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Reduction of carbon monoxide to formaldehyde by the terminal oxidase of the marine bacterium *Pseudomonas nautica* strain 617

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When exposed to CO, the aerobic respiratory system of the marine bacterium *Pseudomonas nautica* strain 617, previously reduced with dithionite, undergoes reoxidation. When dealing with the purified oxidase (dithionite reduced) exposure of the enzyme to CO induces its reoxidation (collapse of its α band). Under our experimental conditions, this form of the oxidase could not be reduced again by dithionite. Addition of formaldehyde to the native oxidized enzyme resulted in full inhibition of the oxidase reduction by dithionite, presumably due to complex formation. We hypothesized a reduction of CO into formaldehyde and a locking of the active site by the reaction product. By using flash photolysis, it was possible to turn over the enzyme, accumulate the reaction product and identify it as formaldehyde. When using the membrane-bound enzyme, formaldehyde accumulated without the help of flash photolysis. This unusual reduction of CO to formaldehyde could be related to the previously reported uncommon features of the *P. nautica* oxidase, in particular O_2 reduction into H_2O_2 as end product [(1989) FEBS Lett. 247, 475–479].

Bacterial oxidase; Carbon monoxide; CO reduction; *Pseudomonas nautica* strain 617

1. INTRODUCTION

In eukaryotic cells, the terminal oxidase of the respiratory system located in the mitochondrial inner membrane is a complex transmembrane enzyme containing 4 redox centres (two *a*-type hemes, *a* and a_3, and two copper atoms, Cu_A and Cu_B). It catalyses the electron transfer from reduced cytochrome *c* to molecular oxygen which is reduced to water. Cytochrome *c* oxidase also takes part in the energy conversion through its proton pump function [1–5]. The initial discovery of this enzyme is linked to its behavior with respect to CO [6,7]. As an analog of O_2, CO can bind to reduced heme a_3, at the sixth coordinate of its iron atom as O_2 does. This CO-liganding is not followed by subsequent electron transfer but gives rise to absorption-band shift towards lower wavelengths. Photolysis of the CO-bound enzyme produces CO dissociation, the extent of this dissociation depending on the light wavelength and energy.

In prokaryotic cells, terminal oxidases can be different from the eukaryotic aa_3-type. They may vary in the nature of the O_2-binding heme, of the electron acceptor heme and they may involve different electron donors (*c*-type cytochrome or quinol). All these differences make presently a broad family which is expanding with investigation of new organisms [8–10]. In order to optically identify terminal oxidases among other heme-containing proteins, spectral changes induced by CO-liganding have been widely used since the pioneering work of Castor and Chance [11]. To identify the oxidase of the marine bacterium *Pseudomonas nautica* strain 617 [12], this approach has been naturally applied and revealed a peculiar behavior of this bacterial oxidase [13]. Indeed, upon exposure of the reduced enzyme to CO, instead of an expected band-shift, the reduced α band collapsed in the same way as upon reoxidation. The capacity of liganding CO, and presumably O_2, was thus detected, but a more complex reaction was occurring. The identified cytochrome, once purified [14], exhibited common features of a terminal oxidase, i.e. O_2 reduction, and NaN_3 inhibition [15,16].

In the present paper we report experimental evidence demonstrating that the purified oxidase from *P. nautica* 617 reduces CO to formaldehyde. This original property is discussed with respect to the characteristics of the purified oxidase [14] and another property which singled out this enzyme, i.e. the reduction of O_2 to H_2O_2 as end product instead of H_2O [15]. The results presented here constitute part of a research thesis [16].

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2. MATERIALS AND METHODS

2.1. *Growth conditions and oxidase purification*

P. nautica strain 617 [12] was grown aerobically at 307 K in a 300-l fermentor on a medium containing artificial sea water [17] and 0.6% yeast extract. Cells were collected in the exponential growth phase by centrifugation and washed with a 20 mM Tris-HCl buffer, pH 6. Cell walls were disrupted with a French press in the presence of DNase. Membrane fractionation and oxidase purification were performed as described by Arnaud et al. [14].

