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Mitochondrial oxygenation of carbon monoxide

Lawrence J. YOUNG* and Winslow S. CAUGHEY
Department of Biochemistry, Colorado State University, Fort Collins, CO 80523, U.S.A.

A variety of mitochondria have been observed to oxidize ^{13}CO to $^{13}CO_2$ in the presence of dioxygen, and on the basis of earlier studies [Young & Caughey (1986) Biochemistry **25**, 152–161; Young (1981) Ph.D. Dissertation, Colorado State University] this activity is attributed to cytochrome *c* oxidase. Implications of these findings in respect of some aspects of the pathological biochemistry of CO poisoning are discussed.

INTRODUCTION

It has been observed [1] that isolated cytochrome *c* oxidase will catalyse an oxygenation of CO to CO_2 at CO/O_2 ratios ranging from 4:1 to 1700:1. It would not be unreasonable, then, to think that the enzyme might be physiologically of consequence in regards to the detoxification of CO. However, the turnover rate of CO is very slow ($0.02\ s^{-1}\cdot mol^{-1}$) for the isolated enzyme, and unless the mitochondrial catalysis is much faster, the oxidase is probably not going to be of major consequence in acute CO poisoning. Cytochrome *c* oxidase could still be an important factor in the adaptive response to chronic CO poisoning, and in this regard it is important to be aware that Luomonmaki & Coburn [3] have shown that humans can oxidatively metabolize small amounts of inhaled CO.

MATERIALS AND METHODS

Bovine and porcine heart mitochondria were prepared by using the methodology of Smith [4], with only the 'heavy' fraction being used in these studies. Bovine brain mitochondria were isolated as described by Cockrell & Bernard [5], whereas the method of Slack & Bursell [6] was used to prepare blowfly (*Sarcophaga* sp.) flight-muscle mitochondria. For rat liver and kidney mitochondria the procedure of Ogata [7] was employed, except for the use of Medium A of Cockrell & Bernard [5]. Isolations were from the freshest tissues possible, except for the rat kidneys, which were stored at −70 °C for 24 h before use. In all cases CO-oxygenation experiments were started immediately after isolation of the mitochondria.

Pelleted organelles were dissolved in a buffered medium (typically a buffered saline/sucrose solution) and divided into two identical fractions. The concentration of cytochrome *c* oxidase present was determined by optical spectroscopy of the first fraction using an oxidized−reduced absorption coefficient of $11.4\ mM^{-1}\cdot cm^{-1}$ at 605 nm [8].

The second fraction was used to demonstrate CO oxygenation. I.r. gas spectroscopy was used as the probe, the exact experimental protocol for which has been outlined in detail elsewhere [1, 2]. The method allows for both an accurate and reproducible measurement of small (nanomolar) amounts of both liquid- and gas-phase CO_2 produced by a suspension of mitochondria. After filling the sample and reference gas cells with a suspension of mitochondria and buffer respectively, both are vigorously flushed with wet N_2 for 5–10 min. Substrate (either pyruvate or succinate) is added and, after a final 2 min N_2 purge, the cells are placed in the i.r. spectrophotometer. Mitochondrial viability is proven with the demonstration of an increase of $^{12}CO_2$ in the sample cell after a brief incubation (~ 15 min) under the residual (post-flush) dioxygen. If such an increase is observed, then ^{13}CO plus air are added and the gas phase is monitored for production of $^{13}CO_2$ and $^{12}CO_2$.

In another set of experiments, bovine heart cytochrome *c* oxidase and Keilin–Hartree particles were used. The purified enzyme was passed through two 0.22 μm-pore-size Millipore filters before being placed in the sterile gas cell. It had been shown previously [1, 2] that bacteria present in the isolated oxidase will produce significant amounts of $^{12}CO_2$ after 8–10 h of incubation of the enzyme under air at 31 °C. Filtration sterilization completely eliminates this artefact. Furthermore, it was found that production of $^{13}CO_2$ by a sample of contaminated oxidase was not inhibited when the appropriate antibiotic was present. In spite of this latter finding and the fact that the incubations were under $O_2/CO/N_2$ (1.1:4.4:94.5) and not $O_2/CO/N_2$ (1:0:4), mitochondrial experiments were allowed to run for only 3.5–5.5 h.

