygen at temperatures between -130 and -60°C again revealed only cytochrome *o* in UNF3502. We conclude that cytochrome *bd*, at least at spectroscopically detectable levels, does not contribute to the measured K_m values for UNF3502. In AN2342, no cytochrome *d* could be detected after photodissociation, but a contribution from cytochrome b_{595} was noted.

Reliability of the deoxygenation method. Validation of the technique utilized in this study may be obtained by comparing our results with other literature values. For example, the K_m determined by us (10) for *A. vinelandii* cytochrome *bd* in cells (4.5 μM) is in good agreement with that obtained recently for the purified enzyme (5.7 μM) by an oxygen electrode method (17). Furthermore, the higher of the two K_m values reported in this paper (0.15 to 0.35 μM) is in excellent agreement with the careful studies of Rice and Hempfling (30), who used a large reaction volume and an electrode covered with an ultrathin membrane to obtain a value of 0.2 μM for cytochrome *o*. Measurements of the K_m for cytochrome *bd* in late-stationary-phase cultures of strain AN2342 (in which this oxidase is dominant) are in agreement with those described in reference 30 in showing cytochrome *bd* to have a higher affinity for oxygen, although the kinetics (11a) reveal additional complexity.

Concluding remarks. The apparent K_m values for cytochrome *o* reported here are 10- to 100-fold lower than those reported by Kita et al. (16), who gave values between 1.4 and 2.9 μM for early-exponential-phase cells and membranes and purified oxidase derived therefrom. Although these differences might be attributed in part to the effective concentrations of reducing substrates presented to the various preparations, we suggest that a major discrepancy results from the use in the study described in reference 16 of an oxygen electrode not modified by use of an ultrathin membrane or a large stirred reaction volume as described by Rice and Hempfling (30). It appears likely that the K_m values reported by Kita et al. (16) have limited significance for understanding oxidase kinetics in vivo. Neither the studies of Kita et al. nor those described in reference 30 would have detected the component with a K_m below 0.1 μM reported in the present paper. An interesting possibility is that the two affinities measured in our study reflect heterogeneity of the oxidases present. Puustinen et al. (29) report that two forms of the oxidase, *bo*′ (bo_3) and *oo*′ (oo_3), are found in different strains and under different growth conditions. The functional properties of these variants await further study.

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