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EVOLUTIONARY ASPECTS OF SUPEROXIDE DISMUTASE: THE COPPER/ZINC ENZYME

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Copper/zinc superoxide dismutase is typically an enzyme of eukaryotes. The presence of the enzyme in the ponyfish symbiont *Photobacterium leiognathi* and some free living bacteria does not have an immediate explanation. Amino acid sequence alignment of 19 Cu/Zn superoxide dismutases shows 21 invariant residues in key positions related to maintenance of the β-barrel fold, the active site structure including the electrostatic channel loop, and dimer contacts. Nineteen other residues are invariant in 18 of the 19 sequences. Thirteen of these nearly invariant residues show substitutions in *Photobacterium* Cu/Zn superoxide dismutase. Copper/zinc superoxide dismutase from the trematode *Schistosoma mansoni* shows an N-terminal sub-domain with a hydrophobic leader peptide, as in human extracellular superoxide dismutase which is a Cu/Zn enzyme. The latter also has a C-terminal sub-domain with preponderance of hydrophilic and positively charged residues. The amino acid sequence of this superoxide dismutase between the N-terminal and C-terminal regions shares many features of cytosolic Cu/Zn superoxide dismutase, including 20 of the 21 invariant residues found in 19 Cu/Zn enzymes, suggesting a similar type of β-barrel fold and active site structure for the extracellular enzyme.

KEY WORDS: Copper/zinc superoxide dismutase, eukaryotes, *Photobacterium leiognathi*, *Schistosoma mansoni*, extracellular superoxide dismutase, amino acid sequences, sequence alignment, evolution.

INTRODUCTION

Superoxide dismutase is one of the most exciting biochemical discoveries of the past two decades (see Ref.[1] for review). The red blood cell copper protein of previously unknown function, called simply haemocuprein, was reported by McCord and Fridovich[2] in 1969 to catalyse the dismutation of superoxide radicals, and the new enzyme came to be known as superoxide dismutase. The enzyme was shown to be a cuprozinc protein, but soon after its discovery it became clear that superoxide dismutase activity was present in a family of metalloproteins comprising also manganese and iron proteins.

The evolution of superoxide dismutase is probably directly connected with the evolution of oxygen in the atmosphere. Changes in the geochemical conditions of the Earth led to the building up of the necessary antioxidant defence mechanisms in living organisms. Superoxide dismutase activity represents the first line of defence against oxygen toxicity. Indeed the enzyme is not only indispensable in an aerobic environment but may also be beneficial in an essentially anaerobic one. Probably the evol-

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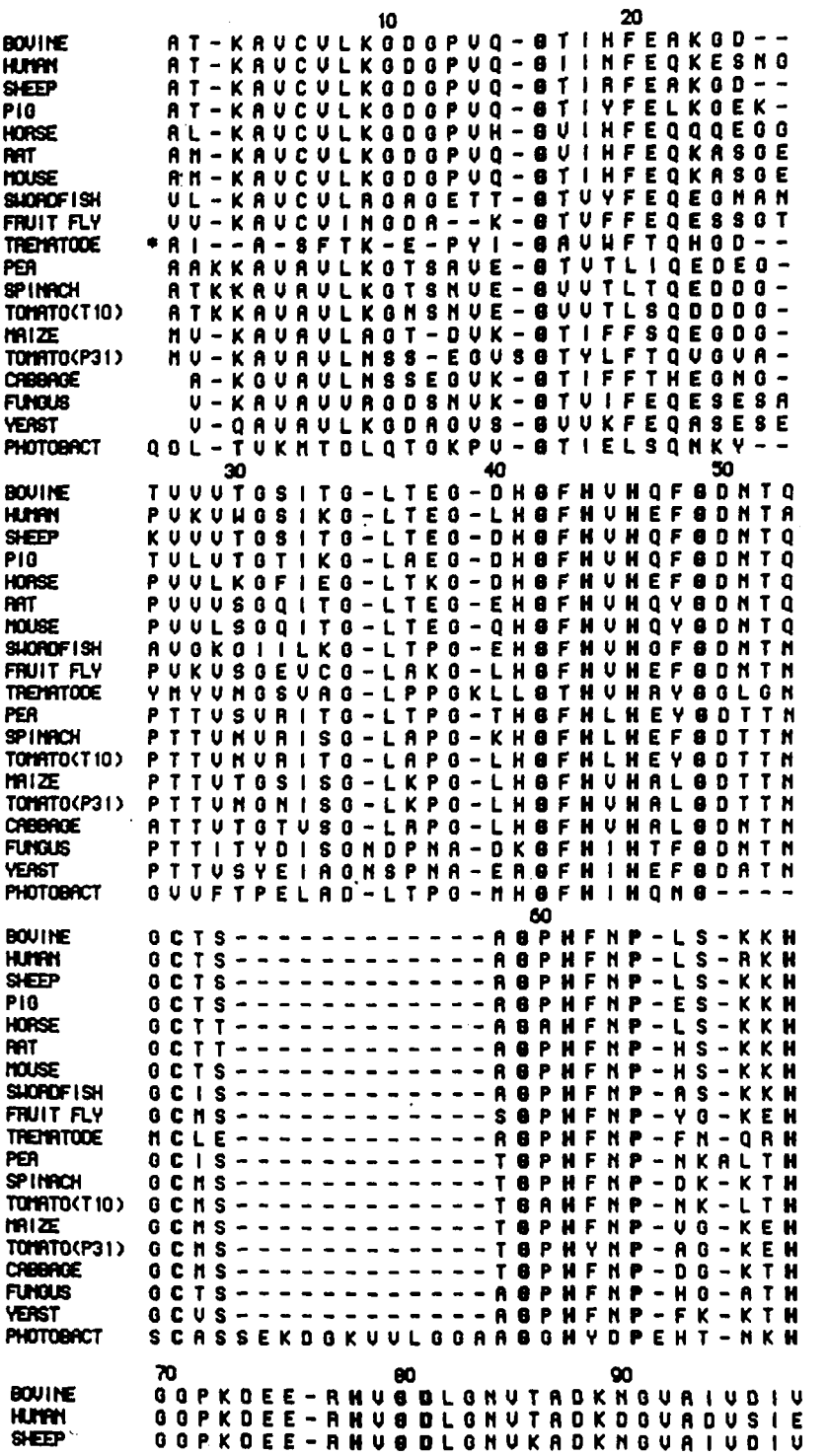

