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Hydrogen peroxide is the end product of oxygen reduction by the terminal oxidase in the marine bacterium *Pseudomonas nautica* 617

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Mitochondrial cytochrome-*c* oxidase as well as several bacterial oxidases are known to reduce dioxygen to water. For the first time, a heme-containing oxidase, the terminal enzyme of the aerobic respiratory system in the marine bacterium *Pseudomonas nautica* 617, is shown to reduce molecular oxygen only to hydrogen peroxide. Whereas the cell content is well protected from H_2O_2 by catalase, the possible efflux of H_2O_2 into seawater could play an important role in the environment.

Bacterial oxidase; Oxygen reduction; Hydrogen peroxide; (*Pseudomonas nautica* 617)

1. INTRODUCTION

Cell respiration in aerobic organisms is characterized by the final electron transfer to molecular oxygen. This flow of reducing equivalents results from a series of substrate-oxidizing metabolisms occurring within the cell [1].

In eukaryotic cells, electron transfer to molecular oxygen is carried out by a complex membranous protein, cytochrome-*c* oxidase (EC 1.9.3.1), also referred to as cytochrome aa_3 [2]. The free energy made available by electron transfer from reduced cytochrome *c* to molecular oxygen is partially conserved by the means of a coupled proton-pumping function [3,4]. The related proton pump generates a proton electrochemical potential difference across the inner mitochondrial membrane which supports ATP production and other cell functions [5]. The O_2 reduction catalyzed by the mitochondrial cytochrome-*c* oxidase consumes 4 electrons, supplied by cytochrome *c* in one-electron steps, and 4 protons, taken up from the matrix space, to give H_2O as the end product.

In prokaryotic cells, this reduction of molecular oxygen to water can be processed by analogous aa_3-type cytochrome-*c* oxidases far simpler in their subunit composition than the complex of mammalian origin: the former comprise 1-3 subunits instead of 13 [6]. However, terminal oxidases in prokaryotes growing under aerobiosis are not restricted to aa_3-type cytochromes. Several distinct groups have been reported in the literature, namely *a*, *o*, *d*, cd_1 or *cd* [7]. Their great diversity in nature as well as structure prevents extrapolation to all bacterial oxidases of the findings established for the intensively investigated aa_3-type cytochrome-*c* oxidase. Nevertheless, it seems reasonable to expect that molecular oxygen is reduced to water by bacterial oxidases containing 4 redox centers like the aa_3-type enzyme [2]. This would be the case for cytochrome cd_{10} in *Pseudomonas aeruginosa*, which involves 2 hemes *c* and 2 hemes *d* [7] and for a cytochrome *o* containing 2 protohemes IX and 2 copper atoms, in *Vitreoscilla* [8] and in *Escherichia*

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coli [9]. Attention is required to avoid confusion as the term cytochrome *o* covers a large variety of oxidases which may differ in metal content like the cytochrome *o* from *Azotobacter vinelandii* which has been found to be devoid of copper [10].

In the absence of detailed information on the redox centers of other identified bacterial oxidases, it cannot be considered as granted that they reduce molecular oxygen to water. The terminal oxidase of the marine bacterium *Ps. nautica* 617 [11] grown under aerobiosis provides the first example of such a non-conventional situation. Indeed, as demonstrated here, the terminal oxidase of *Ps. nautica* reduces O_2 to H_2O_2 and not to H_2O.

2. MATERIALS AND METHODS

2.1. *Biological material and biochemical techniques*

Cells grown aerobically at 305 K, in a medium comprising artificial seawater [11], 0.6% lactate and 0.3% yeast extract, were collected in the exponential growth phase. Cell walls were disrupted by the means of a French press. The membrane fraction, isolated by centrifugation, was washed with 0.5 M NaCl solution to remove loosely bound proteins.

Membrane proteins were successively solubilized in 20 mM sodium phosphate buffer (pH 7.0) with 2% cholate, then in 0.1 M Tris-HCl buffer with 2% Triton X-100 (pH 7.2). Gel filtration was monitored on a Sephadex G-100 column. The final purification step (to be published elsewhere) consisted of ion-exchange chromatography on DEAE 52. The protein content was determined using the method of Lowry et al. [12] adapted for membrane proteins. Electrophoresis was run on polyacrylamide gels in the presence of SDS.

