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In the respiratory chain of *Escherichia coli* cytochromes *bd*-I and *bd*-II are more sensitive to carbon monoxide inhibition than cytochrome bo_3

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ABSTRACT

Bacteria can not only encounter carbon monoxide (CO) in their habitats but also produce the gas endogenously. Bacterial respiratory oxidases, thus, represent possible targets for CO. Accordingly, host macrophages were proposed to produce CO and release it into the surrounding microenvironment to sense viable bacteria through a mechanism that in *Escherichia (E.) coli* was suggested to involve the targeting of a *bd*-type respiratory oxidase by CO. The aerobic respiratory chain of *E. coli* possesses three terminal quinol:O_2-oxidoreductases: the heme-copper oxidase bo_3 and two copper-lacking *bd*-type oxidases, *bd*-I and *bd*-II. Heme-copper and *bd*-type oxidases differ in the mechanism and efficiency of proton motive force generation and in resistance to oxidative and nitrosative stress, cyanide and hydrogen sulfide. Here, we investigated at varied O_2 concentrations the effect of CO gas on the O_2 reductase activity of the purified cytochromes bo_3, *bd*-I and *bd*-II of *E. coli*. We found that CO, in competition with O_2, reversibly inhibits the three enzymes. The inhibition constants K_i for the bo_3, *bd*-I and *bd*-II oxidases are 2.4 ± 0.3, 0.04 ± 0.01 and 0.2 ± 0.1 μM CO, respectively. Thus, in *E. coli*, *bd*-type oxidases are more sensitive to CO inhibition than the heme-copper cytochrome bo_3. The possible physiological consequences of this finding are discussed.

1. Introduction

Along with nitric oxide and hydrogen sulfide, carbon monoxide (CO) serves as a signalling molecule playing a key role in the physiology and patho-physiology of mammals (see [1] and references therein). In higher eukaryotes, CO is endogenously produced during heme degradation by the constitutive (HO-2) and inducible (HO-1) isoforms of heme oxygenase [1] and controls important physiological functions, acting proposedly as a neurotransmitter [2] and exerting vasodilatory, cytoprotective, anti-inflammatory, anti-apoptotic, anti-atherogenic, and anti-proliferative effects [3]. While it is yet unknown if CO acts as a signalling molecule also in bacteria [1], it has been recognized that many bacteria, including pathogens, not only encounter CO in their habitats, but also produce this gas endogenously through the action of homologs of mammalian HOs or phylogenetically unrelated HO-like enzymes [4,5]. These enzymes play a crucial role in iron acquisition by scavenging heme in the extracellular milieu, particularly under iron-limiting conditions, as those which are established during host infection [4]. While the significance of CO in bacterial physiology is yet unknown, several CO-sensing proteins were identified in bacteria, leading to stimulation of CO metabolism and regulation of gene expression (reviewed in [1]).

The role of CO in bacterial virulence needs to be established yet. Of interest is the case of *Mycobacterium (M.) tuberculosis* in which CO was found to have a role in sensing of and adaptation to changes in the host immune status [6,7]. Infection of macrophages by *M. tuberculosis* stimulates expression of the host HO-1, and its product, CO, at physiologic concentrations specifically initiates a *M. tuberculosis* dormancy program [6]. Induction of the CO-producing HO-1 in mice and humans by *M. tuberculosis* infection seems to be a virulence mechanism to enhance inflammation and bacterial growth [7].

As CO is a well-known inhibitor of cellular respiration (it inhibits mitochondrial cytochrome *c* oxidase with an inhibition constant (K_i) of 0.3–0.32 μM [8,9]), bacterial respiratory oxidases represent possible targets not only for extracellular CO which is able to freely diffuse inside the cell, but also for the endogenously produced gas. Information

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on this putative interaction with CO, however, is limited. The sensitivity to CO inhibition is unknown for most bacterial respiratory oxidases and, even for the model bacterium *Escherichia coli*, available information is rather poor.

Of interest, in studies on *E. coli*, CO has been recently proposed to be produced by host macrophages to sense viable pathogenic bacteria in the surrounding environment and promote their killing [10]. Intriguingly, this CO-mediated sensing mechanism was suggested to involve a selective interaction of the gas with one of the terminal oxidases (cytochrome *bd*-I) of the respiratory chain of *E. coli*.

