CRYSTALLINE FERRIC SUPEROXIDE DISMUTASE FROM AN ANAEROBIC GREEN SULFUR BACTERIUM, *CHLOROBIUM THIOSULFATOPHILUM*

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1. Introduction

Superoxide dismutase reduces the steady state concentration of the superoxide radical by catalyzing disproportionation of the radicals, thus protecting cells from oxygen toxicity. Although little superoxide is produced in anaerobes, superoxide dismutase has been found in several anaerobic bacteria [1–5]. The superoxide dismutases from a purple sulfur photosynthetic bacterium, *Chromatium vinosum* [2,3], and from a sulfate-reducing bacterium, *Desulfovibrio desulfuricans* [4] have been characterized as the Fe-enzyme. Aerobic prokaryotes, most algae and protozoa contain Fe- and/or Mn-enzymes and lack Cu,Zn-superoxide dismutase [2,5–7]. This paper reports the isolation of crystalline superoxide dismutase from an anaerobic, green sulfur photosynthetic bacterium, *Chlorobium thiosulfatophilum*, and we describe its properties including metal contents and amino acid composition. The results herein prove that *Chlorobium* superoxide dismutase is the Fe-enzyme, confirming further the absence of the Mn-enzyme in anaerobes.

2. Materials and methods

Cytochrome *c* (Type III), xanthine oxidase and Ampholine (pH 3.5–5.0) were obtained from Sigma, Boehringer and LKB, respectively. The strain of *Chlorobium thiosulfatophilum* was given by Dr T. Akazawa, Nagoya University [8]. Cells were grown anaerobically under illumination from incandescent lamps using the Larsen medium as in [3]. The absence of oxygen in the medium was confirmed using an oxygen-electrode. Cells were harvested at the late logarithmic phase and stored at −20°C until use. Superoxide dismutase was assayed using a modification [9] of the procedure in [10]. Activity is presented in McCord-Fridovich units.

Polyacrylamide disc-gel electrophoresis, locating the activity on the disc, determination of the molecular and subunit molecular weights were performed as in [3]. Metal was analyzed with a Hitachi Perkin-Elmer 303 atomic absorption spectrophotometer equipped with a GA-2 graphite atomizer. Amino acid composition was determined as in [3]. The isoelectric point was measured by isoelectric focusing with Ampholine (pH 3.5–5.0) in a sucrose column. The dry weight of the enzyme was determined after dialysis against water and drying it in an oven at 105°C to constant weight.

3. Results

3.1. *Purification of the enzyme*

Unless specified potassium phosphate buffer was used at pH 7.8. Frozen cells of *Chlorobium thiosulfatophilum* (900 g) were suspended in 10 liters 50 mM phosphate containing 0.5 mM EDTA and were homogenized using a Dyno-mill with glass beads. The homogenates were centrifuged at 14 000 × *g* for 30 min to remove cell debris. Solid KCl was added to the supernatant to give 0.1 M. The solution (300 ml batch) was heated in an 80°C water bath and kept at 60°C for 3 min after reaching 60°C.

After removal of denatured proteins by centrifugation ammonium sulfate was added to make 50% saturation. The precipitate was removed by centrifugation and ammonium sulfate was added to the supernatant to 90% saturation. The precipitate collected by centrifugation was dissolved in and dialyzed against 10 mM phosphate containing 0.1 mM EDTA for 2 days with several changes of buffer. The dialyzed solution was clarified by centrifugation and adsorbed on DEAE–Sephadex A-50 column (9 × 48 cm) pre-equilibrated with 50 mM phosphate containing 0.1 mM EDTA. After the column had been washed with 22 liters equilibrating buffer (cytochrome *c*-553 was eluted following cytochrome *c*-555), a pale yellow band of superoxide dismutase was eluted ahead of a red band of cytochrome *c*-551 on elution with 100 mM phosphate. Superoxide dismutase was collected by centrifugation after the addition of ammonium sulfate and was dissolved in and dialyzed against 50 mM phosphate containing 0.1 mM EDTA for 2 days. After removal of insoluble protein by centrifugation the dialyzed enzyme was adsorbed on a column of DEAE–Sephadex A-50 (2.5 × 41 cm) and linear gradient elution (50–150 mM phosphate) was conducted. The superoxide dismutase fractions eluted at 97 mM phosphate were concentrated to 10 ml in a collodion bag under reduced pressure. The concentrated solution was gel-filtered on a column of Sephadex G-100 (4 × 75 cm) equilibrated and eluted with 20 mM phosphate. Active fractions were pooled and dialyzed against 0.5 mM potassium phosphate, pH 6.0. The dialyzed solution was applied to a small column of hydroxylapatite (BDH Chemicals, 2.5 × 6 cm) pre-equilibrated with 0.5 mM phosphate, pH 6.0. The unadsorbed fraction contained homo-