Reagent-grade chemicals were used as supplied. ^{13}CO (90.7 and 97.0 atom %) was from Prochem, whereas the source of USP (U.S. Pharmacopeia)-grade N_2 (99.9%) and CO_2 (99.95%) was General Air Products.

I.r. spectra were recorded on a Perkin–Elmer Model 180 double-beam spectrometer. An Aminco DW2 optical spectrophotometer measured the oxidized−reduced difference spectra of suspensions of mitochondria.

RESULTS

Fig. 1 shows that $^{13}CO_2$ can be produced from ^{13}CO and O_2 in the presence of mitochondria, Keilin–Hartree particles and purified cytochrome *c* oxidase from bovine heart myocardium. In the case of the mitochondria and Keilin–Hartree particles there has also been production of CO_2 (as a consequence of concomitant tricarboxylic-acid-cycle activity); however, the critical observation insofar as CO oxygenation is concerned is that the natural abundance ratio for carbon (98.9:1.1) has significantly decreased [for each of the runs, spectrum A (Fig. 1) can be taken as the 'pre-^{13}CO plus air' control].

* Present address and address for correspondence and reprint requests: Department of Biochemistry, Duke University, Durham, NC 27705, U.S.A.

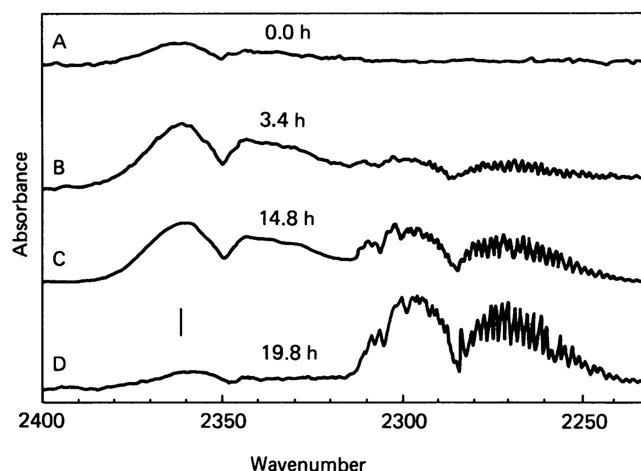

Fig. 1. Production of $^{13}CO_2$ from ^{13}CO and O_2 by bovine heart mitochondria, Keilin–Hartree particles and purified cytochrome oxidase

Oxidase concentrations were 0.900 mM in haem *a* in the case of purified enzyme and 0.045 mM for the other two samples. Reactions were carried out at 33 °C in pH 7.4-buffered media. Times shown are hours after addition of O_2 and 97.0 atom% ^{13}CO to mitochondria (A and B) or 90.7 atom% ^{13}CO to Keilin–Hartree particles (C) and purified enzyme (D). The vertical bar is equivalent to an absorption of 0.08 in C and 0.02 in A, B, and D. In this and the following Figure the raw data have been manually digitized and hence smoothed. No change of information content is either intended or implied.

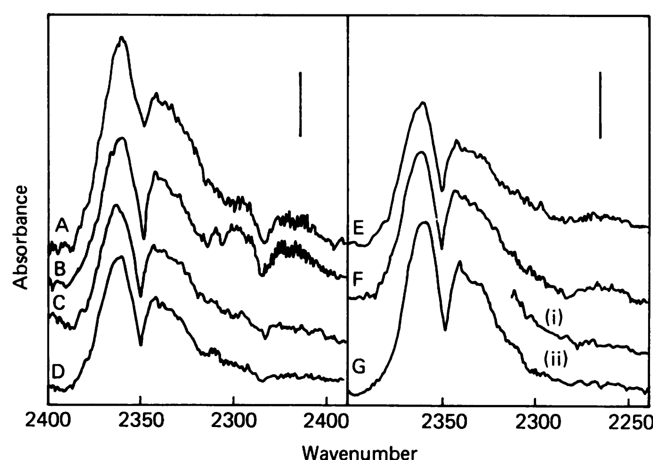

Fig. 2. Production of $^{13}CO_2$ from ^{13}CO and O_2 by a variety of mitochondria

