MICROHETEROGENEITY IN RECOMBINANT HUMAN COPPER, ZINC SUPEROXIDE DISMUTASE PRODUCED IN YEAST

By

VICTOR E. BUCKWOLD

A Thesis submitted to the Faculty of Graduate Studies, in partial fulfilment of the requirements for the degree of Master of Science

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ABSTRACT

Microheterogeneity in recombinant human CuZn-superoxide dismutase (rhSOD) from yeast was investigated and compared to that observed in the natural human red blood cell enzyme (hSOD). The recombinant yeast used in this study contained both the rhSOD-producing plasmid and the yeast's natural dimeric CuZn-SOD. Isoelectric focusing (IEF) of purified rhSOD showed three major active forms with pIs of 4.9, 5.2, and 5.35 which were identical to the charge isomers observed in two different commercial preparations of hSOD. Using IEF, purified yeast CuZn-SOD (pI = 5.2-5.25) or yeast-human CuZn-SOD heterodimers (pIs = 4.6 and 5.85) focussed outside of the pI range of the charge isomers observed in rhSOD. This indicates that yeast CuZn-SOD does not contribute to the charge microheterogeneity observed in rhSOD.

Native polyacrylamide gel electrophoresis (PAGE) of hSOD or rhSOD showed the presence of two major forms of the molecule. Anion-exchange chromatography of extracts of rhSOD-expressing yeast resolved two peaks of superoxide dismutase activity corresponding to these two PAGE isomers. These isomers were collected and named rhSOD I and rhSOD II, according to their chromatographic elution. Sample rhSOD II contained the majority of the protein and activity. The specific activities of rhSOD I and rhSOD II were determined to be 2,040-2,440 U/mg and 3,340-3640 U/mg respectively. Analysis by IEF indicated that rhSOD I was composed of nearly equal quantities of the pI 5.2 and 5.35 rhSOD isoforms, and small quantities of a third pI 5.8 form; rhSOD II consisted of both pI

4.9 and 5.2 forms. Reducing, denaturing SDS-PAGE showed rhSOD I to contain primarily a 20 KD subunit with a small amount of a 21 KD species. Sample rhSOD II showed nearly equal amounts of 21 KD and 22 KD monomers. Other samples of rhSOD or hSOD consisted of mixed dimers of the three subunits with a predominance of the 21 KD form. Circular dichroism (CD) spectra showed rhSOD II to contain a Cotton effect at 240nm not seen in rhSOD I. No differences in the secondary structure of the two rhSOD isomers were observed by CD. The results of these experiments suggest that rhSOD I may be derived from the inactivation of rhSOD II in a manner similar to the mechanism by which CuZn-SOD variants are produced in other species.

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"Don't Waste Clean Thinking on Dirty Enzymes."

Ephraim Racker

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LIST OF ABBREVIATIONS

bSOD	bovine red blood cell CuZn-SOD
CD	circular dichroism
CuZn-SOD	copper zinc superoxide dismutase
dc	dimer contact
DEAE cellulose	diethylaminoethyl cellulose
EDTA	ethylene diamine tetraacetic acid
Fe-SOD	iron SOD
GS	glutamine synthetase
HPLC	high performance liquid chromatography
hSOD	human superoxide dismutase
HP	hydrogen peroxide
IEF	isoelectric focusing
IVS	intervening sequence/intron
kD	kilodaltons
KPi	potassium phosphate
MCO	metal-catalyzed oxidation
Mn-SOD	manganese SOD
MOP	macroxyproteinase
NBT	nitro blue tetrazolium
PAGE	native polyacrylamide gel electrophoresis

pI	isoelectric point
RBC	red blood cell
rhSOD	recombinant human superoxide dismutase
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOD	superoxide dismutase
TEMED	N,N,N',N'- tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
2D gels	two-dimensional gel electrophoresis
UV	ultraviolet
ySOD	yeast CuZn-SOD

INTRODUCTION

Copper, zinc superoxide dismutase is a well-studied enzyme that is known to catalyze the dismutation of it's substrate, superoxide anion, into hydrogen peroxide. A variety of ailments exist in which oxygen radicals are thought to participate in tissue damage, and, thus, superoxide dismutase may be of benefit as a biologic in the treatment of these disorders. ABI Biotechnology Inc. has been developing a recombinant human CuZn superoxide dismutase (rhSOD) produced by a yeast vector. The rhSOD is to be used in the treatment of these clinical manifestations.

Two major difficulties were encountered during the development of this product and they are addressed in this thesis. First, multiple active forms of the enzyme appeared in all preparations, and second, indigenous yeast CuZn superoxide dismutase (ySOD) was detected in the purified rhSOD samples.

Electrophoretic analysis of both the natural and recombinant forms of human CuZn superoxide dismutase (hSOD) show the existance of multiple forms of the enzyme. By native polyacrylamide gel electrophoresis, hSOD prepared in different manners exhibit one to three different active forms, whereas two or more active forms have been observed by isoelectric focusing. The reasons for this observed heterogeneity are not completely understood, but damage to the protein by hydrogen peroxide is known to be responsible, in part, for these differences (Jewett, 1983; Jewett et al, 1989; Salo et al, 1990). In the production of rhSOD as a biologic it was necessary to identify the different forms of the enzyme, to

elucidate the altered structure of the various forms, and to compare these forms with those found in the native hSOD.

A second problem in the production of rhSOD was found in the studies of Campbell (1990). Yeast CuZn-SOD was detected in samples of rhSOD. The presence of ySOD, or that of any other yeast protein, would be highly undesirable in a pharmaceutical-grade product.

Studies were undertaken to purify and characterize the different forms of rhSOD observed, and to compare these proteins with that observed in the natural hSOD. In addition, rhSOD preparations were examined for ySOD contamination.

LITERATURE REVIEW

CuZn-SOD Background

Discovery and Significance of CuZn-SOD

Bannister (1988) discussed the history and significance of the finding of superoxide dismutase (SOD). In 1938, an obscure copper protein was discovered in the red blood cells (RBCs) of ox and it was called haemocuprein. In the years since that discovery, similar proteins were found called hepatocuprein, cerebrocuprein, and erythrocuprein. Eventually these proteins were found to be identical and they were given the name cytocuprein for a short period. Generally, it was believed that these proteins played a role in copper storage or transport. An activity termed tetrazolium oxidase or indophenoloxidase which inhibited the reduction of tetrazolium salts in the presence of light was also known to geneticists since at least 1967 (MIM 14745; McKusick, 1983). Tetrazolium oxidase, was known to contain copper and zinc, but the connection with the cupreins (erythrocuprein) was not made, as cupreins were not known to contain zinc. In 1973 tetrazolium oxidase was found to be copper, zinc superoxide dismutase (CuZn-SOD)(Lippitt and Fridovich, 1973), some time after the superoxide dismuting activity of the protein had been discovered (McCord and Fridovich, 1969). The discovery of an enzyme to remove free radicals brought wide-spread acceptance of the biologic significance of free-radicals. Before this, radicals were only known in biological systems as "caged" or immobilized radicals, such as in the electron transport chain of mitochondria.

Since the original discovery of CuZn-SOD, other unrelated proteins with SOD activity have been described. These include the manganese-containing SOD (Mn-SOD) found in procaryotes and eucaryotes, iron-containing SOD (Fe-SOD) in procaryotes (Parker et al, 1984; Bannister et al, 1987), and the more recently described copper and zinc-containing extracellular SOD of eucaryotes (Marklund, 1982; Halmarsson, 1987). Humans have CuZn-SOD, manganese-SOD, and extracellular forms of SOD (Marklund, 1982; Parker et al, 1984; Bannister et al, 1987), with the CuZn-SOD being responsible for all of the SOD activity of human RBCs. Red blood cell CuZn-SOD has been the focus of most pharmacological research.

Structure of CuZn-SOD

Copper, zinc superoxide dismutase is a dimer of approximately 32 kD composed of identical subunits held together by a strong non-covalent interaction (Bannister et al, 1987). The enzyme is localized in the cytosol and in the mitochondrial intermembrane space, with RBCs containing about 500μg CuZn-SOD/g hemoglobin (Bannister et al, 1987). Each monomer contains an atom each of copper and zinc and a single intrachain disulphide bond. The copper plays a role in catalysis whereas the zinc appears to act in stabilizing the protein's structure. A form of conformational communication exists between subunits in the native dimer, but the effect on catalysis is not understood (Bannister et al, 1987).

The primary structures of all CuZn-SODs are quite similar and 23 invariant

residues have been identified (Getzoff et al, 1989). A comparison of the primary structure of fifteen different CuZn-SODs is shown in **Figure 1**. The N-terminus of CuZn-SODs from mammals is blocked by an acetyl group. By X-ray crystallography the structure of bSOD has been determined to 2Å resolution (Tainer et al, 1982; Tainer et al, 1983; Getzoff et al, 1983). Crystals of the recombinant human enzyme from yeast have been characterized (Parge et al, 1986) and an X-ray crystallographic model will be published soon (J. Tainer, Res. Inst. Scripps Clinic, personal communication). Each monomer is approximately 33 X 36 X 67 Å and shows a high degree of ß-structure (ca 40-60%). Enzyme monomers are composed of eight antiparallel ß-strands which form a Greek key ß-barrel structure. The 2Å backbone drawing and Greek key topology of bSOD are illustrated in **Figure 2**. This Greek key ß-barrel structure is also found in functionally unrelated IgG immunoglobulin (Richardson et al, 1976).

The X-ray model shows the position of the enzyme's metal ions. The catalytic copper sits partially buried at the bottom of a channel in the enzyme surface. The area surrounding the active site channel is believed to create an electrostatic guidance system which directs negatively charged superoxide anion towards the positively charged binding site at the bottom of the channel (Getzoff et al, 1983). The catalytic copper is held in position by four histidine residues. These residues are His 46, His 48, His 63, and His 120 in both the human and bovine enzymes (Getzoff et al, 1989). The residue numbers for these histidine residues are the same for both the human and bovine CuZn-SODs. The zinc

Figure 1. Primary structure and sequence alignment of a variety of CuZn-SODs.

The amino acids are numbered according to the human sequence. Those residues which are identical to the human sequence are boxed. Invariant residues are shaded grey. Roman numerals and solid lines correspond to the seven loops or turns separating the eight ß-strands and the sequences not involved in ß-strands. Residues that are disulphide bridged (S-S), ligate the metals (Cu or Zn), or are involved in dimer contact regions (dc) are shown above the alignment. The locations of the introns (IVS-1 through IVS-4) in the human gene with respect to the coding sequence are shown below the alignment. Regions exhibiting twofold symmetry in the bovine sequence (1 and 1', 2 and 2', 3 and 3', 4 and 4') are marked by numbered horizontal arrows. Identical symmetry-related residues in the bovine sequence (open circles) or among other eucaryotes (closed circles) are indicated.

From Getzoff et al, 1989.

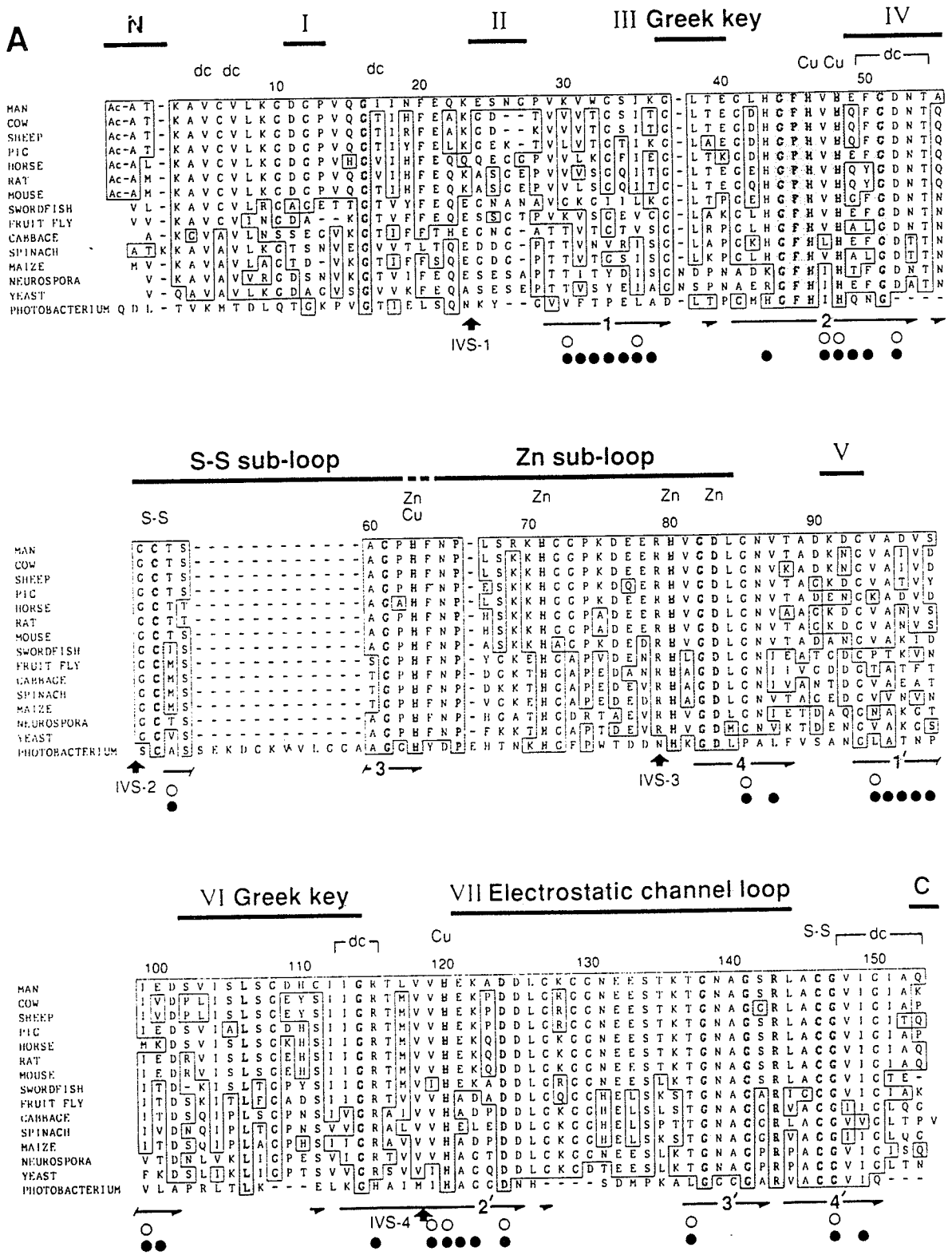
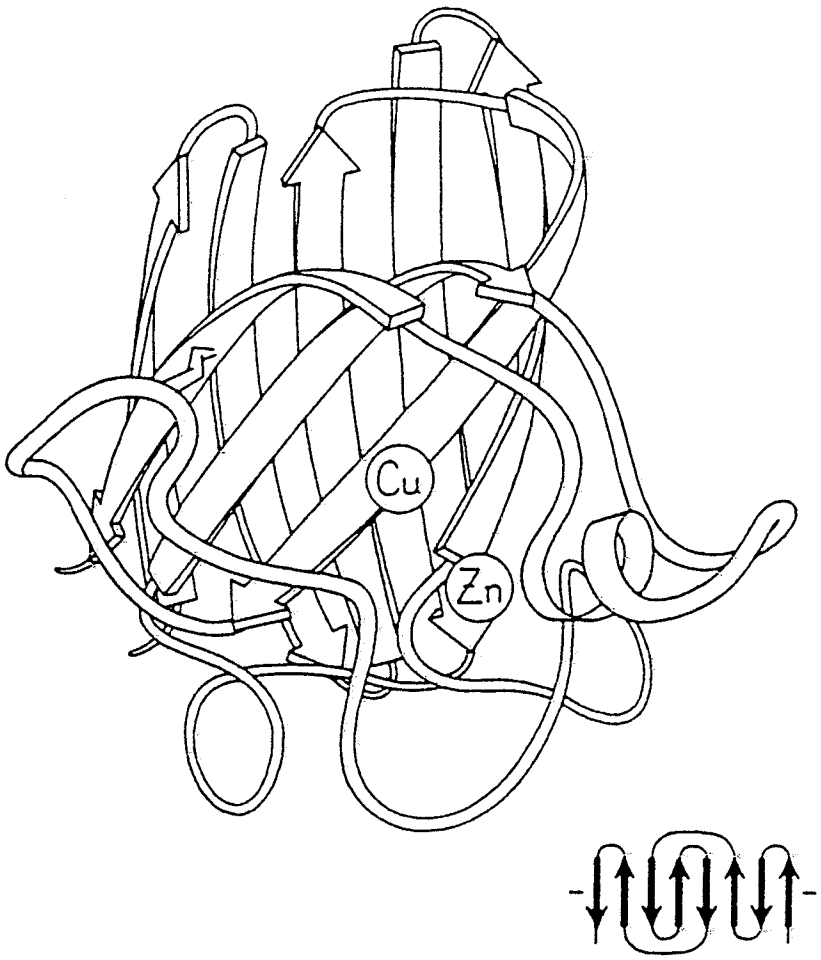

