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The terminal oxidase cytochrome *bd*-I confers carbon monoxide resistance to *Escherichia coli* cells

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ABSTRACT

Carbon monoxide (CO) plays a multifaceted role in the physiology of organisms, from poison to signaling molecule. Heme proteins, including terminal oxidases, are plausible CO targets. Three quinol oxidases terminate the branched aerobic respiratory chain of *Escherichia coli*. These are the heme-copper cytochrome bo_3 and two copper-lacking *bd*-type cytochromes, *bd*-I and *bd*-II. All three enzymes generate a proton motive force during the four-electron oxygen reduction reaction that is used for ATP production. The *bd*-type oxidases also contribute to mechanisms of bacterial defense against various types of stresses. Here we report that in *E. coli* cells, at the enzyme concentrations tested, cytochrome *bd*-I is much more resistant to inhibition by CO than cytochrome *bd*-II and cytochrome bo_3. The apparent half-maximal inhibitory concentration values, IC_{50}, for inhibition of O_2 consumption of the membrane-bound *bd*-II and bo_3 oxidases by CO at ~150 μM O_2 were estimated to be 187.1 ± 11.1 and 183.3 ± 13.5 μM CO, respectively. Under the same conditions, the maximum inhibition observed with the membrane-bound cytochrome *bd*-I was 20 ± 10% at ~200 μM CO.

Carbon monoxide (CO) binds to a pentacoordinate high-spin heme iron in the ferrous form with a high affinity. The formation of a stable heme-CO complex in hemoglobin and other heme proteins underlies its long-known toxicity. Recently it became clear that the physiological role of CO is not so unambiguous. CO, together with nitric oxide (NO) and hydrogen sulfide (H_2S), was found to serve as an endogenous signaling molecule [1,2]. CO places a role in multidirectional communication between the host and the gut microbiome [3]. In higher eukaryotes, it is generated from heme oxygenase-mediated degradation of heme [2]. Bacteria produce CO using both homologs of eukaryotic heme oxygenases and other CO generating enzymes [3].

Since terminal oxidases of the respiratory chains contain hemes, they represent possible targets for CO. These enzymes couple the oxidation of cytochrome *c* or quinol by O_2 to the generation of a proton motive force. There are two structurally and evolutionarily unrelated superfamilies of terminal oxidases, the heme-copper oxidases and the copper-lacking *bd*-type cytochromes [4–7]. CO was reported to be a strictly competitive inhibitor of the purified beef heart cytochrome *c* oxidase toward O_2, with an inhibition constant (K_i) of about 0.3 μM [8,9]. The sensitivity of bacterial terminal oxidases to CO inhibition remains poorly studied. The fact that 100 μM CO inhibits O_2 consumption by membrane particles of wild type *Escherichia coli* by 38% at ca. 75% of O_2 saturation [10] suggests that the terminal oxidases in this bacterium are CO targets.

The branched respiratory chain of *E. coli* contains three terminal quinol oxidases expressed at different O_2 tensions. These are the heme-copper cytochrome bo_3 and two *bd*-type cytochromes, *bd*-I and *bd*-II, encoded by the *cyoABCDE*, *cydABX* and *appCBX* operons, respectively [11–13]. Their 3D structures were determined [14–20]. Cytochrome bo_3 holds a low-spin heme *b*, a high-spin heme o_3, and Cu_B. The latter two redox groups form a binuclear site in which O_2 is reduced to $2H_2O$. Both cytochrome *bd*-I and cytochrome *bd*-II carry three hemes, a low-spin b_{558}, and two high-spin, b_{595} and *d*, but no copper. Heme *d* is the site for the O_2 reduction reaction. Cytochrome *bd*-I endows *E. coli* with resistance to NO [21–24], peroxynitrite [25], ammonia [26]. Both the *bd*-I and *bd*-II oxidases also place the role in the protection of *E. coli* against H_2O_2 [27–30], H_2S [31,32], cyanide [31]. Cytochrome bo_3, in contrast, is much more sensitive to NO, ammonia, H_2S, and cyanide [23,26,31,32]. Cytochrome *bd* is considered as an attractive antibacterial target because it is present in prokaryotes but absent in human mitochondria [33].

