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Localization of the Enzyme System Involved in Anaerobic Reduction of Azo Dyes by *Sphingomonas* sp. Strain BN6 and Effect of Artificial Redox Mediators on the Rate of Azo Dye Reduction

MICHAEL KUDLICH,[1] ANDREAS KECK,[2] JOACHIM KLEIN,[2] AND ANDREAS STOLZ[1]*

Institut für Mikrobiologie[1] and Institut für Industrielle Genetik,[2] Universität Stuttgart, 70569 Stuttgart, Germany

The effect of different artificial redox mediators on the anaerobic reduction of azo dyes by *Sphingomonas* sp. strain BN6 or activated sludge was investigated. Reduction rates were greatly enhanced in the presence of sulfonated anthraquinones. For strain BN6, the presence of both cytoplasmic and membrane-bound azo reductase activities was shown.

Various bacterial strains reduce azo dyes under anaerobic conditions. The most generally accepted hypothesis for this phenomenon is that many bacterial cells possess a rather unspecific cytoplasmic azo reductase which transfers electrons under anaerobic conditions via (soluble) flavins to the azo dyes (7, 12, 30, 32). Recently, it was shown that the naphthalenesulfonate-degrading *Sphingomonas* sp. strain BN6 converted 2-naphthalenesulfonate to some kind of redox mediator (19). It was suggested that these redox mediators enabled the strain under anaerobic conditions to transfer redox equivalents to the azo dyes. In the present study, the location of the enzyme system which is responsible for the reduction of azo dyes by whole cells was determined. The effect of artificial redox mediators on the anaerobic reduction of azo dyes by whole cells from different bacterial strains was also studied.

Bacterial strains and media. The isolation and characterization of *Sphingomonas* sp. strain BN6 (DSM 6383) and the composition of the minimal media have been described before (15, 27).

Reduction of amaranth by resting cells. Strain BN6 was grown in a mineral medium with glucose (10 mM). Cells were harvested by centrifugation and resuspended in 50 mM Na-K-phosphate (pH 7.4) buffer to an optical density at 546 nm of 11. This corresponded to a protein concentration of approximately 1.1 g/liter. The reaction mixture contained, in a final volume of 7.2 ml, 80 µmol of glucose, 400 µmol of Na-K-phosphate buffer (pH 7.4), and different concentrations of the redox mediators. This cell suspension was transferred to a rubber-stoppered serum bottle (30 ml). Oxygen was removed from the medium by at least 15 2-min cycles of evacuation and flushing with nitrogen gas. The reaction was started by the injection of 0.8 ml from an anaerobic stock solution of amaranth (5 or 10 mM). The reduction of the azo dye was determined spectrophotometrically at a λ of 520 nm.

Experiments with activated sludge. The activated sludge was obtained from the aerobic part of the sewage treatment plant of the University of Stuttgart (Büsnau). Particulate material was collected by centrifugation (8,500 × *g*, 30 min), resuspended in Na-K-phosphate buffer (50 mM; pH 7.4), and passed through a sieve with a mesh size of 1.0 mm to eliminate larger particles. The reaction mixture was composed as described above and contained 8.4 mg of protein ml^{-1}.

Experiments with cell extracts. Cell extracts were prepared by passing 40 ml of a suspension of whole cells (protein content, about 20 mg/liter) through a French press as described previously (22). The azo reductase activity was determined anaerobically in cuvettes which were flushed before the assay with nitrogen gas. For the standard assay, 560 µl of an anaerobic stock solution that contained 40 µmol of Tris-HCl buffer (pH 7.8), 48 nmol of amaranth, and 1.25 µmol of 2-anthraquinonesulfonate (AQS) were transferred to a rubber-stoppered cuvette. The cell extract or solubilized cell membranes were added (160 µl, about 100 to 200 µg of protein), and the reaction mixture was flushed again with nitrogen gas for 10 min. Finally, the reaction was started by the addition of 0.64 µmol of NADH from an oxygen-free stock solution. The decrease of absorption at a λ of 520 nm was measured spectrophotometrically. Reaction rates were calculated by use of an extinction coefficient of 27 mM^{-1} cm^{-1}.