A
N
I
II
III Greek key
IV
dc dc
dc
Cu Cu
dc
10
20
30
40
50
MAN
COW
SHEEP
PIG
HORSE
RAT
MOUSE
SWORDFISH
FRUIT FLY
CABBAGE
SPINACH
MAIZE
NEUROSPORA
YEAST
PHOTOBACTERIUM
IVS-1
1
2
S-S sub-loop
Zn sub-loop
V
S-S
Zn
Cu
Zn
Zn
Zn
60
70
80
90
IVS-2
3
IVS-3
4
1'
VI Greek key
VII Electrostatic channel loop
C
dc
Cu
S-S
dc
100
110
120
130
140
150
IVS-4
2'
3'
4'

Figure 2. Schematic backbone drawing of bovine CuZn-SOD subunit and illustration of greek key ß-barrel topology.

Schematic drawing of the bovine CuZn-SOD monomer is based on the 2Å structure of the molecule determined by X-ray crystallography. The ß-strands are shown as arrows and the disulphide bond as a zig zag. The greek key ß-barrel is illustrated (bottom right) as it would be seen when spread out and viewed from the outside.

From Tainer et al, 1982.


Cu
Zn

moiety can interact with the copper but it is not accessible to solvent in the native enzyme (Getzoff et al, 1986). Zinc is not utilized directly in the catalytic cycle. This metal is thought to play a stabilizing role, by ensuring that a certain histidine residue which ligates the copper is protonated; this stabilizes the position of the substrate during electron transfer events (Bannister, 1987). The zinc can be replaced with other metals (eg. Cd(II)) or removed entirely without a significant effect on SOD activity (Fridovich, 1986).

Copper, zinc superoxide dismutase is an exceptionally stable enzyme that is highly resistant towards inactivation by heat, various unfolding and denaturing conditions, and organic solvents (Fielden and Rotilio, 1984). The k_{cat} of CuZn-SOD is one of the highest known for any enzyme.

Function of CuZn-SOD

Superoxide dismutase (SOD; superoxide/superoxide oxidoreductase, EC 1.15.1.1) catalyses the conversion of superoxide radicals into hydrogen peroxide and it is considered the first line of defence against the toxicity of oxygen-centred radicals (Bannister et al, 1987). The superoxide radical is the first reduction product of oxygen and is generated in many biological reactions (Parker et al, 1984). Superoxide radicals will spontaneously undergo dismutation:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

The reaction is rapid and has a rate constant of $8 \times 10^4\ M^{-1}\ s^{-1}$ (Fridovich, 1985). In the presence of SOD, dismutation of superoxide radical into hydrogen peroxide

and oxygen proceeds as follows:

$$\text{Enz-Cu(II)} + O_2^{\bullet-} \rightleftarrows \text{Enz-Cu(I)} + O_2 \qquad (2)$$

$$\text{Enz-Cu(I)} + O_2^{\bullet-} + 2H^+ \rightleftarrows \text{Enz-Cu(II)} + H_2O_2 \qquad (3)$$

The catalytic metal in **Equations (2) and (3)** is shown as copper but the same redox cycling process occurs for the iron or manganese SODs (Parker et al, 1984), although the CuZn-SOD is catalytically the most efficient of these three (Fielden and Rotilio, 1984). The reaction also proceeds in the reverse direction, but the forward reaction is favoured (Hodgson and Fridovich, 1975). This minimal scheme for catalysis is termed the "conventional mechanism" and has been confirmed experimentally (Bannister et al, 1987). Protons are derived from water acting as a general acid (Fee and Bull, 1986), and possibly also from histidine residues which ligate the copper (Parker et al, 1984). The rate constant for the dismutation of superoxide is $2 \times 10^9\ M^{-1}\ s^{-1}$ (Bannister et al, 1987). Both steps of the reaction are catalyzed at identical rates. The rate of this reaction is only an order of magnitude lower than the theoretical limit, itself governed by the rate at which substrate can diffuse to the enzyme's active site.

Both of the active sites in the enzyme appear to function independently (Fridovich, 1986). Also, the activity of the enzyme is known to depend on ionic strength, ionic type, and pH (Cudd and Fridovich, 1982; Barra et al, 1986; O'Neil et al, 1988). Copper, zinc superoxide dismutase is inhibited by phosphate (de Freitas and Valentine, 1984), cyanide, and azide (Bannister et al, 1987).

There may be another role for CuZn-SOD apart from its enzymatic function. Recently, Pluthero et al (1990) found that CuZn-SOD inhibited DNA synthesis and Interleukin 3-dependent proliferation of marrow erythroid cells and cell lines from mice. Further work from his group has shown that this effect is dependent on, but not entirely explained by, the enzyme's activity (F.G. Pluthero, University of Toronto, personal communication). Other SOD types (ie. Fe-SOD, Mn-SOD) also demonstrated this activity.

Clinical Applications For CuZn-SOD

Reactive oxygen metabolites are thought to play an important role in tissue damage occurring in a number of pathological conditions (**Table 1**). The toxicity of superoxide radicals has been questioned based on their chemical reactivity (Sawyer and Valentine, 1981), but no solid evidence is available to support the contention that they are harmless (Bannister et al, 1987). Copper, zinc superoxide dismutase shows great potential as a pharmaceutical in the protection of cells from a variety of radical-mediated injuries. Conditions where CuZn-SOD shows promise include the ischemic syndromes, certain inflammatory disorders, and lung disease (Marcocci et al, 1989).

Ischemic syndromes are those instances were oxygen radicals are formed following the reintroduction of oxygen into tissues that were previously oxygen depleted (post-ischemic tissues). Most of the damage associated with ischemic syndromes is now thought to be derived from oxygen radicals produced after

Table 1. Clinical conditions in which oxygen radicals may be involved.[1]

Inflammatory-immune injury: Glomerulonephritis (idiopathic, membraneous) Vacsculitis (Hepatitis B virus, drugs) Autoimmune disease Rheumatoid arthritis **Ischemia - reflow states:** Stroke/ myocardial infarction Organ transplantation Inflamed rheumatoid joint? **Drug and toxin-induced reactions** **Iron overload:** Idiopathic hemochromatosis Dietary iron overload (Bantu) Thalassemia and other chronic anemias treated with multiple blood transfusions **Nutritional deficiencies (kwashiorkor)** **Alcoholism:** including alcohol-induced iron overload **Radiation injury** **Ageing:** Disorders of premature ageing	**Lung:** Cigarette smoke effects Emphysema Hyperoxia Bronchiopulmonary displasia Oxidant pollutants (O_3) Adult respiratory distress syndrome (some forms) Mineral dust pneumoconiosis Bleomycin toxicity SO_2 toxicity **Heart and cardiovascular system:** Alcohol cardiomyopathy Keshan disease (selenium deficiency) Atherosclerosis Adriamycin cardiotoxicity **Kidney:** Autoimmune nephrotic syndromes Aminoglycoside nephrotoxicity Heavy metal nephrotoxicity **Gastrointestinal tract:** Endotoxin liver injury Halogenated hydrocarbon liver injury (eg. bromobenzene, CCl_4, halothane) Diabetrogenic action of alloxan Pancreatitis Nonsteroidal antiinflammatory drug-induced gastrointestinal tract lesions Oral iron poisoning

Table 1 Continued. Clinical conditions in which oxygen radicals may be involved.[1]

Red blood cells: Phenylhydrazine Primaquine (related drugs) Lead poisoning Protoporphyrin photoxidation Malaria Sickle cell anemia Favism Fanconi's anemia **Brain/nervous system/ neuromuscular disorders:** Hyperbaric oxygen Vitamin E deficiency Neurotoxins Parkinson's disease Hypertensive cerebrovascular injury Neuronal ceroid lipofuscinoses Allergic encephalomyelitis and other demyelinating diseases Aluminum overload Potentiation of tramatic injury Muscular dystrophy Multiple sclerosis	**Eye:** Cataractogenesis Ocular hemorrhage Degenerative retinal damage Retinopathy of prematurity Photic retinopathy **Skin:** Solar radiation Thermal injury Porphyria Hypericin other photosensitizers Contact dermatitis

[1]From Halliwell, 1987.

blood flow is restored rather than from a lack of oxygen in the tissue. Important applications for CuZn-SOD in this area include the preservation of organs for, and during, organ transplantation (or plastic) surgery, and the reduction of ischemic damage associated with heart attack. Some success in these areas have been documented (Greenwald, 1990).

Another important application for CuZn-SOD is for use in certain inflammatory and autoimmune disorders such as articular diseases (osteoarthritis). In Europe, local injection of bovine CuZn-SOD has been shown to be of benefit in the treatment of such disorders (Marcocci et al, 1989).

Preterm infants often require oxygen therapy at birth. Copper, zinc superoxide dismutase has been shown to be of benefit in reducing the severity of oxygen toxicity and bronchiopulmonary dysplasia associated with respiratory distress syndrome in newborns (Greenwald, 1990).

There are other conditions in which CuZn-SOD therapy has been tried or is anticipated to be of some benefit in, but these applications are more speculative. Other potential therapeutic roles for CuZn-SOD have been discussed (Marcocci, 1989; Greenwald, 1990).

CuZn-SOD HETEROGENEITY

Multiple CuZn-SOD Isoforms

Highly purified preparations of CuZn-SOD commonly show multiple active components. Early work showed that hSOD is composed of two or more components (Kimmel et al, 1959; Stansell and Deutch, 1965b). The nature of these different forms is not understood completely, but reduced CuZn-SOD specific activity is often associated with increased heterogeneity. Enzyme heterogeneity is also more pronounced in those tissue-types which are subject to oxidative stress, such as red blood cells (shown for rat and mouse CuZn-SOD: Crosti and Sausa, 1980). This may be related to the known inactivation of CuZn-SOD by its substrate hydrogen peroxide (see OXIDATIVE DAMAGE TO CuZn-SOD below).

Similarly, more CuZn-SOD heterogeneity seems to be found in aged RBCs and in enzyme isolated from older animals. Reduced CuZn-SOD activity is found in older RBCs taken from cows (Bartosz et al, 1978; Bartosz et al, 1981) and rats (Glass and Gershon, 1981). Older human RBCs however, did not show reduced CuZn-SOD activity (Vanella et al, 1982). Bovine CuZn-SOD taken from older RBCs shows an increased proportion of more mobile bands by PAGE (Bartosz et al, 1981). Chronologically older rats' RBC CuZn-SOD has been found to show reduced activity (Reiss and Gershon, 1976; Glass and Gershon, 1981). The studies of Ischiropoulos et al (1990) noted an increased proportion of more mobile PAGE bands with old rats' RBC CuZn-SOD, but Reiss and Gershon (1976) found

no such difference. No differences were noted in the CuZn-SOD of young and old cows (Gartner et al, 1985).

Potential For Yeast CuZn-SOD Contamination of rhSOD

Yeast has its own CuZn-SOD (ySOD) which, in principle, could co-purify with, or form heterologous hybrids with, the recombinant human enzyme to contribute to CuZn-SOD heterogeneity and contaminate the rhSOD preparation. Previous work at ABI Biotechnology Inc. suggested that ySOD was present in the rhSOD preparations (Campbell, 1990). It is possible that ySOD or a yeast-human CuZn-SOD heterodimer could be present in rhSOD samples.

Analysis of ySOD by IEF demonstrated a single band with a pI of approximately 5 (Campbell, 1990). This value is intermediate to the pI range seen in human CuZn-SOD samples (Campbell, 1990). Based on the similarities in surface charge properties of the yeast and human enzymes one would expect co-purification during ion-exchange chromatography, which was one of the steps used in the purification of the rhSOD from the yeast.

The formation of interspecific CuZn-SOD hybrids has been observed before (Tegelstrom, 1975; Edwards et al, 1978; Crosti and Sausa, 1980; Shibata and Ogita, 1986). In one study, it was concluded that combined heating of CuZn-SODs of different species, followed by cooling was the best way to form inter-specific heterodimers (Tegelstrom, 1975). A heating step has also been used to purify rhSOD, which is thermostable (Gartner et al, 1984).

To date, no differences between rhSOD produced in yeast and the natural hSOD have been discovered. Yeast-derived rhSOD is being used for the construction of the X-ray crystallographic model of hSOD (John Tainer, Res. Inst. Scripps Clinic, personal communication). A major post-translational event that occurs during hSOD production is the removal from the protein backbone of the N-terminal methionine required for translational initiation, followed by acetylation of the new N-terminal alanyl residue (Jabusch et al, 1980; Hallewell et al, 1986). The correct intrachain disulphide bond must be formed and the metal ligands must associate with the protein for it to be mature. The rhSOD produced in Escherichia coli is not N^{α}-acetylated (Flohe et al, 1986; Hallewell, 1987; Kajihahra et al, 1988b). E. coli does not posses the enzymes necessary to perform this modification; yeast does, and the rhSOD produced in yeast is properly N^{α}-acetylated (Flohe et al, 1986; Hallewell et al, 1987).

