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Chemical Aspects of the Structure, Function and Evolution of Superoxide Dismutases

M. W. PARKER*

Laboratory of Molecular Biophysics, Dept. of Zoology, University of Oxford, Oxford, U.K.

M. E. SCHININÀ, F. BOSSA

Institute of Biological Chemistry, University of Rome and C.N.R. Centre for Molecular Biology, Rome, Italy

and J. V. BANNISTER

Inorganic Chemistry Laboratory, University of Oxford, Oxford, U.K.

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Introduction

The oxygen atoms available in the atmosphere, oceans and earth's crust have had a complex history. The earth is considered to have been formed from meteoritic material which had solidified in space and was therefore initially composed of pure metals and minerals. It is then hypothesized that a powerful solar wind swept through the inner planetary system carrying away the free gases, volatile elements and the still burning solid particles. Oxygen must also have been lost as gaseous carbon monoxide and water, however, some of it was protected by being locked in refractory minerals such as silicates, titanates, aluminates and oxides which make up the bulk of the meteoritic material that still survives to the present day. The atmosphere and the oceans originated by subsequent outgassing of the outer layers of the earth over geological time and their present composition is due to geochemical and petrochemical processes and relatively recently to the effects of photosynthesis. The most likely abiotic source of free oxygen is the photodissociation of water by ultraviolet sunlight at the top of the atmosphere followed by the escape of hydrogen. The supply of oxygen from photodissociation could not have been too large and the rest of the supply resulted from photosynthesis [1].

Photosynthesis is considered to be the major source of atmospheric oxygen since it can replenish the entire oxygen pool in about 2500 years. Since oxygen will tend to oxidise the metallic elements in the earth's crust all the free oxygen initially liberated was bound to these elements. It took 1.8×10^9 years from the time that oxygen was liberated by photosynthesis to the time that free oxygen appeared and Cloud [2] believes that this time was necessary for the biosphere to build up the necessary antioxidant defences. In fact recently, a hundred years after its discovery, oxygen was recognized as toxic to living elements [3]. Reduced iron is considered to have acted as an external antioxidant during the transition from an anaerobic to an aerobic environment [4]. During this period there was therefore an evolutionary pressure for the development of antioxidant defences. These can be of a varied nature and in the biosphere as many of these as possible are used to combat oxygen toxicity. Investigations on oxygen toxicity have led to two proposals being described regarding its molecular mechanism. The first proposal is that oxygen manifests its toxicity through the generation of oxygen free radicals or reduction products whilst the second proposal which is elaborated from the first is that intrinsic protective mechanisms against oxygen derived free radicals must be present in aerobic cells. The current understanding of oxygen toxicity is based on theoretical and experimental foundations. Michaelis [5] elaborated the theoretical framework which explained the biological generation of free radicals whilst Gerschman *et al.*, [6] following investigations on X-radiation induced toxicity considered that this form of toxicity resembled oxygen toxicity. The superoxide radical is the first reduction product of oxygen. Superoxide radicals are generated in many biological reactions that can reduce molecular oxygen. The toxicity of oxygen has also to be considered in terms of other reduction products such as hydrogen peroxide and also from the reaction between superoxide radicals and hydrogen peroxide —the so called Haber Weiss reaction [7] ($O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^\cdot$) for which a number of metal catalysts have been proposed [8–13]. This reaction produces the highly reactive species—the hydroxyl radical. The first line of defence against oxygen toxicity is therefore the removal of the superoxide radical before the formation of other toxic products [14]. The enzyme superoxide dis-

*Author to whom correspondence should be addressed.

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mutase discovered by McCord and Fridovich [15] scavenges the superoxide radical and is considered the major antioxidant present in cells. The enzyme was first isolated as a green copper protein from bovine erythrocytes and liver by Mann and Keilin [16] and was later found also to contain zinc [17]. The historical events leading to the protein being identified as superoxide dismutase (E.C. 1.15.1.1) are documented by McCord and Fridovich [18]. Further investigations by Fridovich and collaborators led to other forms of superoxide dismutases being identified. There were found to contain either manganese [19] or iron [20].