This chain is branched and contains three terminal quinol:O_2-oxidoreductases: cytochromes bo_3, *bd*-I, and *bd*-II [11]. Cytochrome bo_3 is encoded by the *cyoABCDE* operon [12,13] and expressed when bacteria are grown under conditions of high aeration [14]. It is a member of type A-1 heme-copper oxidase superfamily [15]. The enzyme generates a proton motive force with a high efficiency ($H^+/e^- = 2$) as it is endowed with proton pumping activity [16]. The X-ray structure of the *E. coli* bo_3 oxidase was reported [17]. The enzyme consists of four subunits and has three redox cofactors, a low-spin heme *b*, a high-spin heme o_3 and Cu_B, all located in subunit I. Heme *b* is the primary acceptor of electrons from ubiquinol, whereas heme o_3 and Cu_B form a binuclear active center where the O_2 chemistry occurs. Cytochrome bo_3 contains only one ubiquinol binding site located in subunit I, rather than two as previously thought, that is referred to as the high affinity Q_H site [18,19].

Cytochromes *bd*-I and *bd*-II are encoded by the *cydABX* [14] and *appCBX* [20–23] operons respectively. Cytochrome *bd*-I is expressed under microaerobic conditions when the O_2 tension is from 2 to 15% of air saturation [14,24]. Compared to *bd*-I, cytochrome *bd*-II probably functions under even more O_2-limiting conditions [25] and is preferentially expressed upon entry into the stationary phase and phosphate starvation [26]. The *bd*-I and *bd*-II enzymes are members of the copper-lacking terminal oxidase family of *bd*-type oxidases [27–32]. As such, they lack proton pumping activity and generate a proton motive force with efficiency ($H^+/e^- = 1$) twice as low as compared to cytochrome bo_3 [16,33–38]. Although the X-ray structure of *E. coli bd* enzymes is not available yet, that one of cytochrome *bd* from *Geobacillus thermodenitrificans* K1041 has been reported recently [39]. Cytochrome *bd* contains two large subunits, I and II, and a third, small subunit. Subunit I contains three hemes, the low-spin heme b_{558} and the high-spin hemes b_{595} and *d*, and a quinol binding site known as the "Q-loop". Heme b_{558} is the primary electron acceptor for ubiquinol or menaquinol, whereas heme *d* is the site where O_2 binds and is reduced to $2H_2O$ [40]. The small subunit has been discovered recently and is needed for maintenance of the catalytic activity and stabilization of the hemes [21,22,41–43]. Cytochrome *bd*-I, along with its role in energy conservation, contributes to bacterial resistance to nitric oxide, peroxynitrite, hydrogen peroxide and cyanide [31,44–47]. Both the *bd*-I and *bd*-II oxidases also promote sulfide-resistant O_2-consumption and growth in *E. coli*, whereas cytochrome bo_3 is inhibited by sulfide with half-maximal inhibitory concentration IC_{50} of 1.1 ± 0.1 μM [48,49].

Since *E. coli* bacterial respiratory oxidases are likely exposed physiologically to endogenously produced or extracellularly derived CO, in this work we have assayed at varied O_2 concentration the effects of CO gas on the O_2 reductase activity of the three purified terminal oxidases of *E. coli* to comparatively test their sensitivity to CO inhibition.

2. Materials and methods

2.1. Materials and purification of the terminal quinol oxidases from E. coli

The CO gas was purchased from Air Liquide. Other chemicals were purchased from Sigma. Stock solutions of CO were prepared by equilibrating degassed water with the pure gas at 1 atm and room temperature, yielding 1 mM CO in solution. Cytochromes *bd*-I, *bd*-II and bo_3 were isolated from the *E. coli* strains GO105/pTK1, MB37 and GO105/pJRhisA, respectively, as reported [34,50,51]. The concentration of the cytochromes *bd*-I and *bd*-II was determined from their dithionite reduced-*minus*-'as prepared' difference absorption spectrum, using $\Delta\varepsilon_{628-607} = 10.8\,mM^{-1}\,cm^{-1}$ [52]. The concentration of cytochrome bo_3 was estimated from the Soret absorption band of the oxidized protein, using $\varepsilon_{407} = 182\,mM^{-1}\,cm^{-1}$ [53].

