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Antioxidative Enzymes of Sulfate-Reducing Bacterium *Desulfovibrio desulfuricans*: Superoxide Dismutase and Peroxidases

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Abstract—Extracts of *Desulfovibrio desulfuricans* B-1388 cells grown under anaerobic conditions displayed superoxide dismutase activity. The maximal activity was found during the stationary growth phase. The enzyme was virtually completely located in the periplasm fraction. *D. desulfuricans* B-1388 lacked catalase activity but contained active NADH- and NADPH-peroxidases. The activity of NADH-peroxidase depended on the physiological state of the culture. On changing the growth conditions (the presence of 5% CO in the gaseous phase), the activity of superoxide dismutase decreased.

Key words: *Desulfovibrio desulfuricans*, superoxide dismutase, NADH-peroxidase, NADPH-peroxidase, catalase

For many years sulfate-reducing bacteria were considered to be a very small group of strictly anaerobic bacteria which reduced sulfates to sulfides and oxidized organic acids to acetate. The discovery of new species and also studies on features of early known sulfate-reducing bacteria resulted in significant changes in their systematics and in concepts on metabolism in these microorganisms.

At present, many sulfate-reducing bacteria are shown to be aerotolerant anaerobes. Oxygen plays a certain physiological role in the metabolism of the sulfate-reducing bacteria. It is suggested that these bacteria use oxygen as a final acceptor of electrons in the electron transport chain during the synthesis of ATP [1, 2]. However, it should also be taken into account that oxygen inhibits some enzymes of sulfate-reducing bacteria: lactate dehydrogenase, NAD-dependent alcohol dehydrogenase, etc. [3]. Similarly to oxygen reduction to water [2], autooxidation of many redox proteins in sulfate-reducing bacteria [4] can be associated with production of reactive superoxide radicals as metabolic byproducts. At least two defense mechanisms of sulfate-reducing bacteria against reactive oxygen species have been recently described [5-7].

The present work describes the detection in *Desulfovibrio desulfuricans* B-1388 of antioxidative proteins, such as superoxide dismutase, catalase, and NADH- and NADPH-peroxidases, and the growth conditions have also been studied, in particular, the effect of carbon monoxide on the activities of these enzymes.

MATERIALS AND METHODS

A culture of *D. desulfuricans* strain B-1388 obtained from the All-Union Collection of Microorganisms (Pushchino, Russia) was grown under anaerobic conditions at 30°C in the following medium (in g/liter): KH_2PO_4 (0.5), NH_4Cl (1.0), $Na_2SO_4 \cdot 10H_2O$ (1.0), NaCl (10.0), $MgSO_4 \cdot 7H_2O$ (2.0), calcium lactate (3.5), yeast extract (1.0), $FeSO_4 \cdot 7H_2O$ (0.5); the pH was adjusted to 6.8-7.0 with 20% NaOH. As a reducer, 1% Na_2S in 1% $NaHCO_3$ was used (E_0 of the medium was −230 mV). The inoculate volume was 5% relative to the medium volume. The bacteria were grown in flasks under anaerobic conditions: either 100% Ar or 5% CO + 95% Ar. The gases were sterilized by passing through a bacterial filter.

To prepare extracts, the cells precipitated by centrifugation (5000*g*, 30 min, 4°C) and washed with 0.02 M Tris-HCl buffer (pH 7.6) were suspended in 0.02 M Tris-HCl buffer (pH 7.6) at 2-2.5 g wet weight per 10 ml of the buffer, and broken with a UZDN-2T ultrasonic disintegrator (Russia) (22 kHz, 60 sec/ml, 0°C) with subsequent centrifugation (17,000*g*, 60 min, 4°C). In the subsequent experiments the supernatant was used.

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The cell fractions were prepared by a modified method [5].

The superoxide dismutase (SOD) activity was determined in the cell extract photochemically using a reaction system which contained methionine, riboflavin, and Nitro Blue Tetrazolium [6]. Changes in the absorption were recorded at 560 nm with a Specord M-40 (Germany) over 20 min (each 5 min) in cuvettes with a 1 cm pathlength.

One unit of SOD activity was defined as the amount of protein required to produce 50% inhibition of the reduction rate of Nitro Blue Tetrazolium under the conditions of the assay.

The catalase activity was determined spectrophotometrically by monitoring the decomposition of H_2O_2 recorded at 240 nm [7].

The rate of NADH or NADPH peroxidation was recorded under anaerobic conditions at 340 nm. The reaction was started by addition of 2 mM H_2O_2 [7].

The protein was determined by the Lowry method with modifications [8].

The data were processed by methods of variation statistics [9]. The mean values for three experiments are presented (three independent measurements in each).

