recovery at differing rates. Phosphorylation of inhibitor accompanies inactivation, and hydrolysis of the resulting inhibitor phosphate accompanies reactivation. By comparison, the extremely slow reactivation of GS inactivated with MSO-P suggests that the free energy of binding of the latter is significantly lower than for PPT-P (Ginsburg et al., 1987). The stabilization of GS quaternary structure that accompanies MSO-P binding (Maurizi & Ginsburg, 1982b) may be a function of the greater hydrolytic stability of the sulfoximine phosphate moiety at the enzyme active site. Additional studies exploring the difference in binding of MSO and PPT-based inhibitors are currently in progress.

Registry No. **1**, 53369-07-6; **2**, 115730-43-3; **3**, 119617-93-5; **4**, 119617-94-6; **5**, 119567-65-6; **6**, 121249-46-5; GS, 9023-70-5.

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A Protein Isolated from *Brucella abortus* Is a Cu-Zn Superoxide Dismutase†

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ABSTRACT: *Brucella abortus* contains a protein that elicits an antigenic response in cattle previously exposed to the organism. The amino acid sequence of the recombinant form of this antigenic protein was determined by gas-phase sequencing of the pyridylethylated protein and its peptides obtained by digestion with cyanogen bromide (CNBr), clostripain, and *Staphylococcus aureus* V8 protease. The *Brucella* protein demonstrated 53.6% identity with the Cu-Zn superoxide dismutase (SOD) from *Photobacterium leiognathi*. Residues essential for metal coordination and enzymatic activity and cysteines required for the formation of the intrasubunit disulfide bridge of Cu-Zn SOD were conserved in the *Brucella* protein. The *Brucella* protein also exhibited SOD activity that was inhibited by cyanide, which is characteristic of a Cu-Zn SOD. *Brucella abortus* Cu-Zn SOD is the second prokaryotic Cu-Zn SOD to be sequenced, and the fifth found in prokaryotes. The high degree of conservation between *Photobacterium* and *Brucella* Cu-Zn SOD supports the hypothesis of a separately evolved prokaryotic and eukaryotic Cu-Zn SOD gene.

Brucella abortus contains a group of immunogenic cell surface and periplasmic proteins which have potential value as a vaccine or as a diagnostic reagent for the prevention and diagnosis of bovine brucellosis (Tabatabai & Deyoe, 1984a,b; Tabatabai et al., 1989). One of the proteins with an apparent molecular weight of 20K, as estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions, was purified as previously described (Tabatabai et al., 1989). The present study reports the complete amino acid sequence of the recombinant form of this protein from *B. abortus*. Through homology searches and enzyme activity studies, we have determined that the protein from *B. abortus*

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is a Cu–Zn superoxide dismutase (SOD)[1] (EC 1.15.1.1).

The SODs catalyze the formation of molecular dioxygen and hydrogen peroxide from superoxide radicals. In bacteria, these enzymes are thought to act as a protective mechanism by scavenging superoxide radical anions that arise during normal aerobic metabolism, although disagreement exists on whether SOD provides protection against oxygen poisoning (Fee, 1982). Controversy also exists as to whether superoxide radical anions themselves are directly toxic to cells (Minotti, 1988). However, there is accumulating evidence that superoxide anion may contribute to pathogenesis following mobilization and activation of inflammatory cells (Ward et al., 1988). Several forms of SOD are present in most aerobic and some anaerobic organisms (Fridovich, 1982). The Mn SODs are found in both prokaryotic and eukaryotic species, while Fe SODs are found primarily in bacteria and a few eukaryotes. Conversely, Cu–Zn SOD, until recently, was found only in eukaryotes (Steinman, 1982b).

The SODs may also play an important role in the way some pathogenic bacteria evade destruction by host phagocytes (Johnston et al., 1975; Babior et al., 1975). One of the mechanisms by which phagocytes destroy bacteria is through the production of superoxide radicals during the respiratory burst (Beaman & Beaman, 1984). The superoxide radicals can damage bacterial cell walls, causing loss of cell wall integrity, and eventual death of the bacteria. Some pathogenic bacteria that survive intracellularly in phagocytes either fail to stimulate phagocytic superoxide anion generation (Holzer et al., 1986; Mor et al., 1988) or contain a unique SOD that is secreted extracellularly during growth (Beaman et al., 1983).