2.2. Spectroscopic measurements

UV–visible absorption spectra were recorded using an Agilent Cary 60 UV–Vis or a Varian Cary 300 Bio UV–Visible spectrophotometer. The CO dissociation constant (K_d(CO)) of cytochrome *bd*-II was determined in a sealed cuvette as follows. The enzyme was diluted to a final concentration of 2.3 μM in 50 mM K/phosphate (pH 7.0), 0.1 mM EDTA, 0.02% dodecyl-β-D-maltoside, degassed by nitrogen flushing and reduced with excess dithionite. Aliquots of a 1 mM CO solution were then added anaerobically to the cuvette. After each addition, spectral changes were monitored in real time, and when no more changes were observed, a new addition was immediately made. According to [50], the K_d(CO) value was determined by fitting the data to Eq. (1):

$$\Delta A = 0.5\varepsilon((K_d + [d]_t + [CO]_t) - \sqrt{(K_d + [d]_t + [CO]_t)^2 - 4[d]_t[CO]_t}) \quad (1)$$

where ΔA is $\Delta A_{642-622}$, ε is the extinction coefficient for the heme *d*–CO complex ($\Delta\varepsilon_{642-622}$), $[d]_t$ is total concentration of cytochrome *bd*-II, and $[CO]_t$ is the concentration of added CO. Data analysis was carried out with the use of the software package GIM (Scientific Graphic Interactive Management System) developed by A.L. Drachev in Lomonosov Moscow State University.

2.3. Oxygraphic measurements

Oxygraphic measurements were performed using a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments) equipped with two 1.5-mL chambers. Assays were made at 25 °C in 50 mM K/phosphate buffer (pH 7.0) supplemented with 0.1 mM EDTA and either 0.05% *N*-lauroyl-sarcosine (cytochrome *bd*-I) or 0.02% dodecyl-β-D-maltoside (cytochrome *bd*-II and cytochrome bo_3). The O_2-reductase activity of the terminal oxidases was measured in the presence of 10 mM dithiothreitol (DTT) and 0.25 mM 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone (Q_1). Data analysis was carried out using MATLAB (TheMathworks, South Natick, MA) and Origin (OriginLab Corporation). The apparent IC_{50} values of CO for the O_2-reductase activity of the terminal oxidases were obtained by plotting the percentage inhibition of the oxidases as a function of CO concentration, and fitting the data to the Hill equation [54], assuming a Hill coefficient $n_H = 1$. The inhibition constants (K_i) of the enzymes for CO were obtained plotting, as a function of $[O_2]/K_m(O_2)$, the IC_{50} values measured at different O_2 concentrations, and fitting the data to the equation $IC_{50} = (K_i \cdot [O_2]/K_m(O_2)) + K_i$ [55], assuming O_2 competitive inhibition. Apparent $K_m(O_2)$ values were taken from refs. [56, 57] (0.3 μM for *bd*-I, 2 μM for *bd*-II and 6 μM for bo_3). To be noted that, whereas the $K_m(O_2)$ values reported for *bd*-I and bo_3 were determined working on cell suspensions and thus at unknown quinol concentration [56], the Q_1 concentration used here (250 μM) falls within the quinol concentration range (100–250 μM) used in [57] to determine the $K_m(O_2)$ for *bd*-II working on bacterial membranes.

3. Results

Fig. 1 shows the CO-induced spectral changes measured upon addition of 30 μM CO to the purified dithionite-reduced cytochromes *bd*-I, *bd*-II or bo_3. The difference spectrum of cytochrome *bd*-I (*top panel*) shows a red shift in the visible region and a peculiar W-shaped trough in the Soret band. Very similar spectral changes have also been observed

