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The Single Superoxide Dismutase of *Rhodobacter capsulatus* Is a Cambialistic, Manganese-Containing Enzyme

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The phototrophic bacterium *Rhodobacter capsulatus* contains a single, oxygen-responsive superoxide dismutase (SOD_{Rc}) homologous to iron-containing superoxide dismutase enzymes. Recombinant SOD_{Rc}, however, displayed higher activity after refolding with Mn^{2+}, especially when the pH of the assay mixture was raised. SOD_{Rc} isolated from *Rhodobacter* cells also preferentially contains manganese, but metal discrimination depends on the culture conditions, with iron fractions increasing from 7% in aerobic cultures up to 40% in photosynthetic cultures. Therefore, SOD_{Rc} behaves as a Mn-containing dismutase with cambialistic properties.

Superoxide dismutases (SODs; EC 1.15.1.1) are metalloenzymes that catalyze superoxide (O_2^-) disproportionation to yield H_2O_2 and O_2. They have been found in most organisms, aerobic and anaerobic, and play a key role in cellular protection against oxidative stress conditions (13, 38). These enzymes are generally classified on the basis of the metal cofactor. The iron-containing dismutases (FeSODs) and the manganese-containing dismutases (MnSODs) display a significant degree of sequence and structural similarity and are evolutionarily related, whereas the binuclear copper-zinc enzymes belong to a different lineage (38). In addition, a small group of nickel-dependent SODs has been recently identified in various *Streptomyces* species (18, 19, 47).

Despite their sequence conservation, FeSODs and MnSODs usually attain significant activity with only one of the permitted metal cofactors, although they can frequently bind both. The two types of dismutases have often been distinguished by the presence of some key discriminating amino acids (27) and by their differential sensitivities to H_2O_2 inhibition (4). However, these criteria are sometimes misleading (14, 37), and the identity of the metal cofactor needs to be confirmed by direct analysis of the purified holoproteins. This precaution is particularly relevant since a small group of these enzymes, termed cambialistic, are able to display significant activity with either of the two metals bound at the same active site (23). They have been found in a wide variety of aerotolerant bacteria, including *Propionibacterium freudenreichii* subsp. *shermanii* (26), *Bacteroides fragilis* (15), *Porphyromonas gingivalis* (1), and *Methylomonas* strain J (44).

Rhodobacter capsulatus is a gram-negative phototrophic bacterium that can thrive under a broad range of environmental conditions. When the oxygen tension is low, these microorganisms synthesize ATP through a light-driven, anoxygenic electron transport around a single photosystem (10), whereas in the presence of air they shift to a respiratory metabolism after expression of oxidases and dehydrogenases (9, 31). Little is known about the conditions prevailing in the bacterial cells following the establishment of respiration, but oxygen-centered derivatives such as superoxide and peroxides are expected to be produced by reduced intermediates of the electron transport chain (36). In line with this assumption, a number of protective antioxidant proteins are known to be present in *Rhodobacter* species, including two peroxidases (12) and an oxygen-responsive thioredoxin which is essential for both aerobic and anaerobic growth (28). We have recently identified a single SOD (SOD_{Rc}) in *R. capsulatus* cells cultured under various growth regimes (8). Even though the enzyme could be detected in photosynthesizing anaerobic *Rhodobacter*, its expression was strongly induced on exposure of the cells to air or to the redox-cycling compound and superoxide propagator methyl viologen (8). The gene encoding the *R. capsulatus* dismutase was cloned by genetic complementation of SOD-deficient *Escherichia coli* mutants. The deduced amino acid sequence of the resulting product displayed clear similarity with FeSODs from several microorganisms. However, the low reliability of SOD classifications based solely on sequence comparisons prompted us to investigate the identity of the metal cofactor present at the active site.

Recombinant *Rhodobacter* SOD displays enzymatic activity with both iron and manganese. We first examined the dependence of SOD_{Rc} activity on the nature of the bound metal by expressing the *Rhodobacter sod* gene in *E. coli* under the control of the T7 RNA polymerase promoter. The *sod* coding region was amplified by PCR by using the pA22 plasmid (8) as a template and oligonucleotides 5′-GATCGCTGCCATGGCTTTCGAACTTCC-3′ and 5′-TTTCATCCGTCCATCGAGCTCACATCC-3′ as 5′ and 3′ primers, respectively. The generation of *Nco*I and *Sac*I sites (underlined) permitted cloning of the amplified DNA into compatible sites of the pET-28a expression vector to yield pESOD28. A *Sac*I-*Sph*I fragment of about 900 bp carrying the *sod* gene was excised from pESOD28 and ligated into compatible sites of the pET-32b vector to introduce ampicillin resistance. The resulting plasmid, pESOD3228, was used to transform the *E. coli sodA sodB* strain QC774/DE3 (32).