Superoxide Dismutase as an Antioxidant Defence Enzyme

The fact that superoxide is an important agent of oxygen toxicity and that superoxide dismutase provides the first line of defence is supported by experimental evidence. The enzyme was found to enhance the resistance of several organisms to the lethal effects of hyperbaric oxygen. Facultative organisms such as *Streptococcus faecalis*, *Escherichia coli* and *Saccharomyces cerevisiae* were found to have an increased intracellular accumulation of superoxide dismutase following exposure to oxygen. The iron superoxide dismutase in *Escherichia coli* was found to be present whether the organism was grown in the presence or absence of oxygen. However, transfer of the cells from anaerobic to aerobic conditions resulted in prompt induction of the synthesis of the manganese superoxide dismutase [21]. Also the sea anemone, *Anthopleura elegantissima* containing the symbiotic dinoflagellate *Symbiodinium microadriaticum* in their gastrodermal tissues have superoxide dismutase activities nearly two orders of magnitude greater than in individuals totally lacking symbiotic organisms [22]. The increase in the level of superoxide dismutase activity in these animals was ascribed to the fact that hyperbaric oxygen levels may occur in tissues of organisms that harbour symbionts which in light generate more oxygen than is consumed by the combined host and symbiont. Strong evidence in support of the superoxide theory of oxygen toxicity was obtained through the use of paraquat (methyl viologen). Inside the cell paraquat disrupts the electron transport pathway and is reduced to the semiquinone form which then reacts with oxygen to produce superoxide radicals. Paraquat was found to increase the rate of synthesis of the manganese superoxide dismutase whereas in the absence of oxygen it had no effect on the synthesis of the enzyme [23]. The addition of paraquat to cultures of *Rhizobium japonicum*, an anaerobic bacterium which can fix atmospheric nitrogen, was found to increase the level of superoxide dismutase [24]. Following the discovery of superoxide dismutase it was found that obligate anaerobes did not contain the enzyme [25]. However, this report was shown to be in error [26] and the presence of superoxide dismutase in photosynthetic anaerobes, sulphate-reducing bacteria and fermentative anaerobic bacteria was reported. The presence of superoxide dismutase is to protect against accidental exposure to oxygen. The nitrogenase present in certain nitrogen fixing species is extremely sensitive to oxygen [27]. Tally *et al.* [28] and Carlsson *et al.* [29] have reported superoxide dismutase levels in anaerobic species isolated from clinical specimens and have suggested a correlation between enzyme levels and aerotolerance. The level of superoxide dismutase in the anaerobe *Bacteroides fragilis* was found to increase in the presence of oxygen [30]. However, the induction of superoxide dismutase is not entirely dependent on increased levels of oxygen tensions leading to higher concentrations of superoxide radicals but also on the presence of the appropriate metal in the growth medium. *Escherichia coli* grown in iron-rich media are more resistant to killing by phagocytosis than cells grown in iron-deficient medium [20]. Cultures of fungus, *Dactylum dendroides* contained 80% copper/zinc and 20% manganese superoxide dismutase. Removal of the copper from the growth medium resulted in decreased copper/zinc superoxide dismutase synthesis with a concomitant increase in the level of the manganese enzyme such that the total level of cellular superoxide dismutase activity remained constant [31]. A switching back to normal synthesis occurred when the growth medium was supplemented with copper. The anaerobic bacterium *Propionibacterium shermanii* was found to produce either the iron or manganese superoxide dismutase depending on metal supply [32].

Distribution of Superoxide Dismutases

The distribution of the three types of superoxide dismutase is considered to be a characteristic of the evolutionary stage of the organism and also of the organelle with which it is associated. The distribution has been established by isolation and characterisation, however, a combination of sensitivities and insensitivities of the three enzymes to cyanide and hydrogen peroxide has allowed the determination of the prosthetic metal of the enzyme in crude extracts. Cyanide only inhibits the copper/zinc enzyme whilst the iron enzyme and the copper/zinc enzyme are inhibited by hydrogen peroxide. The three enzymes also exhibit varying degrees of inhibition by azide. The most primitive form of the enzyme is considered to be the iron superoxide dismutase. This is because it is the form present in anaerobic bacteria and has been purified from the anaerobes *Desulphovibrio*

sulfuricans [33], *Chromatium vinosum* [34] and *Propionibacterium shermanii* [36]. In contrast to anaerobic bacteria, aerobic and facultative anaerobes contain either the iron or manganese enzyme or both. However, whether a single form or the two forms of the enzyme are present in anaerobes and aerobes, depends on the growth conditions and remains to be fully investigated. *Bacillus megaterium* has in contrast to being reported to possess an iron enzyme [36] has also been shown to possess a manganese form of the enzyme [37] whilst Teresch and Viganis [38, 39] have described the presence of a manganese superoxide dismutase in *Paracoccus denitrificans* in contrast to an earlier report demonstrating the presence of an iron form of the enzyme.

No cyanide sensitive activity has been detected in unicellular species. The major superoxide dismutase present in prokaryotic blue green algae is the iron enzyme. This form has been purified from *Plectonema boryanum* [40, 58], *Spirulina plataensis* [41] and *Anacystis nidulans* [42]. Eukaryotic algae possess either the iron and/or manganese superoxide dismutase. Both forms have been purified from *Euglena gracilis* [43] and the manganese form from the red algae *Porphyridium cruentum* [44], which is considered to be the most primitive eukaryote. The copper/zinc form also appears to be lacking in protozoa. A predominantly iron form has been purified from *Crithidia fasciculata* [45].

