ELSEVIER

Biochimica et Biophysica Acta 1410 (1999) 71–76

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Evaluation of relative contributions of two enzymes supposed to metabolise hydrogen peroxide in *Paracoccus denitrificans*

Marek Koutný, Luděk Kříž, Igor Kučera *, Ivo Pluháček

Department of Biochemistry, Faculty of Science, Masaryk University, Kotlářská 2, CZ-61137 Brno, Czech Republic

Received 1 October 1998; received in revised form 13 November 1998; accepted 25 November 1998

Abstract

A biosensor exploiting an electrochemically mediated enzyme-catalysed reaction was used to quantify relative contributions of cytoplasmic catalase and periplasmic cytochrome *c* peroxidase to the overall rate of hydrogen peroxide breakdown in cells of *Paracoccus denitrificans*. The effects of antimycin (an inhibitor of electron flow to cytochrome *c* peroxidase), the reaction rate versus substrate concentration profiles for the whole cells and subcellular fractions, and the time courses of oxygen concentration demonstrated a profound decrease in the capacity of cytochrome *c* peroxidase to reduce H_2O_2 under in vivo conditions. The reason is suggested to be a competition for available electrons between the enzyme and terminal oxidases metabolising oxygen produced by catalase.

Keywords: Hydrogen peroxide; Catalase; Cytochrome *c* peroxidase; (*Paracoccus denitrificans*)

1. Introduction

Paracoccus denitrificans can synthesise at least two enzymes capable of scavenging potentially harmful hydrogen peroxide: cytoplasmic catalase and periplasmic cytochrome *c* peroxidase [1]. While the former protein functions autonomously, the latter requires that reduced cytochrome *c* should be supplied by the respiratory chain.

In recent years significant progress has been made in the analysis of structural and catalytic properties of the bacterial two-haem cytochrome *c* peroxidases. The most detailed information is available for the *Pseudomonas aeruginosa* enzyme with the crystal structure determined to 0.24 nm resolution [2]. The enzyme from *P. denitrificans* has been characterised regarding its amino acid sequence, haem redox centres and Ca^{2+} binding [3–8]. Despite these advances, however, no study has drawn attention to H_2O_2 reduction in whole cells, where this process shares the respiratory electron flux with the reduction of oxygen via terminal oxidases of the respiratory chain.

Here we describe for the first time the kinetics and inhibitor sensitivity of H_2O_2 consumption by whole cells as investigated by means of sensitive amperometric measurements. The results, including those obtained with cell-free extracts, are discussed in terms of the ability of cytochrome *c* peroxidase to perform H_2O_2 reduction within the periplasmic space of the cells.

* Corresponding author. Fax: +420-5-4121-1214;
E-mail: ikucera@chemi.muni.cz

0005-2728/99/$ – see front matter
PII: S0005-2728(98)00176-5

2. Materials and methods

2.1. Microorganism and culture conditions

Paracoccus denitrificans NCIB 8944, obtained from the Czech Collection of Microorganisms as CCM 982, was grown at 30°C in gently agitated 3-l conical flasks containing 0.8 l of the succinate minimal medium [9] until the late log phase (18 h). Cells were collected by centrifugation (6200×*g*, 20 min) and washed once with cold 0.1 M sodium phosphate (pH 7.3).

2.2. Preparation of subcellular fractions

Bacteria were fractionated as previously described by Alefounder and Ferguson [10] for type II spheroplasts. Treatment of cells with lysozyme plus EDTA together with a mild osmotic shock, followed by centrifugation, yielded a supernatant designated as the periplasmic fraction. The fraction named cytoplasmic resulted from osmotic lysis of the pelleted spheroplasts, DNAase treatment and one ultracentrifugation step. Both fractions were concentrated by ultrafiltration and checked for their purity by determining the content of periplasmic and cytoplasmic marker enzymes (nitrite reductase and malate dehydrogenase, respectively).