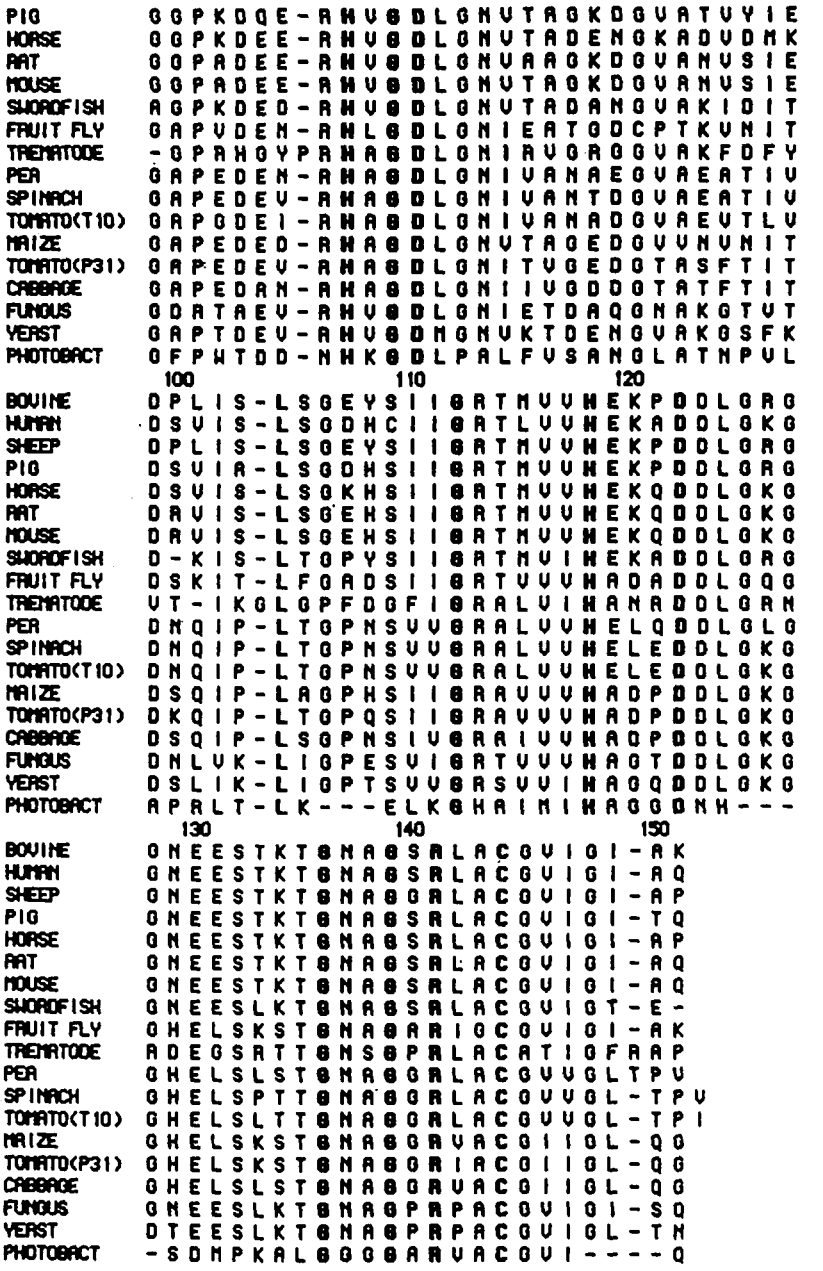

FIGURE 1 Amino acid sequence alignment of 19 Cu/Zn superoxide dismutases. Residue numbers are those of the bovine sequence. Invariant residues are shown in bold type. Asterisk denotes N-terminal extension of trematode superoxide dismutase (see Figure 3). Sequences for Bovine, Human, Sheep, Pig, Horse, Rat, Mouse, Swordfish, Fruit fly (*Drosophila melanogaster*), Trematode (*Schistosoma mansoni*), Pea (chloroplast), Spinach (chloroplast), Tomato T10 (chloroplast) and Tomato P31 (cytosol), Maize (cytosol), Cabbage (cytosol), Fungus (*Neurospora crassa*), Yeast (*Saccharomyces cerevisiae*) and *Photobact* (*Photobacterium leiognathi*) enzymes are from Refs.[22-29,17,20] and [30-37] consecutively.

ution of superoxide dismutase as a metalloprotein is also directly connected with the mode of appearance of transitions metals in the biosphere. It would seem that iron was initially the transition metal selected to be at the active site of the first superoxide dismutase. The metal was abundant and in the reducing environment it existed in the soluble iron(II) form. However, as the level of oxygen in the atmosphere increased the mineral components of the biosphere began to be converted from reduced to oxidised states. This change caused the level of iron(II) to decrease and the response by living organisms was to select also the next available metal for enzymatic dismutation of superoxide. This was manganese(III). The introduction of copper in the active site of superoxide dismutases appears to have taken place when the atmosphere was totally replenished with oxygen. At this stage iron(II) was almost completely unavailable but the insoluble copper(I) was converted into soluble copper(II).[3] However, differences in the electronic structure of the metal necessitated the synthesis of a totally different protein compared to the proteins which contained either iron or manganese at the active site. The iron- or manganese-containing superoxide dismutases are structurally homologous and represent a class of superoxide dismutases totally different from the copper-containing superoxide dismutases. Zinc appears to have been incorporated in the latter together with copper to confer stability to the protein structure.