Enzyme concentrations are 0.022, 0.045, 0.011, 0.015, 0.002, 0.039 mM for porcine heart, bovine heart, bovine brain, rat liver, blowfly (*Sarcophaga* sp.) flight muscle and rat kidney (spectra A–F respectively). All reactions were carried out at 33 °C in pH 7.4–buffered media; 97.0 atom% ^{13}CO was used in all runs. The natural-abundance spectrum (G) was obtained from a sample that contained 99.95% CO_2 and was twice (i and ii) run against an N_2-filled reference cell. The vertical bar is equivalent to an absorbance of 0.02 in all spectra.

The low-M_r $^{12}CO_2$ absorbs between 2380 and 2280 cm^{-1}, with maxima at 2360 and 2340 cm^{-1}, whereas the higher-M_r one ($^{13}CO_2$) absorbs from 2320 to 2220 cm^{-1}, with maxima at 2290 and 2270 cm^{-1}. Also apparent is the well-resolved rotational fine structure in the absorption envelope of the $^{13}CO_2$ species. A scan of the Keilin–Hartree particle sample (results not shown), taken 4.5 h after incubation under ^{13}CO was initiated, demonstrated a degree of isotopic enrichment similar to that seen in mitochondria after a similar period of time.

That oxidation of CO is not a peculiarity of the bovine heart system is shown by the data of Fig. 2. There have been no corrections applied to these spectra for the CO_2 present before addition of ^{13}CO and air or for CO_2 produced as a consequence of tricarboxylic-acid-cycle activity. Furthermore, since the control (spectrum G) contains 15–25% more CO_2 than any sample other than porcine heart (spectrum A), the 'apparent' isotopic enrichment for the other five samples is even further decreased from the 'true' enrichment, i.e. because the ^{13}CO is 97 atom% ^{13}C, the actual amount of $^{12}CO_2$ produced from CO oxygenation is just more than 3% of the $^{13}CO_2$ produced from CO oxygenation. Thus it is apparent that in these experiments nearly all the $^{12}CO_2$ comes from tricarboxylic-acid-cycle activity and nearly all the $^{13}CO_2$ from CO oxygenation. Correction of the raw data to reflect this fact would produce a spectrum similar to spectrum D of Fig. 1.

The evidence then for an increase in the percentage of $^{13}CO_2$ in the raw data of Fig. 2 is 2-fold, e.g., the presence of resolved rotational fine structure between 2320 cm^{-1} and 2220 cm^{-1} as well as a maximum at 2290 cm^{-1} and/or 2270 cm^{-1}. The first criterion was selected because the baseline undulations of spectrum G are random. Since the 'tail' of the $^{12}CO_2$ absorption envelop extends into the first $^{13}CO_2$ maximum, the 2270 cm^{-1} peak is the more reliable of the two.

There are some differences in experimental protocol for the runs of both Figs. 1 and 2 with respect to (i) the amount of oxidase present (enzyme concentrations range from 2 to 45 μM), (ii) the isotopic enrichment of ^{13}CO (90.7–97.0 atom%); (iii) the $^{13}CO/O_2$ ratio (4.2–5.0:1) and (iv) the length of the sample incubation period (3.5–5.5 h). This variability is of no consequence insofar as the qualitative demonstration of 'mitochondrial' oxygenation of CO is concerned.