Transformants were grown aerobically and induced by following the protocols suggested by the pET System manual

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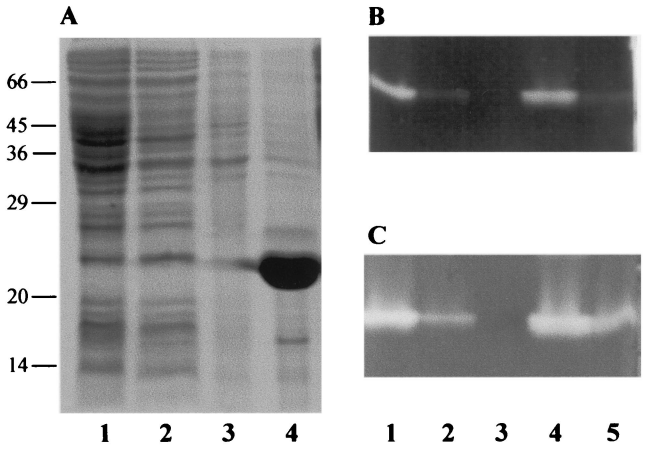

FIG. 1. Reconstitution of recombinant *R. capsulatus* SOD expressed in *E. coli*. Cells of strain QC774/DE3 transformed with plasmid pESOD3228 were induced with isopropyl-β-D-thiogalactopyranoside, collected, and broken by sonic oscillation. (A) Soluble fractions (lanes 1 and 2) and pellets (lanes 3 and 4) corresponding to 50 μl of culture, collected before (lanes 1 and 3) and after (lanes 2 and 4) induction, were resolved by SDS-PAGE and stained with Coomassie brilliant blue. Molecular masses (in kilodaltons) and electrophoretic mobilities of protein standards are shown on the left. (B and C) SOD_{Rc} was isolated from inclusion bodies and diluted in the absence (lanes 3) or in the presence of Mn^{2+} (lanes 4) or Fe^{2+} (lanes 5). Protein contents were determined by a dye binding assay (33). Aliquots corresponding to 1 μg of protein were subjected to native PAGE and in situ activity staining at pH 8.0 (B) or 6.0 (C), essentially as described by Beauchamp and Fridovich (3). Lanes 1 and 2 were loaded with 1 μg of purified aero-SOD_{Rc} and 1 μg of purified photo-SOD_{Rc}, respectively.

(Novagen). Cells were broken by sonic oscillation during 30 s in a Branson Sonifier 250, and the corresponding extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using the buffer system described by Laemmli (20). The results shown in Fig. 1A indicate that most of the recombinant protein sedimented with the inclusion bodies fraction. Pellets were thus collected, washed, and dissolved by following the recommended protocols (pET System; Novagen). All buffers were supplemented with 5 mM dithiothreitol (DTT).

Solubilized apo-SOD was refolded by dilution (1:10) against 0.5 mM $MnSO_4$ or 0.5 mM $Fe(NH_4)_2(SO_4)_2$ in 20 mM Tris-HCl (pH 8.0), 1 mM DTT, and 10% (vol/vol) glycerol. The solution was then loaded onto a Q-Sepharose HP column (Pharmacia Äkta System) and eluted with a NaCl gradient. Protein peaks were dialyzed against 20 mM Tris-HCl (pH 8.0), 5 mM DTT, 5 mM EDTA, 2 mM ascorbic acid, and 10% (vol/vol) glycerol to eliminate unspecifically bound metal. All solutions were previously sparged with N_2.

SOD activity, as determined by in situ staining of native polyacrylamide gels (3), could be recovered by reconstitution with either Mn^{2+} or Fe^{2+}. Although the activity of the Fe form of the enzyme was hardly detectable when developed at pH 8.0, it increased significantly as the pH of the assay mixture was lowered (Fig. 1B and C). A similar behavior has been observed with SODs from other sources (43).