THE COPPER/ZINC SUPEROXIDE DISMUTASES

The Cu/Zn superoxide dismutases are homodimers with a molecular weight of about 32,000. The two identical subunits are associated solely by non-covalent interactions and each subunit contains one copper and one zinc atom. The complete amino acid sequence has been determined for 19 Cu/Zn superoxide dismutases (Figure 1). Apart from the case of *Photobacterium leiognathi*, these superoxide dismutases show a high degree of amino acid sequence homology. The sequence conservation in Cu/Zn superoxide dismutase is almost as great as that of eukaryotic cytochrome *c*.[1] The enzymes from mammals have an acetylated N-terminal group not shown in Figure 1. These enzymes are generally considered to be more stable. However, whether acetylation of the N-terminal amino acid contributes to the increased stability remains to be investigated.

The Cu/Zn superoxide dismutase from *Photobacterium leiognathi* (bacteriocuprein) has attracted considerable attention because this bacterial species, although found free living, is also a symbiont of ponyfish. The molecular size, metal content, specific activity and amino acid sequence classifies *Photobacterium* superoxide dismutase as a Cu/Zn enzyme. Consequently a case of gene transfer from eukaryote to prokaryote species has been considered.[4,5] However, whereas natural gene transfer among prokaryotes and from prokaryotes to eukaryotes is common knowledge and the transfer of retroviral sequences between animal species appears to be a common evolutionary process, the transfer of the Cu/Zn superoxide dismutase gene would constitute the first known example of the transfer of a eukaryotic nuclear gene into a prokaryote.[6] Such a process is not easy to prove and although similarities in the amino acid sequence of swordfish and *Photobacterium* Cu/Zn superoxide dismutase increase from 30 to 44% after point mutations are considered,[5] the evidence is by no means definitive. This is because the increase in homology is not unique to swordfish and *Photobacterium* but spreads to the rest of the Cu/Zn superoxide dismutases analysed. Furthermore the gene transfer theory runs into complications because of the presence

of Cu/Zn enzyme in free living bacteria such as *Caulobacter crescentus*[7] and Pseudomonds.[8] The possibilities could now be that independent evolution of Cu/Zn superoxide dismutase took place in both prokaryotes and eukaryotes, or perhaps the enzyme originated in prokaryotes and was transferred to eukaryotes. This would imply that prokaryote and eukaryote Cu/Zn superoxide dismutase had a common ancestor. However, it is unlikely that the enzyme arose from a common ancestor because the divergence of eukaryotes from prokaryotes took place very early in geological history and at a time when copper was not available. Copper-containing enzymes such as azurin and cytochrome oxidase are mostly found in cyanobacteria and aerobic bacteria suggesting that copper-containing metalloproteins were incorporated into bacterial metabolism in a more or less oxygenated atmosphere.[3] It is therefore clear that definitive evidence for the evolution of Cu/Zn superoxide dismutases in bacteria has to be obtained at the molecular level.

The complete nucleotide sequence of the *Photobacterium* Cu/Zn superoxide dismutase gene has been established.[9] Eukaryotic Cu/Zn superoxide dismutases are typically cytosolic enzymes. In plants they are also found in chloroplasts. It is of interest to

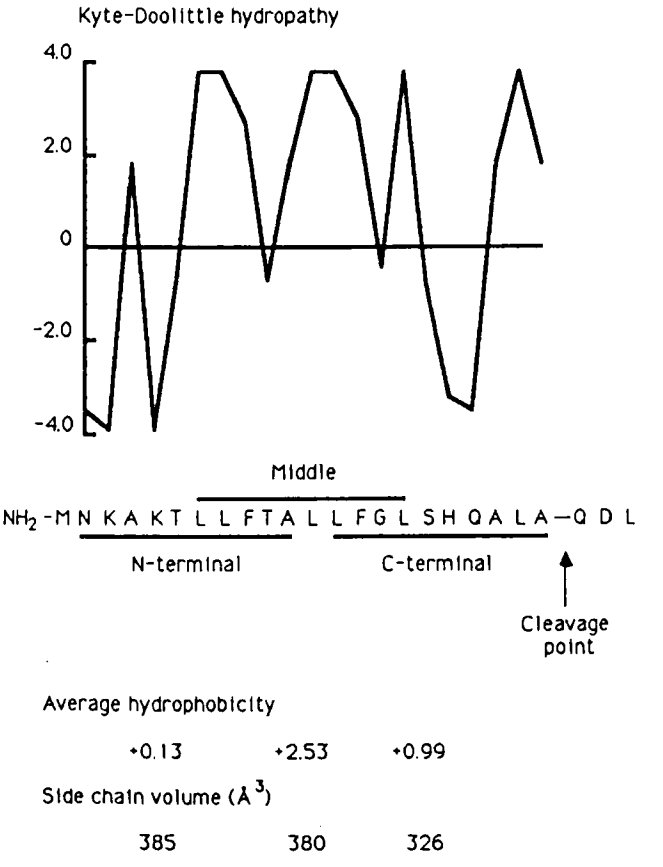

FIGURE 2 Hydropathy plot and average hydrophobicities and side chain volumes for the signal peptide of *Photobacterium leiognathi* Cu/Zn superoxide dismutase. The presentation of data follows the procedures in Ref.[11].