Multicellular organisms in the plant and animal kingdom contain the copper/zinc and the manganese superoxide dismutase. In general terms the distribution of the enzyme can be stated to be that the copper/zinc enzyme is essentially an eurkaryotic enzyme and the iron enzyme is essentially a prokaryotic enzyme. The manganese enzyme can also be considered to be a prokaryotic enzyme. It is, however, also found in the mitochondria of eukaryotes. A number of exceptions to this general rule have come to light. An iron form of the enzyme has been isolated from the seeds of the mustard plant *Brassica campestris* [46] and a survey of 43 plant families for the presence of the iron enzyme showed that it was present in three isolated families [47]. Two bacterial species, *Photobacterium leiognathi* [48] and *Caulobacter crescentus* [49] have been shown to contain the copper/zinc superoxide dismutase. A report also describing the presence of a cyanide sensitive superoxide dismutase in *Paracoccus denitrificans* has now been discounted [50, 51].

Chemical Characterisation of Superoxide Dismutases

The copper/zinc superoxide dismutase is a homodimer of about 32,000 daltons. The molecular weight determined for the enzyme from a variety of sources are all substantially in agreement. The enzyme contains up to 1 g atoms of both copper and zinc per subunit. The iron and manganese enzymes have a subunit molecular weight of about 20,000 daltons. Whilst the iron superoxide dismutase has been shown to be dimeric the manganese enzyme has been found to form tetramers as well as dimers. The extent of polymerisation does not apparently depend on the source of the enzyme. Manganese superoxide dismutase from *Escherichia coli* is a dimer [19] whilst the enzyme from chicken liver [52], *Thermus aquaticus* [53] and yeast mitochondria [54] is a tetramer.

The metal content of the iron and manganese enzymes varies between 1.0 and 2.0 g atoms per dimer (Table I). Stoichiometry requires a maximum of 1 g atom per subunit. Whether this variation is due to loss of metal during the preparation is unclear. Preliminary crystallographic information indicated two binding sites per dimer. A number of discrepancies have also recently appeared in the metal composition of certain iron superoxide dismutases (Table II). These proteins have also been found to contain zinc and two enzymes have also been found to contain manganese. This is the only known superoxide dismutase to contain three metals. The possibility that these enzymes may represent a new class of superoxide dismutases has to be entertained. The cyanide sensitive copper protein in *Paracoccus denitrificans* [50, 51] having superoxide dismutase activity was found to consist of a single polypeptide chain of 33,000 daltons rather than of two subunits of 16,000 daltons.

Mechanism of Action of Superoxide Dismutase

The superoxide dismutases are distinguished by the scavenging of superoxide radicals to produce oxygen and hydrogen peroxide. The catalytic metal whether it is copper, iron or manganese undergoes redox cycling in the process (Table III). The copper present in the copper/zinc enzyme is the most efficient scavenger. The rates of the two half reactions are identical and are the same as that of the overall reaction ($3.5 \times 10^9\ M^{-1}\ s^{-1}$) which is close to the diffusion limit. This rate constant is independent of pH in the range 5–10. This result is relevant to the biological role of the enzyme as the spontaneous dismutation rate and also free metal-catalysed dismutations are strongly pH dependent. Three major lines of investigations have been carried out concerning the mechanism of copper/zinc superoxide dismutase activity. These concerned:

a) the oxidation state of the copper;
b) the source of the proton;
c) the interaction of superoxide with copper.

a) *The Oxidation State of the Copper*

Klug-Roth *et al.* [69] and Fielden *et al.* [70] demonstrated by pulse radiolysis the reaction mech-

TABLE I. Metal Content of Iron and Manganese Superoxide Dismutases.

Source	Metal content	Molecular weight	Ref.
Escherichia coli B	1 g atom Fe	38,000	19
Plectonema boryanum	2	41,700	40
	1	36,500	57
Bacillum megaterium	1	40,000	36
Pseudomonas ovalis	1.4	40,000	58
Desulphovibrio desulfuricans	1.60	43,000	33
Photobacterium leiognathi	1.61	40,660	59
Chromatium vinosum	2	41,000	34
Chlorobium thiosulfatophilum	1.8	43,000	55
Thiobacillus denitrificans	1.35	43,000	56
Anacystis nidulans	1	37,000	42
Crithidia fasciculata	1.4	43,000	45
Strepthococcus mutans	2 g atoms Mn	39,500	60
Escherichia coli	1.6	39,500	19
Pleurotus olearius	2	76,000	61
Chicken liver mitochondria	2.3	80,000	52
Saccharomyces cerevisiae	4	96,000	54
Mycobacterium lepraemurium	1.29	45,000	60
Thermus aquaticus	2	80,000	53
Rat liver mitochondria	4	89,000	62
Bovine heart mitochondria	2	86,000	63
Pisum sativum	1	94,000	64
Paracoccus denitrificans	1.34–2	41,500	39

TABLE II. Anomalous Metal Content of Certain Superoxide Dismutases.