2.2. *Formaldehyde concentration determination*

Formaldehyde concentration was determined by using the procedure of Werringloer [18], derived from the method of Nash [19]. Proteins within the sample were precipitated with trichloracetic acid and spun down at 40 000 × *g* at 4°C for 30 min. The supernatant was collected and supplemented with the Nash reagent. After 10 min incubation at 60°C, the product of the assay, DDL (3,5-diacetyl 1,4-dihydrolutinin), was monitored spectrometrically at 412 nm. The assay was calibrated by using formaldehyde purchased from Sigma.

2.3. *Spectrometric techniques*

Dual-wavelength spectrometry [20] was carried out with a Johnson Foundation spectrometer built from two Bausch and Lomb 250 mm grating monochromators. The recording conditions corresponded to a 4 nm band-width. The transmitted light was monitored by using a fused-silica-window photomultiplier (EMI 9558QB) encompassing all the visible range. A microprocessor-controlled unit performed the data acquisition in a scanning mode with automatic base-line correction. Absorbance variations were plotted on a XY recorder (BD90 Kipp and Zonen). The reference wavelength was 575 nm.

The rapid scan spectrometer CD 66 was used as previously described [21] to monitor, in the visible range, the reaction between CO and the oxidase from *P. nautica* 617.

Flash photolysis was performed with a 500 J xenon flash lamp (Cunow) with a pulse width of 1 ms.

3. RESULTS

The optical characteristics of the purified oxidase in the visible range are represented in Fig. 1 by the difference spectrum between the reduced and oxidized states of the enzyme. Spectral changes following exposure of the reduced purified oxidase to CO were monitored by difference spectrometry. In Fig. 1, the difference spectrum between the state resulting from the CO effect and the initial reduced state presents the same features as the inverse of the reduced minus oxidized spectrum. This optical observation strongly suggests a reoxidation of the enzyme with a concomitant reduction of CO into formaldehyde, a possible derivative of CO reduction. The enzyme state induced by the reaction with CO was unaffected by dithionite added in excess either before or after the exposure to CO.

In order to test if formaldehyde could inhibit the oxidase activity, a sample of the purified oxidase in the oxidized state was supplemented with 1.3 μM formaldehyde in a 20 mM Tris-HCl buffer, 0.1% Triton X-100, pH 7.2. After subsequent addition of dithionite in excess, no reduction of the 1.2 μM oxidase could be observed, suggesting that the liganding of formaldehyde to the heme iron had occurred and was inhibiting further redox change of the oxidase. The action of formaldehyde on the oxidized purified oxidase can thus reproduce the situation resulting from the above CO effect.

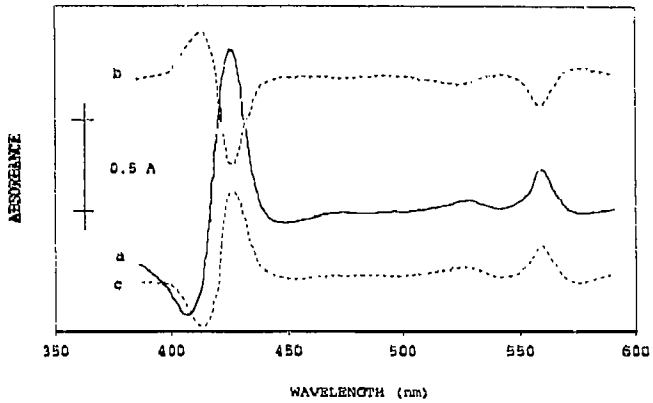


Fig. 1. Difference optical spectra of the purified oxidase from *P. nautica* 617. Spectrum (a) reduced minus oxidized; spectrum (b) CO-reduced minus reduced; spectrum (c) reduced minus CO-reduced. Reference wavelength: 575 nm. Temperature: 293 K. Sample: 6 μM purified oxidase in 5 mM phosphate buffer, pH 7.2, and 0.1% Triton X-100.

From this analogy between inhibitory effects with respect to the enzyme reduction by dithionite, we derived the following hypotheses: (i) the reaction of the reduced oxidase with CO produces formaldehyde, (ii) this end product is not released out of the active site of the purified oxidase but remains liganded to the heme iron, inhibiting further redox change of the oxidase.