2.2. *Spectrometric techniques*

Spectra were recorded at 298 K with the CD66 rapid scan spectrometer [13] (scan speed, 80 spectra per s; digitization time, 7 μs per point, 512 points per spectrum). Each recorded spectrum resulted from the accumulation of 10000 successive spectra in order to benefit from an improved signal-to-noise ratio. Spectra were automatically corrected for apparatus baseline.

2.3. *Oxygen uptake measurements*

Oxygen consumption was monitored with an oxygraph using a Clark-type electrode in a 2 ml measuring chamber maintained at 293 K.

3. RESULTS

The oxygen requirement for hydrocarbon biodegradation [14] drew our attention to the oxidase of the aerobic respiratory system which constitutes a major difference between the enzymatic equipment of the aerobically and anaerobically grown cells.

By a detailed spectrometric investigation at room and low temperatures (not shown), a single oxidase was identified in the aerobic respiratory system. It has been purified as a membrane protein of M_r 130000 as determined by gel filtration. It contains heme *b* as prosthetic group (as judged from pyridine hemochromogen test). Room-temperature optical spectra of this purified *o*-type cytochrome are displayed in fig.1.

The optical features of this cytochrome are quite analogous to those of a *b*-type cytochrome. Exposure of the reduced protein to carbon monoxide changes its optical properties, a classical test for detection of oxidases [15]. However, the oxidase of *Ps. nautica* departs from the usual cytochrome *o* by the fact that CO does not simply ligand the reduced heme iron, which would induce a blue shift of the reduced absorption bands, but rather reacts with it, generating an 'oxidized' spectrum [16]. This reaction is currently under investigation.

3.1. *Hypothesis*

Because of this unusual behavior with CO, we suspected that the reaction with dioxygen may also depart from the usual pattern, i.e. from full reduction of O_2 to H_2O. Considering that both soluble and membrane fractions separated after cell disruption exhibit catalase activity, we derived the following hypothesis summarized by a sequence of 2 reactions catalyzed respectively by the oxidase and the catalase:

$$O_2 + 2H^+ + 2e^- \xrightarrow[\text{step 1}]{\text{oxidase}} H_2O_2 \xrightarrow[\text{step 2}]{\text{catalase}} H_2O + 1/2O_2 \quad (1)$$

The original feature of such a scheme is the reduction of O_2 to H_2O_2 by a heme-containing oxidase, the reaction product being broken down by catalase as soon as it has formed. To test this hypothesis, the oxidase and catalase activities must be handled separately.

Initiation of steps 1 and 2 in the above scheme at different times would generate the theoretical behavior depicted by fig.2A in an oxygraph cell. Addition of the purified oxidase to the respiratory medium containing an artificial electron donor would result in oxygen uptake at rate V of step 1 and in the build-up of H_2O_2 stoichiometrically with respect to the molecular oxygen taken up. Addition of catalase after significant oxygen uptake would initially emphasize step 2 over step 1, generating