Preparation of cell membranes. The cell membranes were isolated by passing about 40 ml of a suspension of whole cells (optical density at 546 nm, about 200) through a French press and three subsequent centrifugation steps at $100,000 \times g$ for 35 min (18, 22). The transparent pellet was resuspended in a volume of about 1 ml in Tris-HCl buffer (50 mM; pH 7.8). For the standard assay of the membrane-bound azo reductase, 300 µl of this preparation was incubated for 5 min with 285 µl of Tris-HCl buffer (50 mM; pH 7.8) and 15 to 75 µl of a solution of Triton X-100 (20%, vol/vol). Finally, 160 µl of this mixture was used for the azo dye reduction as described above.

Determination of protein content. The protein content of cell extracts was determined by the method of Bradford (3) with bovine serum albumin as the standard. The protein contents of whole cells and cell membranes were determined by the Bio-Rad protein assay. The protein content of the activated sludge was determined by a modification of the biuret assay (33).

Analytical methods. Azo dyes and metabolites were analyzed by high-pressure liquid chromatography (HPLC) (consisting of two pumps [type 510], a photodiode array detector [type 994], and an automated gradient controller [Waters Associates, Milford, Mass.]). A reverse-phase column (125 by 4.6 mm [internal diameter]; Bischoff, Leonberg, Germany),packed with 5-µm-diameter particles of Lichrosorb

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* Corresponding author. Mailing address: Institut für Mikrobiologie, Allmandring 31, D-70569 Stuttgart, Germany. Phone: 49 (0) 711 685 5487. Fax: 49 (0) 711 685 5725. E-mail: andreas.stolz@po.uni-stuttgart.de.
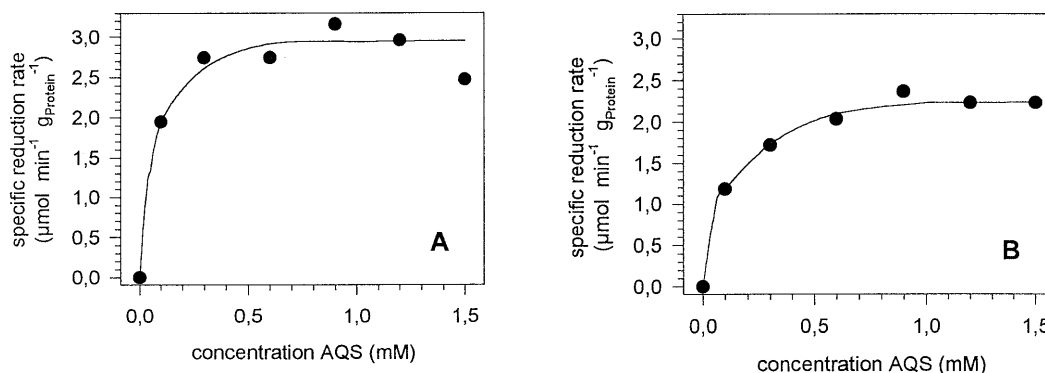
FIG. 1. Decolorization of amaranth in the presence of different concentrations of the redox mediator AQS by whole cells of strain BN6 (A) or activated sludge (B). The cells of strain BN6 were grown aerobically with glucose (10 mM). At the end of the exponential growth phase, cells were harvested by centrifugation and resuspended (protein content, 1.14 g/liter) anaerobically in Na-K-phosphate buffer (pH 7.4; 50 mM). The preparation of the sludge sample is described in Materials and Methods. The assays contained amaranth (0.5 mM), glucose (10 mM), and different concentrations of the redox mediator. The decolorization of the azo dye was determined spectrophotometrically at a λ of 520 nm.

RP18 and equipped with a guard column (Merck, Darmstadt, Germany), was used as the stationary phase. The separated compounds were detected at 210 nm or the wavelength of maximal absorbance of the azo dyes. For the simultaneous analysis of the azo dyes and their respective reduction products, the solvent systems consisted of water, methanol, and an ion-pair reagent (5 mM tetrabutylammonium sulfate; Fluka). Solvent gradients with increasing concentrations of methanol (40 to 90%, vol/vol) were used.

Reduction of amaranth by whole cells of strain BN6 in the presence of different concentrations of AQS. It was previously suggested that the presence of redox mediators could enhance the rate of bacterial azo dye reduction of intestinal bacteria under anaerobic conditions (4, 20). Therefore, whole cells of strain BN6 were incubated under anaerobic conditions with the sulfonated azo dye amaranth in the presence of AQS, a well-known redox mediator (10). Thus, it was found that the addition of AQS significantly increased the rate of decolorization of the azo dye (Fig. 1A). The rate of azo dye decolorization was dependent on the concentration of the mediator added. At a concentration of AQS above about 0.6 mM, however, no significant further increase of reduction rates was observed.