Experimental Procedures

The lyophilized recombinant protein purified by anion-exchange chromatography (Tabatabai et al., 1989) was precipitated with methanol–chloroform–water to remove salts (Wessel & Flugge, 1984). Cysteine residues were modified with 4-vinylpyridine (Fulmer, 1984) in preparation for chemical or enzymatic digestion. The protein (500 μg) was solubilized in 200 μL of 0.01% NH_4HCO_3 in the presence of 6 M urea. The sample was preincubated at 25 °C for 30 min in the presence of 140 mM 2-mercaptoethanol (2 μL) before modification with 270 mM 4-vinylpyridine (6 μL) for 90 min at 25 °C. The amino acid sequence of the recombinant protein was determined by automated Edman degradation of peptides originating from cleavage of the protein at methionine by CNBr (Titani et al., 1972), at arginine by clostripain (Sigma) digestion (Drickamer, 1981), and at glutamic acid by *Staphylococcus aureus* protease V8 (Sigma) digestion (Drapeau, 1977). The CNBr cleavage was performed by dissolving the pyridylethylated protein (500 μg) in 70% formic acid and adding an equal volume of 80 mg of CNBr/mL in 70% formic acid. The reaction was allowed to proceed in the dark at 25 °C for 20 h. Clostripain digestion was performed by activating clostripain in 5 mM DTT and 1.5 mM $CaCl_2$ for 3 h at 25 °C. The pyridylethylated protein (500 μg) was solubilized in 30 mM NH_4HCO_3, 5 mM DTT, 2 mM $CaCl_2$, and 6 M urea. Clostripain was added at a weight ratio of 100:1 (protein: clostripain ratio), and the protein was digested overnight at 37 °C. The *S. aureus* protease digestion was performed by dissolving the protease in 50 mM NH_4HCO_3 and 2 mM EDTA. The pyridylethylated protein (500 μg) was solubilized in 50 mM NH_4HCO_3 and 6 M urea. The *S. aureus* protease was added at a weight ratio of 30:1 (protein:protease ratio), and the protein was digested for 4 h at 37 °C. Peptides were separated by reverse-phase HPLC on a Waters chromatography system using a 5-μm Vydac C-18 column (0.46 × 25 cm) and a linear gradient of 0.1% trifluoroacetic acid (solvent A)/90% acetonitrile (solvent B). Automated Edman degradation was performed on an Applied Biosystems Model 470-A gas-phase sequencer equipped with an on-line microbore HPLC system (Model 120-A) for phenylthiohydantoin-derivatized amino acid identification (Hunkapiller et al., 1983). The protein was hydrolyzed for 1 h at 150 °C using the gas-phase procedure of Bidlingmeyer et al. (1984). The samples were derivatized after dilution with 250 ppm of EDTA with an Applied Biosystems Model 420A derivatizer equipped with an on-line Applied Biosystems Model 130A HPLC system and a Model 920A data system.

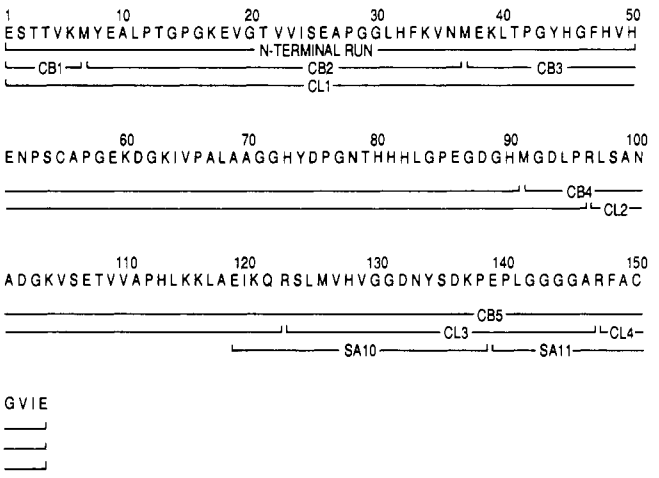

FIGURE 1: Schematic outline of the peptides used to establish the complete amino acid sequence of *B. abortus* Cu–Zn SOD. The following cleavage methods were used: cyanogen bromide (CB); clostripain (CL); and *S. aureus* V8 protease (SA).