2.3. Molecular exclusion chromatography

The periplasmic and cytoplasmic proteins were further fractionated using a 1.4×28.8 cm Superose 12 column equilibrated with 100 mM NaCl/25 mM Tris–HCl (pH 7.23) buffer. The following marker proteins (with their M_r) were used to calibrate the column: chymotrypsinogen A (25 000), ovalbumin monomer (43 000), serum albumin monomer (67 000), aldolase (158 000), catalase (232 000) and ferritin (440 000).

2.4. Electroimmunoassay of nitrite reductase protein

A Laurell-type rocket electroimmunoassay was performed in 1.2% agarose gels with 25 mM sodium barbital–HCl (pH 8.6), 0.01% sodium azide, and swine antibodies against *P. denitrificans* nitrite reductase; the voltage was 1.5–2 V cm^{-1} for 16 h.

2.5. Spectrophotometric assays

All spectrophotometric measurements were made at 30°C with a Cary 118C spectrophotometer in cuvettes of 1 cm light path.

Stock solutions of hydrogen peroxide, prepared by dilution of the commercial H_2O_2 at 30% in distilled water, was determined from the absorbance at 240 nm using an absorption coefficient of 36 $M^{-1}\,cm^{-1}$ [11].

Cytochrome *c* peroxidase activity was measured by following the oxidation of cytochrome *c* at 550 nm. The physiological donor, cytochrome *c*-550 of *P. denitrificans*, was replaced by the commercially available horse heart cytochrome *c*. This substitution is reported to reduce the maximum catalytic-centre activity by 27% [4]. A reaction mixture of 2.0 ml contained 0.1 M sodium phosphate (pH 7.0), 1 mM disodium ethylenediaminetetraacetic acid (EDTA) and 5 μM horse heart ferrocytochrome *c*. After adding a protein sample and recording the endogenous rate for 40–60 s, the final reaction was started by the addition of 20 μM hydrogen peroxide. The activity was calculated on the basis of an absorption coefficient difference (reduced−oxidised) of 21.1 $mM^{-1}\,cm^{-1}$ [12] and a two-electron change for H_2O_2 reduction.

The reaction mixture (2.0 ml) for malate dehydrogenase was composed of 0.1 M sodium phosphate (pH 7.3), 0.15 mM NADH and an appropriate amount of protein. The reaction was started by 0.2 mM oxaloacetate and monitored at 340 nm.

2.6. Electrochemical assays

All electrochemical experiments were performed in a closed 5-ml glass cell with an inlet for reagent addition and for bubbling with argon, thermostatted at 30°C by means of forced water circulation, and stirred magnetically by a glass-coated stirring rod. The operating potential of the working electrode was applied using an ADLC2 amperometric detector (Laboratory Instruments, Prague, CR) or an OH-105 universal polarograph (Radelkis, Budapest, Hungary), the output signal was recorded with the latter apparatus.

A new biosensor for hydrogen peroxide was constructed as follows. The base substrate AC1.WS.RS,

obtained from Krejčí Engineering (Tišnov, CR), consisted of a platinum working electrode (1 mm diameter) surrounded by a silver reference electrode (area 13 mm^2) on a ceramic strip (7 mm × 25 mm). The reference electrode was coated by a silver chloride layer (anodisation in 0.2 M KCl for 30 s at 1.0 V versus Pt counter electrode). The working electrode was covered by a mixture of graphite powder (Sigma-Aldrich Corp., Prague, CR), cellulose acetate (Sigma), ferrocenemonocarboxylic acid (Sigma) and horseradish peroxidase (113 U mg^{-1}, Boehringer Mannheim, Germany) (20:3:4:4, by weight) applied as a thick suspension in cyclohexanone. Cathodic reduction of H_2O_2 proceeded at the operating potential of 0 V vs. the integrated Ag/AgCl reference electrode (0.1 M Tris–HCl (pH 7.3) as the reaction medium). Alternatively, an unmodified simple platinum cathode (2 mm diameter) polarised at +0.65 V (vs. the Ag/AgCl reference) was used for measuring the activity of catalase (0.1 M sodium phosphate (pH 7.3), 4 mM H_2O_2).