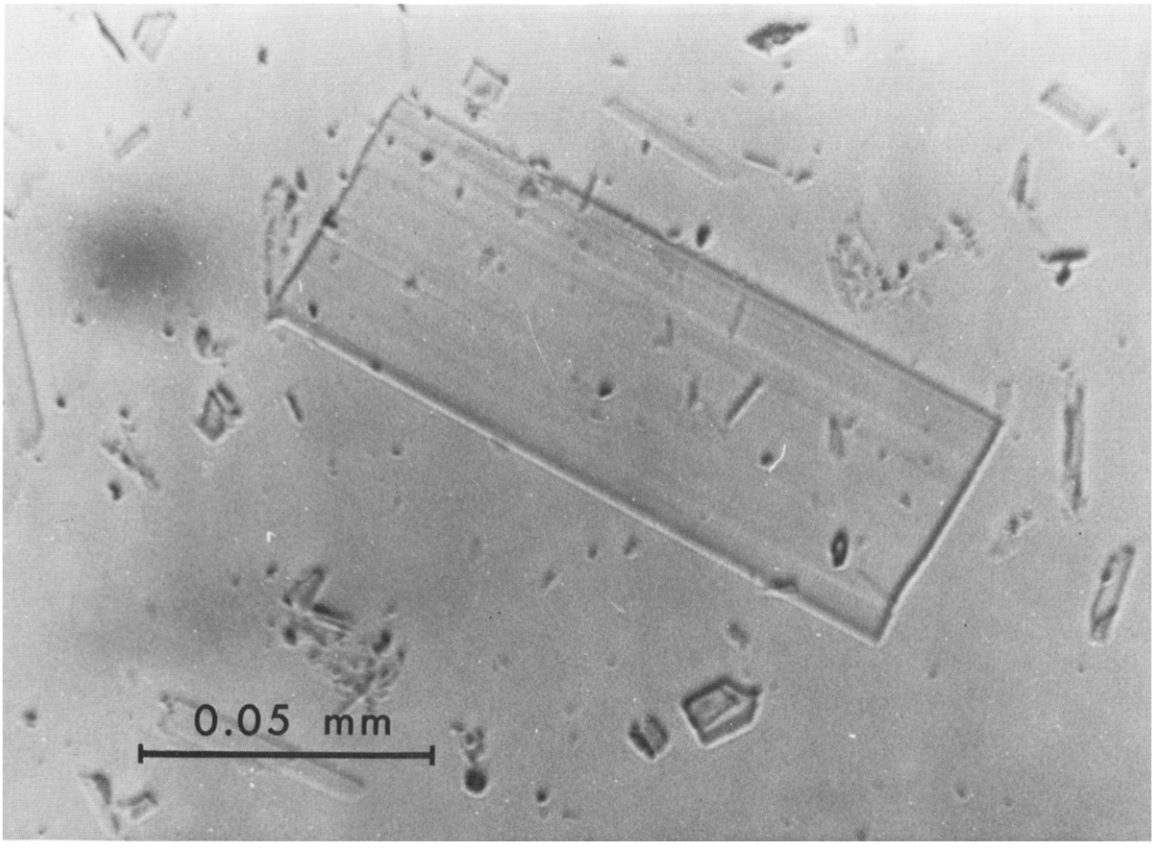

Fig.1. Crystals of *Chlorobium* Fe-superoxide dismutase.

Table 1
Comparison of amino acid compositions of *Chlorobium, Chromatium* and *Desulfovibrio* superoxide dismutases (SOD) in residues/mol

Amino acids	*Chlorobium* Fe-SOD[a)]	*Chromatium* Fe-SOD[b)]	*Desulfovibrio* Fe-SOD[c)]
Lysine	22.29	16	32
Histidine	11.97	8	10
Arginine	4.87	4	8
Aspartic acid	56.61	47	54
Threonine	19.82	23	24
Serine	19.38	32	14
Glutamic acid	42.06	39	36
Proline	17.80	14	16
Glycine	33.49	27	38
Alanine	45.42	42	44
Valine	25.21	28	16
Methionine	5.04	4	4
Isoleucine	9.42	16	14
Leucine	26.49	32	30
Tyrosine	17.91	18	16
Phenylalanine	16.34	17	24
Half-cystine	3.17	2	4
Tryptophan	11.55	12	12

a) The number of residues was calculated for 43 000 g enzyme
b) From [3]
c) From [4]

geneous superoxide dismutase. Specific activity of the purified enzyme (160 mg) was 4136 units/mg enzyme and the yield was about 40%. Faint brown rectangular crystals (fig.1) grew, when the enzyme solution (5 mg/ml) was dialyzed against ammonium sulfate solution at 53% or 56% saturation, at pH 5.1, for several weeks.