The reconstituted enzymes were characterized with respect to quaternary structure and metal content. A single subunit of 22 kDa, corresponding to the deduced molecular mass of the protein encoded by the *R. capsulatus sod* gene (8), was visualized by SDS-PAGE, while gel filtration analysis on a Superdex G 200 HR 10/30 column indicated that the two reconstituted dismutases were dimers of identical polypeptides (data not shown). Atomic absorption photometry revealed that MnSOD contained 0.96 ± 0.05 atoms of Mn per monomer and no detectable Fe (<0.06 atoms per monomer) while FeSOD carried 0.86 ± 0.10 atoms of Fe per monomer with no detectable Mn (<0.01 atom per monomer).

The results indicate that, despite sequence similarity with FeSODs, the single SOD of *R. capsulatus* displays higher enzymatic activity when reconstituted in vitro with Mn^{2+}. Since the enzyme is functional to various degrees with either of the two metals, it is not clear at this stage which cofactor is preferentially bound to SOD in vivo under the different lifestyles of the microorganism.

The culture conditions modulate metal uptake by *Rhodobacter* SOD. To determine the identity of the metal cofactor incorporated by SOD_{Rc} in vivo, the enzyme was purified from both aerobically and photosynthetically cultured *R. capsulatus* 37b4 cells to yield aero-SOD_{Rc} and photo-SOD_{Rc}, respectively. Aerobic or phototrophic conditions were established by growing the cells at 32°C in malate mineral medium (40) either in an air-sparged 15-liter Microferm fermentor (New Brunswick Scientific Co.) under high stirring (500 rpm) or in illuminated screw-cap bottles. Bacteria were grown to an optical density of about 0.8 at 660 nm, harvested by centrifugation, washed, and suspended in 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM DTT, 2 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg of RNase/ml, 10 μg of DNase/ml, and 10% (vol/vol) glycerol. Cells were finally disrupted by sonic oscillation, and lysates were cleared by centrifugation (60 min at 140,000 × *g*). SOD_{Rc} was isolated by ammonium sulfate fractionation between 70 and 90% of saturation, followed by chromatography on a Pharmacia Äkta System (Q-Sepharose High Performance, Phenyl Sepharose CL-B4). SOD_{Rc} was purified 209-fold from aerobic cultures and 380-fold from photosynthetically grown cells, with yields of 34 and 40%, respectively.

Filtration chromatography showed that native SOD_{Rc} isolated from both respiratory and phototrophic cells behaved as a dimer, as had been previously observed for the reconstituted enzymes. Aero-SOD_{Rc} contained 93% manganese and only 7% iron per monomer, but photo-SOD_{Rc} increased its iron content up to 40% (Fig. 2A), suggesting that metal incorporation is influenced by culture conditions, most likely by the oxygen tensions during growth (1, 15). At pH 7.8, the specific activities of purified photo-SOD_{Rc}s (2,500 to 3,966 U/mg) represent 45 to 71% of that displayed by the aerobic form of the enzyme (5,573 U/mg).

Superoxide disproportionation mediated by FeSOD or MnSOD involves a solvation step that makes the reaction pH dependent (6, 34). We therefore measured the catalytic activities of native and recombinant forms of SOD_{Rc} at two different pH values. The reaction rate exhibited by a recombinant SOD_{Rc} reconstituted with Mn was 47-fold higher than that of $FeSOD_{Rc}$ at pH 7.8 (Fig. 2B), while at pH 6.2 this ratio declined to ~3.5 (Fig. 2C). In particular, the specific activity of the SOD reconstituted with Fe increased almost one order of magnitude as the pH of the medium was lowered (Fig. 2C). A