Oxygen concentration was monitored with a conventional Clark-type oxygen electrode (2 mm diameter; Krejčí Engineering, Tišnov, CR).

2.7. Analysis of kinetic data

Half saturation constants $K_{0.5}$ and their standard errors are given as determined by nonlinear regression, using the program EZ-FIT developed by F. Perrella [13].

3. Results

When the microoxically grown cells of *P. denitrificans* were subjected to subcellular fractionation and assayed for catalase and cytochrome *c* peroxidase, the results exemplified by those in Fig. 1 were obtained. As expected from the literature, the first activity was associated with the cytoplasmic fraction (marked by malate dehydrogenase) whereas the second resided in the periplasm (marked by nitrite reductase). From Fig. 1 it follows that the content of cytochrome *c* peroxidase within the periplasmic space of cells might in principle account for approximately one-third of the total H_2O_2 consumption activity observed.

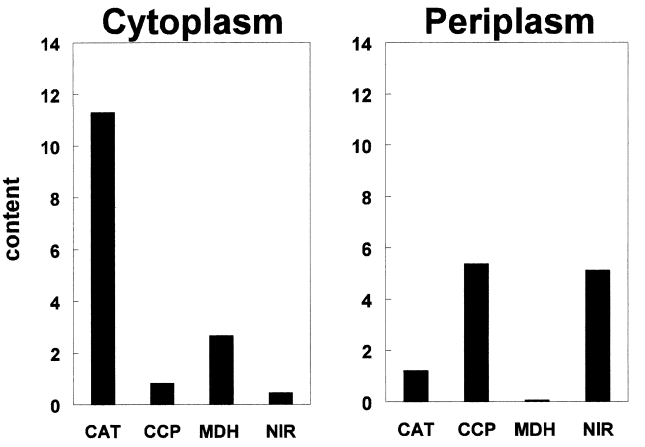

Fig. 1. Subcellular fractionation of enzyme activities. Cytoplasmic and periplasmic fractions prepared from the lysozyme-treated cells (3.6 g dry weight) and assayed for catalase (CAT), cytochrome *c* peroxidase (CCP), malate dehydrogenase (MDH) and nitrite reductase (NIR) as described in Section 2. The units used are nkat (mg dry weight)$^{-1}$ of cells (CAT, CCP, MDH) or μg enzyme protein (mg dry weight)$^{-1}$ of cells (NIR). Data correspond to one of several independent experiments, which usually agreed within ±20%.

Catalase activity present in the cytoplasmic fraction peaked singly upon molecular exclusion chromatography, displaying a relative molecular mass of 210 000 ± 20 000. Similar analysis of the periplasm revealed a single peak of cytochrome *c* peroxidase at an M_r of 42 000 ± 3000. The two partially purified enzymes differed in their dependence on hydrogen peroxide concentration. While the rate of catalase reaction increased linearly with [H_2O_2] over the range up to 1 mM, cytochrome *c* peroxidase showed a saturation kinetics compatible with the $K_{0.5}$ values of 9 ± 4 μM and 2 ± 1 μM for H_2O_2 and reduced cytochrome *c*, respectively.

Having established some of the basic properties of two soluble enzymes possibly involved in the metabolism of H_2O_2, we looked for a method for monitoring H_2O_2 uptake by intact cells. Since the amperometric detection of H_2O_2 based on its reduction at an unmodified platinum electrode did not ensure the sensitivity desired for the micromolar concentration range, we preferred to use an enzyme biosensor, exploiting the mediated peroxidase reaction. This approach took advantage of zero voltage maintained between the working and reference electrode of the biosensor, which led to a marked reduction in the baseline current. Furthermore, the sensitivity (de-

fined as the current per area unit of the cathode exposed to a given concentration of H_2O_2) increased approximately tenfold in comparison with the system lacking the enzyme layer. The biosensor responded linearly for H_2O_2 concentrations below 50 μM; the response time necessary to achieve 90% of the final signal after addition of 10 μM H_2O_2 was about 1 s.