A search for proteins homologous to the recombinant *Brucella* protein was performed using the IFIND (Intelligenetics) and PROSIS (Hitachi) programs utilizing the protein sequence database supplied by the National Biomedical Research Foundation.

The SOD activity was measured by inhibition of xanthine/xanthine oxidase-induced reduction of cytochrome *c* (McCord & Fridovich, 1969) using Cu–Zn SOD (Sigma) from human erythrocytes as a control. The rate of the reaction was measured by the change in absorbance versus time as monitored on a Gilford Response 1 spectrophotometer using the kinetics program. The concentration of KCN that inhibited the Cu–Zn SOD by 50% (I_{50}) was determined from the rate of product formation versus the molar concentration of KCN. Each determination was the average of five replicates.

Results

The first 50 amino acids were determined by N-terminal sequence analysis of the protein as shown in Figure 1. Five peptides were generated from CNBr digestion (CB1, CB2, CB3, CB4, and CB5). CB1 and CB2 were aligned by using the amino acid sequence already known from N-terminal sequence analysis. CB3 was completely sequenced and its order determined by overalapping with the clostripain-digested peptides. CB4 was partially sequenced to residue 122 and CB5 to residue 154. Clostripain digestion resulted in four peptides (CL1, CL2, CL3, and CL4). CL1 was partially sequenced to residue 42, and CB3 was aligned from this sequence. CL2

[1] Abbreviations: kDa, kilodalton(s); HPLC, high-pressure liquid chromatography; SOD, superoxide dismutase; DTT, dithiothreitol.

Table I: Amino Acid Composition of *Brucella abortus* Cu–Zn Superoxide Dismutase and Other Cu–Zn Superoxide Dismutases

	source of Cu–Zn superoxide dismutase			
amino acid	*Brucella*	*Photobacterium*[a]	*Caulobacter*[b]	eukaryotic[c] species
D + N	12[d] (12)[e]	17	16	17–18
E + Q	13 (13)	10	6	11–16
S	7 (5)	6	7	7–11
G	24 (20)	22	19	22–26
H	11 (8)	10	8	6–10
R	3 (4)	2	2	3–4
T	7 (6)	10	11	8–12
A	12 (12)	13	26	8–13
P	13 (12)	9	8	5–8
Y	4 (3)	2	1	0–1
V	13 (11)	10	12	14–17
M	4 (1)[f]	4	0	0–2
I	4 (4)	5	5	6–9
L	11 (11)	14	12	6–9
F	3 (3)	4	3	4–6
K	11 (12)	10	9	10–13
W	0 (ND)[g]	1	1	0–1
C	2 (ND)	2	2	2–4
total residues	154 (137)	151	148	151–153

[a] Amino acid sequence data (Steffens et al., 1983). [b] Amino acid analysis data (Steinman, 1982a). [c] Amino acid sequence data from bovine (Steinman et al., 1974), horse (Lerch & Ammer, 1981), human (Barra et al., 1980), and yeast (Hohansen et al., 1979) Cu–Zn SODs. [d] Amino acid sequence data. [e] Values in parentheses are from amino acid analysis. [f] Partially oxidized during acid hydrolysis; a portion of the oxidation products may coelute with R. [g] Not determined.

was sequenced to residue 122, and CB4 was aligned from this sequence. CL3 and CL4 were completely sequenced, and CB5 was aligned from these two fragments. *Staphylococcus aureus* V8 protease digestion allowed determination of two peptides (SA10 and SA11) at the carboxyl-terminal end of the protein. These two peptides provided information for overlap regions that could not be verified from sequence information obtained from the other peptides.