Source	Metal content		Molecular weight	Ref.
Mycobacterium phlei	1.7 g atoms Mn 1.2 g atoms Fe	0.7 g atoms Zn	80,000	65
Thermoplasma acidophilum	2.0 g atoms Fe	1.0 atoms Zn	82,000	66
Methanobacterium bryantii	2.7 g atoms Fe	1.7 g atoms Zn	91,000	67
Nocardia asteroides	1.2 g atoms Fe 1–2 g atoms Mn	1–2 g atoms Zn	100,000	68

TABLE III. Mechanism of Action of Superoxide Dismutase.

$$E\text{–}Cu(II) + O_2^- \rightarrow E\text{–}Cu(I) + O_2$$

$$E\text{–}Cu(I) + O_2^- \xrightarrow{+2H^+} E\text{–}Cu(II) + H_2O_2$$

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

anism outlined (Table III). A very important point that, however, emerged was that the steady-state level of the oxidised copper in the enzyme which must be 50% in view of the equal rates of the reduction and (re-)oxidation reaction rates was instead found to be approximately 75%. Klug-Roth *et al.* [69] explained this result by assuming that the two copper ions are not initially equivalent but have different molar absorptivities at 680 nm. Fielden *et al.* [70], however, showed that the two sites were spectroscopically equivalent and proposed that only half of the active sites participate in the turnover of the substrate. This indicates anticooperative interaction between the subunits, however, evidence for interactions between subunits in the dimer have been presented [71]. The problem was finally sorted out by Viglino *et al.* [72] who showed that the steady state level of the oxidised copper is actually 50% but can change to 75% in previously lyophilised samples without a concomitant change of activity.

b) *The Source of the Proton*

A possible source of the proton is the imidazole bridging the copper and the zinc. This histidine identified from X-ray investigations to be His 67 in the sequence of the bovine enzyme would release the copper and become protonated in the reduction step. The interaction of the copper and the zinc through a bridging imidazole ligand has recently been challenged using ^{113}cadmium NMR in which the zinc is

substituted with cadmium. Armitage *et al.* [73] found different chemical shifts in the copper-free and in the copper(I) enzyme whilst Bailey *et al.* [74] observed almost identical chemical shifts in both derivatives thereby confirming earlier evidence obtained from spectral changes recorded during a study of the dismutation reaction using enzyme in which cobalt is substituted for zinc. These results clearly demonstrate that rapid protonation of this nitrogen is an important step in the catalytic mechanism.

c) *The Interaction of Superoxide with Copper*

Evidence for complex formation between superoxide and the enzyme copper has been obtained from the saturation effects at high superoxide concentration with an estimated Michaelis constant of 3.6×10^{-4} *M* [75].

From all these lines of investigation a mechanistic scheme has been proposed which involves reversible protonation of proton of the bridging histidine and the displacement of the bound water by superoxide prior to electron transfer from both the reduced and oxidised copper [76, 77]. This mechanism clearly implies a double displacement of the water molecule bound to the copper by superoxide. An alternative mechanism has been proposed [78] because the rate of exchange of water was considered too low and that outer sphere electron transfer from superoxide to copper and *vice-versa* takes place. However ^{19}F NMR relaxation measurements [79] have shown that anionic ligand can be exchanged very fast at the copper centre of the enzyme ($T_M = 4 \times 10^{-8}$ s for ^{19}F) implying that superoxide has actually the time for water displacement and electron donation within the turnover limits of the enzyme.

Amino Acid Sequences of Superoxide Dismutases

The complete amino acid sequence has been determined for five copper/zinc superoxide dismutases [80–84] (Fig. 1). The copper/zinc protein has a number of unusual features. Only the enzymes from higher species have a blocked N-terminal group. These enzymes are generally more stable. However, whether the increased stability is due to acetylation remains to be investigated. Gaps and insertions must be included in the various enzymes, specially in the bacterial protein, to optimize sequence homologies. The enzymes also display considerable variation in the number of tyrosine and tryptophan residues per dimer. Two tryptophans are present in the human and bacterial enzymes. The bovine, swordfish, yeast and bacterial enzymes have no tryptophan but contain tyrosine. The swordfish and bacterial enzymes contain 4 tyrosine in contrast to bovine and yeast enzymes which have two tyrosine. The horse enzyme possesses neither of the residues whilst they are both present in the bacterial enzyme. Finally from the alignments presented the metal binding sites appear to be conserved in all the enzymes. These are His 47, 49, 76 and 134 for the copper and His 76, 85, 94 and Asp 97 for the zinc. Also conserved are Arg 157 which is considered to be essential for activity [85] and the intrachain disulphide bridge formed by Cys 58 and 160 [80]. Only 19 other residues are con-