Reduction of amaranth by sewage sludge in the presence of different concentrations of AQS. In the mitochondrial system, it has been shown that the reduction of soluble quinones is catalyzed by the membrane-bound respiratory NADH:ubiquinone reductase (2, 6). Because of the ubiquity of the isofunctional system in (aerobic) bacteria, whether the addition of AQS also enabled an unadapted sewage sludge to reduce sulfonated azo dyes was determined. Thus, it was found that the addition of AQS resulted in a rapid decolorization of amaranth by the activated sludge. This effect was dependent on the concentration of the mediator added (Fig. 1B).

Comparison of the effect of different redox mediators on the reduction of amaranth. Whole cells of strain BN6 were incubated under anaerobic conditions with different quinones. Thus, it was observed that not only the man-made sulfonated anthraquinones but also the naturally occurring 2-hydroxy-1,4-naphthoquinone significantly increased the rate of amaranth degradation (Table 1). The addition of 100 μM quinones resulted in the complete conversion of more than 1 mM azo dye. This indicated that the quinones did indeed function as mediators for the transfer of reducing power to the azo dye. The

comparison of the reduction rates of amaranth in the presence of different quinones, viologens, or flavin adenine dinucleotide (FAD) demonstrated that the highest reduction rates were found with AQS. Recently, it was shown that humic acids act as extracellular redox mediators in the dissimilatory reduction of ferric iron (24). However, no effect of humic acids (2 mg/ml) on the reduction of azo dyes in our system was found.

Demonstration of two different azoreductase activities in strain BN6. Previously, a soluble FAD-dependent azo reductase was found in cell extracts of strain BN6 (15). The experiments described above were performed with whole cells. Highly polar compounds such as AQS should not penetrate the cell membrane. Therefore, it was probable that the azo reductase activity found with whole cells was present in the cell membranes. Furthermore, externally added FAD had almost no effect on the reaction with whole cells (Table 1), whereas the azo reductase activity of crude extracts was greatly enhanced by the addition of FAD (15). It was therefore tested whether the azo reductase activity which is mediated by the redox compounds studied here was different from the soluble azo reductase described previously. Cells of strain BN6 were separated into a membrane fraction and cell extract. An azo reductase activity was found in the membrane fraction. This

TABLE 1. Effect of different redox mediators on the reduction of amaranth by whole cells of strain BN6[a]

Redox mediator	E_0' (mV)	Specific reduction rate (μmol min^{-1} g of protein^{-1})
Ethyl viologen	−480	0.2
Methyl viologen	−440	0.3
Benzyl viologen	−358	0.4
AQS	−225	2.1
2,6-Anthraquinonedisulfonate	−184	1.3
2-Hydroxy-1,4-naphthoquinone	−137	2.0
FAD	−219	0.3
None		0.2

[a] The oxygen-free reaction mixtures contained, in a total volume of 8 ml, 50 mM Na-K-phosphate buffer, 10 mM glucose, 0.5 mM amaranth, and resting cells of strain BN6. After different time intervals, aliquots were removed and the remaining concentration of amaranth was determined spectrophotometrically. The redox mediators were added in concentrations of 100 μM each. The E_0' values were taken from Fultz and Durst (10).

TABLE 2. Effect of different compounds on the reduction of
amaranth by a membrane preparation or a cell extract from
strain BN6[a]

Compound added (concn in test)	Sp act (μmol min^{-1} g of protein^{-1})	
	Membranes	Cell extracts
None	6	18
Triton X-100 (0.01%)	41	18
Triton X-100 (0.03%)	34	20
Triton X-100 (0.05%)	38	19
Triton X-100 (0.01%) + p-chloromercuribenzoate (10 μM)	1	19
Triton X-100 (0.01%) + p-hydroxymercuribenzoate (10 μM)	1	20
Triton X-100 (0.01%) + diphenyliodonium (100 μM)	1	3
Triton X-100 (0.01%) + diphenyliodonium (10 μM)	18	15

[a] The assays were performed under anaerobic conditions. The enzyme activities were determined spectrophotometrically.

activity was significantly increased after the addition of Triton X-100 (Table 2). In contrast, no effect of Triton X-100 was found in the soluble fraction. By using different potential inhibitors, it was demonstrated that diphenyleneiodonium and rotenone, which are known inhibitors of the mitochondrial NADH:ubiquinone oxidoreductase (13, 25, 34), were only weak inhibitors of both the membrane-bound and the cytoplasmic azo reductases. The thiol-specific inhibitor p-hydroxymercuribenzenesulfonate almost completely inactivated the membrane-bound azo reductase. In contrast, this compound had almost no effect on the cytoplasmic azo reductase (Table 2). Thus, the membrane-bound and the cytoplasmic azo reductases are probably two different enzyme systems.