determine the localisation of the Cu/Zn enzyme in *Photobacterium*, which also has an iron superoxide dismutase presumably in the cytoplasm from the evidence for *Escherichia coli*.[10] *Photobacterium* Cu/Zn superoxide dismutase has a signal peptide. The physicochemical properties of the amino acids constituting the N-terminal, middle and C-terminal regions of the signal peptides are a good guide to the localisation of various proteins in *Escherichia coli*.[11] Based mainly on the average hydrophobicity of the N-terminal, middle and C-terminal segments of the signal peptide of *Photobacterium* Cu/Zn superoxide dismutase, particularly the low average hydrophobicity of the N-terminus (Figure 2), the enzyme appears to be a periplasmic and/or inner membrane protein.

The alignment of 19 Cu/Zn superoxide dismutase amino acid sequences given in Figure 1 is one which maximises the number of invariant (in 19/19 sequences) or nearly invariant (in 18/19 sequences) residues. The invariant residues are listed in Table I, and the nearly invariant ones are listed in Table II. The numbering of the residues is that of the bovine enzyme. Other notation given is that of Tainer *et al.*[12] The 21 invariant residues (Table I) include the copper ligands (His-44, His-46, His-61 and His-118); the zinc ligands (His-61, His-69, His-78 and Asp-81); the cysteines forming the intrachain S–S bond (Cys-55 and Cys-144); four residues in the electrostatic channel loop 7,8 between β-strands 7g and 8h (Asp-122, Gly-136, Gly-139 and Arg-141); one residue in β-strand 2b (Gly-16); one residue in β-strand 6d (Gly-42); four residues in loop 6,5 between β-strands 6d and 5e (Gly-49 and Gly-59 in the disulphide region and Pro-64 and Gly-80 in the zinc ligand region of the loop); and

TABLE I
Invariant residues in 19 Cu/Zn superoxide dismutases

Bovine sequence position	Residue	Structural location	Remarks
16	Gly	β-strand 2b	
42	Gly	β-strand 6d	
44	His	β-strand 6d	Cu ligand
46	His	β-strand 6d	Cu ligand
49	Gly	loop 6,5 disulphide region	
55	Cys	loop 6,5 disulphide region	intrachain S–S bond
59	Gly	loop 6,5 disulphide region	
61	His	loop 6,5 Zn ligand region	Cu/Zn ligand
64	Pro	loop 6,5 Zn ligand region	
69	His	loop 6,5 Zn ligand region	Zn ligand
78	His	loop 6,5 Zn ligand region	Zn ligand
80	Gly	loop 6,5 Zn ligand region	
81	Asp	loop 6,5 Zn ligand region	Zn ligand
104	Leu	loop 4,7 (Greek key loop)	
112	Gly	loop 4,7 (Greek key loop)	
118	His	β-strand 7g	Cu ligand
122	Asp	loop 7,8 (electrostatic channel)	
136	Gly	loop 7,8 (electrostatic channel)	
139	Gly	loop 7,8 (electrostatic channel)	
141	Arg	loop 7,8 (electrostatic channel)	
144	Cys	β-strand 8h	intrachain S–S bond

TABLE II

Nearly invariant residues in 19 Cu/Zn superoxide dismutases defined as found in 18/19 sequences. Substitutions in human extracellular superoxide dismutase (EC-SOD) are also indicated

Bovine sequence position	Residue	Structural location	Substitution
35	Gly	β-strand 3c to β-strand 4f turn	Asp in *Photobacterium*
43	Phe	β-strand 6d	Thr in trematode Ile in EC-SOD
63	Asn	loop 6,5 Zn ligand region	Asp in *Photobacterium*
72	Pro	loop 6,5 Zn ligand region	Arg in *Neurospora*
77	Arg	loop 6,5 Zn ligand region	Asn in *Photobacterium* Gln in EC-SOD
82	Leu	loop 6,5 Zn ligand region	Met in yeast Phe in EC-SOD
83	Gly	β-strand 5e	Pro in *Photobacterium*
84	Asn	β-strand 5e	Ala in *Photobacterium*
91	Gly	β-strand 4f	Cys in fruit fly Ser in EC-SOD
113	Arg	β-strand 7g	His in *Photobacterium*
116	Val	β-strand 7g	Met in *Photobacterium*
123	Asp	loop 7,8 (electrostatic channel)	Asn in *Photobacterium*
124	Leu	loop 7,8 (electrostatic channel)	His in *Photobacterium*
130	Glu	loop 7,8 (electrostatic channel)	Asp in *Photobacterium* Gln in EC-SOD
132	Ser	loop 7,8 (electrostatic channel)	Pro in *Photobacterium*
135	Thr	loop 7,8 (electrostatic channel)	Leu in *Photobacterium* Asn in EC-SOD
137	Asn	loop 7,8 (electrostatic channel)	Gly in *Phototbacterium*
143	Ala	β-strand 8h	Gly in fruit fly
145	Gly	β-strand 8h	Ala in trematode Cys in EC-SOD

two residues in Greek key loop 4,7 between β-strands 4f and 7g (Leu-104 and Gly-112).