DISCUSSION

This is the first report of a CO oxygenase activity in isolated mitochondria, thereby demonstrating that it is neither an artefact of the purified oxidase nor a peculiarity of the bovine heart system. It might be argued that some mitochondrial enzyme other than the oxidase is turning over the CO. This is thought to be highly improbable, since studies with intact and homogenized heart muscle [9, 10] as well as the isolated enzyme [1, 2] show that inhibitors of cytochrome *c* oxidase prevent oxidation of CO. The results reported here support the original observations of Fenn [11] concerning the phenomenon of CO oxygenation and provide the final link in the chain of evidence which extends from live humans [3], through isolated muscle [11], crude muscle homogenate [9], mitochondria and thence to the isolated oxidase [1, 2].

The oxygenation studies done with the isolated oxidase used one- and two-electron-reduced enzyme, and thus it was not known if the mitochondrial oxidase would be able to turn over the CO, i.e., if such partially reduced

forms could not be produced in the mitochondrion or if three- or four-electron-reduced enzyme would not oxygenate CO, then no isotopic enrichment of CO_2 would have been observed. The fact that enrichment was seen does not, of course, indicate which, if either, possibility is correct.

This study is not of the quantitative comparative type for several reasons: (*a*) the minor differences in protocol alluded to above; (*b*) succinate was the 'exogenous' substrate for bovine and porcine heart mitochondria, whereas pyruvate was used for the others; (*c*) although all the mitochondria studied were functional, i.e., they showed tricarboxylic-acid-cycle activity, there is no knowledge in an absolute sense of the degree of functionality; (*d*) it is not known what the reduction state of the mitochondrial oxidase was during CO oxygenation.

The data of Fig. 1 do, however, suggest that both mitochondria and Keilin–Hartree particles are more effective than the isolated enzyme in detoxifying CO, i.e., at 1/20 of the concentration of the purified oxidase, the mitochondrial enzyme after only 3.4 h has produced about 1/5 the amount of $^{13}CO_2$ produced by the former after 19.8 h. This observation should not be over-interpreted, since each sample in Fig. 1 was isolated from different ox hearts and cholic acid (a known oxidase inhibitor) was used to extract the oxidase from the mitochondrial inner membrane.

As mentioned above, it is unlikely that cytochrome *c* oxidase will have a significant role in ameliorating the effects of a rapid accumulation of inhaled CO. Similarly, a sudden increase in tissue CO not caused by inhalation of the gas (such as might occur during a haemolytic crisis of whatever aetiology) will probably be dissipated much more quickly by diffusion of CO to the blood, to the lungs and thence to the atmosphere, than by anything that the oxidase might do.

It is well known that tolerance for low levels of inhaled CO is possible [12], although aside from polycythaemia, the basis for this acclimation is not known. In the context of a role for the oxidase, it is conceivable that tolerance could be achieved by a mitochondrial 'branching' mechanism. This phenomenon was first observed by Chance [13] in studies on the effects of CO on mitochondrial respiration. It appears to arise from the ability of individual respiratory chains to shuttle reducing equivalents between each other via the mobile carriers coenzyme Q and cytochrome *c* when one of the chains is terminally blocked.

In this regard, the data of Fig. 2 show that both oxygenation of CO and tricarboxylic-acid-cycle decarboxylations occur during the course of the incubation, something which would not happen if NADH were accumulating, i.e., a chain that is oxygenating CO is similar to one that is inhibited by CO in that the catalytic-centre activity for CO is almost the same as the k_{off} for CO (0.01 s^{-1}), and the best evidence to date [1] suggests that there is no electron flow during CO oxygenation. If electron transport in all the chains was blocked, by either ligation of CO or oxygenation of CO, the excess NADH would inhibit the tricarboxylic acid cycle.

If most of the chains are functioning normally, albeit at an increased rate of electron flow, this will not occur. Therefore 'branching' during CO oxygenation provides a mechanism for developing a tolerance to small amounts of CO (inhaled or produced endogenously), by providing a means for electrons to bypass chains that are oxygenating CO.

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