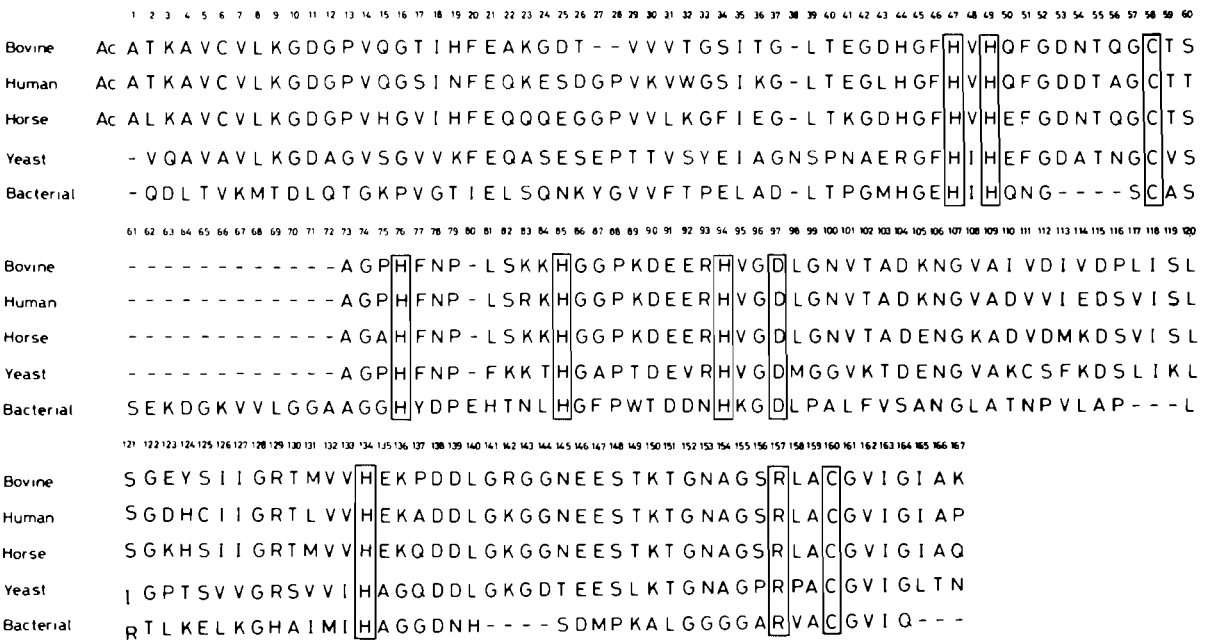

Fig. 1. Amino acid sequences of copper/zinc superoxide dismutases.

served in all the 5 sequences and it is noteworthy, and evidently connected with the three dimensional tight packing of the molecule, that 8 out of these are glycine residues.

A high degree of sequence homology is evident between the various vertebrate species (Table IV). The yeast enzyme exhibits between 50 to 60% sequence homology indicating conservation of structure. However, very little identity is evident between the bacterial and the other eukaryotic enzymes. This raises the question as to whether the bacterial enzyme is a new copper/zinc form. Gene transfer has been predicted on the basis of amino acid compositions [86] however because of the low sequence homology between the bacterial and the other enzymes this possibility has to be ruled out and an independent evolutionary line has to be considered for this form of enzyme. This can only be confirmed when other bacterial copper/zinc enzymes are sequenced. At this stage it is also perhaps important to point out that no detailed comparative catalytic investigations have as yet been carried out on this class of enzymes. The manganese superoxide dismutase has been sequenced from four species [87–90] (Fig. 2). No iron enzyme has as yet been sequenced. However, a comparison of the N-terminal residues [91, 92, 32] (Fig. 3) indicates a high degree of sequence homology between the iron and manganese enzymes. Interestingly the iron or manganese forms isolated from the same species grown under different conditions appear to have the same N-terminal sequence. The four manganese enzyme sequences exhibit various degrees of sequence homologies (Table V). These do not appear to be as high as the identities between the copper/zinc enzyme in vertebrates. In the absence of crystallographic data the ligands to the manganese are still unknown, but from recently published data on the crystal structure of the iron enzyme [93, 94] and assuming a high degree of sequence homology between the iron and manganese enzyme it is possible to make some prediction from secondary structure determinations. These were predicted using three independent methods [95–97]. The subunit of the iron superoxide dismutase appears to contain six helices (Fig. 4)

TABLE V. Sequence Homologies of Manganese Superoxide Dismutases.[a]