The amino acid composition of the *Brucella* protein obtained by amino acid analysis as shown in Table I was in good agreement with the amino acid composition obtained from the sequence data with the exception of glycine, histidine, and methionine. Table I also compares the *Brucella* protein amino acid analysis and sequence data with those of Cu–Zn SOD from *Photobacterium leiognathi*, *Caulobacter crescentus*, and eukaryotic species (bovine, equine, human, and yeast).

On the basis of amino acid sequence data, the molecular weight of the *Brucella* protein was 16071. The amino acid chain length for the *Brucella* protein was 154 amino acids.

A search for homology was performed with the 154 amino acid *Brucella* sequence using the IFIND program and accessing the protein sequence database. The IFIND search using the Wilbur and Lipman (1983) algorithm gave a match score of 34 for the Cu–Zn SOD protein sequence from *P. leiognathi*. The Intelligenetics program reported a 53.6% identity in 140 amino acid overlap between the *Brucella* protein and *P. leiognathi* SOD. The *Brucella* protein was less related to human and bovine Cu–Zn SODs, demonstrating 27.4% and 27.4% identity in 140 amino acid overlap, respectively. Figure 2 demonstrates the homology between the protein from *B. abortus* and Cu–Zn SODs from *P. leiognathi*, bovine, and human species.

The *Brucella* protein was assayed for its ability to inhibit the production of superoxide radical, a measure of SOD activity. The *Brucella* protein demonstrated an activity of 130 units/mg, compared with 8700 units/mg for the human SOD. Only Cu–Zn SOD activity is significantly inhibited by 1 mM KCN (Crapo et al., 1978). The protein demonstrated SOD activity that was inhibited 50% by the addition of 0.29 mM KCN. In comparison, the human Cu–Zn SOD used as a control was inhibited 50% by 0.35 mM KCN.

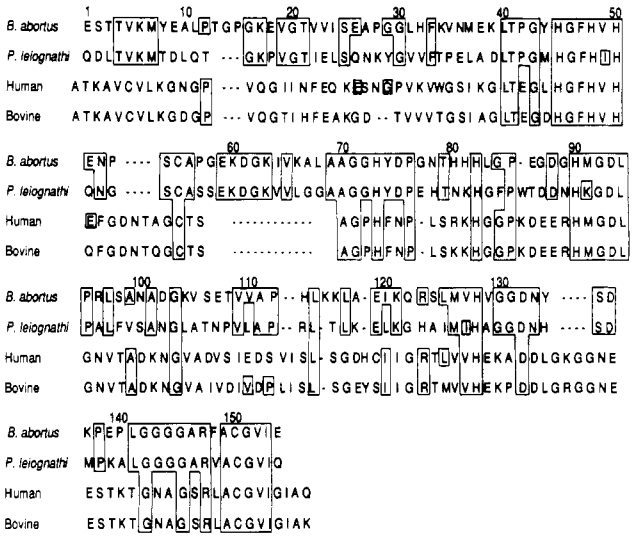

FIGURE 2: Comparison of *B. abortus* Cu–Zn SOD with *P. leiognathi*, human, and bovine Cu–Zn SOD. The amino acid sequences were arranged in order to obtain maximum sequence homology. Areas of homology between *Brucella* Cu–Zn SOD and the other Cu–Zn SODs are indicated by boxes.

Discussion

We have demonstrated by sequence homology and enzyme activity studies that a recombinant *B. abortus* protein is a Cu–Zn SOD.

The molecular weight of the *Brucella* protein, M_r 16071 is in agreement with that of the subunit, M_r 15780, of the Cu–Zn SOD from *P. leiognathi* (Steffens et al., 1983). The amino acid chain length for the *Brucella* protein was 154 amino acids, compared with 151 amino acids for the *P. leiognathi* SOD.

One of the distinguishing characteristics of the *P. leiognathi* Cu–Zn SOD is a very basic isoelectric point (p*I*) of 8.25 (Puget & Michelson, 1974). The p*I* of 8.6 determined for the *Brucella* protein (Tabatabai, unpublished experiments) is similar to that of *P. leiognathi*.