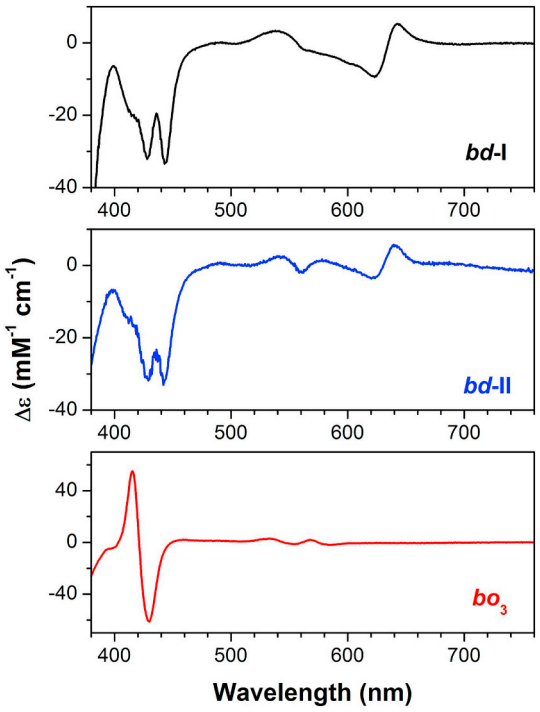

Fig. 1. Spectral changes induced by CO in *E. coli* terminal oxidases. Difference (dithionite-reduced CO-treated *minus* dithionite-reduced) absorption spectra of the isolated cytochromes *bd*-I (*top panel*), *bd*-II (*middle panel*) and bo_3 (*bottom panel*) normalized to the enzyme concentration. Buffer: 50 mM K/phosphate (pH 7.0), 0.1 mM EDTA and either 0.05% *N*-lauroyl-sarcosine (*bd*-I) or 0.02% dodecyl-β-D-maltoside (*bd*-II and bo_3).

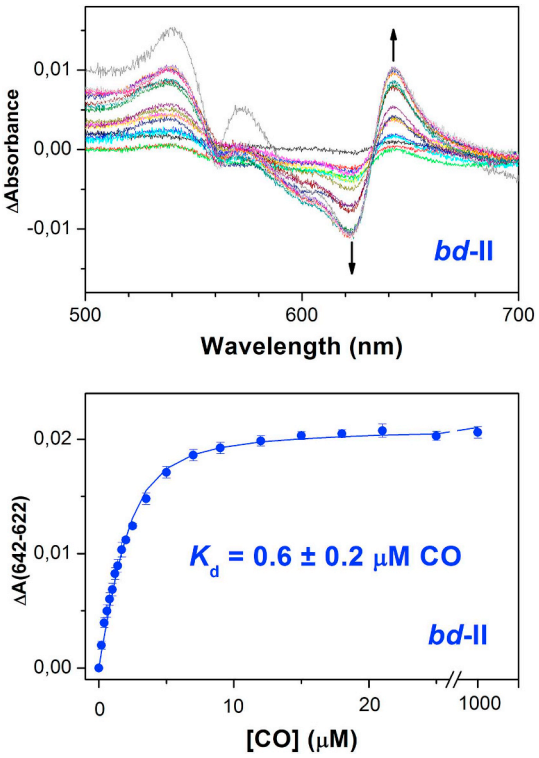

Fig. 2. Determination of apparent K_d for CO binding to the reduced *E. coli* cytochrome *bd*-II. *Top panel*: Difference (dithionite-reduced CO-treated *minus* dithionite-reduced) absorption spectra acquired upon titration of the isolated cytochrome *bd*-II (2.3 μM) with CO. The arrows depict the direction of absorbance changes at increasing [CO]. *Bottom panel*: Absorbance changes measured at 642 nm *minus* 622 nm as a function of [CO]. Experimental data (*filled circles*) are shown together with their best fit (*solid line*) to Eq. (1) (see Materials and methods), yielding the depicted K_d value (mean ± standard deviation, $n = 3$). Buffer as in Fig. 1.

in previous studies on the *bd* enzymes from *E. coli* and *Azotobacter vinelandii* [50,52,58–63]. These spectral changes most likely reflect the formation of the ferrous heme *d*-CO adduct associated to a small spectral perturbation of heme b_{595} [61], the CO binding to *b*-type hemes being minor (if any) at micromolar concentration of the gas, according to MCD work [52].