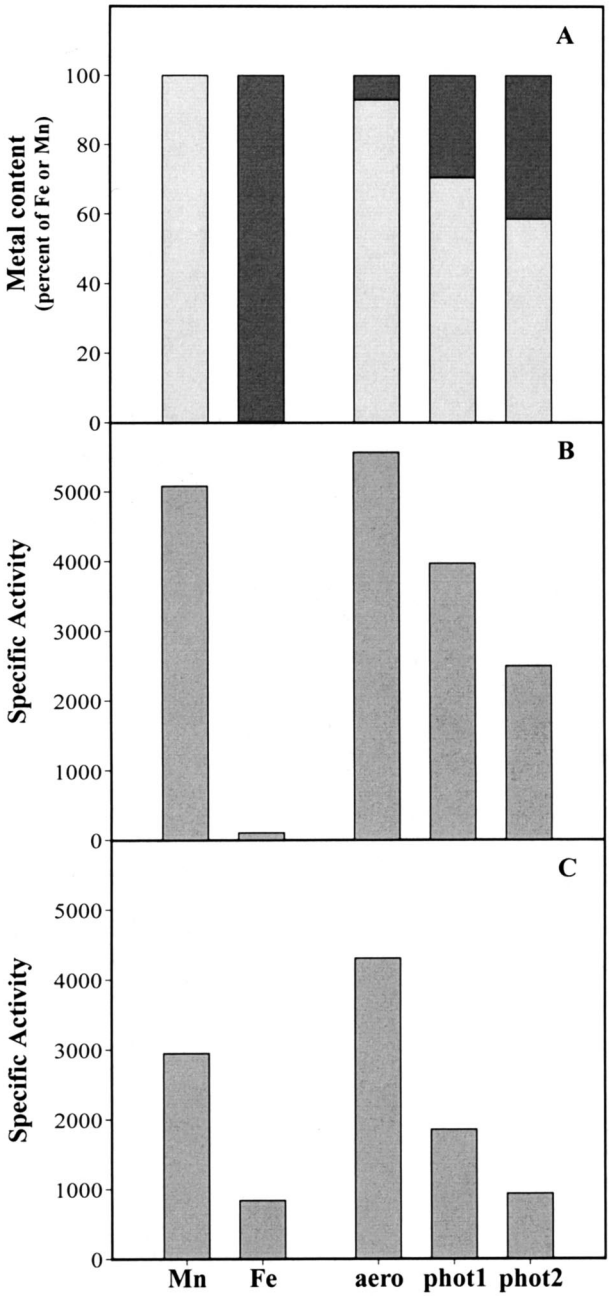

FIG. 2. Correlation between the catalytic activity and the metal present in SOD of *R. capsulatus.* Native and reconstituted SOD_{Rc} forms were prepared as indicated in the text. (A) Manganese (light gray) and iron (dark gray) contents of the various SOD forms were measured by atomic absorption spectrometry. (B and C) The activities of SOD_{Rc} proteins isolated from aerobic cultures (aero) and from two independent photosynthetic cultures (phot1 and phot2) and those obtained from recombinant SOD_{Rc} reconstituted with either Mn or Fe were determined by published procedures (25) at pH 7.8 (B) or 6.2 (C). Bars represent the specific activities of each dismutase form at the two pH values. Standard errors in metal determination and activity measurements were less than 5 and 10%, respectively.

comparison of the properties of reconstituted dismutases with those displayed by aero-SOD_{Rc} and photo-SOD_{Rc} suggests that the latter enzymes are made up of mixtures of homogenous dimers (MnSOD or FeSOD) and heterodimeric forms in various proportions. However, the fall in catalytic competence experienced by all native SOD_{Rc} species at acidic pH (Fig. 2B and C) could not be accounted for by any simple combination of enzyme variants and metal distribution. It is likely that heterodimers have specific activities that are different from those of single-metal dismutases and affected by the pH in an unpredicted way, but further research is required to substantiate this assumption. The results also suggest that knowledge about the effects of pH on dismutase activity might contribute to an understanding of the molecular basis of metal discrimination and function in the FeSOD and MnSOD families, although the physiological significance of this pH dependence is not obvious. Internal pH is probably regulated within narrow limits as *R. capsulatus* shifts among different growth conditions, but little is known about the homeostatic mechanisms operating in these phototrophic bacteria under changing environments.

The monomeric unit of MnSOD and FeSOD is characterized by two domains (11, 21, 35). The N-terminal portion of the molecule includes two α-helices in close contact, while the C-terminal domain forms a globular-shaped structure surrounding a core of three β-strands. The active-site metal is bound at the interface between apolar side chains contributed by the β-sheet and the N-terminal α-helices. The metal cofactor presents a bipyramidal trigonal geometry and is ligated by two histidines and an aspartate in the equatorial plane, with a third histidine and a coordinated solvent as axial ligands.