	B. stearo	*E. coli*	*S. cerevisiae*	Human liver
B. stearo	–	59.1	40.9	47.9
E. coli	59.1	–	38.1	40.9
S. cerevisiae	40.9	38.1	–	43.3
Human liver	47.9	40.9	43.3	–

[a]Values are given as percentage of sequence homologies.

and the iron atom is co-ordinated by four protein ligands. Histidine 26 has been identified as one of the ligands and analysis of the X-ray and primary structure predicats residues 69, 148 and 152 as the remaining ligands. Histidine 26 which in aligned N-terminal sequences becomes His 29 is present in all the iron and manganese enzymes except in the iron enzyme from *Photobacterium leiognathi* where it is substituted for glycine (Fig. 3). When the secondary structure of the manganese enzymes is predicted from the amino acid sequences (Fig. 4) and compared with the secondary structure obtained for the iron enzyme it is seen that the α-helices and β-strands align quite well. The other residues likely to be ligands to the iron are found to be in the region 50–60 and 170–185 in the aligned secondary structures. Turning to the amino acid sequences obtained for the manganese enzymes, the most likely ligand in this region seemed Glu 55 until it was realized that this residue was absent in the human enzyme. The possibilities in the 170–185 region are likely to be His 179 and Tyr 182. Other possibilities could also be Asp 175, Glu 178 and Tyr 181 for ligand 148 and 152.

The crystal structure of bovine copper/zinc superoxide dismutase has been determined [98] (Fig. 5). Each enzyme subunit is composed primarily of eight antiparallel β-strands that form a flattened cylinder plus three external loops (Fig. 6). The copper and zinc on each subunit are very near to each other, about 6 Å apart. The exterior of the barrel contributed three ligands to the copper, His 44, 46 and 118 and one to the zinc, Asp 81. The interior is lined with

TABLE IV. Sequence Homologies of Copper/Zinc Superoxide Dismutases.[a]

	Bovine	Human	Horse	Yeast	Swordfish	Bacterial
Bovine	–	81.8	81.2	52.6	70.1	27.9
Human	81.8	–	79.9	52.6	65.6	25.3
Horse	81.2	79.9	–	56.5	66.9	27.3
Yeast	52.6	52.6	56.5	–	53.2	26.0
Swordfish	70.1	65.6	66.9	53.2	–	28.6
Bacterial	27.9	25.3	27.3	26.0	28.6	–

[a]Values are given as percentage of sequence homologies.

Fig. 2. Amino acid sequences of manganese superoxide dismutases.

Fig. 3. Comparison of the N-terminal sequences of manganese and iron superoxide dismutases.

hydrophobic residues. Two loops of non repetitive structure project out of the barrel: the first loop includes the first Lys of the intra-chain disulphide bridge and also contributes the zinc ligands His 61, 69 and 78. The second loop is very hydrophobic and contains the small percentage of α-helix (5%) present.

The three-dimensional structure of copper/zinc superoxide dismutase revealed a striking similarity of the subunit folding to the immunoglobulin domain [99] in spite of the absence of any sequence homologies. The loops extending out of the cylinder are in the same place as the bends which form the

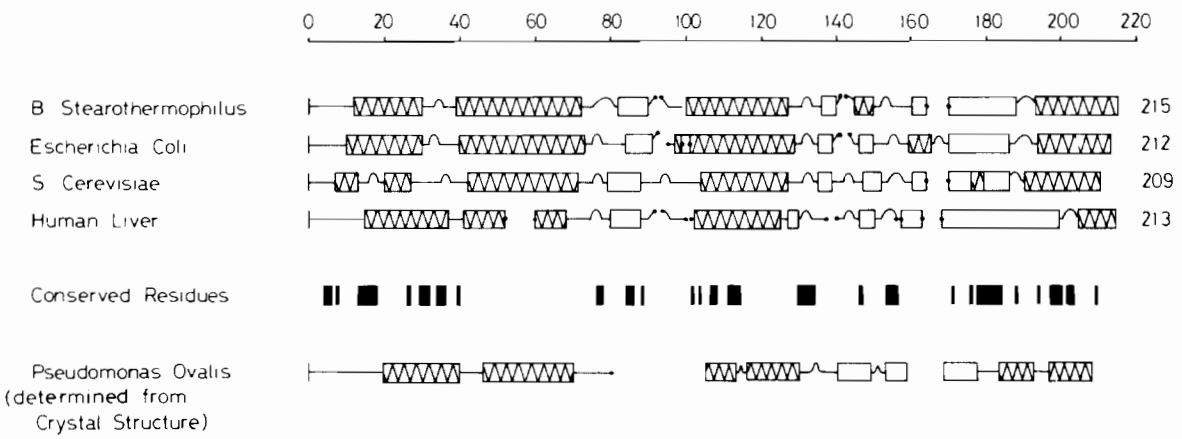

Fig. 4. Comparison of the secondary structures of manganese and iron superoxide dismutases. □ β-strand, ⌇⌇ α-helix and Ω β-turn.