It can be deduced from the known structure of bovine Cu/Zn superoxide dismutase[12,13] that among the 21 invariant residues in the sequence alignment of Figure 1, those that are not directly involved as metal ligands are involved in side chain packings and interactions responsible for maintaining the structure and function of the active site by correct orientation of the actual metal ligands and positioning of the electrostatic channel loop (including Asp-122, Gly-42, Gly-59, Gly-80, Gly-136, Gly-139 and Pro-64); for maintaining dimer contacts (Gly-49, Gly-112 and the intrachain S–S bridge cysteines Cys-55 and Cys-144); and for maintaining the β-barrel fold (Gly-16 and Leu-104). The S–S bridge between cysteines Cys-55 and Cys-144 stabilises the disulphide region of loop 6,5 which is involved in dimer contact by binding it covalently to the β-barrel. As regards maintenance of the β-barrel fold, a problem arises with the substitution of Phe-43 by Thr in the trematode superoxide dismutase, and probably also with the substitution of Gly-145 by Ala in the same enzyme but perhaps mutation of Phe-43 (and/or Gly-145) is not as problematic as one might think from structural considerations for the bovine superoxide dismutase. Phenylalanine-43 participates in one of two cross-barrel interactions that serve to stabilise the β-barrel fold, namely, those between Phe-43 and Ile-18 and between Val-117 and Ile-33, in the bovine enzyme.[13] Being adjacent to copper ligands His-44,

His-46 and His-118, residues Phe-43 and Val-117 serve to anchor the active site through the β-barrel to the more regular opposite side of the structure. Trematode superoxide dismutase has a Val in bovine position 18 in the sequence alignment of Figure 1. It is not clear that the Thr substituting Phe-43 interacts with this Val residue. Note there are two Leu residues in bovine positions 40 and 41 close to Thr in position 43 in the trematode enzyme which might substitute for the mutated Phe-43 in function. Other considerations on the substitution of Phe-43 by Thr in the trematode superoxide dismutase are given below. Valine-117 is unchanged and presumably it can interact with the Val substituting Ile-33.

There remains Arg-141. This invariant residue seems to be an essential part of the superoxide binding pocket above the catalytic copper ion. The positive charge of the guanidinium group of Arg-141 participates in short-range pre-collision electrostatic guidance of the negatively charged superoxide radical. Long-range electrostatic guidance for the radical is thought to be provided by Lys-134 and Glu-131.[14,15] The former is substituted by Ser in fruit fly, pea, maize, tomato cytoplasm (P31) and cabbage; by Thr in spinach and tomato chloroplast (T10); and by Ala in *Photobacterium* superoxide dismutase. The latter is substituted by Leu in fruit fly, pea, spinach, tomato chloroplast (T10), maize, tomato cytoplasm (P31) and cabbage; by Gly in trematode; and by Met in *Photobacterium* superoxide dismutase. If one looks for possibly coupled positively and negatively charged side chains that might take over the function of bovine Lys-134 and Glu-131 in the electrostatic channel loop, one finds these as His-129 and Glu-130 in fruit fly, pea, spinach, tomato chloroplast (T10), maize, tomato cytoplasm (P31) and cabbage; as Arg-128 and Asp-129 (and/or Glu-130) in trematode; and as His 124 and Asp-130 in *Photobacterium* superoxide dismutase.

The 19 nearly invariant residues (Table II) include eight residues in the β-strands (Phe-43 in β-strand 6d; Gly-83 and Asn-84 in β-strand 5e; Gly-91 in β-strand 4f; Arg-113 and Val-116 in β-strand 7g; and Ala-143 and Gly-145 in β-strand 8h); one residue in the turn connection between β-strands 3c and 4f (Gly-35); four residues in the zinc ligand region of loop 6,7 (Asn-63, Pro-72, Arg-77 and Leu-82); and six residues in the electrostatic channel loop 7,8 (Asp-123, Leu-124, Glu-130, Ser-132, Thr-135 and Asn-137). Thirteen of the 19 amino acid substitutions occur in the *Photobacterium* superoxide dismutase. The rest occur in the fruit fly, trematode, *Neurospora* and yeast enzymes.

The most remarkable alteration of a residue expected to remain invariant is the substitution of Phe-43 by Thr in the trematode enzyme, as already noted for cross-barrel anchorage of the active site. Phenylalanine-43 is located in β-strand 6d and seems also be involved in orienting the copper ligand residues His-44 and His-46 of the active site. This supposition has to be weighed against the fact that Thr is only half as bulky as Phe. The apparent insertion of a Lys residue in β-strand 6d (between bovine positions 39 and 40) of the trematode superoxide dismutase might compensate for the lower bulk of Thr. Additionally, Thr is a hydrophilic residue whereas Phe is hydrophobic and it is not immediately clear how this change is accommodated in the local structure.

Figure 1 shows the trematode enzyme as the only Cu/Zn superoxide dismutase with a deletion in the body of a β-strand (corresponding to bovine position 5 in β-strand 1a). This superoxide dismutase has an N-terminal sub-domain (see Figure 3). It is not clear where the N-terminal extension actually starts. In any case, shortening of β-strand 1a might be compensated for by a shift in the alignment of the strand. It might be thought on general grounds that β-strands would permit sequence variation

