CHARACTERIZATION OF DIMETHYLSULFOXIDE REDUCTASE, A NOVEL MOLYBDO-ENZYME
FROM PHOTOSYNTHETIC BACTERIA, Rhodobacter sphaeroides f.s. denitrificans

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Abstract - Dimethylsulfoxide reductase (DMSO-R), a novel molybdo-enzyme isolated from photosynthetic bacteria, Rhodobacter sphaeroides f.s. denitrificans, have been studied in relation to structure and activity. On limited proteolysis with trypsin and V8 protease, the DMSO-R activity increased more than twofold. Higher order structure was maintained by disulfide bonds even after nicking by proteases and was changed to be more susceptible to substrate.

Key words DMSO reductase, Mo-enzyme, limited proteolysis, structure-activity relationship

Photosynthetic bacteria, Rhodobacter sphaeroides f.s. denitrificans, which was isolated by one of the present authors (T.S.) from waste water of "tofu" factory in Nagano pref., Japan, was found to grow anaerobically under the light using dimethylsulfoxide (DMSO) as a terminal electron acceptor in the respiratory chain. DMSO reductase (DMSO-R) which reduces DMSO to dimethylsulfide (DMS) was chromatographically purified and found to be a molybdenum-containing enzyme. In this study, structure-activity relationship of DMSO-R based on the results obtained by limited proteolysis will be described.

MATERIALS AND METHODS

Rhodobacter sphaeroides f.s. denitrificans was cultured anaerobically under the visible light in the presence of DMSO for 24 h. DMSO reductase was purified according to the method described previously[1]. Mo and other element analyses were done by a Seiko SPS 1200A inductively coupled plasma emission spectrometer. Enzymatic activity was measured using benzyl viologen as an electron donor. Neurospora crassa nit-1 mutant was kindly supplied by Fungal Genetic Stock Center of Kansu University. Reconstitution of nitrate reductase activity was performed by the method of Nason[2]. Limited proteolysis was done using trypsin and staphylococcal V8 protease. Time course analyses were followed by Native- and SDS-polyacrylamide gel electrophoresis together with capillary zone electrophoresis. Amino acid sequences of proteolyzed fragments were obtained by a gas-phase amino acid sequencer after they were electroblotted to PVDF membrane.

RESULTS AND DISCUSSION

The molybdenum-containing enzymes (Mo-enzymes) have been widely distributed in biosphere, though not known so many kinds. Molybdenum locates unexceptionally in the active center of the enzymes and plays important roles as a redox center on nitrogen and sulfur metabolisms. In the Mo-enzymes known so far, Mo forms a specific cluster, called Mo-cluster, which consists of iron, labile sulfur and sulfhydryl groups of cysteine residues. Except for nitrogenase, a pterin derivative is also involved as an indispensable constituent. However, the analytical data shown in Table 1 indicated that DMSO-R which has m.w. of

82,000 Da with a single polypeptide chain contains per mole of enzyme 1 g atom Mo, 4-5 g atoms P, 31 g atoms S as determined by ICP spectrometry. No Fe was detected, which suggested that, different from other Mo-enzymes, Fe-S clusters are depleted in this enzyme.

Heat denaturation of DMSO-R gave the fluorescent derivatives, which were identified by fluorescent spectrometry as already known pterin derivatives common in other Mo-enzymes. To confirm this pterin further, reconstitution of the nitrate reductase (NR) activity was checked by adding the pterin containing fraction to the extract of *Neurospora crassa* nit-1 mutant which contains non-active apo-NR. The success in reconstitution suggested that the cofactor has common structural characteristics with that of nitrate reductase.