Glycation of CuZn-SOD

Copper, zinc superoxide dismutase can be glycated in vivo but the contribution of this modification to enzyme heterogeneity is not known. Human CuZn-SOD has been resolved into glucosylated (ca 20% of enzyme) and non-glucosylated forms (Arai et al, 1987a). Patients with diabetes, and older RBCs, showed a higher proportion of the glucosylated form of the enzyme. Certain reactive lysine residues were found to be glycated, and the enzyme was found to be inactivated when Lys 122 and/or Lys 128 was glucosylated (Arai et al, 1987b).

This reaction was shown to proceed non-enzymatically after incubation with glucose. Covalently-attached sugar residues have been observed in hSOD preparations (Kimmel et al, 1959; Stansell and Deutsch, 1965b), but others have not confirmed this (Hartz and Deutsch, 1969). Several other CuZn-SODs lack detectable carbohydrate content (bSOD, Bartoz et al, 1981; Saunders et al, 1986; ySOD, Goscin and Fridovich, 1972; wheat germ CuZn-SOD, Beauchamp and Fridovich, 1973; pea leaf CuZn-SOD, Duke and Salin, 1983). In the case of bSOD, glycation was not seen even when the enzyme was incubated with glucose in vitro (Bartoz et al, 1981). Enzymic glycosylation of CuZn-SOD might not be expected because it is a resident protein of the cytoplasm (cytosol). The amount of glycated CuZn-SOD formed is related to the levels of glucose in the environment, and to the extent of exposure to it. This is the case too, with non-enzymatic glycation of hemoglobin (Spicer et al, 1979).

ISOLATION AND CHARACTERIZATION OF CuZn-SOD ISOFORMS

Two Major Forms of CuZn-SOD

Several investigators have observed two major peaks of CuZn-SOD activity eluting from anion-exchange columns during purification of CuZn-SOD from a variety of sources (**Table 2**). When these two peaks of CuZn-SOD activity were analyzed by PAGE, they appeared homogeneous and differed in electrophoretic mobility. The first peak that was eluted from the column showed a slower mobility relative to the second peak in native gels. For convenience and clarity, CuZn-SOD

Table 2. Examples of two peaks of CuZn-superoxide dismutase activity eluting from anion-exchange columns.

Enzyme Source	References
Human Red Blood Cells	Hartz and Deutch, 1969 Marklund et al, 1976 Bannister et al, 1977 Briggs and Fee, 1978a Arai et al, 1986
Bovine Red Blood Cells	Civalleri et al, 1982 Gartner et al, 1985 Kato et al, 1985
Rat Liver	Reiss and Gershon, 1976
Ponyfish	Martin and Fridovich, 1981
Bajra Seedlings	Reddy et al, 1986
Iris pseudoacorus Rhizomes	K. Fagerstedt, University of Helsinki, personal communication.
Kidney Bean Leaves	Kono et al, 1979

isomers with these properties are referred to as SOD I and SOD II (after the nomenclature of Bannister et al, 1977). Material taken from the first peak is SOD I and that from the second peak is SOD II.

Bannister et al (1977) were the first to describe the properties of hSOD I and hSOD II. The two forms were found to have essentially the same subunit molecular weight, amino acid composition, and metal content. They differed in chromatographic behaviour, electrophoretic mobility in native gels (even in metal-depleted isomers), tryptophan fluorescence, activity, absorption spectra, and in secondary structure as determined by circular dichroism (CD). The hSOD II was found to have a higher specific activity than hSOD I. The differences in the absorption spectra were primarily in a band at about 320nm that was present in hSOD I and absent from hSOD II. This band was also observed in the CD spectra. The band was removed by treatment with a variety of sulfhydryl-containing reagents (eg. dithiothreitol) but the behaviour of the treated enzyme by PAGE was not mentioned.

The next report in which hSOD I and hSOD II-like components were isolated was from Briggs and Fee (1978a, 1978b). One major, and several minor, peaks of SOD activity were eluted from an anion-exchange column. Only small differences in the mobility of hSOD I and hSOD II components were observed on native gels, and the different forms were incompletely resolved. Some conversion of hSOD I to hSOD II occurred after rechromatography, but the converse reaction was not observed. The hSOD II maintained a slightly higher specific activity than

did hSOD I. Fractions eluting later from the anion-exchange column showed less 320nm absorbance, and the 320nm band was found to be destroyed by reaction with a variety of thiophiles and reducing agents.

The presence of the 320nm absorption band had been noted in several early studies of hSOD. The band was shown to be an artifact of the methods used to purify the enzyme (Briggs and Fee, 1978b). The band was caused by a modification to the free sulfhydryl groups on the enzyme (Calabrese et al, 1975; Briggs and Fee, 1978b). As the 320nm band has not been observed in CuZn-SODs from other sources it is believed to arise from the extra cysteine residue (Cys-111) found in the human enzyme. The band arises from the formation of a mixed disulphide or of R-S-$(S)_n$-R' ($n \geq 0$) on this residue (Lontie and Groeseneken, 1983). It seems unlikely that the 320nm band arises from a physiochemical difference between SOD I and SOD II components, as these forms have been characterized from sources where no 320nm band has been observed.

The first case where two peaks of bSOD activity were isolated and their properties examined was in work by Civalleri et al (1982). Three forms of CuZn-SOD could be distinguished on native gels or by isoelectric focusing (IEF). By IEF, pI 5.2, 4.9, and 4.7 forms could be distinguished. Two major forms of CuZn-SOD were separated by anion-exchange chromatography or preparative IEF. These forms exhibited differences in SDS-PAGE patterns, metal content, antigenicity, electron spin resonance spectra, and UV or visible spectra. Native gel electrophoresis of the bSOD I component showed a homogenous slower

moving band, and the bSOD II component a more mobile one. The bSOD I possessed slightly higher specific activity than bSOD II. Isoelectric focusing analysis of bSOD I demonstrated a single molecular form with a pI of 5.2 and bSOD II a single form with a pI of 4.9. The two forms also differed in the extent of activity loss by heat treatment. When heat treated bSOD I was rerun on IEF gels, it still showed the single pI 5.2 band. Heat treated bSOD II however, showed all three pI 5.2, 4.9, and 4.7 bands. Metal-depleted enzyme showed different properties altogether. It was concluded that the differences observed in PAGE mobility of the forms was primarily due to conformational differences in the enzyme. These authors suggested that the pattern arose from the segregation of subunits made non-identical from an asymmetric post-translational modification to the original protein. Here the modification would affect only one subunit per dimer.

Later, Gartner et al (1985) also separated out two forms of bSOD using chromatofocusing. The bSOD I component showed a doublet pattern by PAGE and a single component of pI 5.2 by IEF. A bSOD II showed a single faster moving band by PAGE and a single pI 4.9 component by IEF. The bSOD I was slightly higher in activity than bSOD II. The two forms showed no differences in copper content, amino acid composition, X-ray photoelectron spectra (oxidized sulphur), and electron paramagnetic resonance spectra. By CD, bSOD I did show twice the 261nm ellipticity of bSOD II. This indicates a difference in the position of a chromophore which absorbs in this region. They then compared these

isomers to those generated from hydrogen peroxide-treated samples and concluded that the CuZn-SODs generated by this method were different from the isomers that they had purified.

These SOD I and SOD II-like isomers have also been found in kidney bean leaves (Kono et al, 1979), bajra seedlings (Reddy et al, 1986), rat liver (Reiss and Gershon, 1976), and Iris pseudoacorus rhizomes (K. Fagerstedt, University of Helsinki, personal communication). Each of these CuZn-SODs showed the characteristic dual peaks of CuZn-SOD activity eluted from anion-exchange columns with each peak corresponding to an electrophoretic isomer by PAGE. The rat liver SOD I, however, showed two slower moving bands relative to the single band of SOD II. The doublet pattern seen for SOD I may have been an artifact of the PAGE system used (discussed below).

Two peaks of chromatographically resolved CuZn-SOD activity were also characterized from wheat germ (Beuchamp and Fridovich, 1973), corn (Baum and Scandalios, 1981), Xenopus (Capo et al, 1990), and peas (Duke and Salin, 1983), but their properties were so different that they appeared to be unique gene products. Multiple CuZn-SOD genes are known for corn (Baum and Scandalios, 1979) and for Xenopus (Schinina et al, 1989).

Electrophoretic Isomers of CuZn-SOD

Two CuZn-SOD isomers which differ in mobility by PAGE have been intensely studied. These studies, in contrast to those presented above, were undertaken with heterogeneous preparations of enzyme. These CuZn-SODs appear to be the SOD I and SOD II isomers discussed above.

Generally, electrophoretic CuZn-SOD isomers have been shown to undergo a one way interconversion of form induced by gel filtration chromatography (hSOD, Hartz and Deutsch, 1969), or ageing of the solution (hSOD, Stansell and Deutsch, 1965a; pea & corn seed CuZn-SOD, Giannopolitis and Ries, 1977). Aged solutions of rat and mouse CuZn-SOD did not show this property (Crosti and Sausa, 1980). Chronological ageing, as well as solution ageing, may contribute to the heterogeneity.

By using Ferguson plots (Hedrick and Smith, 1968), electrophoretic variants of CuZn-SOD have been shown to be size isomers (hSOD, Bannister and Wood, 1970; bSOD, Bannister et al, 1971) and charge isomers (hSOD, Crosti, 1978; watermelon cotyledon CuZn-SOD, Sandalio and Del Rio, 1986; mouse RBC, liver, kidney, lung, brain and muscle CuZn-SOD, Bloor et al, 1983, Crosti and Sausa, 1980; Photobacter leiognathi CuZn-SOD, Martin and Fridovich, 1981; rat RBC, liver, and kidney CuZn-SOD, Crosti and Sausa, 1980). Electrophoretic studies are often difficult to interpret as a variety of PAGE, and sometimes agarose, systems and conditions have been used. It is known that different numbers of electrophoretic components (1-3 or more species) are detected when the same

preparation is run using different buffers, or the same buffers differing in ionic strength or pH (hSOD, Stansell and Deutsch, 1965a; Hartz and Deutsch, 1969; Carrico and Deutsch, 1969; bSOD, Zepp et al, 1980). Sometimes a single CuZn-SOD band may be resolved into two components under certain conditions of electrophoresis. In general, enzyme preparations appear more homogeneous in high ionic strength buffers. Charge isomers of CuZn-SOD are frequently observed by IEF analysis while the existence of size isomers is less certain.

In a study of the electrophoretic isomers of hSOD, Crosi (1978) electrophoresed hSOD, eluted the two isomers from the gel, and then electrophoresed the two isolated forms of CuZn-SOD. Partial conversion of one form to another occurred and a double-banded pattern was observed for each sample. It was theorized that the isomers represent two (post-translational) active forms of the molecule which were probably in equilibrium. Such behaviour during electrophoresis would also be expected when heterogeneity is not inherent but arises from some type of interaction during the separation process (Cann, 1972; Ressler, 1973).

Multiple Forms of CuZn-SOD

Apart from the isolation and characterization of the two major forms of CuZn-SOD, a few studies have been undertaken where multiple charged forms of CuZn-SOD were isolated and their biophysical properties examined.

Three differently charged forms of rhSOD from Escherichia coli were

isolated using weak ion-exchange chromatography, although no chromatogram was shown (Kajihara, 1988a). An IEF of the purified isomers showed forms of pI 5.14, 5.06, and 4.99. The gel clearly shows that the separation of the three forms was incomplete. No differences in specific activity, UV spectra, electron spin resonance spectra, amino acid composition, metal content, or disulphide bond position were noted for the isomers. They did display some differences in their CD spectra (260nm chromophore) and in their elution pattern by reverse phase HPLC. After reduction and carboxymethylation, the isomers all showed a single form by IEF (pI≈ 5.14). The authors claim that reduction of the enzyme alone produced the same effect, although the gel indicated that only the pI 4.99 form was affected. Mutants were then constructed in which either the Cys 6 or Cys 111 residues on the protein were changed to a serine residue. The ^{6}Cys→Ser mutant appeared identical to native rhSOD while the ^{111}Cys→Ser mutant lacked the pI 4.99 isomer yet still showed some of the pI 5.06 form. It was concluded that the isomers arose from an alteration in secondary or tertiary structure derived from a structural alteration around Cys 111.

The significance of these last findings in relation to other CuZn-SOD types is unclear. E. coli rhSOD is not N^{α}-acetylated as is the natural hSOD or the rhSOD produced in yeast (Flohe et al, 1986; Hallewell, et al, 1987; Kajihara et al, 1988b). This difference is thought to be responsible for the pI differences seen between hSOD isomers and the rHSOD isomers produced in E. coli (Kajihara et al, 1988b).

Different forms of murine RBC CuZn-SOD have been isolated and studied (Shibata and Ogita, 1986). Three forms of CuZn-SOD were seen by IEF with pIs of 6.4 (A), 5.7 (B), and 5.0 (C). Two bands were observed by native PAGE. SDS-PAGE of all samples showed a single band. Two dimensional gels run under native conditions showed that each of the three different forms observed by IEF was resolved into two bands on the native gel. Most of the A form was associated with the slower moving band while most of the C form was associated with the faster moving band. The B form showed nearly equal amounts of both bands. This suggests that each pI form has two alternate and distinguishable forms. The CuZn-SODs were then eluted from gels and then rerun under a variety of conditions. Forms A and B showed a single band after IEF. When these CuZn-SODs were heated for one hour at 60°C before refocusing, the A form was resolved into bands A, B, and small quantities of a new form (D, pI≈6.5). The IEF pattern of form B was unaffected by heat treatment. This would imply that form B and D are homodimers and that form A is a heterodimer. Findings on the C form were not discussed. The charge modified heterodimer was confirmed by heating mixtures of form A or B with hSOD. The hSOD-A mixture showed the presence of two new hybrid forms while the hSOD-B mixture showed only one new hybrid. Since form D was not seen in the original sample it was suggested that the original B form of CuZn-SOD was modified on one subunit of the dimer to produce form A.

Three forms of CuZn-SOD were isolated from rat lungs by either preparative

IEF or by chromatofocusing (pIs= 5.15, 4.88, 4.75), although no chromatogram was shown (Ischiropoulos et al, 1990). The pI 4.88 form showed the highest specific activity, was the most resistant against inactivation by heat, hydrogen peroxide, diethyldithiocarbamate, and cyanide, and showed a higher metal content than the other forms studied. Diethyldithiocarbamate and cyanide are copper chelators. The other two forms of CuZn-SOD studied appeared to be partially metal depleted. In older rats the pI 4.88 form was found to be less abundant and the proportion of the other forms increased.

In another study, eight charge variants of rat liver CuZn-SOD were isolated (Yano, 1990). The specific activities of the forms correlated well with their metal content. The ratio of the results of protein level determination in the Coomassie blue protein assay of Bradford relative to that determined by the Lowry method was different for these CuZn-SODs. This ratio was found to be inversely related to specific activity. This suggests that the protein is assuming a more relaxed (open) structure as more aromatic amino acid residues become available for reaction with the Bradford dye, and that this is accompanied by a reduction in specific activity. The isomers, in order of highest to lowest specific activities, had pIs of 4.80, 4.75, 5.15, 4.70, 4.65, 4.60, and 4.50 forms. The forms also differed in specific activity at pH 10. The SDS-PAGE pattern of all forms was identical. Yano speculated that the isomers with pI values less than 4.88 were derived from an unfolding of the pI 4.88 form. This could explain the reduction in pI, the increased reaction of accessible amino acids with Coomassie blue, and the

reduced enzymatic activity. The unfolding was thought to result from oxidative damage to the enzyme. This interpretation is consistent with what is known about free radical (oxidative) damage to CuZn-SOD (discussed below). No explanation was offered about the presence of the pI 5.15 form of the enzyme.

