Isolation and Properties of Trimethylamine N-Oxide/Dimethylsulfoxide Reductase from the Purple Bacterium *Rhodospirillum rubrum*

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Among 4 strains of the purple bacterium *Rhodospirillum rubrum* only one did not contain trimethylamine N-oxide/dimethylsulfoxide reductase and was unable to grow under anaerobic dark conditions in fructose media supplemented with trimethylamine N-oxide (TMAO) or dimethylsulfoxide (DMSO). By growing the bacteria in media treated with charcoal or supplemented with tungstate (2 mM), TMAO/DMSO reductase activity was lowered to about 10% of the value of the untreated control. The enzyme was purified from the periplasmic protein fraction of strain S 1 and was shown to contain 2 subunits (mol. wt. of 40,000 and 36,000, respectively) in a 1:1 stoichiometry. Although the affinity of the reductase to DMSO (K_m = 0.67 mM) was much higher than to TMAO (K_m = 24 mM), the catalytic activity with the latter substrate was about 10 times as high (90–110 μmol/min × mg protein) as with DMSO.

Some purple bacteria are able to grow under anaerobic dark conditions by using dimethylsulfoxide (DMSO) or trimethylamine N-oxide (TMAO) as terminal electron acceptors [1, 2]. The TMAO/DMSO reductase from *Rhodobacter sphaeroides* was highly purified and reported to consist of a single polypeptide with the molecular weight of 82,000 [3]. By contrast, a protein with TMAO reductase activity solubilized with SDS from the intracytoplasmic membranes of *Rb. capsulatus* had only a molecular weight of 46,000 [4]. Like other bacterial TMAO/DMSO reductases the enzyme from *Rb. sphaeroides* contains a molybdenum cofactor [5]. Data concerning the occurrence of a distinct TMAO/DMSO reductase in *Rhodospirillum rubrum* are lacking. In this paper we report of the purification of a molybdenum-dependent TMAO/DMSO reductase from the periplasmic protein fraction of *R. rubrum* and describe some of the properties of the enzyme.

The *R. rubrum* strains used in this study (FR 1 = DSM 1067; Ha = DSM 107; S 1 = DSM 467; 1761-1 a) are kept in the culture collection of the institute. The bacteria were grown photosynthetically or dark anaerobically at 30 °C. For photosynthetic growth, completely filled 500 ml screw cap flasks or a 1 l bioreactor gassed with N_2 were used. Illumination was provided by 60 W tungsten lamps (2 lamps per flask or bioreactor) placed in a distance of 25 cm from the surface of the culture vessels. For photosynthetic growth the malate-$(NH_4)_2SO_4$ medium of [6] with and without DMSO (75 mM) or TMAO (40 mM) was used. For dark anaerobic growth in a N_2-flushed 1 l fermentor equipped with pH control, the medium described in [6] (without malate) was supplemented with 15 mM D-fructose and 75 mM DMSO or 40 mM TMAO. For some experiments, the culture media were treated with charcoal according to the method described in [7] in order to remove traces of molybdate. Bacterial growth was monitored by measuring the optical density at 660 nm. Cellular protein was analyzed by the Lowry method after extraction of photopigments with an acetone–methanol (7:2) mixture.

DMSO and TMAO reductase activities of cell-free extracts were measured at 30 °C in N_2-flushed reaction mixtures using dithionite-reduced benzylviologen as electron donor [8]. The reaction was started by addition of enzyme and electron acceptor and measured spectrophotometrically by following the decrease in absorbancy at 600 nm. Enzyme activity is expressed in units (U). 1 U is the activity catalyzing the oxidation of 1 μmol reduced benzylviologen/min. 1 U = 16.67 nkat. Polyacrylamide gel electrophoresis (PAGE) of native proteins was performed according to [9]. Protein bands were made visible by silver staining [10] and TMAO/DMSO reductase activity bands by the method described in [11]. SDS-PAGE was performed according to [12]. Biochemicals were purchased from Boehringer, Mannheim; Serva, Heidelberg, and Sigma, München. Chromatographic material was obtained from Pharmacia-LKB, Freiburg; and all other chemicals from Merck, Darmstadt.

Abbreviations: R., Rhodospirillum; Rb., Rhodobacter; SDS, sodium dodecylsulfate; U, unit.

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Among the four *R. rubrum* strains tested (S 1, FR 1, 1761-1 a, Ha) only strain Ha was unable to grow under anaerobic dark conditions in fructose media supplemented with DMSO or TMAO (Table I). The inability of strain Ha to use TMAO or DMSO as terminal electron acceptors under anaerobic dark conditions coincided with the absence of significant TMAO/DMSO reductase activity. The other strains contained DMSO reductase activity at levels of 40–50 (S1); 45–50 (FR 1); and 30–40 (1761-1 a) mU/mg protein. The enzyme from strain S 1 (type strain of *R. rubrum*) was characterized further. The specific reductase activity of cells grown photosynthetically or dark anaerobically in the presence and absence of DMSO or TMAO was about the same. Thus, contrary to the inducible nature of the enzyme in *E. coli* [11], the TMAO reductase of *R. rubrum* S 1 is constitutive. For convenience, all further experiments were therefore conducted with cells grown photosynthetically in the standard malate-ammoniumsulfate medium. When the bacteria were grown in a culture medium treated with charcoal in order to remove traces of molybdate [7] or in a medium supplemented with 2 mM sodium-tungstate in order to achieve a phenotypical molybdenum limitation [13], DMSO reductase was reduced from activity levels of 40–50 to about 3–5 mU/mg protein. This finding lends support to the assumption that the *R. rubrum* enzyme, like other bacterial TMAO reductases [14], contains a molybdenum cofactor.