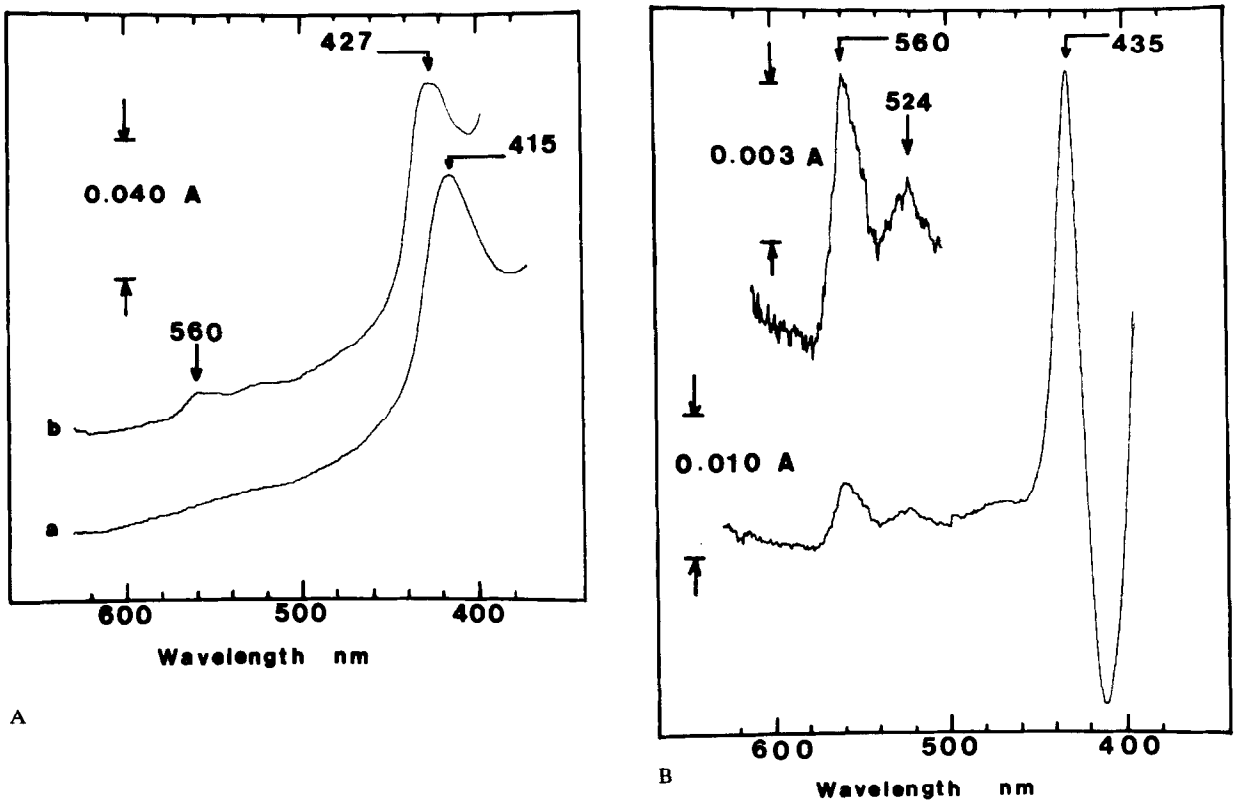

Fig.1. Optical properties of the purified heme *b* oxidase. (A) Absolute spectra of the oxidized (trace a) and reduced (trace b) purified enzyme. (B) Difference spectrum (reduced minus oxidized) of the purified enzyme. Note difference in absorbance scale. Sample: 0.1 M Tris-HCl buffer (pH 7.2), 0.1% Triton X-100, 2.3 μM oxidase (M_r 130000).