OXIDATIVE DAMAGE TO CuZn-SOD

Metal Catalyzed Oxidation of Proteins

Glutamine synthetase (GS, L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2) from E. coli and Klebsiella aerogenes has served as a model for understanding the mechanisms by which protein oxidation and degradation occurs. A variety of other enzymes including CuZn-SOD have also been shown to be affected in this manner. The oxidation that occurs is dependent upon NAD(P)H, O_2, and Fe(III) or Cu(II) and occurs by a mixed function oxidation type mechanism by both enzymic and non-enzymic systems. The mechanisms by which this metal-catalyzed oxidation (MCO) of proteins occurs has recently been reviewed (Stadtman, 1986; Stadtman, 1990; Stadtman and Oliver, 1991).

The metal-catalyzed oxidation of proteins is a site-specific process involving the interaction of hydrogen peroxide and a Fe(II) (or other metal ion) at the metal binding site on the protein. Oxygen in the presence of transition metals can generate H_2O_2 and hydroxyl radicals (Kim et al, 1985). Oxygen will react with reduced transition metals such as Fe(II) or Cu(I) to yield superoxide radical (**Equation 4**) which dismutates to form H_2O_2 (**Equation 5**). H_2O_2 can then react

with the reduced metal ions to generate highly reactive hydroxyl radicals (•OH) via the Fenton reaction (**Equation 6**).

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\bullet -} \quad (4)$$

$$O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

$$Fe(II) + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- \quad (6)$$

Hydroxyl radicals may also be formed in the absence of metals according to the Harber-Weiss reaction (**Equation 7**):

$$H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + {}^{\bullet}OH + OH^- \quad (7)$$

However, this direct reaction is generally considered to be far too slow to be of biological importance (Lontie and Groeseneken, 1983).

The activated oxygen species (hydroxyl radical) then reacts with amino acid side chains proximal to the metal binding site. The reaction itself is viewed as a "caged" one as the activated oxygen species does not leave the site but reacts preferentially with functional groups at or near the metal binding site. With oxidized E. coli GS for example, a single histidine residue per subunit is first attacked and converted to an aspartate residue (Levine, 1983). Another histidine residue is then modified to an as yet unidentified derivative marking the enzyme for proteolytic degradation. The oxidatively modified GS is then subject to attack by a specific protease (Roseman and Levine, 1987). The native GS is not a substrate for this protease. Rat and mouse livers were also found to contain different proteases which specifically degrade oxidized E. coli GS (Rivett, 1985a;

Rivett, 1985b). In some cases of oxidant damaged proteins, the radical species may itself cleave the protein backbone (Lin and Davies, 1985; Kim et al, 1985; Davies, 1987). There is evidence that targeted degredation of MCO-marked proteins is a major mechanism by which protein turnover during normal metabolism and ageing occurs (Stadtman, 1986; Starke-Reed and Oliver, 1989).

The MCO-system typically results in the conversion of amino acid residues into carbonyl derivatives. Indeed the generation of protein carbonyl groups is used as a measure of MCO-mediated modification of proteins (Levine et al, 1990). Levels of protein carbonyl groups increase with cellular or chronological ageing and are associated with a concomitant loss of enzyme activity (Oliver et al, 1987). Histidine, proline, arginine, and lysine are particularly susceptible to MCO-reactions. Histidine is known to be converted primarily to asparagine or aspartate residues by the MCO system (Amici et al, 1989).

Generation of CuZn-SOD Heterogeneity in Vitro

Early work with bSOD showed that CuZn-SOD was inactivated by its product H_2O_2 (Fielden et al, 1974), and that a single histidine residue was destroyed in the process (Bray et al, 1974). A mechanism was proposed for the inactivation reaction (Hodgson and Fridovich, 1975):

$$Enz\text{-}Cu^{2+} + H_2O_2 \rightleftarrows Enz\text{-}Cu^{+} + O_2^{\bullet -} + 2H^{+} \quad (8)$$

$$Enz\text{-}Cu^{+} + H_2O_2 \rightleftarrows Enz\text{-}Cu^{2+}\text{-}OH^{\bullet} + OH^{-} \quad (9)$$

$$Enz\text{-}Cu^{2+}\text{-}OH^{\bullet} + ImH \rightarrow Enz\text{-}Cu^{2+} + Im^{\bullet} + H_2O \quad (10)$$

Here enzyme-bound copper is reduced by H_2O_2, followed by a Fenton-type reaction with the reduced copper and H_2O_2. This forms an oxy radical (Cu^{2+}-$OH^{\bullet}$ or Cu^{2+}-$O^{\bullet -}$) which oxidatively attacks the imidazole moiety (ImH) of a histidine residue which ligates the active-site copper. Also, in the absence of catalase, CuZn-SOD can catalyze the formation of free hydroxyl radicals directly without exogenous metals (Yim et al, 1990). Human CuZn-SOD was shown to be inactivated by H_2O_2 (Sinet and Garber, 1981). This study implicated a bound hydroxyl radical species as the inactivating agent. Yeast CuZn-SOD was also found to be inactivated by H_2O_2 with concomitant modification of a single histidine (Blech and Borders, 1983). They proposed that hydroperoxy anion, and not H_2O_2 per se, was the inactivating reagent. Subsequently this group confirmed these findings with bSOD (Fuchs and Borders, 1983). Hydrogen peroxide-induced inactivation of CuZn-SOD with histidine modification has since been described in bajra seedling CuZn-SOD (Reddy and Venkaiah, 1988). The hydrogen peroxide-induced inactivation of CuZn-SOD has also been observed for murine (Shibata and Ogita, 1986) and rat liver enzyme (Yano, 1990). Copper, zinc superoxide dismutase is inactivated by an MCO reaction in which H_2O_2, the product of the enzyme's catalytic mechanism, reacts with the enzyme's copper to produce a hydroxyl radical which destroys a copper-ligating histidine at the enzyme's active site. It is as yet not known which histidine residue is attacked. The alteration to the histidine residue has not been investigated, but likely it is converted to an

asparagine or aspartate residue as is GS (Amici et al, 1989).

The first indication that H_2O_2-induced alterations might contribute to CuZn-SOD heterogeneity came from Bartoz et al (1981). They found that bovine CuZn-SOD showed increased electrophoretic mobility by PAGE (and reduced activity) after treatment with H_2O_2. This treatment though, seemed to produce a different pattern of isomers than that observed in CuZn-SOD from old RBCs. The effect of H_2O_2 on CuZn-SOD was next studied by Jewett (1983). She showed that the reaction of H_2O_2 with bSOD was associated with the formation of the more mobile forms of the enzyme by PAGE. Three forms of CuZn-SOD were observed which were shifted towards the faster-migrating forms after this treatment. Furthermore, the inactivation of enzyme was related to the copper content. Copper was lost from treated enzyme and copper content was related to the heterogeneity of enzyme preparations. Zinc was also found to be removed from CuZn-SOD, but only at high ratios of H_2O_2/active site.

The production of electrophoretic variants of bSOD by H_2O_2 treatment has been confirmed (Mavelli et al, 1983; Gartner et al, 1985; Viglino et al, 1985; Jewett, 1986; Gartner et al, 1986; Davies, 1987; Jewett et al, 1989; Salo et al, 1990). It has been shown that H_2O_2-treated CuZn-SOD shows less affinity for it's copper ligand (Jewett et al, 1989; Salo et al, 1990). Thus a weaker protein-metal interaction appears to precede copper loss from CuZn-SOD. However, Gartner et al (1985; 1986) found that the electroforms generated by H_2O_2 were different from the natural isomers by CD, electron paramagnetic resonance-, and UV-spectroscopy.

Oxidatively Modified CuZn-SOD is Specifically Degraded in Vivo

Just as oxidatively modified GS is specifically degraded in vivo, so is CuZn-SOD. Bovine CuZn-SOD that has been exposed to H_2O_2 or superoxide radicals is degraded by a specific protease termed macroxyproteinase (MOP)(Salo et al, 1990). When CuZn-SOD was exposed to either H_2O_2 or superoxide anions, an inactivation occurred associated with charge changes, partial denaturation (unfolding), and copper loss from the enzyme (Davies, 1987; Salo et al, 1990). After these events the enzyme became a substrate for MOP. This protease is a 670 kD ATP-independent complex found in RBCs and other cell types, which degrades oxidatively modified proteins. It may be the same protease complex that degrades oxidatively modified GS (Rivett, 1985a; Rivett, 1985b). These proteases are thought to recognize a variety of oxidized substrates which have undergone a conformational change endowing them with increased hydrophobicity. MOP will also act on oxidatively modified bovine serum albumin and hemoglobin (Davies and Goldberg, 1987a and 1987b; Pacifi and Davies, 1988).

MATERIALS AND METHODS

Materials

Polypropylene glycol PPG 2025 antifoaming agent was obtained from BDH. Bacto-agar and yeast nitrogen base without amino acids were from Difco Laboratories (Detroit, MI). Glass beads were obtained from Glen Mills Inc. (Maywood, NJ); cytochrome c was horse heart type III from Sigma; xanthine oxidase was from Boehringer Mannhein; ampholytes were from Pharmacia (Pharmalytes pH 4-6.5 range). Standard human red blood cell CuZn-SOD was a gift from Dr. W.H. Bannister, University of Malta. He originally acquired this enzyme from Carlsberg Biotechnology of Denmark. Another human red blood cell CuZn-SOD standard was from Rh Pharmaceuticals Inc. (WinSOD). Yeast CuZn-SOD was a generous gift from C. Campbell (formerly ABI Biotechnology, Inc.). All other materials used were standard laboratory grade.

Methods

Fermentative Growth of Recombinant Yeast

The recombinant yeast used in this study contain a plasmid expressing human superoxide dismutase (rhSOD). This strain is known as AH22p37HSOD. The construction and properties of this strain have been described (Campbell, 1990). The rhSOD is expressed constitutively, and the yeast can be grown selectively in media lacking leucine. For large scale fermentations, a complex medium such as this, is impractical in terms of cost and labour. Therefore, non-

selective medium was used in the fermenter.

Yeast were grown in progressively larger volumes over a period of time. Preparation of yeast for the fermenter started with the transfer of a loopfull of organisms taken from stock cultures and spread onto plates of medium lacking leucine. This selective medium is called YNB/AAB-L. One litre contains 6.7g Difco yeast nitrogen base without amino acids, 0.1M sodium succinate, 2% glucose, and 50μg/mL (final concentration) of amino acid solution (500mg each of phenylalanine, histidine, tyrosine, tryptophan, lysine, adenine, and uracil, dissolved in one litre of water as a 10X concentrated stock), pH 5.8. After two days of incubation at 29°C, a loopfull of this material was transferred to a test tube containing 3mL of YNB/AAB-L and incubated (29°C) for 24 hours. This suspension was then placed into a 250mL shake flask containing 40mL YNB/AAB-L and incubated for 24 hours at 29°C in an orbital shaker (New Brunswick Scientific Co. Series 25D Incubator shaker) at 200 R.P.M. agitation. Next, 10mL of these cells were put into a 2L shake flask containing 400mL of YEPD medium. This non-selective (complete) medium contains 1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose (added from a pre-sterilized 40% stock solution), pH6.0. This flask was incubated (24 hours, 29°C, 200 R.P.M.) and then loaded into a fermenter (Bioengineering L1523, Switzerland, 13L working volume) containing 11.7L YEPD lacking glucose and 0.1% PPG 2025 antifoaming agent. Glucose was added from a 20% feed solution at 48mL/hour for 5 days. The fermenter was run at 200 R.P.M. and 30°C. Dissolved oxygen was maintained at around 70% (200mm Hg). Foaming was a problem

throughout fermentation and an additional 30mL of antifoaming agent PPG 2025 were added. Samples of yeast were taken either two or three times a day during the fermentation and the absorbance (600nm), and pellet weight (wet weight yeast) determined. Samples of yeast cells were also lysed and supernatants examined for protein content and SOD activity. Fermentation continued for a total of 122.5 hours. Yeast were harvested by centrifugation of the ferment at 13,680 g (9000 R.P.M.) for 10 minutes at 15°C. Pellets were washed once with 0.85% NaCl (saline) and centrifuged again as above. Pellets were pooled, weighed, and stored at -80°C. All work was performed in accordance with NIH guidelines for research involving recombinant DNA molecules.

Plating of Yeast

Yeast samples were diluted with saline and 0.1mL samples plated on selective (YNB/AAB-L) and non-selective (YEPD) media which was made up to 1.5% agar. All samples were plated in duplicate and the average counts per plate were used when there were between 30-300 organisms per plate.

Absorbance Measurements

Yeast samples were diluted with saline to give absorbance readings of between 0.2-0.3 at 600nm on a Shimadzu UV-160 UV-visible recording spectrophotometer. The average value of three separate readings at this dilution was used to determine the optical density. Optical density was defined as the

average absorbance times the dilution factor.

Absorption spectra of CuZn-SOD samples were taken from scans from 200-800nm.

Wet Weight Yeast

Yeast pellet weight was determined by centrifuging 10mL samples in pre-weighed tubes at 2800 R.P.M. (3000g) for 10 minutes at room temperature. The pellets were then washed with 10mL of saline and centrifuged as above. Wet weight of yeast is the weight of the tube with the pellet less the weight of the tube.

Cell Disruption

Small samples (ca 10mL) of yeast were lysed essentially by the method of Harlow and Lane (1988). Pellets obtained from 10mL samples of yeast (see wet weight yeast above) were resuspended in 2mL extraction buffer (20mM Tris-HCL, 10mM $CuSO_4$, pH 8.2) and enough 0.5mm glass beads were added so as to be just under the meniscus. Each tube was then vortexed ten times, for 2 min. at a time at full speed on an American Scientific Products S/P vortex mixer. The tubes were cooled on ice between runs.

The extent of cell lysis was checked microscopically. Samples of treated yeast were added to an equal volume of 0.02% Trypan Blue and the extent of exclusion of the dye from cells observed. If cell breakage was less than about 90%, disruption was continued until this level was reached.

Lysed cells were then centrifuged at 2000g for 5 minutes at 15°C. Supernatants were collected and the volumes recorded.

Protein Assay

Protein concentration was determined with the BCA (bicinchoninic acid) assay according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL); bovine serum albumin was used as a standard . The protein concentration of highly purified material was determined using the micro-BCA assay. This required only one tenth the materials and was performed in microtitre plates. Absorbance measurements (570nm) were taken on a Titertek ELISA plate reader.