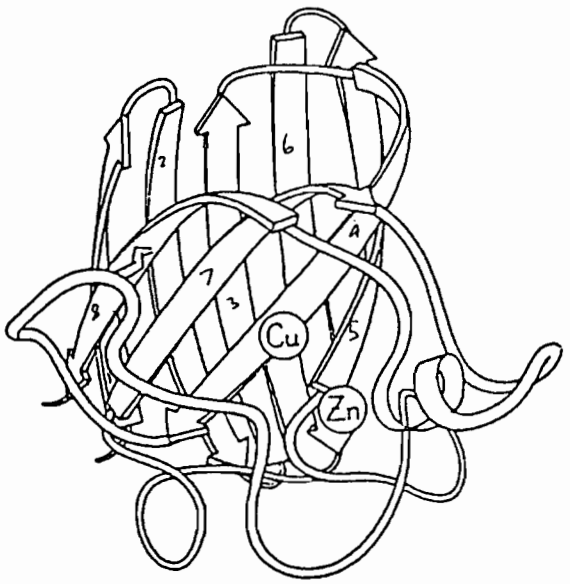

Fig. 5. X-ray structure of bovine copper/zinc superoxide dismutase (reproduced with permission from ref. 98).

antigen-binding loops in immunoglobulin variable domains and are usually referred to as the hypervariable region.

Since with the exception of the bacterial copper/zinc enzyme the remaining eukaryotic enzymes were found to have a high degree of sequence homology, then similar three-dimensional structures are therefore to be expected. A comparison of the predicted secondary structure of all copper/zinc enzymes with that determined for the bovine enzyme from the crystal structure indicates considerable identity (Fig. 7). The major difference between the predicted and the crystal structure is a predicted interruption in the 4th β-strand and a predicted extension of the 7th β-strand.

The first, second, third, seventh and eighth β-strands are highly conserved in all the eukaryotic superoxide dismutases. Notable differences are:

a) absence of the fifth β-strand in the yeast enzyme.

b) the replacement of the sixth β-strand by an α-helix and an extra β-strand between the fourth and fifth β-strand in the horse enzyme.

c) bacterial superoxide dismutase has only about 25% sequence homology with the other superoxide dismutases and this is demonstrated in the predicted structure. An α-helix replaces the fourth and half of the seventh β-strand. The 12 residue insertion between position 61 and 72 starts with a β-turn followed by a β-strand. Therefore the major expected difference between bacterial and eukaryotic copper/zinc superoxide dismutase could be a significant amount of α-helix and the presence of an extra β-strand.

Prediction of the Antigenic Determinants of Superoxide Dismutases

Antigenic determinants were predicted by the method of Hopp and Woods [100]. This method predicts the location of the antigenic determinants by finding areas of highest local average hydrophilicity in a given amino acid sequence. The highest positive peak average has been found to correspond to a determinant whereas the second and third highest points were found to have less than 50% success rate based on data for 12 different proteins [100]. The results obtained for the four highest peaks for the superoxide dismutases are given in Table VI according to the aligned sequences presented for the copper/zinc (Fig. 1) and for the manganese (Fig. 2) proteins. In general the sequence position of the highest peak varied with protein but all

the highest peaks are conserved within the form of superoxide dismutase analysis. The peaks at residues 88–96 and 134–151 are present in the copper/zinc superoxide dismutases analysed whilst for the manganese superoxide dismutases the peaks are at residues 104–110, 117–121, 185–192 and 203–208. The peak representing residues 117–121 is absent in the *Saccharomyces cerevisiae* manganese enzyme. Sequence hypervariability has been proposed as a criterion for the determination of antigenic sites. In the peak region of copper/zinc superoxide dismutases, the bovine, human and horse enzymes are very homologous whereas there are significant differences between these and yeast and bacterial enzyme. There are more significant differences between peak region residues in the manganese superoxide dismutases (*e.g.* residues 117–121). It should be noted that this is not necessarily a correlation between antigenic sites and hypervariability of sequences. The external loop of bovine copper/zinc superoxide dismutases occurs in a position equivalent to the hypervariable region loop of immunoglobulin [99]. The immunoglobulin loop forms the antigenic binding site whilst loop 7, 8 (Fig. 6; equivalent to residues 135–158 in Fig. 1) and the latter part of loop 6, 5 (Fig. 6); equivalent to residues 76–98 in Fig. 1) are predicted to be antigenic sites in the present investigation.

