JOURNAL OF BACTERIOLOGY, Dec. 1981, p. 991-994
0021-9193/81/120991-04$02.00/0

Vol. 148, No. 3

Electron Transport System of an Aerobic Carbon Monoxide-Oxidizing Bacterium

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Received 22 May 1981/Accepted 31 July 1981

Experiments with crude extracts of *Pseudomonas carboxydohydrogena* revealed that a quinone is necessary for CO oxidation, and that cytochromes of the *a*, *b*, and *c* types and functional terminal oxidase(s) are found in cells grown on CO as the sole source of carbon and energy. CO dehydrogenase delivers electrons to the electron transport system at the level of quinone, and nicotinamide adenine dinucleotide (phosphate) is not involved in CO oxidation.

It has been reported that several aerobic CO-oxidizing bacteria which can use CO as a sole source of carbon and energy possess cytochromes of the *b*, *c*, *o*, and *a* types, indicating that their electron transport system (ETS) may participate in CO oxidation. These bacteria include: *Pseudomonas carboxydoflava* (5a, 14), *Pseudomonas gazotropha* (5a, 14), *Comamonas compransoris* (5a, 14), *Achromobacter carboxydus* (14), *Seliberia carboxydohydrogena* (14), *Pseudomonas carboxydovorans* (9), an *Azotobacter* sp., and *Azomonas* strains (S. Kirkconnell, Ph.D. thesis, Indiana University, Bloomington, 1978).

Studies of artificial electron acceptors for CO oxidation with purified CO oxidizing enzymes from *P. carboxydovorans* (10) and *Pseudomonas* (*Seliberia*) *carboxydohydrogena* (5) and crude extracts (2) from *P. carboxydoflava*, *C. compransoris*, *A. carboxydus*, and three other unidentified strains suggested that ubiquinone is a possible physiological electron acceptor. Experiments with crude extracts from *Azotobacter* spp. and *Azomonas* spp. strains also suggested that a quinone is a strong candidate as a physiological electron acceptor during oxidation of CO to CO_2 (Kirkconnell, Ph.D. thesis).

The present work was carried out to learn whether a "typical" bacterial ETS functions in *P. carboxydohydrogena* during growth on CO and to determine whether electrons from CO are delivered at the level of quinone. The latter question was approached by reconstitution experiments with UV-irradiated crude extracts of *P. carboxydohydrogena* and by measuring the effects of several ETS inhibitors.

P. carboxydohydrogena DSM1083 was the gift of J. Schmidt and was grown under standard culture conditions with CO as the carbon and energy source as described before (5). Crude extracts were prepared from exponentially growing cells (5). Protein concentration in crude extracts was determined after treatment with NaOH by the biuret method (9).

It is known that quinones in cell extracts are labile to UV irradiation (3). Based on this fact, an experiment was performed to test involvement of quinones in CO oxidation. The CO-dependent reduction of cytochrome *c* by crude extract after UV irradiation was almost negligible, but activity was largely restored when the irradiated extract was incubated with ubiquinone 10, indicating that a quinone is necessary for CO oxidation (Fig. 1). In preliminary experiments it was observed that the purified carbon monoxide dehydrogenase (CO:acceptor oxidoreductase; CO-DH) (5) could reduce ubiquinone 10, but not NAD, FAD, or cytochrome *c* under standard assay conditions. Taken together with the fact that the purified enzyme can use several dyes which have redox potentials approximating that of quinone (5), it appears that a quinone is not only necessary but also a physiological electron acceptor in CO oxidation.

The nature of the ETS in CO-grown cells was also investigated by means of visible light difference spectra (Fig. 2). The reduced minus oxidized difference spectrum of the particulate fraction clearly disclosed the presence of cytochromes of the *b* (peaks at 562 and 530 nm), *c* (peaks at 552 and 523 nm), and *a* (peak at 604 nm) types. After NaCN treatment, peaks for cytochrome *c* disappeared, and several new peaks which represent cyanide-bound cytochrome oxidase appeared (peaks at 437 [data not shown], 544, and 577 nm). When the crude extract was flushed with CO, peaks for cytochromes *b* and *c* disappeared, and two new peaks and one trough (peaks at 545 and 574 nm, trough at 560 nm) appeared, strongly supporting the