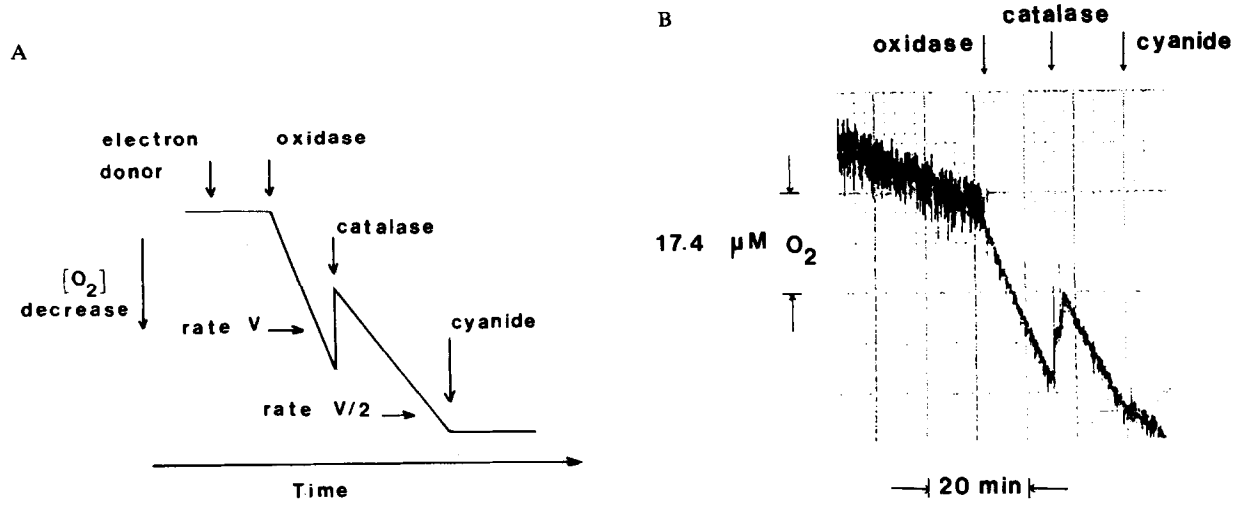

Fig.2. Oxygen uptake by the purified heme *b* oxidase in the absence and in presence of catalase. (A) Theoretical pattern according to the scheme in reaction 1. (B) Experimental recording. Electron donors: 7.8 mM ascorbate, 1.3 mM *p*-phenylenediamine dihydrochloride. Sample: 3.46 μM purified oxidase; same buffer as in fig.1; 0.15 mg · ml^{-1} catalase (Sigma); 25 μM cyanide. For oxidase addition, 0.9 ml medium was replaced by 0.9 ml purified oxidase. Data have been corrected to account for this dilution.

one half of the oxygen previously consumed. Then both activities would proceed in sequence according to the reaction of scheme 1 in such a way that the overall rate of oxygen uptake would decrease to half of the initial value. The addition of cyanide is merely a reminder that we are concerned with a respiratory terminal oxidase which should be fully inhibited by cyanide just as the whole cells are.

3.2. *Experiments*

These experiments have been run with the purified oxidase from aerobically grown *Ps. nautica* in an oxygraph using a Clark-type electrode. Fig.2B presents a typical output on following the protocol in fig.2A. The electron donors used are oxygen-sensitive which accounts for the observed oxygen drift before oxidase addition. Supplying catalase in excess results in the restoration of about one half of the oxygen consumed by the oxidase, thereafter the oxygen uptake proceeding at a lower rate. Addition of cyanide results in inhibition of oxidase activity and the residual oxygen consumption is taken as a baseline for quantitating enzyme activities. The specific activity of the oxidase assessed prior to catalase addition is 12.4 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. This corresponds to a turnover number of 0.03 s^{-1}. Catalase restores half of the oxygen reduced by the oxidase, as predicted by the theoretical diagram of fig.2A, which demonstrates that the oxidase of *Ps. nautica* behaves effectively according to the scheme of reaction 1, i.e. it reduces molecular oxygen to hydrogen peroxide and not to water. Comparison of the rates of oxygen uptake in the absence and presence of catalase, correcting for dilutions, yields a specific activity of H_2O_2 production of 13.1 nmol $H_2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$.

Analogous experiments have been performed with the cytochrome-*c* oxidase purified from beef heart mitochondria. Oxygen uptake by the mammalian enzyme was unaffected by addition of catalase (not shown).

4. DISCUSSION

The above results fully confirm the working hypothesis expressed by the reaction scheme (eqn 1), in which hydrogen peroxide is the end product of molecular oxygen reduction by the oxidase of *Ps. nautica*.

To the best of our knowledge, this is the first heme-containing oxidase which has been shown to reduce molecular oxygen to hydrogen peroxide in contrast with other oxidases reducing oxygen to water.

The bacterial cell content seems to be well protected by catalase against this synthesized hydrogen peroxide. It is reasonable to consider that a significant amount of hydrogen peroxide, produced as shown above, could be released by *Ps. nautica* before being broken down by catalase, contributing thereby to the steady level of H_2O_2 in seawater [17].

Within the context of basic mechanisms, this heme-containing oxidase should provide a very useful model to study the early steps in molecular oxygen reduction (transfer of 2 electrons and 2 protons to O_2), since its action stops halfway along the course of the reaction catalyzed by the mitochondrial cytochrome-*c* oxidase. Indeed, hydrogen peroxide formation occurs as a transient state in the aa_3-type oxidase whereas it can accumulate as an end product of molecular oxygen reduction by this bacterial oxidase (fig.2B). The present finding emphasizes the difficulty in classifying bacterial oxidases [18].

Another question of interest, presently under investigation, is the possible role of the reported reaction in hydrocarbon biodegradation.

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