The interaction of cytochrome *bd*-II with CO has not been studied yet, except a report of the absolute and difference absorption spectra measured after bubbling the dithionite-reduced enzyme with CO for 2 min [64]. In agreement with [64], the spectral changes measured in the present study upon CO binding to cytochrome *bd*-II (*middle panel*) display a red shift with $\lambda_{max} = 640$ nm and $\lambda_{min} = 622$ nm and a broad maximum at about 541 nm with a minimum at 560 nm in the visible region, along with a W-shaped trough with minima at 429 and 442 nm in the Soret. This spectrum is similar to that one of the *bd*-I oxidase (*top panel*). On this basis, we propose that, as for cytochrome *bd*-I, the spectral changes observed upon addition of 30 μM CO to the reduced cytochrome *bd*-II are mainly (if not exclusively) due to binding of the ligand to the ferrous heme *d*.

The difference spectrum measured on CO binding to cytochrome bo_3 (*bottom panel*) displays two peaks at 533 and 568 nm in the visible region and a blue shift in the Soret band with $\lambda_{max} = 415$ nm and $\lambda_{min} = 430$ nm. This spectrum is very similar to those reported previously [65–67]. Note that the changes in the Soret band are about 23-times higher in magnitude (maximum to minimum) than those in the visible region, in line with the conclusion that in cytochrome bo_3 CO binds to the high-spin heme o_3 in the reduced binuclear center [53,67].

Next, we determined the dissociation constant for CO binding to cytochrome *bd*-II by optical anaerobic titration of the enzyme with increasing concentrations of the ligand. Spectral changes collected along the titration are shown in Fig. 2 (*top panel*). The observed titration profile was analysed as described in the Materials and Methods, yielding $K_d(CO) = 0.6 \pm 0.2$ μM (Fig. 2, *bottom panel*).

Finally, we compared the effect of CO gas on the O_2 reductase activity of the three purified terminal oxidases of *E. coli*, cytochromes bo_3, *bd*-I and *bd*-II. In these experiments, O_2 consumption by the enzymes was sustained by DTT and Q_1 as the reducing system. Fig. 3 shows that 25.3 μM CO, added at $[O_2] = 150$ μM, rapidly inhibits the O_2 reductase activity of any of the three oxidases, but to a different extent - bo_3 by 31%, *bd*-I by 52% and *bd*-II by 66%. For any of the three oxidases, the inhibition by CO is fully reversible. A virtually complete recovery of the O_2 reductase activity of the enzymes is indeed observed if CO is removed from solution by sample reoxygenation (data not shown).

To comparatively evaluate the sensitivity of the three oxidases to CO inhibition, we measured the apparent half-maximal inhibitory concentration values (IC_{50}) for CO inhibition of each oxidase at four different concentrations of O_2 (50, 100, 150 and 200 μM). The data are shown in Fig. 4 and reported in Table 1. It can be seen that the IC_{50} values for CO are proportional to the O_2 concentration at which they were determined. This suggests that CO binds to each of the three cytochromes in competition with O_2, i.e. that, as expected, in turnover conditions each oxidase populates an O_2-reactive species able to reversibly bind CO.

To estimate the inhibition constants (K_i) for CO, we plotted the IC_{50} values measured at different O_2 concentrations as a function of $[O_2]/K_m(O_2)$ and fitted the data, assuming competitive inhibition (see Materials and methods). For this analysis, we used the following $K_m(O_2)$ values previously measured by others: 6 μM for bo_3, 0.3 μM for *bd*-I and 2 μM for *bd*-II [56,57]. In this way, the following K_i values for CO were obtained: 2.4 ± 0.3 μM for bo_3, 0.04 ± 0.01 μM for *bd*-I and 0.2 ± 0.1 μM for *bd*-II (Fig. 5). Thus, under the tested experimental conditions, of the three oxidases, cytochrome bo_3 is the least sensitive to