Reduction of different azo dyes and identification of the corresponding amines as reduction products. Cells of strain BN6 were incubated anaerobically with AQS and different azo dyes, and the supernatants were analyzed by HPLC. Thus, it was found that the addition of the mediator led to a significant increase in the reduction rates of all dyes (Table 3). Stoichiometric amounts of at least one of the corresponding amines were found with naphthol blue black and acid red 1 (aniline), sunset yellow (4-aminobenzenesulfonate), and amaranth (4-aminonaphthalene-1-sulfonate).

Various eukaryotic and prokaryotic biological systems are able to reduce the azo bond under anaerobic conditions (1, 5, 36). In eukaryotes, the azo reductase activity in the liver was found to be catalyzed predominantly by NADPH- and NADH-

TABLE 3. Reduction of different azo dyes by whole cells of strain
BN6 in the presence of AQS as the redox mediator

Azo dye	Specific reduction rate (μmol min^{-1} g of protein^{-1}) with:	
	300 μM AQS	600 μM AQS
Amaranth	3.3	4.5
Acid red 1	1.7	2.2
Sunset yellow	4.3	6.6
Naphthol blue black	3.0	3.2

[a] The reaction mixtures contained, under anaerobic conditions, resting cells of strain BN6 (protein content, 1.14 g liter^{-1}), glucose (10 mM), AQS (0.3 mM or 0.6 mM), Na-K-phosphate buffer (pH 7.4; 50 mM), and the respective dyes (1 mM each). Within a period of 10 h, no azo reduction was measured in control assays without the mediator.

dependent components of the microsomal monooxygenase system (9, 16, 17, 28). The bacterial azo reductase activity has been generally related to soluble cytoplasmatic enzymes. This was based mainly on the observations that (i) cell extracts of various bacteria show azo reductase activity and (ii) cell extracts show higher azo reductase activity than intact cells do. For the highly polar sulfonated azo dyes, it was therefore generally assumed that the reduction of the dyes was limited by the permeation of the dyes through the cell membrane. This hypothesis was supported by the observation that lysis of cells by aging or the addition of membrane-active compounds such as toluene resulted in an increased rate of azo dye reduction (26, 30). For the actual reduction of the dyes in the cytoplasm of the cells, the existence of an unspecific process has been repeatedly proposed. The reduction was assumed to be catalyzed by soluble or enzyme-bound flavines (7, 12, 30, 32). Recently, different bacterial strains which seem to excrete a flavin-dependent extracellular azo reductase were isolated from the human intestine (29).

The results obtained during the present study suggest a different mechanism for the reduction of azo dyes. Obviously, different redox mediators can transfer reduction equivalents from the periplasmically orientated parts of the respiratory chain to the azo dyes. This mechanism does not require the penetration of the azo dyes through the cell membranes and therefore explains the ability of bacteria to reduce highly polar sulfonated azo dyes or polymeric azo dyes. Because various quinoid structures are found among naturally occurring products, it seems probable that a redox mediator-catalyzed process is also (at least partially) responsible for the reduction of azo compounds in the intestine and other anaerobic environments (11, 20, 31, 36).

The following various lines of evidence suggest that in the aerobic bacteria studied here, the NADH:ubiquinone oxidoreductase of the respiratory chain is responsible for the reduction of azo dyes by the cell membranes and intact cells. (i) The redox potentials (E_0') of AQS (-225 mV) (10), amaranth (about -250 mV), and different parts of mammalian or fungal mitochondrial NADH:ubiquinone oxidoreductase (-330 to -50 mV) (38) are within the same range. (ii) This part of the respiratory chain should be ubiquitous among aerobic bacteria. (iii) It has been shown that the isolated mitochondrial NADH:ubiquinone reductase (complex I) reduced soluble quinones (2, 6).

An interesting parallel to the mediator-catalyzed reduction of highly polar (and presumably polymeric) azo compounds under anaerobic conditions is found with the aerobic degradation of highly polymeric (lignin-like) material. It was recently proposed that the action of lignin peroxidase and laccase is at least partly due to mediators such as veratryl alcohol, manganese ions, and 3-hydroxyanthranilate (8, 14, 35).

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