SOD Activity Assay

Superoxide dismutase activity was determined using the classical cytochrome c method with which the enzyme's activity was originally described (McCord and Fridovich, 1969). Raw data were analyzed by the method of Flohe and Otting (1984) and Ysebaert-Vanneste and Vanneste (1980). The cytochrome c based assay is an indirect assay of SOD activity. A xanthine/xanthine oxidase reaction produces a constant flux of superoxide radicals. Cytochrome c (III) acts as a detector. It's reduction by superoxide radicals is monitored at 550nm, where the difference in molar extinction of the reduced and oxidized cytochrome c is 18,300-21,000 $M^{-1}s^{-1}$ (Goldstein et al, 1988). Any SOD present interferes with cytochrome c reduction by competition for superoxide. One unit of enzyme activity

is defined as that amount of SOD which causes 50% inhibition of the initial rate of reduction of cytochrome c under the conditions specified (McCord and Fridovich, 1969). These conditions are 50mM KPi, 0.1mM EDTA, 50μM xanthine, 10μM ferricytochrome c, and enough xanthine oxidase (ca 6nM) to cause a change in absorbance at 550nm = 0.025 AU/min, at pH 7.8 and 25°C in a total volume of 3 mL. Ultrapure SOD preparations typically give activities of 3000 U/mg in this assay. All data were derived from average values obtained from duplicate determinations at each dilution used.

Purification of rHSOD

Recombinant human CuZn-SOD was purified by disruption in a bead beater, heat treatment of the supernatant, and ion-exchange chromatography. Purification of these SODs are described below. Different batches of standard rhSOD were purified using the same method, sometimes followed by an additional step of copper chelate affinity chromatography (Weselake et al, 1986a). Here, proteins are retained in the column as a ternary complex of the matrix, iminodiacetate, the metal, copper, and the protein. Interaction of the protein with the matrix occurs primarily through interactions with histidyl residues (Arnold, 1991). Proteins are eluted from the column using high concentrations of salt.

Yeast were lysed as follows. A 20.0g wet weight sample of recombinant yeast was thawed from a frozen batch and disrupted mechanically in an 80mL bead beater (Biospec Products, Bartlesville, OK). Yeast were suspended in 60mL

of extraction buffer (20mM Tris-HCl, 2mM $CuSO_4$, pH 8.2), 0.1-0.15mm diameter glass beads were added to bring the level of the meniscus to three quarters volume full, and then more extraction buffer was added to fill the chamber. The bead beater was run for 1 minute on and three minutes off in a 4°C cold cabinet for a total of twelve times. The extent of lysis was checked microscopically (see cell disruption section above). The disrupted material was then strained through wire mesh.

Heat treatment followed by anion-exchange chromatography was used to quickly purify thermostable SOD (Gartner et al, 1984). The disrupted material was placed in sealed tubes and then into a water bath at 65°C for 2 hours. After this, the solution was centrifuged at 1245g (3500 R.P.M.) for 10 minutes and the supernatant passed through a 0.2μM filter. This material was then dialyzed against 10mM Tris-HCl, pH 7.5.

The material collected after the heat treatment step was loaded directly onto a 25mL (2.5X7cm) column of Q-Sepharose which had been equilibrated with 10mM Tris-HCl, pH 7.5. Several column volumes of start buffer (10mM Tris-HCl, 0.075M NaCl, pH 7.5) was used to elute contaminants. This was followed by a linear gradient from the start buffer (0.075M NaCl) to 10mM Tris-HCl, 0.5M NaCl, pH 7.5 at 1.78mL/min. at 1 absorbance unit full scale with the chart recorder at 1mm/minute. Ninety-nine fractions were collected.

Gel Electrophoresis

Electrophoresis was performed either in an LKB Vertical electrophoresis unit or in Pharmacia Mini Protean II cells. Native polyacrylamide gel electrophoresis (PAGE) was run using 12% resolving gel, either according to the method given in the Hoefer catalogue, or according to Ausubel et al (1989). The latter method was found to give clearer resolution. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% resolving gels according to the Mini Protean II instruction manual after the method of Laemmli (1970). Lyophilized samples were placed 1:1 into 2X dithiothreitol (DTT)-containing sample buffer (immediately before use, 0.124g of DTT was added to 10mL of a solution of 10mL SDS, 6.25mL 0.5M Tris-HCl, pH 6.8, 5mL glycerol, 0.01g bromophenol blue) and placed into a boiling water bath for 5 minutes. Monomer molecular weights of unknown samples were determined from plots of log molecular weight of the standard proteins versus distance of migration from the origin. The Pharmacia low molecular weight electrophoresis calibration kit was used. This mixture contained phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

Gel Staining

Gels were stained with Coomassie blue by placing them into 0.1% Coomassie Brilliant Blue R-250 in destain (30% ethanol or methanol, 10% glacial

acetic acid) for about an hour followed by successive changes of destain until the background stain had cleared sufficiently.

Gels were stained for SOD activity by the method of Beuchamp and Fridovich (1971). In this method, photochemically reduced flavin generates superoxide radicals which reduce nitro blue tetrazolium (NBT) to blue formazan (blue colored). Superoxide dismutase inhibits the reduction of NBT and is visualized as achromatic zones on otherwise uniformly blue gels. Gels were placed into a 0.1% NBT solution for about 20 minutes on a gel shaker. The gels were then transferred into a solution of 0.32% TEMED and 0.008% riboflavin in 10mM potassium phosphate buffer, pH 7.4, for 20 minutes on a gel shaker. Finally the gels were placed on a dry dish and illuminated on a gel viewing box for about 10 minutes. The reaction was then stopped by placing the gels into distilled water.

Isoelectric Focusing

Isoelectric focusing of samples was performed using the LKB 2117 Multiphor II electrophoresis unit with the LKB 2297 Macrodrive 5 constant power supply essentially according to the method recommended by the manufacturer. Gels were cast using the 0.5mm spacer and contained 4.3mL of 29:1 acrylamide:bis-acrylamide, 14mL of 16.8% sucrose, and 1mL each of pH range 4-6.5 ampholines (Pharmalyte). This solution was degassed before use and then allowed to polymerize for at least an hour after the addition of 1mL of freshly prepared 1% ammonium persulfate. The cathode strip was wetted with a solution of 1M sodium

hydroxide and the anode strip with a solution of 1M phosphoric acid. The polymerized gel was then pre-focused for 1.5 hours at 1000V, 40mA, 5W. Samples were brought to the same volume (usually 10μL), loaded onto sample pads (LKB), and focused for 3.5 hours as above. Sample pads were removed about one hour after focusing had begun. Isoelectric points were determined by comparing the mobility of unknown proteins with a standard curve of mobility verses pI made with pI standards. The Pharmacia low pI calibration kit was used. It contained human carbonic anhydrase (pI 6.55), bovine carbonic anhydrase B (pI 5.85), ß-lactoglobulin A (pI 5.2), soybean trypsin inhibitor (pI 4.55), glucose oxidase (pI 4.15), and methyl red dye (pI 3.75).

Circular Dichroism

Light polarized in a plane is broken down into two components which are circularly polarized. These two components' electric vectors travel in right-handed and left-handed orientations and they are equal in magnitude. These components are absorbed to different extents by optically active molecules such as proteins. The vectors are no longer equal in magnitude and the light leaving the sample is elliptically polarized. Circular dichroism (CD) measures ellipticity (Θ), which is proportional to the difference in the absorbance of left- and right-handed circularly polarized light. Circular dichroism is used as an experimental measure of secondary structure.

Proteins are composed of a variety of secondary structure elements such

as α-helix, ß-sheet, and unordered (or random coil) structures. Other distinctions can be made, for example, β-sheets can be described as being parallel or antiparallel, or as being composed of ß-sheet and ß-turn elements. The observed ellipticity of a protein at a given wavelength $[\Theta_\lambda]$ can be considered as the sum of the ellipticities of the individual secondary structure components of which it is constructed (**Equation 11**):

$$[\Theta_\lambda] = f_\alpha[\Theta_\lambda]_\alpha + f_\beta[\Theta_\lambda]_\beta + f_\gamma[\Theta_\lambda]_\gamma \quad (11)$$

Where f values represent the fraction of α-helix (f_α), ß-sheet (f_β), and random coil (f_γ) structures, and $[\Theta_\lambda]$ values represent the corresponding ellipticities at a certain wavelength λ. Analysis of secondary structure involves the simultaneous solution of these three (or four) equations at each wavelength tested, and comparison to a series of spectra for known secondary structure generated from a database.

Circular dichroism measurements were carried out at room temperature using a Jasco J-500A spectropolarimeter which had been calibrated with epi-Androsterone. The optical path was 0.101 cm in a quartz cuvette, and spectra were scanned from 270 to 200 nm. Protein was at a concentration of between 0.094-0.151 $mg.mL^{-1}$ in 50mM sodium phosphate buffer, pH 7.5. The protein concentration was determined just after CD measurements were taken using the Pierce BCA assay using BSA as a standard. Each spectrum was recorded in triplicate and corrected for the base line contribution of the buffer. The ellipticity values from the CD spectra were digitized manually at 3nm intervals from 270-201nm and converted to mean residue ellipticity $[\Theta]_{MR}$ ($degree.cm^2.decimole^{-1}$) from

Equation 12:

$$[\Theta]_{MR} = \Theta M/10lc \qquad (12)$$

where Θ is the measured ellipticity, M is the mean residue weight = 103.3 defined as the average molecular weight of the protein per amino acid residue (molecular weight/number of amino acid residues) calculated from the composition of the protein (Hallewell et al, 1985), l is the optical path length in cm, and c is the protein concentration in mg/mL.

Circular dichroism spectra were analyzed to estimate protein structure by a number of different methods (Chen et al, 1978; Bolotina et al, 1980; Brahms and Brahms, 1980). These algorithms use a least-squares method to fit the CD spectrum of the protein of interest to a series of reference spectra based on the known secondary structure of proteins determined by X-ray crystallography (reviewed in Yang et al (1986)). In the method of Bolotina et al (1980), ß-structures are defined as ß-sheet and ß-turn elements.

Heterodimer Formation

Yeast-human CuZn-SOD heterodimers were produced in a manner similar to that of Tegelstrom (1975). Equal quantities of human and yeast CuZn-SODs were vortexed and then placed into a 65° C water bath for two hours. After this the tubes were kept at 4° C till required (hours-days).

RESULTS

Fermentative Growth of Recombinant Yeast

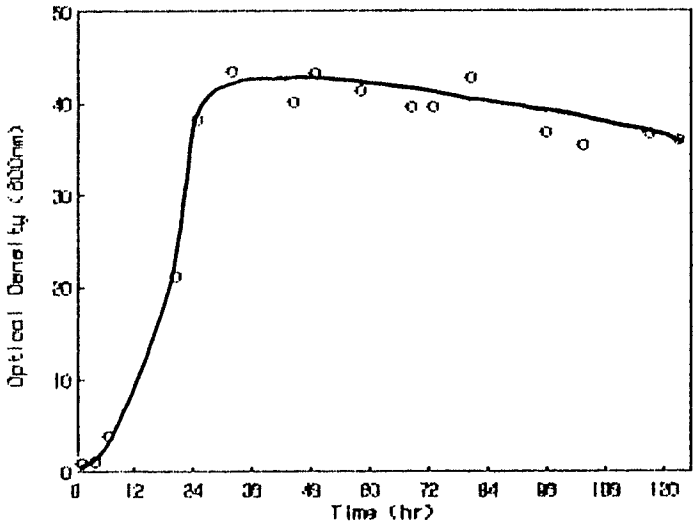
Recombinant yeast were fermented for a total of 122.5 hr. resulting in the harvesting of 644g (wet weight) yeast from 12.5L of ferment. Graphs illustrating the change in wet weight yeast, absorbance, soluble protein, and SOD activity during a typical fermentation are presented in **Figure 3.** Log phase growth continued until between 36-48 hr. of fermentation as illustrated by the linear increases in wet weight yeast, absorbance, soluble protein, and SOD activity during this period. Each of these assays was performed twice. Much of the fermentation was performed under conditions of stationary phase growth. No significant changes in wet weight, absorbance, soluble protein, or SOD activity occurred after about 48 hours of fermentation. Plating of the yeast near the end of the fermentation (72.5 hr.) on selective (YNB/AAB-L) and non-selective (YEPD) agar plates showed $5.5X10^8$ organisms/mL and $7.8X10^8$ organisms/mL respectively. The reduced levels of growth on plates under selective conditions may indicate plasmid loss from a proportion of the population. Other experiments at ABI Biotechnology Inc. in which rhSOD-containing yeast from single colony isolates were grown in shake flask cultures also had reduced growth on selective verses non-selective media (H.A. Kaplan, ABI Biotechnology Inc., personal communication).

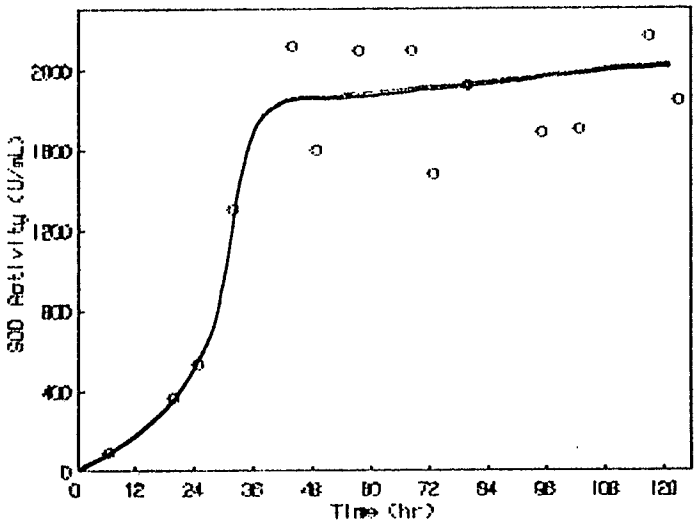
Isoelectric Focusing of Yeast and Human SODs

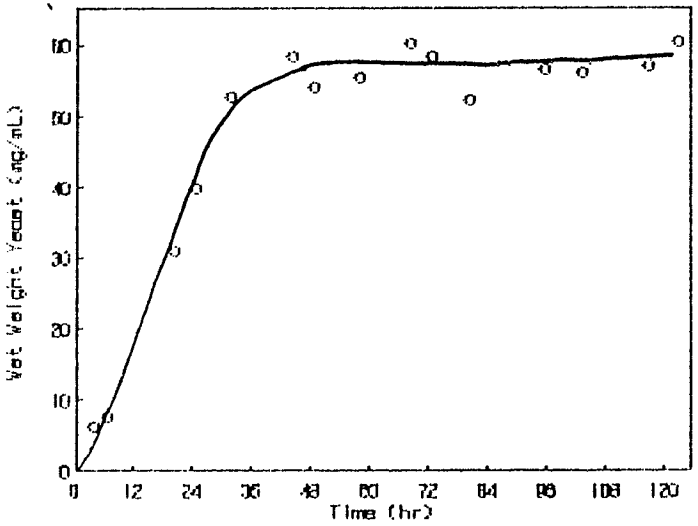
Yeast CuZn-SOD (ySOD) and human CuZn-SOD (hSOD) or recombinant

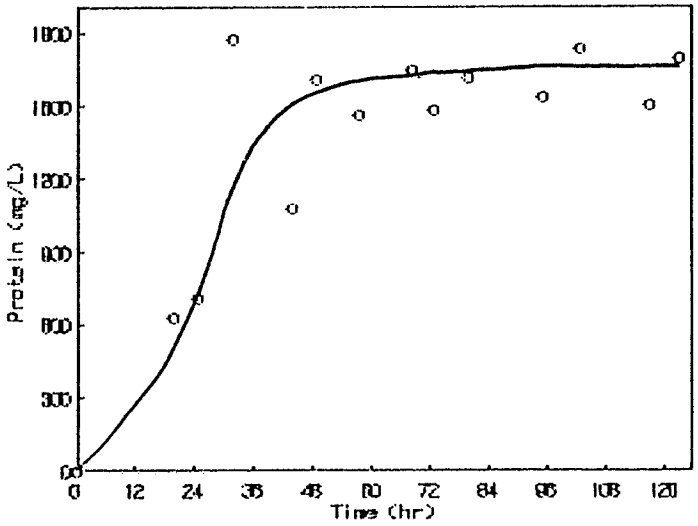
Figure 3. Growth of recombinant yeast during fermentation.