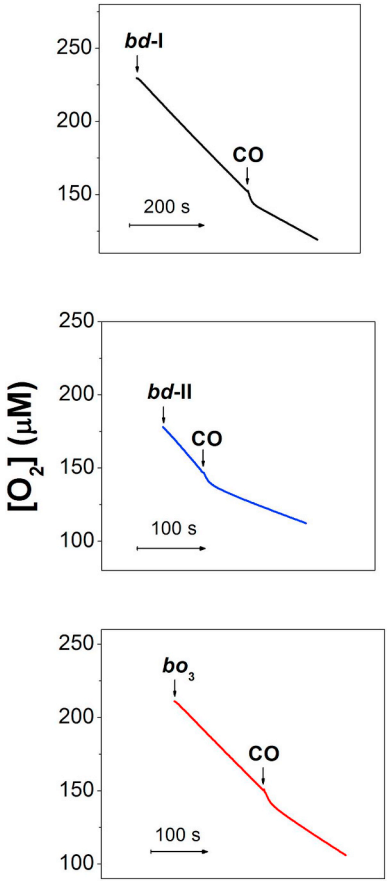

Fig. 3. Effect of CO on the quinol oxidase activity of *E. coli* terminal oxidases. O_2 consumption traces, measured at 25 °C in the presence of DTT (10 mM), Q_1 (0.25 mM) and the isolated cytochromes *bd*-I (3.5 nM), *bd*-II (1.1 nM) or bo_3 (5.4 nM), in which CO (25.3 μM) was added at $[O_2]$ = 150 μM. Buffer as in Fig. 1.

inhibition by CO gas.

4. Discussion

In this work, we compared the sensitivity to CO inhibition of the three terminal quinol oxidases of *E. coli* - the two copper-lacking *bd*-type cytochromes, *bd*-I and *bd*-II, and the heme-copper cytochrome bo_3. The enzymes are all inhibited by CO (Fig. 3). This is consistent with the information reported by Jesse et al. [68] that O_2 consumption by wild type *E. coli* membrane particles is inhibited by 38% following the addition of 100 μM CO at ~75% of O_2 saturation. We found that for each oxidase CO inhibition is fully reversible since CO removal from solution through reoxygenation of the sample restores the initial O_2 consumption rates measured prior to CO addition (data not shown). The inhibition is $[O_2]$-dependent, with the IC_{50} values for CO increasing with O_2 concentration (Table 1). This suggests that CO competes with O_2 for the binding to reduced heme *d* in the *bd*-type oxidases or reduced heme o_3 in cytochrome bo_3. A competitive inhibition by CO was also suggested for the purified beef heart cytochrome *c* oxidase [8]. In cytochrome *c* oxidase, like O_2, CO can bind to the ferrous iron of heme a_3 only if the adjacent Cu_B in the binuclear center is also reduced [69]. The same is most likely the case for cytochrome bo_3. Quite on the contrary, CO and O_2 can bind to the ferrous heme *d* both in one-electron-reduced (MV) and in the fully reduced (R) state of the Cu-deficient *bd*-type oxidase [61,70–73].

As mentioned in the Introduction, cytochrome *bd* has a higher resistance to nitric oxide, hydrogen sulfide, peroxynitrite, hydrogen peroxide and cyanide, as compared to heme-copper oxidases. In the case of

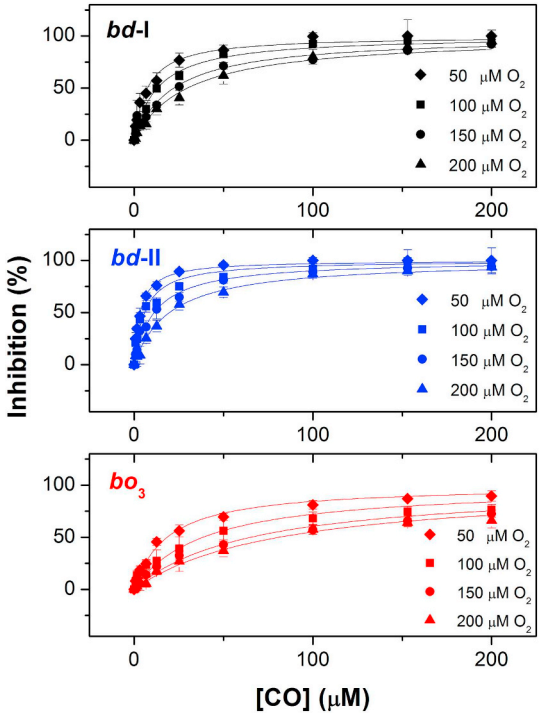

Fig. 4. Determination of apparent IC_{50} values for CO inhibition of *E. coli* terminal oxidases. Percentage inhibition of the O_2 reductase activity of the isolated cytochromes *bd*-I (3.5 nM), *bd*-II (1.1 nM) and bo_3 (5.4 nM), measured at increasing [CO] in the presence of DTT (10 mM) and Q_1 (0.25 mM), following the addition of CO at $[O_2]$ = 50, 100, 150 or 200 μM. The values represent the mean ($n \geq 3$) ± standard deviations. Conditions as in Fig. 3.