If a part of the H_2O_2 consumption capacity of the intact cells derives from the respiratory chain-linked cytochrome *c* peroxidase, as seems likely from the fractionation studies, then it might be expected that respiratory inhibitors would alter the observed rate of H_2O_2 disappearance. However, the traces presented in Fig. 2 demonstrate that antimycin was without significant inhibitory effect even at the titre sufficient to block electron flow through the bc_1 complex [14]. The time courses remained the same after a fourfold dilution of the Tris buffer or with mucidin (another inhibitor of the bc_1 complex) instead of antimycin (results not shown). These findings may imply a negligible activity of the periplasmic enzyme within the cells. The same conclusion could be ar-

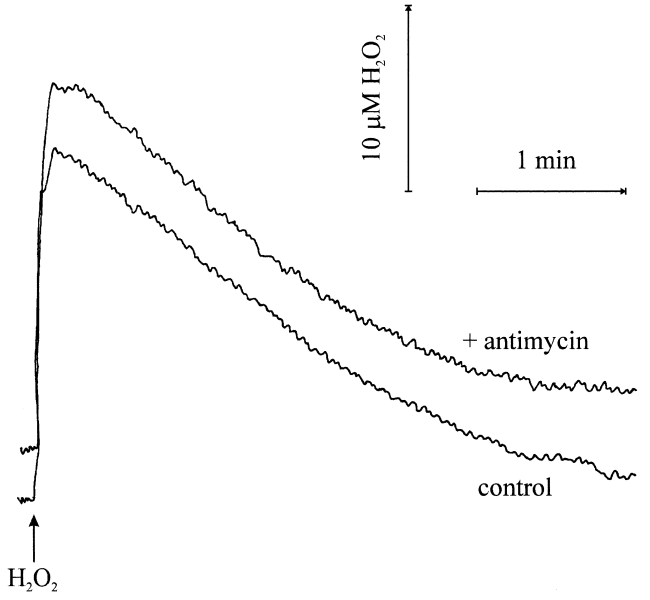

Fig. 2. Disappearance of hydrogen peroxide from a suspension of *P. denitrificans* and the effect of antimycin. Washed intact cells (2.5 mg dry weight) were resuspended in 5.1 ml of the argon-flushed medium containing 0.1 M Tris chloride (pH 7.3), and 5 mM sodium succinate, placed in a closed chamber fitted with a H_2O_2 biosensor. When indicated, 25 μg antimycin was also added. The mixture was incubated 5 min at 30°C prior to addition of 20 μM H_2O_2.

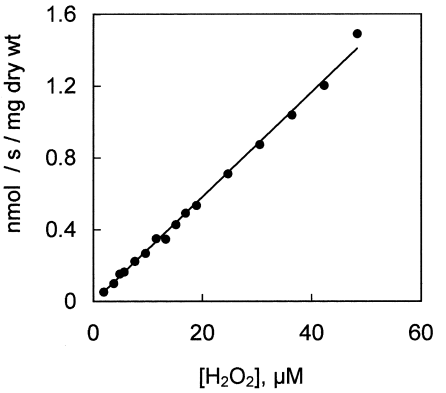

Fig. 3. Dependence of the initial rate of H_2O_2 uptake by *P. denitrificans* on the concentration of H_2O_2. Conditions were as specified for Fig. 2 except that the reaction was started by different concentrations of hydrogen peroxide and the mixture did not contain antimycin. Rates were calculated from the slopes of the initial parts of the reaction progress curves.

rived at from a clear-cut linear dependence between initial velocity and H_2O_2 concentration (Fig. 3), comparable with that found for the partially purified *P. denitrificans* catalase (see above). It is evident that the dependence in Fig. 3 lacks a hyperbolic component expected to originate from gradual saturation of cytochrome *c* peroxidase by micromolar concentrations of H_2O_2.