In order to accumulate the assumed product of the reaction between the reduced bacterial oxidase and CO, samples containing dithionite-reduced enzyme exposed to CO either through bubbling or by addition of CO-saturated buffer, were photolysed repetitively. Two experiments were run under these conditions with the purified oxidase (Table I). The samples were submitted to

Table I

Formaldehyde production through CO reduction by the purified or membrane-bound oxidase of *Pseudomonas nautica* strain 617

Sample		Control	Purified oxidase		Membrane fraction
		oxidase concentration μM			
		0	1.77	0.78	0.38
Analysed subsample		formaldehyde concentration μM			
A	Measured	0	55*	33*	138
	Calculated		71	78	
B	Measured	0	151*	105*	327
	Calculated		142	156	
C	Measured	0		356	

*Formaldehyde production under the control of photolysis (see text).

series of 5–25 flashes with a 20 s interval between flashes. The absorption spectrum of the samples was recorded after each series of flashes to look for possible spectral changes. Subsamples were taken at 3 different stages (named A, B, C in Table I) to determine the amount of formaldehyde produced, as described in Section 2. In the absence of oxidase, formaldehyde was never detected.

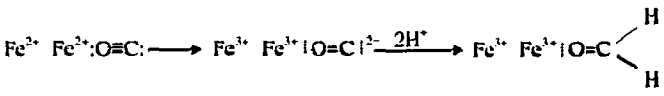
Assuming a single turnover of the oxidase after photolysis, theoretical values of product formation at stages A and B were calculated in the two experiments involving the purified enzyme. With initial oxidase concentrations of 1.77 and 0.78 μM and after 40 and 100 flashes respectively, the analysis revealed the production of 55 and 33 μM formaldehyde in the corresponding subsamples. Making the assumption – one flash, one turnover – the efficiency of the assumed process would be respectively 77 and 42%. In the following subsamples, corresponding to additional series of 120 and 100 flashes, the related contents of formaldehyde were found to be 151 and 105 μM whereas theoretical values amounted to 142 and 156 μM. The corresponding efficiency of the flash-controlled formaldehyde production raised to 106 and 67%. A third subsample in the experiment run with 0.78 μM purified oxidase was further monitored spectrally for 75 min in the absence of photolysis, but supplemented with CO and dithionite in excess. The final formaldehyde concentration was 356 μM.

A membrane fraction containing 0.38 μM oxidase was reduced with dithionite, exposed to CO and spectrally monitored in a similar way except that the sample was not submitted to flash photolysis. Two subsamples were taken at two different times, about 14 and 30 min, and the analysis revealed a significant production of formaldehyde, respectively 138 and 327 μM.

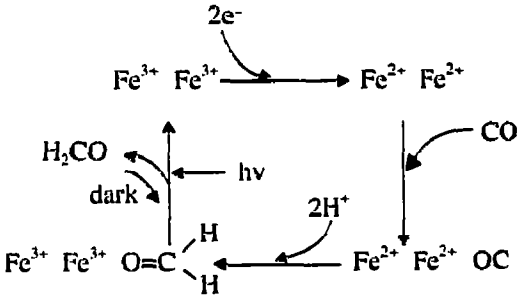
4. DISCUSSION

The initial observation that addition of CO to the dithionite-reduced respiratory system of the marine bacterium *P. nautica* 617 grown under aerobiosis results in cytochrome reoxidation [13], is finally accounted for by the above results. Indeed, experiments with the purified oxidase as reported in Table I, clearly demonstrate that the reduced oxidase from *P. nautica* 617 would not plainly bind CO as the other known oxidases do, but further reacts with it to produce formaldehyde in a 2 electron- 2 proton-reaction. For some reason, in the above experiments, the reaction product would keep binding to the heme iron, inhibiting further redox change of the oxidase. This was confirmed by the control experiment in which addition of formaldehyde to the oxidized oxidase effectively could not be followed by reduction of the heme centres in the presence of dithionite. The locking of the active site by the oxidase–formaldehyde complex is not specific to the enzyme in its natural environment since it does not occur when using the membrane fraction (Table I). This would suggest a subtle conformational change induced by the enzyme purification and which could be released after a large number of flashes as shown for subsample C in Table I. Flash photolysis turned out to be a good means for compensating the artefact induced by the purification procedure. Indeed, it helped the enzyme turnover with a reasonable efficiency, 77 and 42% for the concerned subsamples A (Table I). The efficiency increase to 106 and 67% (subsamples B, Table I) could be accounted for by the occurrence of more than one turnover after some flashes, a preliminary step to the freely reacting enzyme of subsample C (Table I). The locking of the active site by formaldehyde as soon as it is produced would also account for the fact that the corresponding Soret band (422 nm) does not superimpose on the one of the native oxidized states (417 nm) [14]. This formaldehyde-induced band shift would also explain the smaller amplitude of the related difference spectrum in the Soret range (Fig. 1b) than that of the reduced minus oxidized one (Fig. 1a).