TABLE 1. Characteristics of DMSO Reductase

- 1) M.W. 82,000 No Subunit
- 2) Cofactor including Molybdenum
- 3) Elemental Composition

Element	g atoms/mol of enzyme
Mo	0.92
W	<0.001
Fe	<0.001
P	4.5
S	31
Se	<0.01

- 4) Substrate

Substrate	Km (μ M)	Vmax (unit/mg protein)
DMSO	15	15
TMAO*	1,000	48
Met.sulfoxide**	200	13
Chlorate	1,100	16

*TMAO:trimethylamine N-oxide

**Met.sulfoxide:methionine sulfoxide

Fig. 1. Time Course of DMSO-R Activation by Trypsin and V8 Protease

On limited proteolysis with trypsin and/or staphylococcal V8 protease, the DMSO-R activity increased two- to threefold when incubated at 30 °C (Fig.1). Native PAGE and SDS PAGE were done to confirm the proteolyzed products. On Native PAGE in each protease treatment was given a single band with the same position as that of DMSO-R as shown in Fig. 2. Whereas on SDS-PAGE it gave two bands around 42,000 and 40,000 (Ta and Tb for trypsin/ Va and Vb for V8 treatment, respectively, in Fig. 2). These suggest that both proteases nicked almost the same position at the center part of the peptide chain of DMSO-R and furthermore the enzyme behaved on Native PAGE as if it were not cleaved in the absence of SDS. To confirm this, DMSO-R was treated with or without

Fig. 2. Native- and SDS-PAGE of Limited Proteolysis Products of DMSO-R

Fig. 3. SDS-PAGE of DMSO-R with or without Trypsin and/or 2-ME Treatments

trypsin digestion in the presence or absence of 2-mercaptoethanol (2-ME) and the results were shown in SDS PAGE (Fig. 3). The original enzyme (without any treatment, lane c) appeared at around 40,000 Da, the half value of its native molecular weight, 82,000 Da which appeared in lane a treated with 2-ME. When the enzyme was digested with trypsin but not treated with 2-ME (lane d), it gave the similar value (40,000 Da) as the native enzyme. Finally in lane b, the trypsin digested sample was treated with 2-ME, where two bands, Ta and Tb appeared. These data were summarized; (i) the native DMSO-R behaves as if an apparent m.w. is as half as the original one, (ii) the nicked enzyme (lane d) also gives the same m.w. as the native enzyme, (iii) Treatment with 2-ME gives the original m.w. (iv) Both trypsin and 2-ME treatments give the fragment bands. From these data together with the facts that trypsin cleaves peptide bond, 2-ME reduces disulfide bond and SDS dissociates hydrophobic interaction among amino acid residues, it is concluded as shown in Fig. 4 that the native DMSO-R is folded by disulfide bonds and hydrophobic interaction as the volume looks to be a half, trypsin cleaves almost the center part of the peptide chain to give two equal fragments when disulfide bonds are cleaved by 2-ME, but to give a single band when disulfide bonds are

Fig. 4. Limited Proteolysis of DMSO-R by Trypsin

present. In the latter case proteolysed DMSO-R retains the higher order structure by disulfide bonds to enable to enhance the reactivity. The involvement of disulfide bonds was further confirmed by two dimensional electrophoresis; run in the first dimension without 2-ME and in the second with 2-ME. As shown in Fig. 5, the activity of DMSO-R and trypsin proteolyzed enzyme decreased with time in the presence of 2-ME, which suggested that disulfide bonds are a key factor to maintain the active higher order structure. These were further confirmed by capillary zone electrophoresis, in which the signal shift occurred in the presence of 2-ME.

Fig. 5. Reduction of DMSO-R activity in the Presence of 2-ME

Fig. 6. Protease Cleavage sites in DMSO-R

Protease cleavage sites were analysed by a gas phase sequencer. Interestingly, trypsin and V8 proteases cleaved the chain at the quite near positions as shown in Fig. 6. The fact that cleavage enhanced the activity suggests that this portion of the chain is very important for the substrate to access the Mo-pterin active center. Details will be clarified by analyses of protein sequence together with the location of Mo-pterin in the sequence.

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