The available data on the sensitivity of *bd*-type cytochromes to CO are somewhat equivocal. Working on the isolated enzymes, Forte et al.

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reported that in *E. coli*, both *bd*-type oxidases are more sensitive to CO inhibition than cytochrome bo_3 [34]. The situation turned out to be the opposite in cell cultures of *Mycobacterium smegmatis*. The latter contains two terminal oxidases, cytochrome *bd* and cytochrome *bcc-aa*$_3$ supercomplex, encoded by the *cydAB* and *qcrCAB* operons, respectively. Bayly et al. found that the Δ*cydbd* mutant shows complete inhibition of O_2 consumption upon addition of CO, whereas the Δ*qcrCAB* mutant is not significantly inhibited by this gas [35]. Here we show that in *E. coli* cell suspensions, cytochrome *bd*-I is much more resistant to inhibition by CO than cytochromes *bd*-II and bo_3.

We compared the effect of CO on O_2 consumption of cell suspensions of the three *E. coli* mutant strains each expressing a single terminal oxidase: cytochrome *bd*-I or cytochrome *bd*-II or cytochrome bo_3.[1] In these experiments, no external electron donor was added and cell respiration was sustained by endogenous respiratory substrates. Fig. 1 shows that 96.3 μM CO added at $[O_2] = 150$ μM inhibits O_2 consumption of *bd*-II-only and bo_3-only cells to a similar extent, by 35 ± 4% and 31 ± 4%, respectively. Under the same conditions, the CO inhibition of respiration of *bd*-I-only cells is minor, 6 ± 10%. The apparent half-maximal inhibitory concentration values, IC_{50}, for inhibition of *bd*-II- and bo_3-only cells at $[O_2] = 150$ μM were determined to be 187.1 ± 11.1 and 183.3 ± 13.5 μM CO, respectively. (Fig. 2). We were unable to estimate IC_{50} for *bd*-I-only cells due to insignificant inhibition of respiration by CO. The maximum inhibition percentage observed with *bd*-I-only cells at [CO] = 196,3 μM turned out to be 20 ± 10% (Fig. 2). Thus, we can conclude that under the conditions tested, in *E. coli* cells, the *bd*-I oxidase is much less sensitive to inhibition by CO than the *bd*-II and bo_3 enzymes. The latter two display similar susceptibility to this gas.

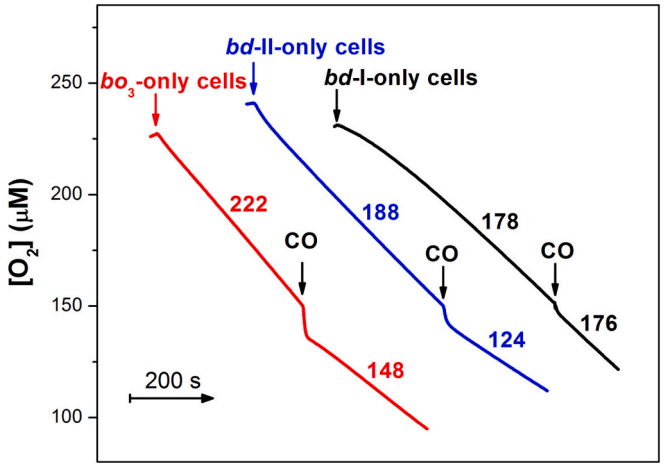

Fig. 1. Effect of CO on O_2 consumption by cell suspensions of the *E. coli* mutant strains expressing cytochrome *bd*-I, cytochrome *bd*-II or cytochrome bo_3 as the only terminal oxidase. Shown are selected O_2 consumption traces in which 96.3 μM CO was added to the chamber at $[O_2] = 150$ μM. O_2 consumption rates (nM O_2/s) measured before and after addition of CO are shown adjacent to each trace. Concentrations of cytochromes *bd*-I, *bd*-II, and bo_3 were 55, 58, and 64 nM, respectively.