3.2. *Properties of the enzyme*

Polyacrylamide disc-gel electrophoresis of the purified enzyme, at pH 8.9, gave a single protein band corresponding to the enzyme-active zone of the crude extract, indicating that no modification of the enzyme occurred during the purification. The enzyme was estimated to be mol. wt 44 00 by gel filtration with Sephadex G-100. Sedimentation equilibrium centrifugation was conducted at 0.79 mg enzyme/ml in 10 mM phosphate, pH 7.8, containing 0.1 M KCl at 23 276 rev./min at 20°C. A mol. wt 43 000 was obtained from the slope of the log (fringe displacement) plotted against the square of the distance from the center of rotation. A partial specific volume (0.719) was calculated from the amino acid composition (table 1). When exposed to sodium dodecylsulfate (SDS) and subjected to SDS–polyacrylamide disc-gel electrophoresis the enzyme gave one band corresponding to a position of mol. wt 22 000 in the presence and absence of 2-mercaptoethanol. Thus, *Chlorobium* superoxide dismutase is a dimer composed of equal subunits without a disulfide bridge.

The ultraviolet spectrum of the enzyme exhibits an absorption maximum at 280 nm with shoulders at 260 nm and 290 nm (fig.2). $A^{1\%}_{1\,cm}$ and the absorbance coefficient at 280 nm were estimated to be 19.10 and 82 100 M^{-1} cm^{-1}, respectively, based on mol. wt 43 000. The visible spectrum shows a weak and broad absorption from 330–600 nm with a shoulder around 350 nm which is characteristic of Fe-superoxide dismutase. The absorbance coefficient at 350 nm is 3530 M^{-1} cm^{-1}.

The *Chlorobium* enzyme contained 1.80 ± 0.05 atoms Fe/mol enzyme on the basis of mol. wt 43 000. The contents of Mn, Cu and Zn were insignificant.

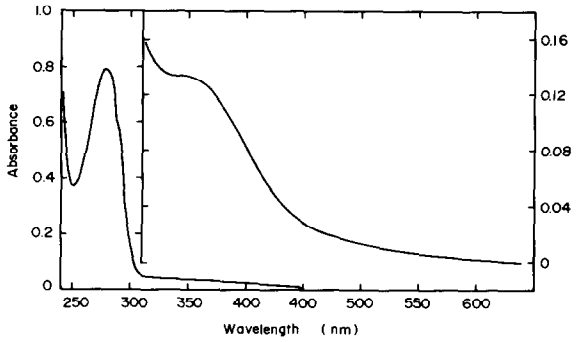

Fig.2. Absorption spectrum of *Chlorobium* superoxide dismutase. The ultraviolet and visible spectra were recorded at 0.415 and 1.659 mg enzyme/ml, respectively, in 10 mM potassium phosphate, pH 7.8, at 25°C. The light path was 10 mm.

No sulfide was detected by acidification of the enzyme. Thus, *Chlorobium* superoxide dismutase is the Fe-enzyme.

Isoelectric focusing was performed in 1% ampholine (pH 3.5–5.0) stabilized by a sucrose gradient using a 30 ml column at 500 V for 24 h followed by 800 V for 48 h at 4°C. After the pH-gradient had been established 1 ml fractions were collected and measured for pH and enzyme activity, which revealed an isoelectric point of 4.17.

Table 1 shows the amino acid composition of the *Chlorobium* Fe-superoxide dismutase, with those of the Fe-enzymes from other anaerobes. The composition is very similar to compositions of the Fe-enzymes from anaerobic and aerobic bacteria. In spite of the similar amino acid compositions, antibodies against the *Plectonema* Fe-superoxide dismutase [5] and the *Mycobacterium* Fe- and Mn-enzymes [11] dit not affect the activity of the *Chlorobium* enzyme nor cross-react with the enzyme in Ouchterlony double immunodiffusion. The antibody against the spinach Cu,Zn-enzyme [5] also showed no reaction.

4. Discussion

The absorption spectrum, metal contents, amino acid composition, molecular weight and subunit structure of *Chlorobium* superoxide dismutase are very similar to those of the Fe-enzymes isolated from aerobic and anaerobic bacteria. Green sulfur photosynthetic bacteria contain only this form of enzyme as do other anaerobes, i.e., sulfate-reducing and purple sulfur bacteria [2–4]. Thus, all three anaerobes including sulfate-reducing bacteria and photosynthetic sulfur bacteria contain only Fe-superoxide dismutase. To our knowledge Mn-superoxide dismutase has not been found in anaerobic bacteria but in aerobic bacteria such as purple nonsulfur bacteria and blue–green algae. In this respect it is interesting to note that *Escherichia coli* contains only the Fe-enzyme when the cells are cultured under an extremely low partial pressure of oxygen while the cells cultured in air or oxygen contain the Mn-enzyme in addition to the Fe-enzyme [12]. A comparison of the energy metabolism and the amino acid sequence of *c*-type cytochromes suggests that aerobic bacteria have evolved from anaerobic photosynthetic bacteria [13]. The present results support the proposal [3,6] that the Fe-superoxide dismutase in anaerobic bacteria is the ancestor of the Fe- and Mn-superoxide dismutases in aerobes. The physiological function of the enzyme in anaerobes is not yet understood, however.

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