The high degree of sequence and structural conservation among dismutases of these two families precludes the use of standard sequence homology analyses, which are unable to identify subtle conformational differences, such as small variations in distance among important residues in the folded protein or minor changes in metal coordination chemistry. However, careful comparison of aligned sequences of MnSODs and FeSODs combined with assessment of structural data led several authors to identify a few specific amino acids, located close to the cofactor binding site, as major determinants in metal discrimination (17, 27). In particular, the glutamine residue present in the outer coordination sphere that forms a hydrogen bond with the active-site solvent molecule has been singled out as a "crucial factor" for metal selectivity (41). For typical FeSODs, this glutamine is contributed by the α2-helix (corresponding to position 68 in the *E. coli* MnSOD), whereas in the majority of MnSODs, the isofunctional residue (Gln146 in *E. coli* FeSOD) belongs to the C-terminal end of the β2-strand. However, the atypical SOD from *Sinorhizobium meliloti* displays an iron-type sequence but preferentially incorporates Mn in vivo (30) and the cambialistic SOD of *P. gingivalis* has the solvent-coordinating glutamine at the N-terminal position (1, 2). Moreover, the results obtained by exchanging this functional residue between the α2-helix and β2-sheet locations through site-directed mutagenesis indicate that it is not the only determinant of metal selection in FeSODs and MnSODs (16, 22, 32).

At the metal binding site of *R. capsulatus* SOD, the crucial glutamine interacting with the coordinated solvent is most likely provided by the N-terminal portion of the protein, as in canonical FeSODs (8). However, the present study shows that the native dismutases found in both aerated and photosynthetic cultures contain Mn as the predominant cofactor (Fig. 2)

and that purified recombinant SOD_{Rc} is indeed more active when reconstituted with Mn (Fig. 1B and C and 2). The cofactor environment of Mn-reconstituted SOD_{Rc} appears to be very similar to that of *E. coli* MnSOD, the major difference being a higher degree of symmetry in the equatorial ligand-metal interactions of the *Rhodobacter* dismutase, as revealed by high-field electron paramagnetic resonance (39). The overall results indicate that SOD_{Rc} is a MnSOD with cambialistic properties and that the corresponding gene should be renamed *sodA*. They also show that structural and functional predictions inferred from sequence comparisons have to be handled with caution for this class of SODs.

Another criterion used to classify SODs within the Fe and Mn families is their differential sensitivities to certain inhibitors. Rapid inactivation of FeSOD, but not MnSOD, by H_2O_2 has been ascribed to the oxidation of an essential tryptophan residue in the vicinity of the iron binding site (4, 42, 46), although the actual identity of the reactive amino acid is still a matter of debate (7, 27, 37, 45). A recombinant SOD_{Rc} reconstituted with Mn was virtually insensitive to H_2O_2, whereas Fe incorporation led to a holoenzyme that was inhibited 60 to 70% by a similar treatment (data not shown). On the other hand, native SOD_{Rc} was shown to be affected by H_2O_2 exposure, although metal contents were not determined at the time (8). When purified dismutases of known metal compositions were exposed to H_2O_2, the observed inactivation levels always exceeded those predicted from the theoretical fractions of FeSOD in the mixtures (data not shown), once again suggesting the presence of heterodimers that were sensitive to H_2O_2 deleterious action.

Previous reports have shown that metal incorporation in cambialistic SODs depends on metal availability and contingent growth conditions (24, 26, 44). Even in *E. coli*, competition between iron and manganese for nascent MnSOD polypeptide chains occurs in vivo, leading to copurification of variously enriched dismutases (5). In other facultative bacteria, manganese is preferentially incorporated in the presence of oxygen, whereas anaerobiosis facilitates iron binding (1, 15). This is indeed the behavior exhibited by *R. capsulatus* cells, which accumulate Fe-enriched SOD under phototrophic conditions and mostly MnSOD in aerated media (Fig. 2). This trend might be facilitated by Fe release from bacterioferritin under photosynthetic culture regimes (29). Then, *Rhodobacter* fluctuations between aerobiosis and the microaerobic environment prevailing during photosynthetic growth, together with the concomitant changes in metal availability, might have encouraged the evolution of a cambialistic SOD that can bind (and function with) either metal, depending on the external conditions imposed upon the growing bacteria.

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