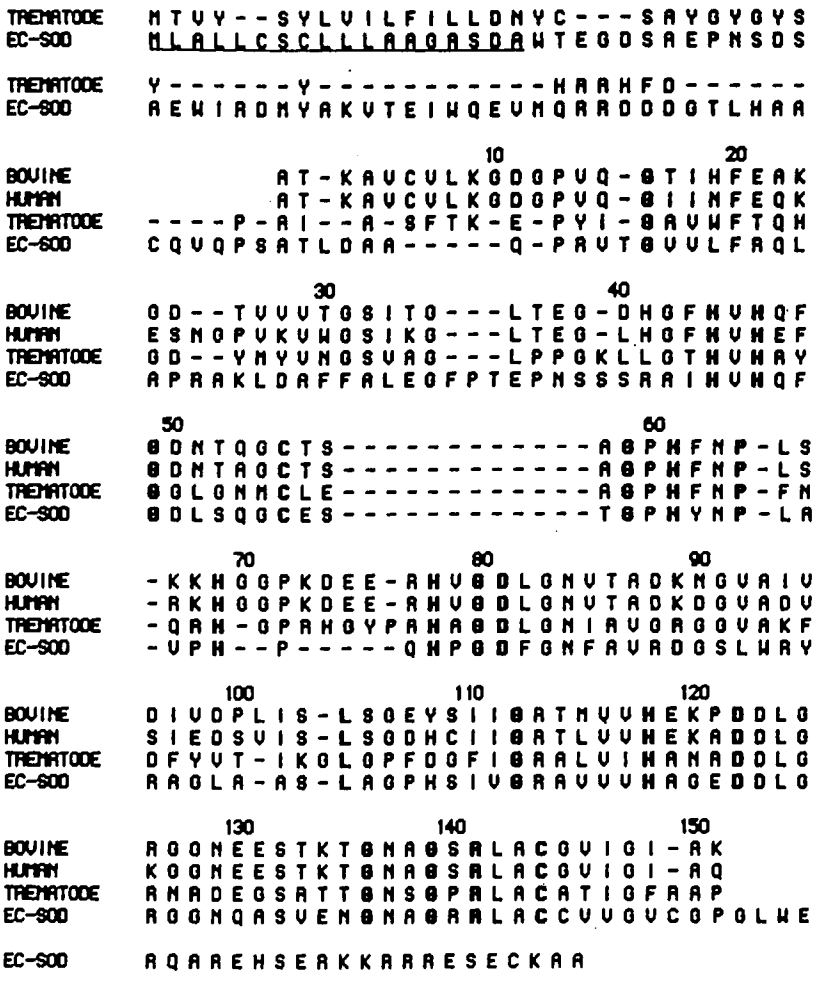

FIGURE 3 Amino acid sequence alignment of bovine, human and trematode (*Schistosoma mansoni*) Cu/Zn superoxide dismutases and human extracellular superoxide dismutase (EC-SOD). Residue numbers are those of the bovine sequence. Invariant residues are shown in bold type and gaps in the alignment of bovine, human and trematode sequences of Figure 1 are retained. The leader peptide of EC-SOD is underlined. Sequences are from Refs.[22,23] and [20] for Bovine, Human and Trematode enzymes and from Ref.[21] for EC-SOD.

but not insertions or deletions within their body. This seems to hold quite well. As regards single residue insertions these might be accommodated by β-bulge formation. The sequence alignment of Figure 1 indicates the insertion of a Lys residue in β-strand 6d (between bovine positions 39 and 40) of the trematode superoxide dismutase as already mentioned; the insertion of a Ser residue in β-strand 2b (between bovine positions 15 and 16) of the tomato cytoplasmic (P31) superoxide dismutase; the insertion of an Arg residue in β-strand 8h (between bovine positions 149 and 150) of the trematode enzyme; and the insertion a Thr residue in β-strand 8h (also between bovine positions 149 and 150) of the pea superoxide dismutase.

The accumulation of Cu/Zn superoxide dismutase amino acid sequences continues to show two hypervariable regions: one from bovine positions 17 to 34 and the other from bovine positions 86 to 101 at the most. The first region includes part of β-strand 2b and β-strand 3c, while the second includes part of β-strand 5e, β-strand 4f, and part of Greek key loop 4,7. The former was noticed when the sequences for bovine, human, horse and yeast Cu/Zn superoxide dismutases were available.[16] The latter was noticed when the sequence of *Drosophila* Cu/Zn superoxide dismutase became additionally available.[17] The β-strands in these regions are connected by β-hairpins exposed to solvent (at bovine positions 23 to 26 between β-strands 2b and 3c and positions 89 to 91 between β-strands 5e and 4f).[18] The bovine residue 86 to 101 region contains Asn-96 of *Drosophila* Cu/Zn superoxide dismutase, which is replaced by Lys in the slow electrophoretic variant of the superoxide dismutase.[19] Copper/zinc superoxide dismutase polymorphisms may thus occur in the hypervariable regions.

EXTRACELLULAR SUPEROXIDE DISMUTASE

The trematode Cu/Zn superoxide dismutase discussed here is that of the blood fluke *Schistosoma mansoni*.[20] Although put with the cytosolic and chloroplast Cu/Zn superoxide dismutases in Figure 1, this superoxide dismutase has an N-terminal sub-domain with a hydrophobic leader peptide (Figure 3) that might function as a signal sequence for uptake by the endoplasmic reticulum and some secretion of the enzyme. That the superoxide dismutase may be secreted is shown by the fact that the product of *in vitro* translation of the mRNA is immunoprecipitable with sera pooled from patients with chronic schistosomiasis.[20]