For enzyme purification, 15 g cells (wet weight) grown photosynthetically in malate-$(NH_4)_2SO_4$ medium (supplemented with 0.5 g yeast extract per liter) were washed once in 50 mM Tris-HCl (pH 7.5), resuspended in 300 ml lysis buffer (0.5 M sucrose and 1.5 mM EDTA in 50 mM Tris-HCl, pH 8) and incubated with 240 mg lysozyme at 30 °C for 90 min. The sphaeroplasts were removed by centrifugation and the supernatant (= fraction of periplasmic proteins) was subjected to fractionation by $(NH_4)_2SO_4$. Protein precipitating between 40–70% saturation was dissolved in 5 ml NGT buffer (0.5 M NaCl and 20 vol.% glycerol in 50 mM Tris-HCl, pH 7.5) and subsequently filtered through a column of Sephacryl S-200 (type 16/60 HR, Pharmacia) equilibrated and eluted with NGT buffer. Peak fractions were pooled, concentrated by ultrafiltration through Milex-GV membranes and finally subjected to chromatography on a MonoQ column of 1 ml void volume (Pharmacia). Adsorbed proteins were eluted with a NaCl gradient ranging from 0–0.30 M and collected in fractions of 1 ml. The purification procedure increased the specific activity of the enzyme (DMSO as substrate) from 0.13 (periplasmic fraction) to 10.2 U/mg protein (peak fractions of MonoQ eluate). The highly purified enzyme was electrophoretically homogeneous (PAGE of native enzyme) and yielded 3 bands in SDS-PAGE corresponding to molecular weights of 83,000; 40,000 and 36,000; respectively. The 83 kDa band, very likely, is the undissociated enzyme. Note, that the native protein was eluted from Sephacryl S-200 gels at a position indicating a mol. wt. (M_r) of 87,000. The other two bands are interpreted to be subunits (α and β) of the enzyme (see Table II). The only protein band seen in native PAGE gels corresponded to the position of the activity band. The fact that a significant fraction of the *R. rubrum* reductase did not dissociate in SDS gels makes it unlikely that the enzyme of *Rb. sphaeroides* (mol. wt. 82,000) consists of only one polypeptide chain [3].

Table I. Effect of $NaHCO_3$, DMSO and TMAO on anaerobic dark growth in fructose medium of *Rhodospirillum rubrum* strains. N_2-gassed media containing 15 mM D-fructose and $NaHCO_3$ (10 mM), DMSO (75 mM) or TMAO (50 mM) as indicated were inoculated (3% inoculum) with phototrophically grown cells and incubated for 5 days in the dark under anaerobic conditions. Biomass was calculated from cellular protein assuming a protein content of cellular biomass of 55%. Data represent mean values from 3 different experiments. Variation was less than 20%.

Strain	Biomass [g/liter culture medium]			
	no add.	+$NaHCO_3$	+DMSO	+TMAO
S1	0.06	0.15	0.25	0.33
FR1	0.04	0.13	0.27	0.54
1761-1 a	0.04	0.10	0.19	0.23
Ha	0.03	0.04	0.05	0.04

Table II. Properties of TMAO/DMSO reductase purified from *R. rubrum* S 1.

Mol. wt. (native)	gel filtration: 87,000 ± 5,000 gel electrophoresis: 83,000 ± 5,000
Subunits	α: 40,000 ± 2,500, β 36,000 ± 2,000
K_m values	TMAO: 24 mM DMSO: 0.67 mM
V_{max} values (30 °C)	TMAO: 95–110 U/mg protein DMSO: 9–10 U/mg protein
Mo content	+ (deduced from inhibition of enzyme synthesis by tungstate and reduction of enzyme levels in charcoal-treated media)

The highly purified *R. rubrum* enzyme was active with TMAO, L-methionine-sulfoxide and DMSO (relative activities of 100, 26 and 9%, respectively), but not with nitrate. The affinity of the reductase was much higher to DMSO than to TMAO (see Table II), but the maximum catalytic activity with TMAO was about 10 times as high as with DMSO. Although molybdenum has not been shown, by direct chemical analysis, to be a constituent of the enzyme, its presence can be inferred from the growth experiments with media treated with charcoal or supplemented with tungstate. The presence of a constitutive Mo-dependent TMAO/DMSO reductase in *R. rubrum* is in accord with the earlier finding that a subcellular fraction of this organism restores nitrate reductase activity in *Neurospora* mutants lacking a functional Mo-cofactor [15].

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