Figure 2 demonstrates the high degree of sequence homology between *B. abortus* and *P. leiognathi* Cu–Zn SOD. Only one 3 amino acid gap between residues 12 and 14 in the *P. leiognathi* sequence and one 1 amino acid gap in the *B. abortus* sequence were required in order to align the two sequences. As can be seen in Figure 2, the *Brucella* Cu–Zn SOD demonstrates 53.6% identity with *P. leiognathi* Cu–Zn SOD, compared with 27.4% identity with eukaryotic human and bovine Cu–Zn SODs.

Like the Cu–Zn SOD from *P. leiognathi*, the *Brucella* Cu–Zn SOD contained in equivalent positions all amino acid residues previously identified by X-ray crystallography as essential for metal coordination and enzymatic activity (Steinman, 1982b; Dunbar & Johansen, 1981). Aspartic acid residue 93 and histidine residues at positions 48, 50, 73, 82, 90, and 128 are known Cu–Zn binding sites in bovine cu–Zn SOD (Steinman, 1982b). Cysteines at positions 55 and 150 are homologous to those forming the intrasubunit disulfide bridge in the human, bovine, and *P. leiognathi* enzymes. The arginine residue at position 147, known to be important for catalytic activity (Borders & Johansen, 1980; Borders et al., 1985), is also conserved.

Although *Brucella* Cu–Zn SOD activity was 130 units/mg compared with 8700 units/mg for human Cu–Zn SOD, others have found variable Cu–Zn SOD activity in the limited number of bacterial species studied. Steinman (1982) has reported 2800 units/mg activity for purified *Caulobacter* Cu–Zn SOD. Conversely, Steinman (1985) found less than 20 units/mg Cu–Zn SOD activity in sonicated cell supernatants of *Pseudomonas diminuta* and *Pseudomonas maltophilia*. Recent studies in our laboratory have shown that *Brucella* Cu–Zn SOD can be stimulated 2-fold by 75 μM Cu^{2+}. Other differences in prokaryotic Cu–Zn SOD activity appear to be species-related.

It was originally believed that the gene for *Photobacterium* Cu–Zn SOD was transferred from the ponyfish, the symbiotic host of *P. leiognathi* (Martin & Fridovich, 1981). Now that Cu–Zn SODs have also been discovered in *B. abortus*, *C. crescentus* (Steinman, 1982a), *Paracoccus dentrificans* (Vignais et al., 1982), *P. dimunuta* (Steinman, 1985), and *P. maltophilia* (Steinman, 1985), the existence of a separate gene for prokaryotic Cu–Zn SODs must be considered. The major evidence for this hypothesis is the existence of more than one species of bacterial Cu–Zn SOD and the high degree of sequence homology between the *Brucella* and *Photobacterium* Cu–Zn SODs. Using the PROSIS maximum homology program, which takes into consideration equivalent amino acids between sequences, 71% alignment occurred between the two sequences. As mentioned previously, the sequence identity between the bacterial Cu–Zn SODs and human and bovine dismutases is only 27.4%. Amino acid sequence data from the other bacterial Cu–Zn SODs are needed to fully understand the evolution of prokaryotic Cu–Zn SOD.

There are various reports in the literature suggesting that bacterial SODs are involved with an intracellular pathogenic bacterium's ability to survive in host phagocytes (Johnston et al., 1975; Babior et al., 1975). Some intracellular pathogens induce the respiratory burst in phagocytes, causing the production of free radicals, yet these bacteria are not destroyed by free radicals. One of these organisms, *Nocardia asteroides*, contains a unique SOD associated with the outer cell wall that is secreted into the growth medium (Beaman et al., 1983). Because the addition of exogenous SOD has been shown to protect some bacteria against phagocytic killing (Johnston et al., 1975; Babior et al., 1975; Yost & Fridovich, 1974), it seems reasonable to suggest that a secreted SOD could also provide protection against phagocytic attack. Similar to *Nocardia*, *Brucella* is an intracellular parasite that survives in phagocytes (Smith, 1977). Recent reports, however, have questioned the role of SOD as a protective enzyme (Fee, 1982; Minotti, 1988). The significance for a role of SOD in bacterial survival or pathogenicity, therefore, is not clear. Preliminary DNA sequence data indicate the presence of an N-terminal leader sequence which is absent from the mature *Brucella* Cu–Zn SOD polypeptide (S. M. Halling, personal communication). These data strongly suggest that *Brucella* Cu–Zn, SOD, like *Nocardia* SOD, is localized in the periplasm. Therefore, the function of Brucella Cu–Zn SOD may be determined by its cellular location. Work is in progress to confirm its cellular location using immunogold-labeled antiserum raised against the *Brucella* Cu–Zn SOD. Currently, studies are being conducted to ascertain the role of *Brucella* Cu–Zn SOD in bacterial evasion of host phagocytic defenses.