The N-terminal sub-domain of *Schistosoma* Cu/Zn superoxide dismutase shows the general features of the N-terminal sub-domain of the extracellular superoxide dismutase found so far in humans and mammals.[21] Extracellular superoxide dismutase is a tetrameric glycoprotein with an apparent subunit molecular weight of about 30,000, containing one copper and one zinc atom per subunit. In the blood vesels it is found bound to endothelial cell surfaces with membrane-bound heparan sulphate as the likely receptor. The amino acid sequence of human extracellular superoxide dismutase type 3 with high affinity binding to heparin-Sepharose is given in Figure 3.[21] Besides an N-terminal sub-domain with a hydrophobic leader peptide, extracellular superoxide dismutase also has a C-terminal sub-domain which is very hydrophilic and contains a number of positively charged amino acids (Figure 3). This sub-domain is probably responsible for the affinity of extracellular superoxide dismutase for heparin and heparan sulphate.[21] Extracellular superoxide dismutase probably evolved from cytosolic Cu/Zn superoxide dismutase. The amino acid sequence between the N-terminal and the C-terminal sub-domains is aligned with the sequences of bovine, human and trematode Cu/Zn superoxide dismutase in Figure 3. Apart from problems with β-strand 1a and the tight turn in the zinc ligand region of loop 6,5 involving Gly-71, Pro-72, Lys-73 and Asp-74 in bovine Cu/Zn superoxide dismutase, of which residues only one (Pro-72) was found for extracellular superoxide dismutase in the sequence alignment of Figure 3, it would seem that the β-barrel and active site structure of Cu/Zn superoxide dismutase have been retained in extracellular Cu/Zn enzyme. Twenty of the 21 invariant residues in the sequence alignment of Cu/Zn superoxide dismutases of Figure 1 can be found in the same place in extracellular superoxide dismutase. The residue that is substituted is the Gly in bovine position 42

which is replaced by Ala. Of the 19 nearly invariant residues (in 18/19 superoxide dismutases) in the Cu/Zn superoxide dismutase sequences aligned in Figure 1, 12 are retained in extracellular superoxide dismutase. The seven substituted residues are indicated in Table 2. On the other hand, only six of the 19 nearly invariant residues are retained in *Photobacterium* Cu/Zn superoxide dismutase, and by this criterion extracellular superoxide dismutase is closer in sequence identity to cytosolic (and chloroplast) Cu/Zn superoxide dismutases than *Photobacterium* Cu/Zn superoxide dismutase.

Of interest in the amino acid sequence of extracellular superoxide dismutase as aligned in Figure 3 is the substitution of Phe-43 by Ile which might make a cross-barrel hydrophobic linkage with the Val present in the position corresponding to bovine Ile-18. Valine-117 is retained and its cross-barrel linkage partner (Ile-33 in bovine superoxide dismutase) is similarly hydrophobic being a Leu. Arg-141 is also retained. The Lys-134 and Glu-131 residue pair probably responsible for long-range electrostatic guidance of the superoxide radical is substituted by Glu in position 134 and Ala in position 131 in the sequence alignment of Figure 3. The nearest positively charged residue to the Glu in the extracellular superoxide dismutase sequence is an Arg in bovine position 126. Further extracellular superoxide dismutase amino acid sequences are awaited with interest, as are bacterial Cu/Zn superoxide dismutase sequences, as a means of solving the conundrum of the evolution of the entire class of copper- and zinc-containing superoxide dismutases.

References

1. J.V. Bannister, W.H. Bannister and G. Rotilio (1987). Aspects of the structure, function, and applications of superoxide dismutase. *CRC Critical Reviews in Biochemistry*, **22**, 111–180.
2. J.M. McCord and I. Fridovich (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *The Journal of Biological Chemistry*, **244**, 6049–6055.
3. E.-I. Ochiai (1983). Copper and biological evolution. *Biosystems*, **16**, 81–86.
4. J.P. Martin, Jr. and I. Fridovich (1981). Evidence for a natural gene transfer from the ponyfish to its bioluminescent bacterial symbiont *Photobacter leiognathi*. The close relationship between bacteriocuprein and the copper-zinc superoxide dismutase of teleost fishes. *The Journal of Biological Chemistry*, **256**, 6080–6089.
5. J.V. Bannister and M.W. Parker (1985). The presence of copper/zinc superoxide dismutase in the bacterium *Photobacterium leiognathi*: A likely case of gene transfer from eukaryotes to prokaryotes. *Proceedings of the Natural Academy of Sciences of the USA*, **82**, 149–152.
6. J.A.M. Leunissen and W.W. de Jong (1986). Copper/zinc superoxide dismutase: How likely is gene transfer from ponyfish to *Photobacterium leiognathi*? *Journal of Molecular Evolution*, **23**, 250–258.
7. H.M. Steinman (1982). Copper-zinc superoxide dismutase from *Caulobacter crescentus* CB15. A novel bacteriocuprein form of the enzyme. *The Journal of Biological Chemistry*, **257**, 10283–10293.
8. H.M. Steinman (1985). Bacteriocuprein superoxide dismutases in pseudomonads. *Journal of Bacteriology*, **162**, 1255–1260.
9. H.M. Steinman (1987). Bacteriocuprein superoxide dismutase of *Photobacterium leiognathi*. Isolation and sequence of the gene and evidence for a precursor form. *The Journal of Biological Chemistry*, **262**, 1882–1887.
10. L. Britton and I. Fridovich (1977). Intracellular localization of the superoxide dismutases of *Escherichia coli*. *Journal of Bacteriology*, **131**, 815–820.
11. M. Sjöstrom, S. Wold, Å. Wieslander and L. Rilfors (1987). Signal peptide amino acid sequences in *Escherichia coli* contain information related to final protein localization. A multivariate data analysis. *The EMBO Journal*, **6**, 823–831.
12. J.A. Tainer, E.D. Getzoff, K.M. Beem, J.S. Richardson and D.C. Richardson (1982). Determination and analysis of the 2Å structure of copper, zinc superoxide dismutase. *Journal of Molecular Biology*, **160**, 181–217.
13. E.D. Getzoff, A.J. Olson and J.A. Tainer (1986). Anatomy of an enzyme: Computer graphics views