Further information about the contributions made by individual enzymes acting on H_2O_2 came from measurements using the oxygen probe. Addition of hydrogen peroxide to cells resulted in an immediate burst of oxygen concentration, followed by a slower decrease (Fig. 4). From the peak of the oxygen trace (corrected for simultaneous consumption by extrapolating back to the point of hydrogen peroxide addition) and the known amount of H_2O_2 added, the O_2/H_2O_2 values close to 0.5 could be estimated. This again supports the notion of catalase as the primary contributor toward H_2O_2 removal.

4. Discussion

Isolated cytochrome *c* peroxidases catalyse the reduction of hydrogen peroxide by the periplasmic electron donors cytochrome *c* or azurin/pseudoazurin and the same activity has previously been implicitly assumed to occur in vivo. Contrary to this expectation, three lines of evidence (Figs. 2–4) suggest

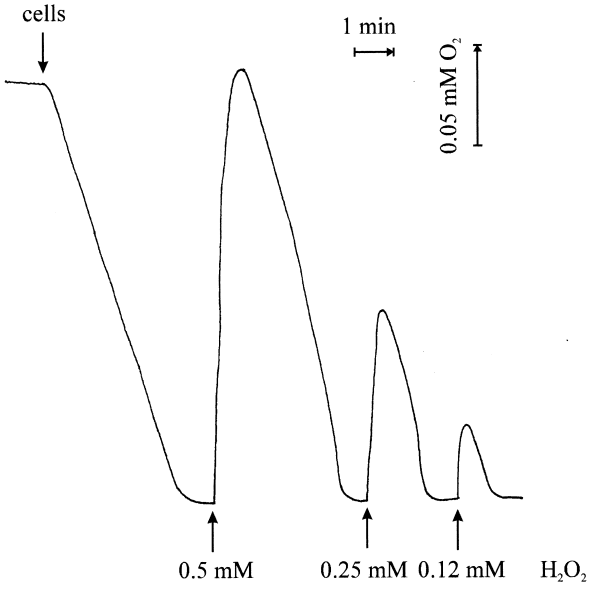

Fig. 4. Production of oxygen from H_2O_2 by *P. denitrificans*. The Clark electrode chamber contained, at 30°C, 2.1 ml of 0.1 M sodium phosphate (pH 7.3) and 5 mM sodium succinate. The additions of cells (1.0 mg dry weight) and hydrogen peroxide (final concentrations given) were as indicated by arrows.

that the enzyme, although abundantly present in the bacterial cells examined (Fig. 1), does not participate significantly in the metabolism of the external hydrogen peroxide. This inability can reflect a restricted electron supply to the periplasm since the operation of cytoplasmic catalase generates oxygen whose reduction to water by the membrane-bound terminal oxidases consumes a substantial part of electrons coming from the respiratory substrates. Competitive phenomena of this kind are known to operate when the periplasmic reduction of nitrite and nitrous oxide ceases following oxygen addition. In this case, oxygen effect can be overcome by an adequate supply of reduced cytochrome *c*, the physiological electron donor to both reductases [15].

Expression of *P. denitrificans* cytochrome *c* peroxidase requires activation of the FnrP protein [16] and therefore takes place only at low oxygen tensions. This may be because the enzyme works exclusively in the breakdown of trace amounts of H_2O_2 formed under microoxic conditions whereas oxygen concentrations sufficient to saturate terminal oxidases preclude it from functioning. Alternatively the cytochrome *c* peroxidase protein may somehow be involved in the anaerobic electron transport as an electron carrier. The latter possibility was already considered in context of the finding that a fraction of periplasmic proteins enriched in cytochrome *c* peroxidase mediated electron flow from membrane vesicles to nitrite reductase [17], but recent results from this laboratory suggest that the observed activity may be due to the presence of pseudoazurin (M. Koutný, I. Kučera, unpublished data).