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Registry No. Superoxide dismutase, 9054-89-1; superoxide dismutase (*Brucella abortus* protein moiety reduced), 124098-23-3.

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Monofunctional Chorismate Mutase from *Bacillus subtilis*: Purification of the Protein, Molecular Cloning of the Gene, and Overexpression of the Gene Product in *Escherichia coli*†

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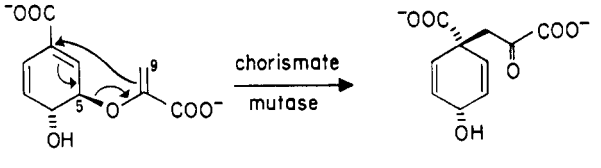
ABSTRACT: The monofunctional chorismate mutase from *Bacillus subtilis* has been purified 2200-fold to homogeneity. The enzyme is a homodimer of subunit M_r = 14 500 and is the smallest natural chorismate mutase that has been characterized. The purified enzyme follows Michaelis–Menten kinetics with a K_m of 100 μM and a k_{cat} of 50 s^{-1}, carries no other associated enzymic activities, and is unaffected by any of the aromatic amino acids. The N-terminal amino acid sequence of the protein has been determined, and this information has been used to construct a precise oligonucleotide probe for the gene by means of in vitro DNA amplification from total chromosomal DNA by the polymerase chain reaction. The cloned *aroH* gene encodes a protein of 127 amino acid residues and is expressed in *Escherichia coli*. The cloned gene product is indistinguishable from that purified from *Bacillus*. The *aroH* coding region was directly subcloned into a phagemid expression vector by means of the polymerase chain reaction. The resulting construct, with the *aroH* gene positioned behind efficient transcription and translation initiation sequences of *E. coli*, results in the production of the monofunctional mutase at levels of 30–35% of the soluble cell protein in *E. coli* transformants. Chorismate mutases comprise a set of functionally related proteins that show little sequence similarity to each other. This diversity stands in contrast to other chorismate-utilizing enzymes.

Chorismate mutase catalyzed the rearrangement of chorismate (**1**) to prephenate (**2**), and the enzyme lies at the branch point of the biosynthetic pathway leading to the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan. The



chorismate **1** prephenate **2**

catalyzed reaction is formally a Claisen rearrangement and is the only example of what appears to be a pericyclic process in primary metabolism.

The uncatalyzed reaction occurs readily, though this transformation is accelerated by the enzyme by more than 10^6 at 25 °C (Andrews et al., 1973; Görisch, 1978). The nonenzymic process has been extensively studied and is believed to proceed via a concerted, asynchronous reaction (Dewar, 1984) in which bond breaking is far in advance of bond making at the transition state (Addadi et al., 1983). In contrast, little is known about the mechanism of the enzyme-catalyzed rearrangement. The absence of secondary tritium kinetic isotope effects at C-5 and C-9 of chorismate (**1**) suggests that some transition state other than that involving the chemical transformation is rate limiting (Addadi et al., 1983). This conclusion leaves obscure the origins of the enzyme-mediated rate enhancement. The stereochemical course of the enzymic rearrangement has been established (Sogo et al., 1984) and involves a transition state of chairlike geometry, as has also been found for the nonenzymic process (Copley & Knowles, 1985).

The most thoroughly studied chorismate mutases are the P and T proteins of *Escherichia coli*, which are both bifunctional enzymes of subunit M_r near 40 000, the mutase activity

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