The present data is inconsistent with the earlier observation that, being isolated, the *bd*-I and *bd*-II oxidases are more sensitive to inhibition by CO than cytochrome bo_3 [34]. We hypothesize that the discrepancy is due to different enzyme environments (native membrane lipids vs. detergent micelles) that could modulate its sensitivity to CO. Consistently, recent studies on membrane proteins suggest that lipid membranes can significantly affect their local structure, dynamics, and activity [38–40]. Furthermore, in *E. coli* membranes, cytochrome *bd*-I probably forms a supercomplex with other respiratory chain enzymes [41] that in turn may also affect its susceptibility to CO.

It can be suggested that in *E. coli* cells both CO and O_2 bind competitively to the high-spin ferrous heme (*d* in *bd*-I and *bd*-II, and o_3 in bo_3) in an enzyme's active site. However, their binding affinity is different in such a way that in cytochrome *bd*-I CO binding to the heme is outcompeted by O_2, whereas in cytochromes *bd*-II and bo_3 the opposite occurs. This would be consistent with the fact that CO dissociates from cytochrome *bd*-I at an unusually high rate ($k_{off} = 6\ s^{-1}$) [22]. The difference between the enzymes in the affinity for CO and O_2 may be due to structural differences in their heme active sites and the surrounding

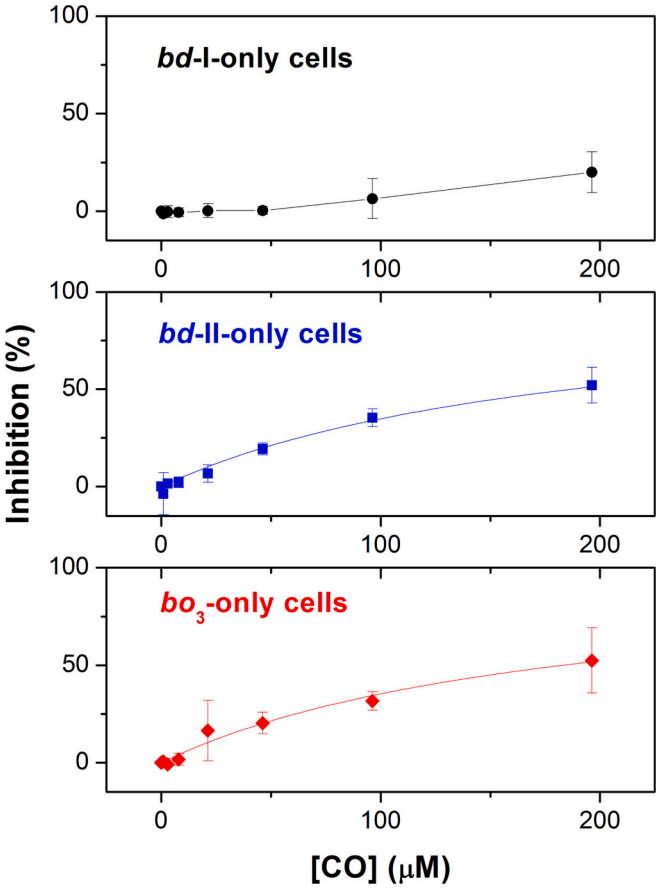

Fig. 2. Percentage inhibition of O_2 consumption by cell suspensions of the *E. coli* mutant strains expressing cytochrome *bd*-I, cytochrome *bd*-II or cytochrome bo_3 as the only terminal oxidase, measured at increasing concentration of CO added to the chamber at $[O_2] = 150$ μM. Concentrations of cytochromes *bd*-I, *bd*-II, and bo_3 were 65 ± 11, 57 ± 22 and 59 ± 12 nM, respectively. Values represent the mean ($n = 3$) ± standard deviations.