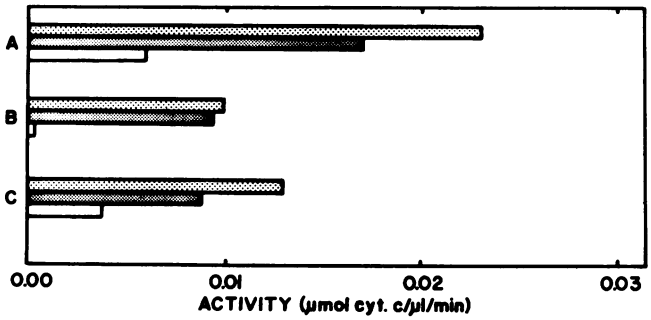

FIG. 1. *Restoration of CO-dependent cytochrome c reduction in UV-irradiated cell extracts. A 3-ml sample of crude extract (1.95 mg of protein per ml) was placed in a glass petri dish on ice and subjected to UV irradiation under a Mineralight lamp (model R52; Ultra-Violet Products, Inc.) for 15 min at a distance of 10 cm. A portion of the irradiated extract was removed for assay, 1.5 ml of ubiquinone (bovine heart; Sigma)-saturated ethanol was added to the irradiated extract, and the mixture was left for 2.5 h at room temperature for reactivation of the crude extract by the added quinone. The CO-DH activity of non-irradiated (A), irradiated (B), and reconstituted (C) extracts was measured by the standard enzyme assay method (5), except that 50 μM cytochrome c (horse heart; Sigma) was used as the electron acceptor and that enzyme activity was calculated as rate of reduction of cytochrome c. ϵ_{550}(red-ox) for cytochrome c was taken to be 18.5 $mM^{-1} \cdot cm^{-1}$ (7) for all experiments. Symbols: presence of CO (▨), absence of CO (▩), and CO-dependent reduction (═). Activity was measured as micromoles of cytochrome c reduced per microliter (1.94 μg of protein per μl) of enzyme per minute.*

conclusion that cytochrome *o* functions as a terminal oxidase (4) in cells grown with CO, and that there may be a branched ETS in *P. carboxydohydrogena*.

CO is known to inhibit the functioning of terminal oxidase in respiratory organisms. The existence of terminal oxidase in CO-autotrophically grown cells on solid medium was examined by using the "Nadi" reaction described by Marrs and Gest (8). α-Naphthol and dimethyl-*p*-phenylenediamine were purchased from Sigma Chemical Co. After treatment with Nadi reagent, colonies of cells grown with CO stained a deep blue color within 30 s (data not shown), indicating that the terminal oxidase is synthesized in this organism under CO. These results imply that a typical ETS exists in cells of *P. carboxydohydrogena* grown with CO.

Some well-known ETS inhibitors were used to test whether the ETS functions in cells growing under CO. One type of experiment used CO-dependent reduction of cytochrome *c* in extracts (Table 1), and the other used CO-dependent oxygen uptake by particulate fractions (Table 2). Among the ETS inhibitors tested with crude extracts, thenoyltrifluoroacetone, antimycin A, and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide were effective inhibitors of the CO-dependent reduction of cytochrome *c*, but rotenone was found to be completely ineffective. Inhibition of O_2 consumption in crude extracts in the presence of the inhibitors showed the same results, except that inhibition was not complete even with 10 mM cyanide.

These observations suggest that the existing ETS functions during CO oxidation, and that electrons are delivered from CO-DH to the ETS at the level of quinone. NAD(P) is not involved during growth with CO, as is also true of succinate dehydrogenase (13).

It is well known that CO is inhibitory for virtually all aerobic respiratory organisms. Even in aerobic carboxydobacteria it has been reported that high concentrations of CO reduce the growth rate and cellular yield (11, 12, 14) indicating that CO tolerance is involved in the ability to use CO at higher concentrations. In *Bacillus megaterium* cytochrome *o* is responsible for a small portion of cellular respiration and has a lower affinity for CO than does cytochrome a_3 (1). If a similar ETS in *P. carboxydohydrogena* acts together with the CO-DH which reduces the CO concentration in the cell by oxidizing CO as an energy source, it may be partly responsible for CO tolerance. It has been reported that cytochrome *o* also seems to be less sensitive to cyanide inhibition than cytochrome a_3 (6), perhaps explaining why 10 mM KCN was not sufficient to completely inhibit oxygen consumption by crude extracts of *P. carboxydohydrogena*.