Table 1
Apparent IC_{50} values (μM CO) for CO inhibition of *E. coli* terminal oxidases at pH 7.0 and T = 25 °C.

	bd-I	*bd*-II	bo_3
50 μM O_2	7.3 ± 0.7	3.3 ± 0.3	18.5 ± 1.2
100 μM O_2	13.0 ± 0.9	5.7 ± 0.6	38.6 ± 3.8
150 μM O_2	22.1 ± 2.2	11.2 ± 0.7	65.3 ± 3.9
200 μM O_2	29.7 ± 2.3	19.8 ± 1.5	81.2 ± 5.1

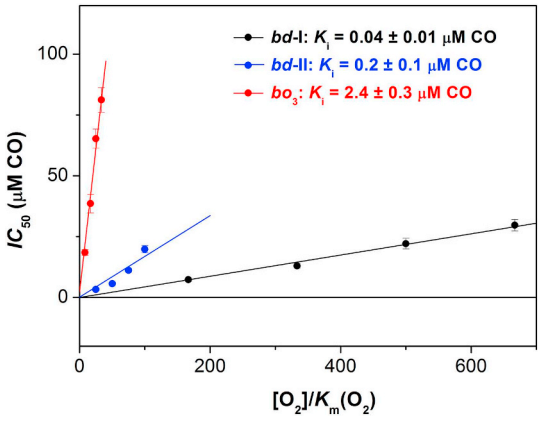

Fig. 5. Determination of K_i values for CO inhibition of *E. coli* terminal oxidases. K_i values for CO inhibition were obtained from the experimentally determined apparent IC_{50} values, assuming competitive inhibition. Apparent $K_m(O_2)$ values were taken from the literature: 0.3 μM (cytochrome *bd*-I) and 6 μM (cytochrome bo_3) are from ref. [56], and 2 μM (cytochrome *bd*-II) is from ref. [57].

NO, the reason for the lower sensitivity of cytochrome *bd*-I is likely to be the unusually high *off*-rate, much greater than those characteristic of the bo_3 oxidase and the large majority of other heme proteins [56,74]. In contrast, in the case of CO, both *E. coli bd* oxidases appear to be more sensitive to inhibition than cytochrome bo_3. The calculated K_i (CO) values (0.04 μM for *bd*-I, 2.4 μM for bo_3 and 0.2 μM for *bd*-II) are in fairly good agreement with the corresponding apparent K_d(CO) values for the fully reduced enzymes reported previously (80 nM [50] and 1.7 μM [53], for *bd*-I and bo_3, respectively) or determined here (0.6 μM for *bd*-II, Fig. 2). Seeking an explanation for the remarkably different K_d (and K_i) values between bo_3 and *bd*-I, it seems reasonable to compare their k_{off} and k_{on} values for CO. The comparison shows that CO binds/dissociates to/from reduced heme *d* significantly faster ($k_{on} = 8 \times 10^7$ [75], $k_{off} = 6\,s^{-1}$ [74]) than to/from reduced heme o_3 ($k_{on} = 6.1 \times 10^4\,M^{-1}\,s^{-1}$ [53], $k_{off} = 0.08\,s^{-1}$ [76]). This is likely because Cu_B in heme–copper oxidases serves a gate that controls ligand binding to the high-spin heme in the binuclear center [77]. However, as expected, the k_{off}/k_{on} ratio is in the case of cytochrome *bd*-I (75 nM) much smaller than for cytochrome bo_3 (1.3 μM) and in both cases consistent with both the previously reported K_d values and the K_i values determined in the present study. Unfortunately, it is not possible to make such a comparison for *bd*-II because the k_{off} and k_{on} values for the reaction of this oxidase with CO are unknown. Of interest, K_i (CO) for the mitochondrial cytochrome *c* oxidase (0.3–0.32 μM [8,9]) is markedly lower than that measured here for cytochrome bo_3 (2.4 μM). The k_{on}(CO) of cytochrome *c* oxidase ($8 \times 10^4\,M^{-1}\,s^{-1}$, [78]) is very similar to that of cytochrome bo_3 ($6.1 \times 10^4\,M^{-1}\,s^{-1}$, [53]). However, the k_{off}(CO) for cytochrome *c* oxidase ($0.02\,s^{-1}$, [78]) is 4-fold smaller than that for cytochrome bo_3 ($0.08\,s^{-1}$, [76]), explaining, at least in part, such a difference.