Small samples of yeast collected during the fermentation were assayed for absorbance (600nm), pellet weight, soluble protein, and superoxide dismutase activity. Average values of triplicate determinations are shown as a function of time of fermentation.


Optical Density (600nm)
Time (hr)


SOD Activity (U/mL)
Time (hr)


Wet Weight Yeast (mg/mL)
Time (hr)


Protein (mg/L)
Time (hr)

hSOD produced in yeast (rhSOD) were clearly resolved by isoelectric focusing (IEF, **Figure 4**). Yeast CuZn-SOD showed a single molecular form with a pI of approximately 5.25. Human RBC CuZn-SOD from two different commercial sources (WinSOD and Carlsberg SOD) and rhSOD showed three forms with pIs of 4.9 (range 4.9-4.95), 5.2 (range 5.1-5.2), and 5.35 (range 5.26-5.45). The Carlsberg SOD showed a nearly equal distribution between the three isomers; WinSOD however, showed predominantly the pI 4.9 and pI 5.2 forms, and very little of the pI 5.35 form. The ySOD always appeared at a position between the hSOD pI 5.2 and pI 5.35 form. The ySOD focused at a distinct pI after mixing it with rhSOD before IEF. This indicates that yeast CuZn-SOD is distinguishable from the human isomers by IEF.

To investigate whether any of the three forms of rhSOD were derived from heterodimer formation between subunits of recombinant human and yeast CuZn-SOD monomers, samples of the recombinant human and yeast enzymes were mixed and then heated for two hours at 70° C. An IEF of this material is shown in **Figure 5.** The focusing pattern of yeast CuZn-SOD was unaffected by this heating. Human CuZn-SOD showed a reduction in the amount of the pI 4.9 form, and the presence of a new band with a pI of 5.4-5.5. Two faint bands were observed in samples of yeast and human CuZn-SOD which were mixed and heated before focusing, that were not observed in either yeast or human CuZn-SOD samples which were also heated before focusing. These yeast-human heterodimers focus at pI 4.6, and pI 5.85, at positions distinct from that of the other CuZn-SOD isomers.

Figure 4. Isoelectric focusing of yeast, human, and recombinant human CuZn-superoxide dismutases.

CuZn-SOD samples were analyzed by IEF on a pH 4-6.5 gradient at 15µg/lane. The gel was stained with Coomassie blue. The pI's of some standard proteins are shown on the right.

Lane 1 shows a preparation of recombinant human CuZn-SOD.

Lane 2 shows yeast CuZn-SOD.

Lane 3 shows a mixture of yeast CuZn-SOD and recombinant human CuZn-SOD.

Lane 4 shows human red blood cell CuZn-SOD (Carlsberg SOD).

Lane 5 shows human red blood cell CuZn-SOD (WinSOD).

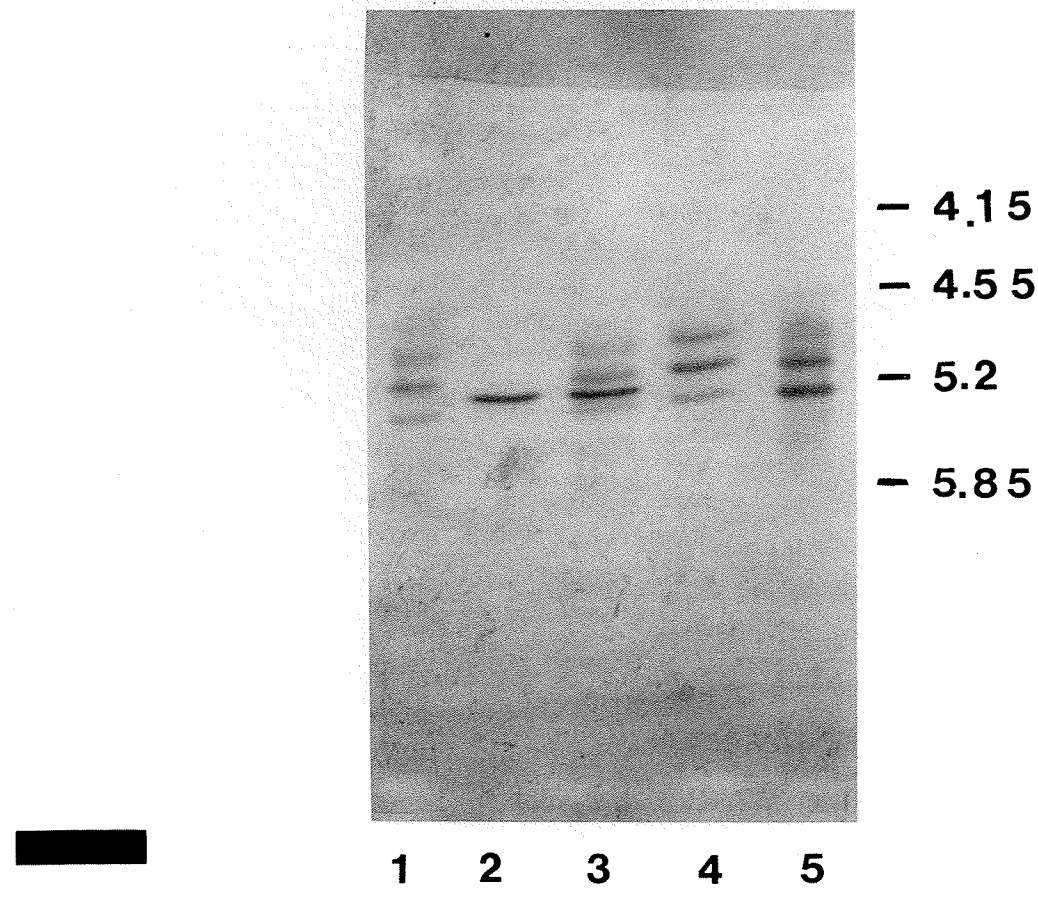

4.15
4.55
5.2
5.85
1
2
3
4
5

Figure 5. Isoelectric focusing of yeast-human heterodimers.

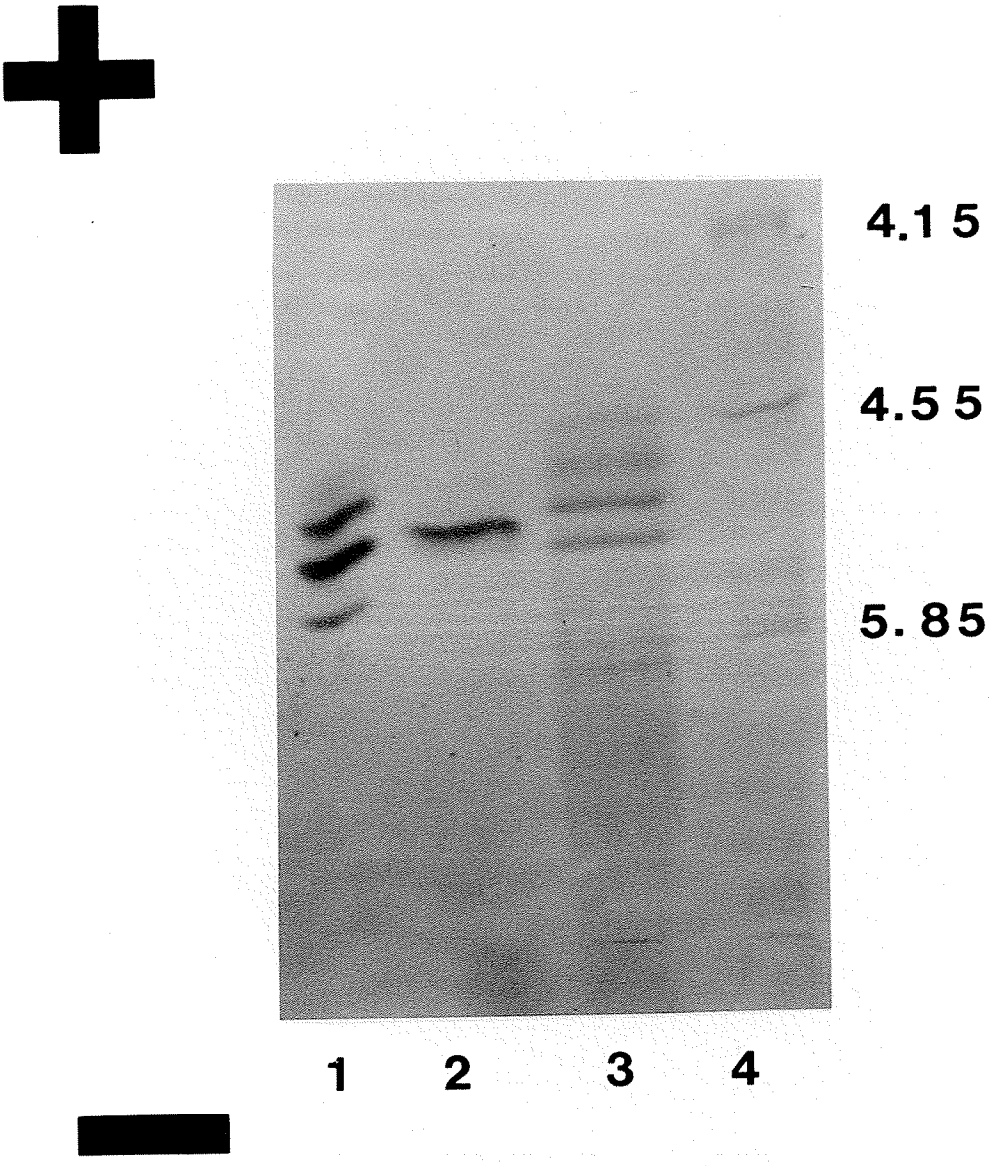
Equal amounts of yeast and human CuZn-SOD were heated for two hours at 70°C and then run on a pH 4-6.5 gradient isoelectric focusing gel along with samples of human and yeast CuZn-SODs. Each lane contains 15μg protein. The gel was stained with Coomassie blue.

Lane 1: human CuZn superoxide dismutase.

Lane 2: yeast CuZn superoxide dismutase.

Lane 3: Mixture of heated human and yeast CuZn superoxide dismutases.

Lane 4: pI standard proteins; pIs are indicated on the right.


4.15
4.55
5.85
1
2
3
4

Also, no increased staining was evident in the three positions occupied by the hSOD isomers or yeast CuZn-SOD. This suggests that no yeast-human heterodimers were formed which co-focused at these positions.

Purification of Two Major Forms of rHSOD

Recombinant yeast were heat treated for two hours at 65°C. Q-sepharose chromatography of a heat treated extract of the recombinant yeast resolved two major peaks and one minor peak. Five fractions were isolated and pooled (I-V, **Figure 6**). Pools I-V each contained SOD activity, though pool V had a low specific activity. Unfortunately, there was not enough pool V material available for further characterization. The material in the second peak showed a higher specific activity than material taken from the first peak. **Table 3** summarizes the data collected during rhSOD purification. A combination of bead beating, heat treatment, and ion-exchange chromatography resulted in a 12.6-63 fold purification of rhSOD with a 23% recovery of the SOD activity present in the original extract. A total of 6.52mg rhSOD was purified from 20.9g rhSOD-containing yeast.

Characterization of Two Forms of rhSOD

Native PAGE of the fractions collected by ion-exchange chromatography revealed that separation of the two major native forms of rhSOD was achieved (**Figure 7**). Fractions I and IV were catalytically active forms of rhSOD which differed most markedly in PAGE mobility (**Figure 8**). These fractions contained the

Figure 6. Q-sepharose chromatography of recombinant human CuZn-superoxide dismutase.

Yeast extract (84.5mL) was loaded onto a 25mL column of Q-Sepharose which had been equilibrated with buffer (10mM Tris-HCl, 0.075M NaCl, pH 7.5). This was followed by a linear gradient to 0.5M NaCl. Five pools containing SOD activity (I-V) were collected separately. Pool I contained fractions 8-15, II contained fractions 16-21, III contained fractions 22-29, IV contained fractions 30-36, and pool V contained fractions 37-40.