of Cu, Zn superoxide dismutase. In *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine* (ed. G. Rotilio). Elsevier Science Publishers, Amsterdam, pp. 135–140.
14. J.A. Tainer, E.D. Getzoff, J.S. Richardson and D.C. Richardson (1983). Structure and mechanism of copper, zinc superoxide dismutase. *Nature*, **306**, 284–287.
15. E.D. Getzoff, J.A. Tainer, P.K. Weiner, P.A. Kollman, J.S. Richardson and D.C. Richardson (1983). Electrostatic recognition between superoxide and copper, zinc superoxide dismutase. *Nature*, **306**, 287–290.
16. D. Barra, F. Martini, J.V. Banniste, M.E. Schininà, G. Rotilio, W.H. Bannister and F. Bossa (1980). The complete amino acid sequence of human Cu/Zn superoxide dismutase. *FEBS Letters*, **120**, 53–56.
17. Y.M. Lee, D.J. Friedman and F.J. Ayala (1985). Superoxide dismutase: An evolutionary puzzle. *Proceedings of the National Academy of Sciences of the USA*, **82**, 824–828.
18. E.J. Milner-White and R. Poet (1986). Four classes of β-hairpins in proteins. *Biochemical Journal*, **240**, 289–292.
19. Y.M. Lee and F.J. Ayala (1985). Superoxide dismutase in *Drosophila melanogaster*. Mutation site difference between two electromorphs. *FEBS Letters*, **179**, 115–119.
20. M.C. Simurda, H. van Keulen, D.M. Rekosh and P.T. LoVerde (1988). *Schistosoma mansoni*: Identification and analysis of an mRNA and a gene encoding superoxide dismutase. *Experimental Parasitology*, **67**, 73–84.
21. K. Hjalmarsson, S.L. Marklund, Å. Engström and T. Eklund (1987. Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase. *Proceedings of the National Academy of Sciences of the USA*, **84**, 6340–6344.
22. H.M. Steinman, V.R. Naik, J.L. Abernethy and R.L. Hill (1974). Bovine erythrocyte superoxide dismutase. Complete amino acid sequence. *The Journal of Biological Chemistry*, **249**, 7326–7338.
23. L. Sherman, J. Lieman-Hurwitz and Y. Groner (1983). Nucleotide sequence and expression of human chromosome 21-encoded superoxide dismutase mRNA. *Proceedings of the National Academy of Sciences of the USA*, **80**, 5465–5469.
24. M.E. Schininà, D. Barra, S. Gentilomo, F. Bossa, C. Capo, G. Rotilio and L. Calabrese (1986). Primary structure of a cationic Cu, Zn superoxide dismutase. The sheep enzyme. *FEBS Letters*, **207**, 7–10.
25. M.E. Schininà, D. Barra, M. Simmaco, F. Bossa and G. Rotilio (1985). Primary structure of porcine Cu, Zn superoxide dismutase. *FEBS Letters*, **186**, 267–270.
26. K. Lerch and D. Ammer (1981). Amino acid sequence of copper-zinc superoxide dismutase from horse liver. *The Journal of Biological Chemistry*, **256**, 11545–11551.
27. G.J. Steffens, A.M. Michelson, K. Puget and L. Flohé (1986). The amino-acid sequence of rat Cu-Zn superoxide dismutase. *Biological Chemistry Hoppe-Seyler*, **367**, 1017–1024.
28. G.C. Bewley (1988). cDNA and deduced amino acid sequence of murine Cu-Zn superoxide dismutase. *Nucleic Acids Research*, **16**, 2728.
29. H.A. Rocha, W.H. Bannister and J.V. Bannister (1984). The amino-acid sequence of copper/zinc superoxide dismutase from swordfish liver. Comparison of copper/zinc superoxide dismutase sequences. *European Journal of Biochemistry*, **145**, 477–484.
30. J.R. Scioli and B.A. Zilinskas (1988). Cloning and characterization of cDNA encoding the chloroplastic copper/zinc superoxide dismutase from pea. *Proceedings of the National Academy of Sciences of the USA*, **85**, 7661–7665.
31. Y. Kitagawa, S. Tsunasawa, N. Tanaka, Y. Katsube, F. Sakiyama and K. Asada (1986). Amino acid sequence of copper, zinc superoxide dismutase from spinach leaves. *Journal of Biochemistry*, **99**, 1289–1298.
32. R. Perl-Treves, B. Nacmias, D. Aviv, E.P. Zeelon and E. Galun (1988). Isolation of two cDNA clones from tomato containing two different superoxide dismutase sequences. *Plant Molecular Biology*, **11**, 609–623.
33. R.E. Cannon, J.A. White and J.G. Scandalios (1987). Cloning of cDNA for maize superoxide dismutase-2 (SOD2). *Proceedings of the National Academy of Sciences of the USA*, **84**, 179–183.
34. G.J. Steffens, A.M. Michelson, F. Ötting, K. Puget, W. Strassburger and L. Flohé (1986). Primary structure of Cu-Zn superoxide dismutase of *Brassica oleracea* proves homology with the corresponding enzymes of animals, fungi and prokaryotes. *Biological Chemistry Hoppe-Seyler*, **367**, 1007–1016.
35. K. Lerch and E. Schenk (1985). Primary structure of copper-zinc superoxide dismutase from *Neurospora crassa*. *The Journal of Biological Chemistry*, **260**, 9559–9566.
36. J.T. Johansen, C. Overballe-Petersen, B. Martin, V. Hasemann and I. Svendsen (1979). The complete amino acid sequence of copper, zinc superoxide dismutase from *Saccharomyces cerevisiae*. *Carlsberg Research Communications*, **44**, 201–217.

37. G.-J. Steffens, J.V. Bannister, W.H. Bannister, L. Flohé, W. Günzler, S.-M.A. Kim and F. Ötting (1983). The primary structure of Cu-Zn superoxide dismutase from *Photobacterium leiognathi*: Evidence for a separate evolution of Cu-Zn superoxide dismutase in bacteria. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie*, **364**, 675–690.

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