The notable difference between *E. coli bd* oxidases and cytochrome bo_3 in the affinity for CO and NO is intriguing and expected to result from structural differences in the O_2-binding hemes of these enzymes (heme *d* and heme o_3, respectively) and their surrounding protein pockets. Several features are indeed known to modulate the affinity of heme proteins for exogenous gaseous ligands such as NO, CO, and O_2, including the chemical identity of endogenous axial heme ligand(s), the geometry of the iron coordination sphere, but also the ability of residues in the heme pocket to establish electrostatic interactions or H-bonds with the gaseous ligand or to sterically hinder its access to the heme iron [79]. However, while the 3D structure of *E. coli* cytochrome bo_3 was solved [17], that one of *E. coli bd* oxidases is yet unknown, which prevents at this stage to gain insights into the structural determinants accounting for the different affinities displayed by these enzymes for CO and NO.

The higher sensitivity to CO inhibition of cytochrome *bd*-I compared to *bd*-II and bo_3 oxidases may appear inconsistent with the finding by Jesse et al. [68] that an *E. coli* mutant with cytochrome *bd*-I as the only terminal oxidase is less sensitive to inhibition by the CO-releasing molecule CORM-3 ($Ru(CO)_3Cl$(glycinate)), both in terms of cell growth and respiration, compared to mutants expressing either *bd*-II or bo_3 as the sole terminal oxidase. However, it is now established that CO gas and CO-releasing molecules have different modes of action, the potent antimicrobial and cytotoxic properties of CORM-3 being due to the Ru (II) ion, rather than to the released CO itself [80].

Given both the O_2 competitive nature of CO inhibition and the higher (than cytochrome bo_3) sensitivity of *bd*-type oxidases to CO inhibition, exposure of the respiratory chain of *E. coli* to CO is expected to produce largely different effects in aerobic versus microaerobic conditions. Under the latter conditions, not only CO is a more potent inhibitor, but *E. coli* preferentially expresses the *bd*-type oxidases which are more sensitive to CO inhibition. This is possibly consistent with the largely O_2-dependent effects caused by CO on the *E. coli* transcriptome and, more specifically, on the expression of the genes encoding the respiratory oxidases [81]. Indeed, while CO was found to induce an increase in *cydAB* expression under both aerobic and anaerobic conditions, perhaps as a consequence of the CO-induced iron depletion, the gas caused opposite effects on the *cyo* operon depending on O_2 availability (decreased expression under aerobic and increased expression under anaerobic conditions).

The higher sensitivity to CO inhibition of *bd*-I and *bd*-II oxidases, compared to cytochrome bo_3, is of interest also in light of the proposed role of CO in bacterial sensing by macrophages [10]. These cells were proposed to produce CO via HO-I to sense viable pathogens in their surroundings. According to [10], upon interaction with CO, *E. coli* bacteria release ATP which then, acting as a danger molecule, enhances bacterial killing by macrophages. Interestingly, this CO-mediated sensing mechanism was found to require cytochrome *bd*-I, but not cytochrome bo_3, in line with our finding that the former oxidase has markedly higher affinity for CO than the latter.

In conclusion, we have shown here that the O_2 reductase activities of *bd*-type and heme/copper-type oxidases from *E. coli* are affected by CO differently, in line with the different reactivity of the gas with the O_2-reactive heme in their active site. This notably different sensitivity of the oxidases to CO inhibition, together with the O_2-competitive nature of inhibition and the largely adjustable expression of the oxidases, is expected to enable a fine modulation of the sensitivity of aerobic respiration to CO, which could be advantageous for *E. coli* under specific physiological conditions.

Transparency document

The Transparency document associated with this article can be found, in online version.

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