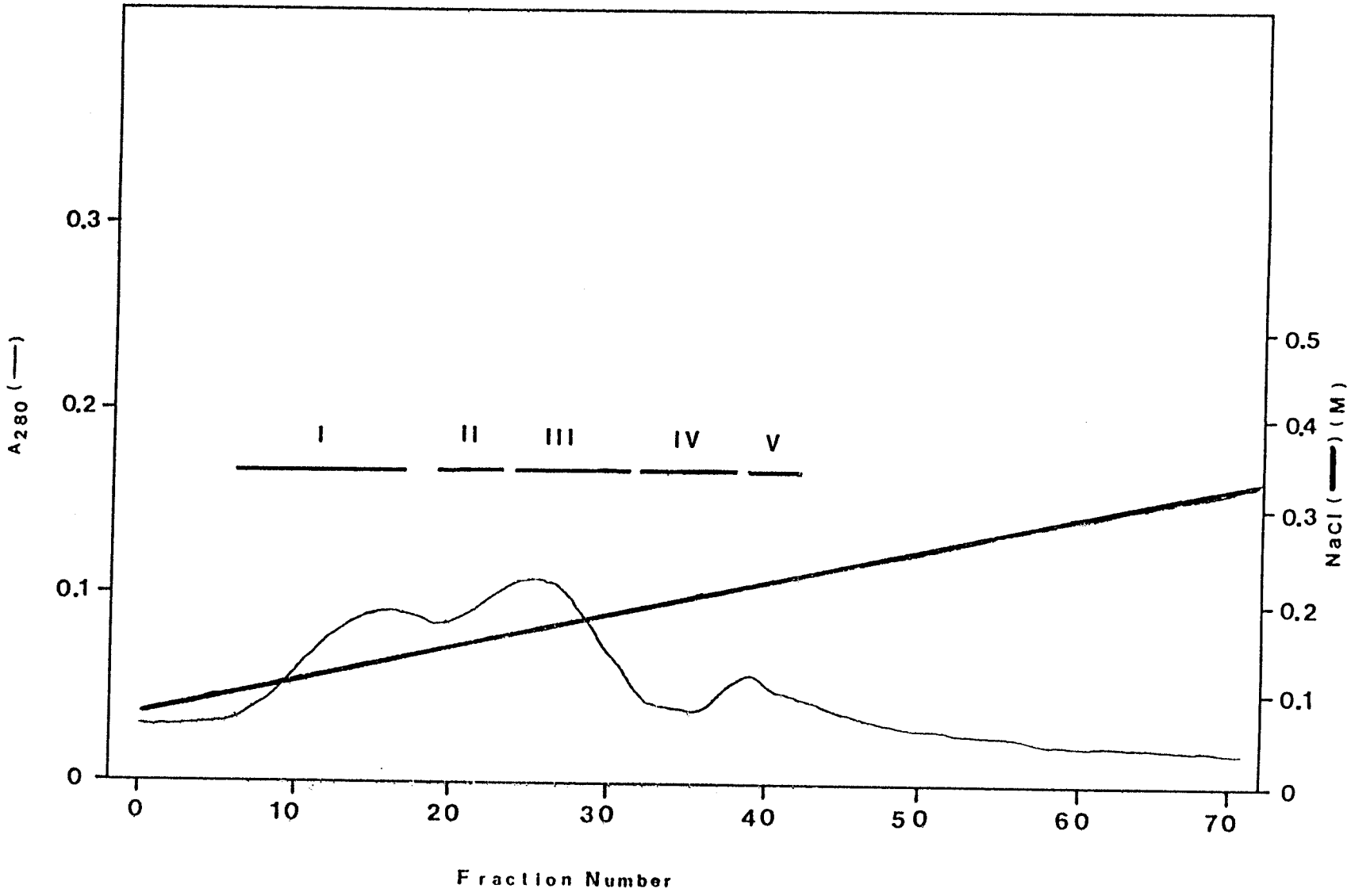

0.3
0.2
0.1
0
A280 (—)
I
II
III
IV
V
0.5
0.4
0.3
0.2
0.1
0
Nacl (—) (M)
0
10
20
30
40
50
60
70
Fraction Number

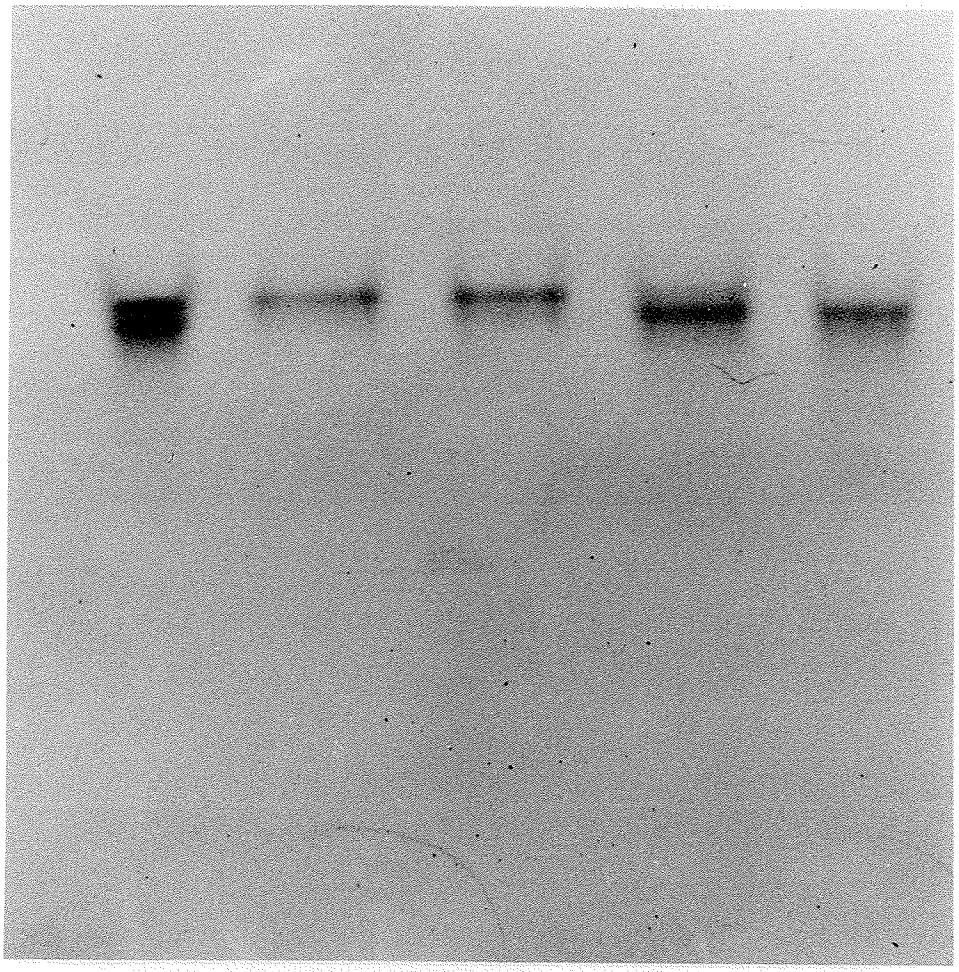
Table 3. Summary of data from purification of recombinant human CuZn-superoxide dismutase.

STEP	VOLUME (mL)	PROTEIN (μg/mL)	TOTAL PROTEIN (mg)	SOD ACTIVITY (U/mL)	TOTAL SOD ACTIVITY (U)	SPECIFIC ACTIVITY (U/mg)	PURIFI-CATION FACTOR (FOLD)	YIELD (%)
Bead Beating	112	12,200	1,370	708	79,300	59.7	1.00	100
Heat Treatment	85.0	626	53.2	364	30,900	581	10.0	39.0
Dialysis against Tris	84.5	440	37.2	220	18,600	500	8.60	23.0
Q-Sepharose Chroma-tography								
Fraction I	22.0	46.1	1.01	93.5	2,060	2,040	35.0	2.60
Fraction II	15.5	123	1.91	300	4,660	2,440	42.0	5.90
Fraction III	22.0	97.0	2.13	356	7,820	3,670	63.0	9.90
Fraction IV	18.5	59.1	1.09	197	3,640	3,340	58.0	4.60
Fraction V	10.5	35.8	0.376	26.1	274	730	12.6	0.350
Total:	88.5	NA	6.52	NA	18,500	NA	NA	23.0

NA = not applicable.

Figure 7. Native polyacrylamide gel electrophoresis of fractions collected by Q-sepharose chromatography.

Lanes 1 contains a standard preparation of recombinant human CuZn-SOD. Lanes 2-5 show recombinant human CuZn-SOD Q-sepharose chromatography fractions I-IV. The gel was stained with Coomassie blue and each lane contains 4μg protein.

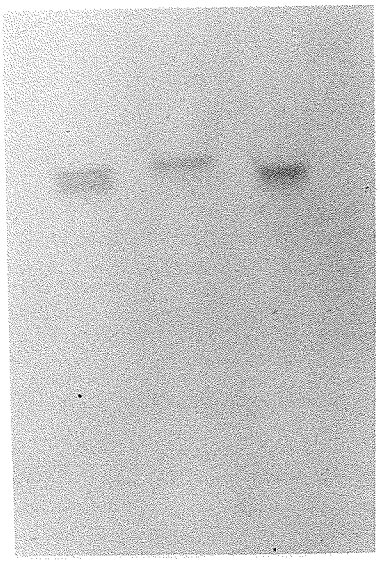


1 2 3 4 5

Figure 8. Native gel electrophoresis of rhSOD I and rhSOD II.

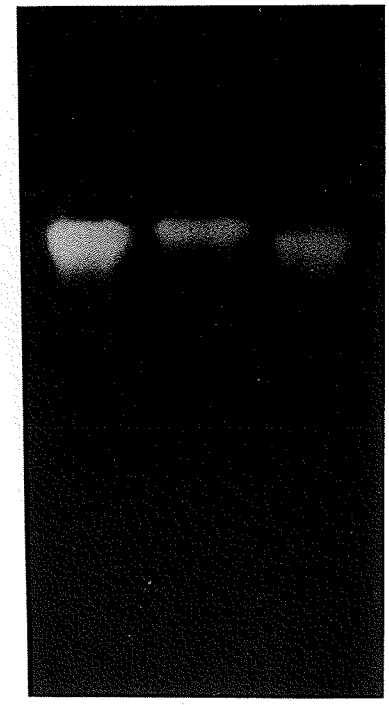
Lanes 1 shows a standard preparation of recombinant human CuZn-SOD. Lanes 2 and 3 contains fractions 1 (rhSOD I) and 4 (rhSOD II) taken from Q-sepharose chromatography respectively. Gel A was stained with Coomassie blue and gel B was stained for SOD activity. Each lane contains 4μg protein.

A



1 2 3

B



1 2 3

least carry-over from incomplete separation of the two major peaks during chromatography. These samples were chosen for investigations into the structure of these different CuZn-SOD forms. For convenience, fraction I was referred to as rhSOD I and fraction IV as rhSOD II. A standard sample of rhSOD had both of these isomers (**Figures 7 and 8**).

No major differences were noted in a comparison of the absorption spectra of rhSOD I and rhSOD II (data not shown). Using the cytochrome c activity assay, rhSOD I had a specific activity of 2,040 U/mg and rhSOD II a specific activity of 3,640 U/mg. Pure bovine or human CuZn-SOD generally gives a specific activity of approximately 3,000 U/mg in this activity assay. An IEF of these isomers is shown in **Figure 9**. Standard rhSOD was resolved into three distinct forms with pI's of 4.9, 5.2, and 5.35 in Coomassie stained gels. The rhSOD I had the pI 5.2 and 5.35 forms, and a small amount of a pI 5.8 form. The rhSOD II had the presence of pI 4.9 and 5.2 forms. These forms of rhSOD were active when IEF gels were stained for SOD activity (data not shown).

SDS-PAGE indicated that the two SODs differed in subunit composition (**Figure 10**). The standard rhSOD had three components of subunit molecular weight 20 kD, 21 kD (predominant species), and 22 kD. The rhSOD I was primarily composed of the 20 kD form, and small amount of the 21 kD form, while rhSOD II had 21 kD and 22 kD forms, with a predominance of the 21 kD form. Similar results were observed with hSOD (data not shown).

Circular dichroism (CD) of rhSOD, rhSOD I, and rhSOD II was undertaken

Figure 9. Isoelectric focusing of rhSOD I and rhSOD II.

Samples of recombinant human CuZn-SOD were analyzed by isoelectric focusing using a pH 4-6.5 gradient with 15μg protein/lane. The pI of some standard proteins are shown on the right. The gel was Coomassie stained.

Lane 1 contains recombinant human CuZn-SOD.

Lane 2 contains rhSOD I.

Lane 3 contains rhSOD II.

Lane 4 contains pI standards; the pIs are shown on the right.

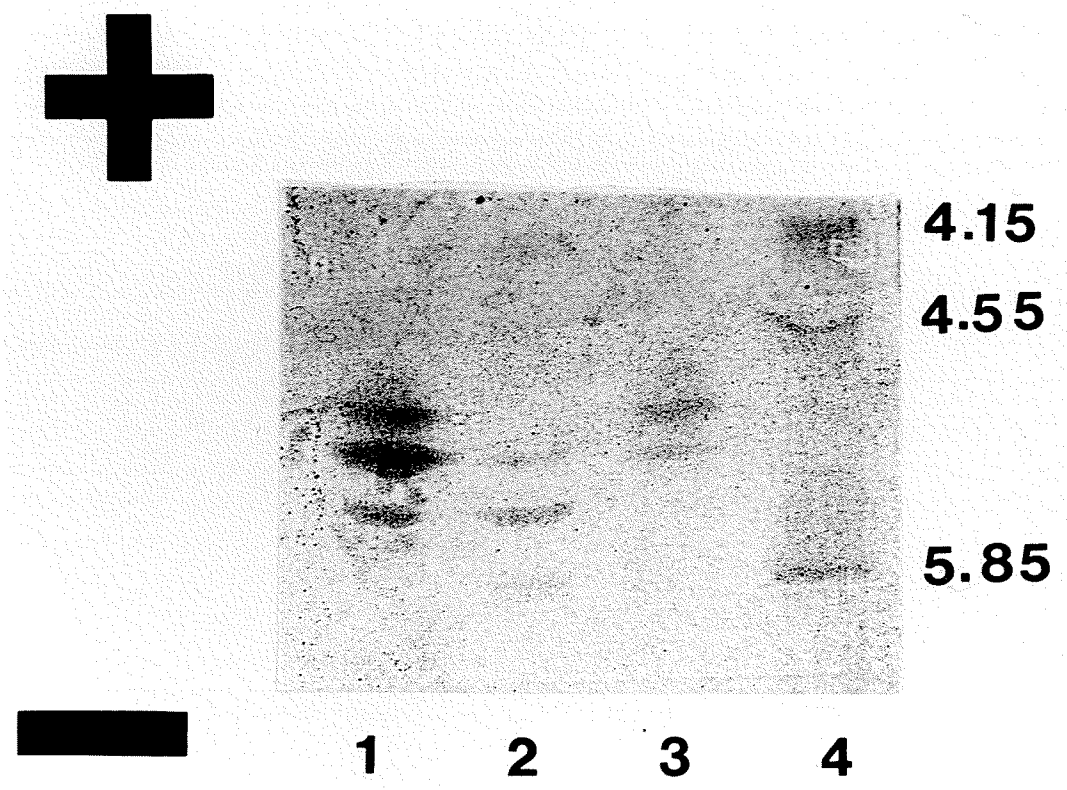

4.15
4.55
5.85
1
2
3
4

Figure 10. SDS-PAGE of recombinant human CuZn-superoxide dismutase samples.

Samples were boiled for five minutes and then run on 15% reducing and denaturing polyacrylamide gels at 4μg/lane. The migration of molecular weight standards (kD) is shown on the right. The gel stained with Coomassie blue.