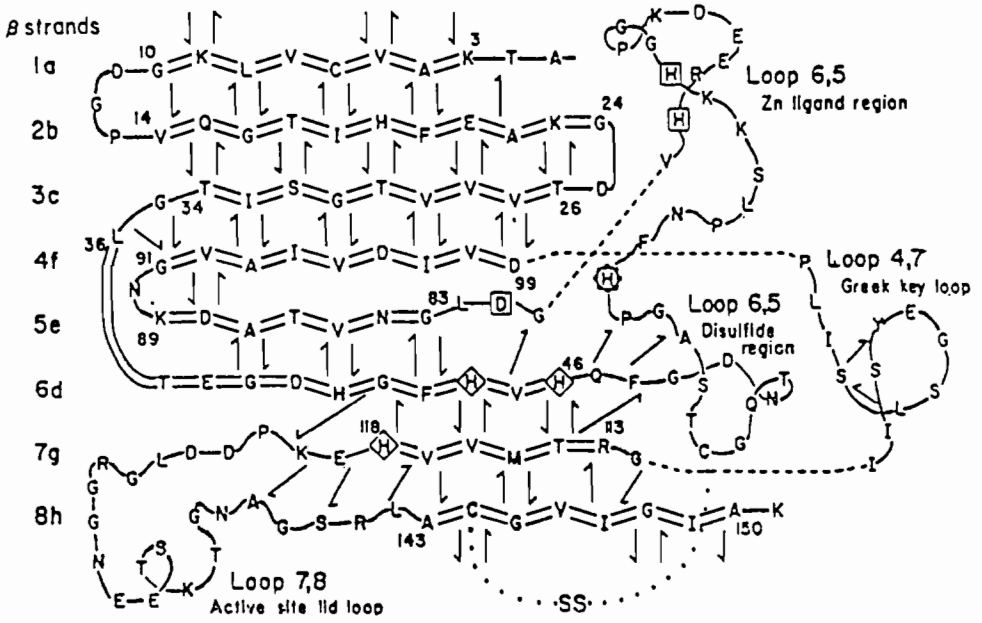

Fig. 6. Schematic diagram of the X-ray structure of bovine copper/zinc superoxide dismutase. (Reproduced with permission from ref. 98).

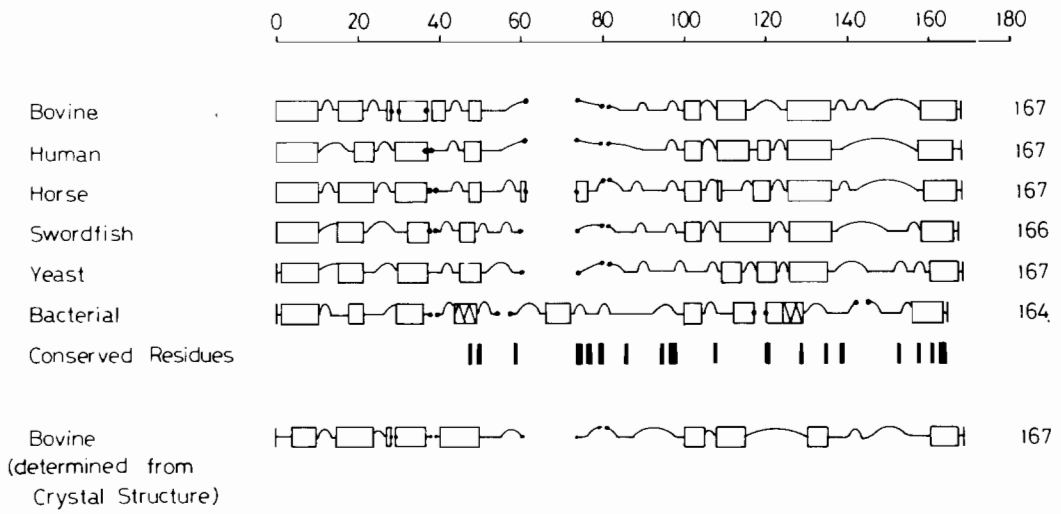

Fig. 7. Comparison of the secondary structures of copper/zinc superoxide dismutases. □β-strand, ⌇⌇⌇ α-helix, Ω β-turn.

TABLE VI. Amino Acid Residues in Superoxide Dismutase Sequence with greatest Average Hydrophilicity.

	1st peak	2nd peak	3rd peak	4th peak
Copper/zinc enzyme				
Bovine	81–96	132–143	141–152	20–26
Human	85–97	19–28	133–143	147–152
Horse	85–97	133–142	78–87	141–152
Yeast	141–151	99–107	88–95	134–143
Bacterial	58–68	90–98	123–129	137–147
Manganese enzyme				
B. stearothermophilus	203–207	117–124	185–192	105–110
E. coli	185–193	117–124	62–71	104–110
S. cerevisiae	202–219	186–192	104–110	–
Human liver	114–121	38–48	104–110	185–192

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