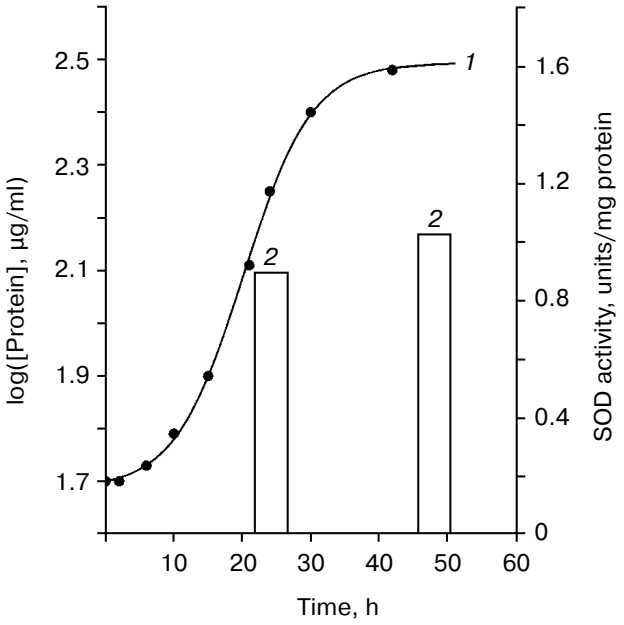

Fig. 1. Growth curve (*1*) and the superoxide dismutase activity (*2*) of *D. desulfuricans* B-1388 in lactate-containing mineral medium.

RESULTS AND DISCUSSION

The presence of superoxide dismutase was reported in the bacteria *D. desulfuricans* [10] and in *D. vulgaris* [11] in the 1970s and 1980s. However, real information flow about enzymes protecting anaerobic bacteria against reactive oxygen species has occurred only in the last four years [12-15]. Superoxide dismutase was found in *D. gigas* [16], and SOD-active iron-containing proteins, such as desulfoferrodoxin in *Desulfoarculus baarsii* [3] and in *D. desulfuricans* ATCC 27774 [17] and nilaredoxin in *D. gigas* [13], were described.

We have found superoxide dismutase activity in *D. desulfuricans* B-1388 cells grown under anaerobic conditions. This finding suggests that the enzyme is generated constitutively during the growth of the microorganism in lactate-containing mineral medium, and this does not contradict the literature data [16]. The enzyme activity does not depend on the physiological state of the cells and is about 1.03 units/mg protein in the stationary growth phase (Fig. 1).

There are only scattered reports on the SOD localization in cells of sulfate-reducing bacteria. It has been shown in [12] that SOD of *D. vulgaris* has the periplasmic localization while rubredoxin oxidoreductase which has superoxide reductase activity rather than superoxide dismutase activity was found in the cytoplasmic fraction.

To determine the localization of superoxide dismutase in the *D. desulfuricans* cells, fractions of the periplasm and of the membrane-containing cytoplasm were prepared. Virtually all SOD (100%) was found in the periplasm fraction (Table 1).

The periplasm space is especially significant for defense of growing cells against reactive oxygen species which can enter the cell from outside or be produced on the membrane surface. The conversion of hydrogen peroxide into the more aggressive $OH^{\cdot}$ radical can be prevented by catalase and peroxidases.

We have shown lack of catalase activity in *D. desulfuricans* cells. Note that catalase activity was not found in some *Desulfovibrio* species studied. The strains *D. vulgaris*, *D. gigas*, and *Desulfomicrobium norvegicum* contained catalase, whereas the strains *D. salexigens* and *D. desulfuricans* Essex 6 were catalase-free. Some authors suggest that the catalase lack can be associated either with a deficiency of the gene encoding this protein or with the gene expression only under certain conditions [7].

Table 1. Localization of antioxidative enzymes in the *D. desulfuricans* B-1388

Fraction	Superoxide dismutase, %	NADH-peroxidase, %	NADPH-peroxidase, %
Periplasm	100	60	70
Cytoplasm + membranes	0	40	30

Table 2. NADH- and NADPH-peroxidase activities of *D. desulfuricans* B-1388 depending on the physiological state of the bacteria under different growth conditions

Growth conditions	NADH-peroxidase, μmol/min per mg protein		NADPH-peroxidase, μmol/min per mg protein	
	exponential growth phase	stationary growth phase	exponential growth phase	stationary growth phase
Mineral medium + lactate	40.5	66.9	35.3	39.6
Mineral medium + lactate + 5% CO	64.5	121.9	72.5	31.0

NADH- and NADPH-peroxidases have been found in sulfate-reducing *Desulfovibrio* bacteria lacking catalase activity (except *D. desulfuricans* ATCC 27774) [7]. We found in the *D. desulfuricans* cells active NADH- and NADPH-peroxidases catalyzing the cleavage of hydrogen peroxide. These enzymes were found in both the periplasm and cytoplasm membrane-containing fractions (Table 1). NADHP-peroxidase is mainly localized in the periplasm (70%), whereas NADH-peroxidase is nearly equally distributed in the periplasm and in the cytoplasm membrane-containing fractions (60 and 40%, respectively). As in the case of the SOD activity, the activity of NADPH-peroxidase does not depend on the physiological state of the culture. The NADH-peroxidase activity of cells in the stationary growth phase is higher than the enzyme activity in cells during the exponential growth phase (Table 2).