To explain this unexpected reduction of CO by a heme-containing terminal oxidase, we would like to suggest that carbon monoxide binds to the ferrous iron via the oxygen atom, at variance with all known hemeproteins in which the carbon atom of CO is bound to the ferrous iron. Such a binding is likely to facilitate the formation of the 2 electron-reduced CO intermediate:



Under these conditions, the simplest mechanism for the observed turnover induced by flash photolysis would be as follows:



To our knowledge, this is the first terminal oxidase shown to reduce CO to formaldehyde in contrast to other investigated oxidases which tightly bind CO and are subsequently fully inhibited.

The CO reduction to formaldehyde is reminiscent of the original dioxygen reduction to hydrogen peroxide

by this peculiar bacterial oxidase [15]. Indeed, both reactions imply a mechanism involving 2 electrons and 2 protons.

The capacity of this novel oxidase to undergo other original redox reactions is presently under investigation.

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REFERENCES

[1] Wikström, M., Krab, K. and Saraste, M. (1981) in: Cytochrome oxidase. A synthesis, Academic Press, London.
[2] Denis, M. (1986) Biochimie 68, 459–470.
[3] Brunori, M., Antonini, G., Malatesta, F., Sarti, P. and Wilson, M. (1987) Eur. J. Biochem. 169, 1–8.
[4] Capaldi, R.A. (1990) Annu. Rev. Biochem. 59, 569–596.
[5] Chan, S.I. and Li, P.M. (1990) Biochemistry 29, 1–12.
[6] Keilin, D. (1925) Proc. R. Soc. London Ser. B 98, 312.
[7] Keilin, D. and Hartree, E.F. (1939) Proc. R. Soc. London Ser. B 127, 167.
[8] Anraku, Y. and Gennis, R.B. (1987) Trends Biochem. Sci. 12, 262–266.
[9] Anraku, Y. (1988) Annu. Rev. Biochem. 57, 101–132.
[10] Schäfer, G., Lüben, M. and Anemüller, S. (1990) Biochim. Biophys. Acta 1018, 271–274.
[11] Castor, L.N. and Chance, B. (1959) J. Biol. Chem. 154, 1587–1592.
[12] Bonin, P., Gilewicz, M. and Bertrand, J.C. (1987) Ann. Inst. Pasteur Microbiol. 138, 371–383.
[13] Arnaud, S., Malatesta, F. and Denis, M. (1988) Bioenerg. Conf. Rep. 5, 93.
[14] Arnaud, S., Malatesta, F., Guigliarelli, B., Gayda, J.P., Bertrand, P., Miraglio, R. and Denis, M. (1991) Eur. J. Biochem. 198, 349–356.
[15] Denis, M., Arnaud, S. and Malatesta, F. (1989) FEBS Lett. 247, 475–479.
[16] Arnaud, S. (1990) Thesis, University of Aix-Marseille II, Marseille.
[17] Bauman, P. and Bauman, L. (1971) in: The Prokaryotes. A Handbook on Habitats, Isolation and Identification of Bacteria (Starr, M.P., Stolp, H., Trüper, H.G., Balows, A. and Schlegel, H.G. eds.) pp. 1302–1330, Springer-Verlag, New-York.
[18] Werringloer, J. (1987) Methods Enzymol. 52, 297–302.
[19] Nash, T. (1953) Biochem. J. 55, 416.
[20] Chance, B. and Graham, N. (1971) Rev. Sci. Instrum. 42, 941–945.
[21] Denis, M., Neau, E. and Leveau, M. (1988) Eur. Biophys. J. 16, 259–265.