P. carboxydohydrogena and other aerobic CO-utilizing bacteria cannot obtain reducing power directly through the reduction of $NAD(P^+)$ to NAD(P)H with the CO-DH enzyme. If electrons from CO are delivered from the CO-DH at the level of quinone, reduced pyridine nucleotide must be generated by re-

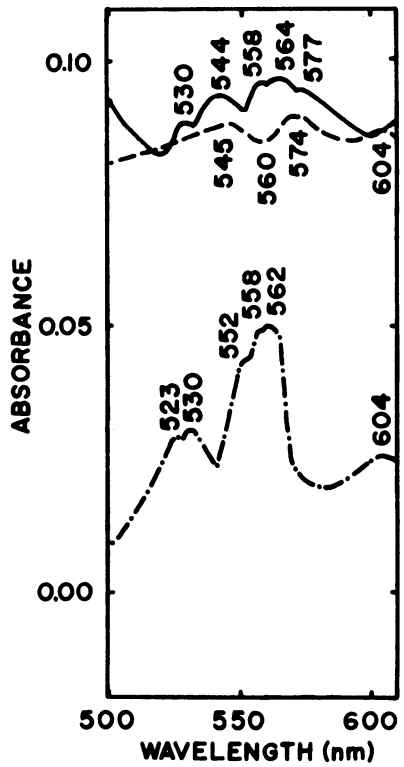

FIG. 2. *Absorption spectra of particulate fractions from P. carboxydohydrogena. Crude extract was centrifuged at 100,000 × g for 90 min at 4°C. The sediment was washed by resuspension in cold standard buffer (5) and recentrifuged. After washing, the sediment was suspended in 30% bovine serum albumin (Sigma), and a drop of 30% H_2O_2 was added to oxidize the samples, or a few crystals of sodium hydrosulfite to reduce the samples. To examine effects of cyanide or CO, a few crystals of NaCN were added, or the extract was flushed with CO for 6 min, respectively. The absorption spectra of samples were recorded from 400 to 610 nm at room temperature (Cary 14 spectrophotometer). Symbols: reduced versus oxidized (-·-), cyanide versus oxidized under air (——), and CO versus oxidized under air (- - -). CO-reduced difference spectra gave the same pattern as CO-oxidized under air, except that the two peaks and a trough for cytochrome o appeared at 570, 537, and 555 nm, respectively.*

verse electron transport, a process which is inefficient compared with direct reduction of $NAD(P^+)$ by substrate. This requirement may explain why *P. carboxydohydrogena* grows very slowly (t_d = 25 h) with CO (5) and why the efficiency of conversion of CO carbon to cellular material is only about 4% (14). It seems that *P. carboxydovorans*, which converts 16% of the CO oxidized to cellular carbon (9), has a more efficient ETS than *P. carboxydohydrogena* and that *Azotobacter* spp. and *Azomonas* spp.

TABLE 1. *Effects of inhibitors on CO-dependent cytochrome c reduction*[a]

Inhibitor	Final concn (μM)	Activity (nmol/μg/min)
None (+CO)[b]		5.8
None (−CO)[b]		4.4
Rotenone	4	5.4
Thenoyltrifluoroacetone	4	4.2
Antimycin A	4	3.6
HOQNO[c]	4	3.7

[a] Stock solutions of inhibitors (Sigma) were prepared in absolute ethanol and mixed with crude extracts (1.6 mg of protein per ml). Portions of extract, each treated with a different inhibitor, were left for 1 h at room temperature and then assayed for CO-DH activity by the standard method (5) with cytochrome *c* as the electron acceptor.

[b] Total activity with no inhibitor added in the presence (+CO) and absence (−CO) of carbon monoxide as substrate.

[c] HOQNO, 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide.

TABLE 2. *Effects of inhibitors on O_2 consumption in cell extracts*[a]

Inhibitor	Final concn (μM)	Remaining activity[b] (%) after:	
		1 h	2 h
None (+CO)[c]		100	100
None (−CO)[c]		0	0
Rotenone	4	100	100
Thenoyltrifluoroacetone	4	86	73
Antimycin A	4	88	74
HOQNO[d]	4	86	75
NaCN	1×10^4	33	13

[a] Inhibitors, prepared as described for Table 1, except that NaCN was prepared in water, were each added to a separate sample of crude extract (3.5 mg of protein per ml) and left for 1 to 2 h at room temperature. Oxygen consumption by each sample was measured at 30°C using a Yellow Springs Instruments model 53 biological oxygen monitor in conjunction with a strip chart recorder.

[b] O_2 consumption (about 300 μl) without inhibitor after 1 or 2 h is taken to be 100%.

[c] Total activity with no inhibitor added in the presence (+CO) and absence (−CO) of carbon monoxide as substrate.

[d] HOQNO, 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide.

strains (Kirkconnell, Ph.D. thesis) are still less efficient because they incorporate only 2% of the CO oxidized into the cellular material when grown autotrophically with CO. These differences imply, as suggested by Zavarzin and Nozhevnikova (14), that there are quantitative rather than qualitative variations in the ETS among utilitarian aerobic CO oxidizers.

We thank J. Schmidt for bacterial strains and H. Gest for helpful suggestions.

This work was supported by National Science Foundation research grant PCM 78-12482.

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