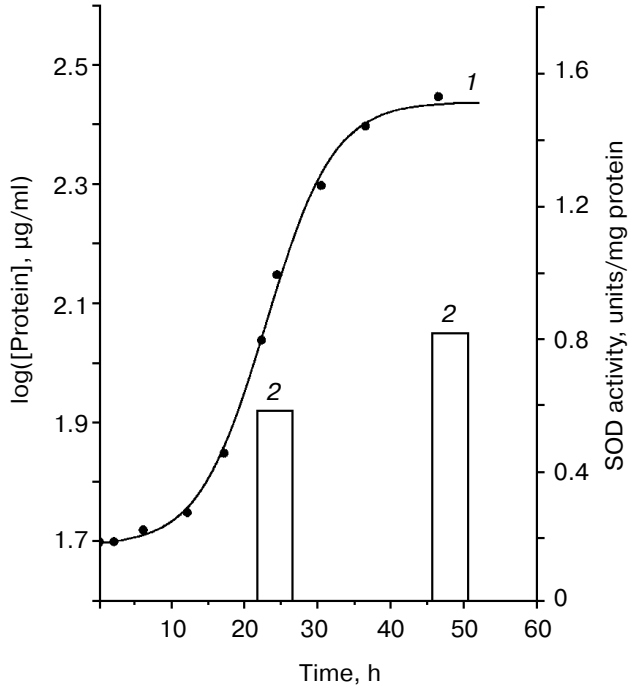

Fig. 2. Growth curve (*1*) and the superoxide dismutase activity (*2*) of *D. desulfuricans* B-1388 in lactate-containing mineral medium in the presence of 5% carbon monoxide.

Studies on the effect of unfavorable growth conditions on the defense mechanisms of the cell seem to be especially interesting. We have earlier shown that CO at the concentration of about 5% induces a "nonspecific stress" in the sulfate-reducing bacteria *D. desulfuricans* B-1388. Physiological features of *D. desulfuricans* growing with CO suggested multiple changes in the cell metabolism: the specific growth rate and the economic coefficient were decreased, the intracellular contents of cytochromes *c* and of reduced pyridine nucleotides were increased, and the cell energy was exhausted [18, 19].

On changing the growth conditions (Postgate B medium in atmosphere of 5% CO and 95% Ar) the SOD activity in the *D. desulfuricans* cells decreased ~1.5-fold (Fig. 2): the enzyme activity in the exponential growth phase and in the stationary growth phase were 0.58 and 0.82 unit/mg protein, respectively. The inhibiting effect of carbon monoxide on enzymes is known to be caused by complexing with metal atoms of their active sites.

Because superoxide dismutases are metal-containing enzymes [13, 14, 16], the inhibiting effect of CO on the superoxide dismutase activity of the *D. desulfuricans* was studied. Preincubation of the cell extracts with 0.067 mM CO for 20 min noticeably (50%) inhibited the superoxide dismutase activity of *D. desulfuricans*.

D. desulfuricans cells grown in the presence of CO displayed higher NADH- and NADPH-peroxidase activities. The activity of NADH-peroxidase during the exponential growth phase increased from 40.5 to 64.5 μmol/min per mg protein and during the stationary growth phase it increased from 66.9 to 121.9 μmol/min per mg protein (Table 2). The activity of NADPH-peroxidase during the exponential growth phase increased from 35.3 to 72.5 μmol/min per mg protein.

Overall, the experimental findings suggest that the sulfate-reducing bacteria *D. desulfuricans* B-1388 contain active antioxidative enzymes: superoxide dismutase and NADH- and NADPH-peroxidases. The expression of superoxide dismutase and of NADH- and NADPH-peroxidases in the sulfate-reducing bacteria seems to protect

the cells against oxygen radicals which are reactive by-products of their metabolism. Growth of the sulfate-reducing bacteria in the presence of carbon monoxide decreases the activity of superoxide dismutase and, thus, increases the cell vulnerability to reactive oxygen species. Superoxide dismutase and peroxidases are suggested to be a necessary system of defense elaborated by the cell during evolution.

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