[1] The *E. coli* strains TBE025 (MG1655 Δ*cydB nuoB appB::kan*), TBE026 (MG1655 Δ*cydB nuoB cyoB::kan*) and TBE037 (MG1655 Δ*appB nuoB cyoB::kan*), which respectively express bo_3, *bd*-II and *bd*-I as the only terminal oxidase, were used [31]. The cells were grown in 50 mL-Falcon tubes, in 5 mL Luria Bertani (LB) medium supplemented with 30 μg/mL kanamycin, at 37 °C and 200 rpm. Oxygraphic measurements were performed at 25 °C in 50 mM K/phosphate pH 7.0, using a high-resolution respirometer (Oxygraph-2 k, Oroboros Instruments) equipped with two 1.5-mL chambers. Data analysis was carried out using Origin (OriginLab Corporation). The apparent IC_{50} values of CO were estimated by plotting the percentage inhibition of O_2 consumption of cell suspensions as a function of CO concentration, and fitting the data to the Hill Eq. [36], assuming a Hill coefficient $n_H = 1$. The level of protein present in each strain was estimated from the dithionite-reduced-*minus*-ferricyanyde-oxidized difference absorption spectrum of sonicated cells using $\Delta\varepsilon_{561-580}$ of 21 $mM^{-1}\ cm^{-1}$ (*bd*-I and *bd*-II) [37] and 16.3 $mM^{-1}\ cm^{-1}$ (bo_3). The latter value corresponds to $\varepsilon_{407} = 182\ mM^{-1}\ cm^{-1}$ in the absolute absorption spectrum of the oxidized bo_3 protein [34].

environment. Indeed, it is known that exogenous ligand affinity of heme proteins is modulated by the nature and geometry of the proximal heme ligand and distal amino acid residues which can stabilize bound exogenous ligand via electrostatic interactions and the donation of hydrogen bonds or, conversely, introduce steric hindrance to ligand binding [42]. Cytochrome *bd*-I after 100 μM CO starts to exhibit an increase of inhibition because, although the enzyme's affinity for CO in cells is probably lower than that for O_2, CO acts as a competitive inhibitor, meaning that at high concentrations CO starts to bind to the active site instead of O_2.

The difference in the sensitivity to CO between the two *bd*-type cytochromes, *bd*-I and *bd*-II, is intriguing. In this regard, it should be noted that cytochrome *bd*-I contains four subunits (CydA, CydB, CydX, CydY) while cytochrome *bd*-II only three (AppC, AppB, AppX). Interestingly, in the *bd*-I oxidase, CydY shields heme b_{595} from the membrane lipid environment thereby blocking access of gaseous molecules to the heme [17,18]. In contrast, in the *bd*-II oxidase, lack of CydY enables direct access of such ligands from the membrane interface [19,20]. For this reason, in cytochrome *bd*-II, CO might bind to the ferrous heme b_{595} in turnover resulting in the inhibition of electron transfer between heme b_{558} and heme *d* via heme b_{595}, i.e. the inhibition of the *bd*-II enzyme activity. This is not possible in cytochrome *bd*-I due to the presence of CydY. It is also worth noting that in both structural studies on cytochrome *bd*-II there is a consensus that the axial ligand of heme *d* is His^{19} of AppC [19,20]. On the contrary, data on the axial heme *d* ligand in cytochrome *bd*-I is contradictory. Safarian et al. [17] claim that it is also His^{19} (of CydA, homolog to AppC) but according to Theßeling et al. [18], Glu^{99} of CydA in cytochrome *bd*-I serves as the axial heme *d* ligand. Note that His^{19} and Glu^{99} are located on opposite sides of the heme macrocycle plane. If the axial ligand of heme *d* in cytochromes *bd*-I and *bd*-II in *E. coli* cells is indeed different, this could also be one of the reasons for the difference in their resistance to CO. Incidentally, perhaps due to the different nature of the axial ligand, heme *d* in cytochrome *bd*-II appears to have a much higher redox potential than in cytochrome *bd*-I, +440 mV vs +258 mV [19]. Further studies under varying experimental conditions are needed to get a full and consistent picture of the interactions between the *bd*-type oxidase and CO.

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CRediT authorship contribution statement

Martina R. Nastasi: Investigation, Methodology, Data curation. **Vitaliy B. Borisov:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Elena Forte:** Conceptualization, Data curation, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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