There appears to be a discrepancy between our results and the conclusions drawn recently by Richardson and Ferguson [18]. These authors observed a strong inhibition of nitrate respiration by H_2O_2 in several denitrifying strains and ascribed it to a partitioning of electrons between nitrate reductases and periplasmic cytochrome *c* peroxidases. Such an association must be regarded as tenuous for the present, however, since neither the time courses of H_2O_2 and O_2 concentrations nor the actual capacity of the cytochrome *c* peroxidase pathway were examined. Moreover, H_2O_2 in itself might disturb nitrate reductase or other proteins involved in electron transport to nitrate. Further studies employing the amperometric technique described here can help to clarify this issue.

Acknowledgements

This work was supported in part by Grant 204/93/2054 from the Grant Agency of the Czech Republic.

References

[1] C.F. Goodhew, I.B.H. Wilson, D.J.B. Hunter, G.W. Pettigrew, Biochem. J. 271 (1990) 707–712.
[2] V. Fülöp, C.J. Ridout, C. Greenwood, J. Hajdu, Structure 3 (1995) 1225–1233.
[3] R. Gilmour, C.F. Goodhew, G.W. Pettigrew, S. Prazeres, I. Moura, J.J.G. Moura, Biochem. J. 294 (1993) 745–752.
[4] R. Gilmour, C.F. Goodhew, G.W. Pettigrew, S. Prazeres, J.J.G. Moura, I. Moura, Biochem. J. 300 (1994) 907–914.
[5] R. Gilmour, S. Prazeres, D.F. McGinnity, C.F. Goodhew, J.J.G. Moura, I. Moura, G.W. Pettigrew, Eur. J. Biochem. 234 (1995) 878–886.
[6] S. Prazeres, J.J.G. Moura, I. Moura, R. Gilmour, C.F. Goodhew, G.W. Pettigrew, N. Ravi, B.H. Huynh, J. Biol. Chem. 270 (1995) 24264–24269.
[7] D.F. McGinnity, B. Devreese, S. Prazeres, J.J. Van Beeumen, I. Moura, J.J.G. Moura, G.W. Pettigrew, J. Biol. Chem. 271 (1996) 11126–11133.

[8] W. Hu, G. Van Driessche, B. Devreese, C.F. Goodhew, D.F. McGinnity, N. Saunders, V. Fülöp, G.W. Pettigrew, J.J. Van Beeumen, Biochemistry 36 (1997) 7958–7966.
[9] I. Kučera, I. Mat'chová, V. Dadák, Biocatalysis 4 (1990) 29–37.
[10] P.R. Alefounder, S.J. Ferguson, Biochem. J. 192 (1980) 231–238.
[11] H.U. Bergmeyer, K. Gawehn, M. Grassl, in: H.U. Bergmeyer (Ed.), Methoden der Enzymatischen Analyse, vol. 1, Verlag Chemie, Weinheim, Germany, 1970, p. 440.
[12] P. Jensen, R. Aasa, B.G. Malmström, FEBS Lett. 125 (1981) 161–164.
[13] F. Perrella, Anal. Biochem. 174 (1988) 437–447.
[14] I. Kučera, R. Hedbávný, V. Dadák, Biochem. J. 252 (1988) 905–908.
[15] P.R. Alefounder, A.J. Greenfield, J.E.G. McCarthy, S.J. Ferguson, Biochim. Biophys. Acta 724 (1983) 20–29.
[16] R.J.M. Van Spanning, A.P.N. De Boer, W.N.M. Reijnders, H.V. Westerhoff, A.H. Stouthemer, J. Van der Oost, Mol. Microbiol. 23 (1997) 893–907.
[17] I. Mat'chová, I. Kučera, O. Janiczek, R.J.M. Van Spanning, L.F. Oltmann, Arch. Microbiol. 159 (1993) 272–275.
[18] D.J. Richardson, S.J. Ferguson, FEMS Microbiol. Lett. 132 (1995) 125–129.