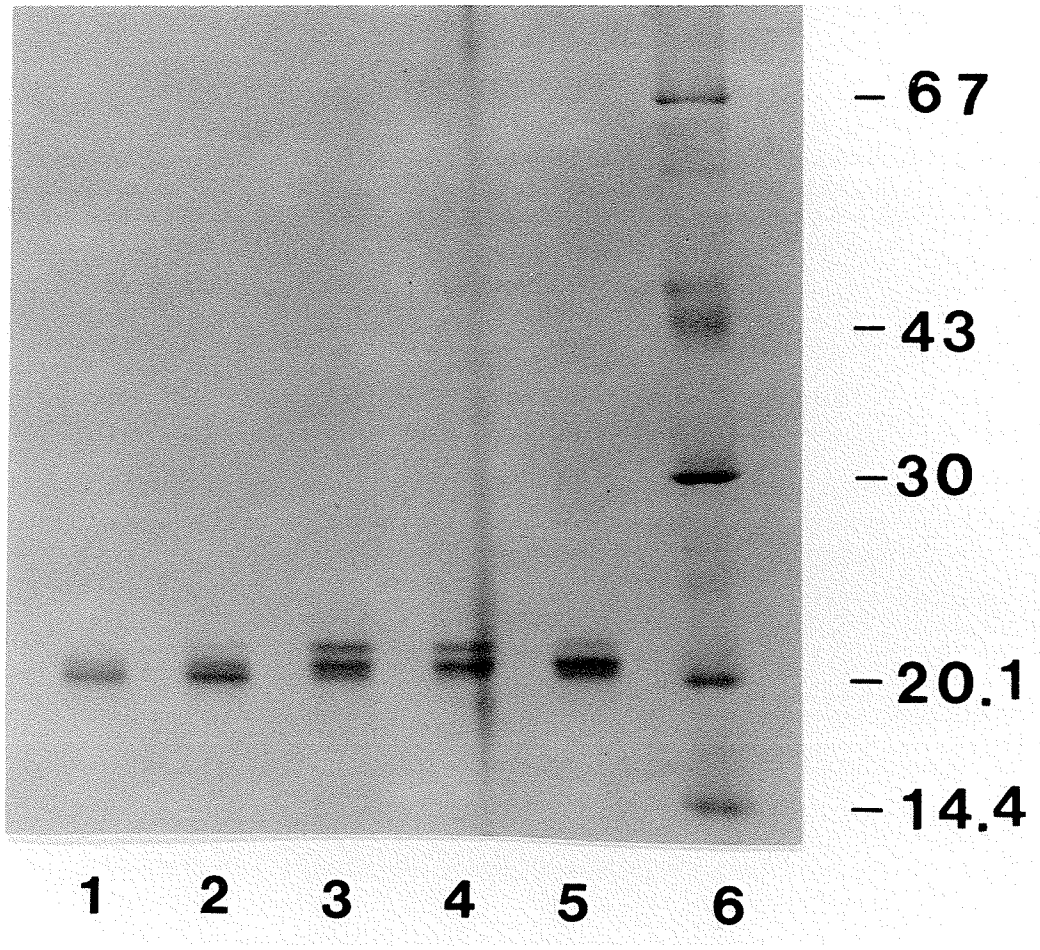
Lane 1 contains rhSOD I (Q-sepharose chromatography pool I).

Lane 2 contains Q-sepharose chromatography pool II.

Lane 3 contains Q-sepharose chromatography pool III.

Lane 4 contains rhSOD II (Q-sepharose chromatography pool IV).

Lane 5 contains standard recombinant human CuZn-superoxide dismutase.


67
43
30
20.1
14.4
1
2
3
4
5
6

to look for any informative spectral features and to determine if any alterations in protein secondary structure were evident in the samples. The CD spectra are given in **Figure 11**. The CD spectra of the samples were similar. A notable difference was that rhSOD II had a negative Cotton effect at 240nm not observed in rhSOD or rhSOD I. Samples of rhSOD appeared similar to rhSOD I at 240nm.

Secondary structure analysis of the rhSODs from an analysis of the CD data indicated some differences between the samples. A variety of algorithms were used to estimate the secondary structure of the proteins, and the method of Bolotina et al (1981) was found to give the lowest sum of squares error when compared to the other algorithms. The data from the secondary structure analysis of rhSOD samples based on two experiments are given in **Table 4**. Using this method, rhSOD and rhSOD I had similar secondary structures. Samples of rhSOD showed 9.40 ± 1.98% (mean ± 2s) α-helical content, 26.3 ± 1.84% ß-sheet, 24.4 ± 1.70% ß-turn, and 40.0 ± 1.56% unordered (or random) secondary structure. Sample rhSOD I showed 6.65 ± 4.10% α-helix, 25.5 ± 6.08% ß-sheet, 25.4 ± 3.82% ß-turn, and 42.6 ± 5.80% unordered structure. Sample rhSOD II had 7.40 ± 1.13% α-helix, 23.9 ± 1.98% ß-sheet, 24.4 ± 2.40% ß-turn, and 44.4 ± 5.52% unordered structure. The results of these analyses indicate no significant differences in secondary structure among rhSOD, rhSOD I, or rhSOD II.

Figure 11. Circular dichroism spectra of recombinant human CuZn-superoxide dismutase samples.

Samples of CuZn-SOD between 0.094-0.151 $mg.mL^{-1}$ in 50mM sodium phosphate buffer, pH 7.4 were scanned from 270-200nm. Spectra of rhSOD (−), rhSOD I (○), rhSOD II (Δ). All spectra were recorded in triplicate and corrected for background absorbance. Secondary structure was estimated from 270-201nm mean residue ellipticity by a variety of methods (see Materials and Methods).

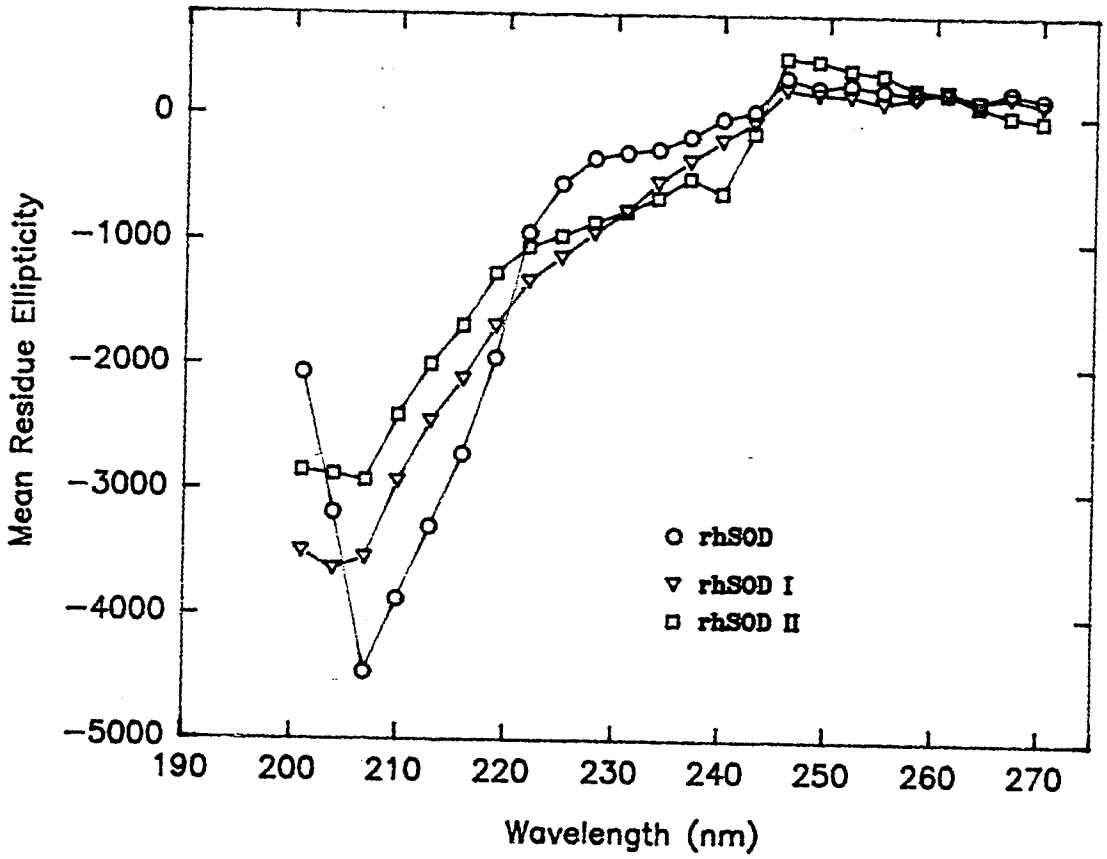

Mean Residue Ellipticity
0
−1000
−2000
−3000
−4000
−5000
190
200
210
220
230
240
250
260
270
Wavelength (nm)
rhSOD
rhSOD I
rhSOD II

Table 4. Secondary structure analysis of rhSOD samples using circular dichroism[1].

Enzyme	Method of Estimation	Helix (%)	ß-sheet (%)	ß-turn (%)	Unordered Structure (%)	Sum of Squares Error
rhSOD	method of Bolotina[2]	8.70 10.1	26.9 25.6	25.0 23.8	39.4 40.5	13.95 10.09
	method of Chen 1974[3]	2.00 4.50	54.7 51.4	NA NA	43.3 44.1	48.45 36.91
	method of Brahms[4]	0.00 0.00	52.5 52.2	NA NA	47.5 47.8	108.2 72.79
rhSOD I	method of Bolotina[2]	8.10 5.20	23.3 27.6	24.0 26.7	44.6 40.5	23.41 43.65
	method of Chen 1974[3]	2.30 0.00	49.5 54.1	NA NA	48.2 45.9	61.15 107.7
	method of Brahms[4]	0.00 0.00	51.0 52.0	NA NA	49.0 48.0	124.0 232.6
rhSOD II	method of Bolotina[2]	7.80 7.00	24.6 23.2	25.2 23.5	42.4 46.3	34.93 17.54
	method of Chen 1974[3]	12.0 15.0	41.6 49.0	NA NA	46.4 36.0	82.44 52.03
	method of Brahms[4]	0.00 0.00	52.0 50.2	NA NA	48.0 49.8	155.6 129.4

NA = not applicable.

[1]CD spectra were recorded in triplicate and corrected for the base line contribution of the buffer. Ellipticity values were digitized manually at 3nm intervals from 270-201nm and converted to mean residue ellipticity as outlined in Materials and Methods. The table shows the results of two seperate experiments.

[2]secondary structure estimated by the method of Bolotina et al (1981).

[3]secondary structure estimated by the method of Chen et al (1974).

[4]secondary structure estimated by the method of Brahms and Brahms (1980).

DISCUSSION

Microheterogeneity in recombinant human CuZn superoxide dismutase (rhSOD) produced in yeast was examined by isoelectric focusing (IEF). Earlier studies had shown the presence of between three and five different forms of human CuZn superoxide dismutase (hSOD) by IEF (Hartz and Deutsch, 1969; Arai et al, 1986; Weselake et al, 1986b). The standard rhSOD used in this study showed essentially the same focusing pattern as human red blood cell (RBC) CuZn-SOD (hSOD) from two different commercial sources and purified in different manners. The rhSOD used in this study was purified by a combination of heat treatment and anion-exchange chromatography (Gartner et al, 1984), sometimes followed by an additional step of copper chelate affinity chromatography (Weselake et al, 1986a). By IEF, these preparations were indistinguishable. Natural hSOD was purified by either cation-exchange chromatography (Carlsberg SOD)(Johansen, 1983, United States Patent 4,388,406) or by chloroform/ethanol precipitation of hemoglobin followed by ion-exchange and metal chelate chromatography (WinSOD)(McCord and Fridovich, 1969; Weselake et al, 1986b). Each of these hSODs showed three isomers with pIs of approximately 4.9, 5.2, and 5.35. The three isomers were found in equal abundance in all samples except for that of WinSOD which showed less of the pI 5.35 isomer and nearly equal quantities of the other two forms. WinSOD has shown four forms by IEF in other work (Weselake et al, 1986b).

As yeast CuZn-SOD was detected in these preparations of rhSOD but not in hSOD (Campbell, 1990), experiments were performed to determine whether any

of the rhSOD isomers was due to the presence of the yeast enzyme. The presence of yeast CuZn-SOD in the rhSOD would be expected as rhSOD was purified on the basis of heat stability and surface charge properties which are very similar to that of the yeast enzyme. Yeast CuZn-SOD was not detected in Coomassie-stained IEF gels of rhSOD. In IEF gels, the yeast CuZn-SOD showed a single form of pI 5.25. The yeast enzyme's pI was distinct from that of the multiple charged forms of rhSOD or hSOD (pIs = 4.9, 5.2, 5.35) and the yeast CuZn-SOD was clearly resolved from the hSOD isomers when yeast and hSODs were mixed before focusing. Furthermore, yeast-human CuZn-SOD heterodimers were found to focus at a different position (pI = 4.6 and 5.85) than the yeast or hSOD isomers and no increased staining at the position of the three hSOD isomers was observed. No yeast-human CuZn-SOD heterodimers were detected in the rhSOD samples analyzed, and by IEF, rhSOD preparations appeared nearly identical to two different commercial preparations of hSOD. These findings disagree with the earlier studies of Campbell (1990). These studies utilized a monoclonal antibody which could detect very minute quantities of yeast CuZn-SOD. Also, it was suggested that yeast CuZn-SOD and rhSOD represented approximately 0.2% and 3.5% respectively of total extractable protein from stationary phase cells (Campbell, 1990). This implies that rhSOD is 17.5-fold more abundant than yeast CuZn-SOD in soluble protein extracted from the recombinant yeast. Possibly, the methods used here were not sensitive enough to detect such low levels of contaminating yeast protein. Additional research will be required to address the question of how

much yeast CuZn-SOD and other yeast proteins contaminate the rhSOD preparations. Clearly yeast CuZn-SOD was not responsible for any of the microheterogeneity observed in rhSOD. The level of yeast contaminants in the rhSOD that will be deemed acceptable will depend on what the product is actually to be used for. If repeated injections of rhSOD are required over long periods, low levels of yeast contaminants could prove to be unsafe, and may provoke a potentially lethal immune reaction. Alternatively if the enzyme is only needed rarely, or once, as for example with its use in the storage of organs for transplantation (Marcocci et al, 1989), the level of yeast impurities would not be as threatening. Superoxide dismutase has proved to be very safe. In Europe, bovine CuZn-SOD is routinely used for a variety of ailments. In patients injected with bovine CuZn-SOD, only minor episodes of itching/skin irritation at the injection site have been noted (Greenwald, 1990). No major toxicities have been reported (Carson et al, 1973), indicating that bovine CuZn-SOD is not very immunogenic.

The largest loss of rhSOD during purification occurred at the heating step. This step resulted in a 61% loss of total activity. The large loss of activity is undesirable, but the ease and speed with which this step can be performed makes it desirable for large scale protein purification. Also, the recovery of rhSOD may have been underestimated. The total activity of the yeast homogenate is used as the standard by which the extent of purification is compared. A crude homogenate though, contains a variety of low-molecular-weight compounds which can interfere with the SOD enzyme assay by chemically reducing the indicator cytochrome c

(Crapo et al, 1978). Thus the SOD specific activity in the original homogenate may be less than that reported.

During purification of rhSOD, two peaks of superoxide dismutase (SOD) activity were resolved by anion-exchange chromatography. Native polyacrylamide gel electrophoresis (PAGE) of the fractions showed that the proteins within the two peaks eluted by chromatography were homogeneous and that they had different mobilities. Two forms of hSOD were previously isolated chromatographically which also showed this property (Bannister et al, 1972). The two forms of rhSOD isolated were named rhSOD I and rhSOD II. Sample rhSOD I was the form with a slower mobility by PAGE and rhSOD II showed a faster migrating band. No discussion of microheterogeneity, or any investigations on these isomers in rhSOD produced in yeast has appeared in the literature.

In this study, rhSOD II had the majority of the activity and protein. The specific activity of rhSOD II was higher than that of rhSOD I. Sample rhSOD I showed a relatively low specific activity of 2,040 U/mg compared to the high specific activity of 3,340-3,640 U/mg observed for rhSOD II. Higher specific activity in the later eluting second major peak has been observed before after the chromatographic isolation of two major CuZn-SOD peaks (hSOD, Bannister et al, 1977; Briggs and Fee, 1978a and 1978b; bovine CuZn-SOD, Gartner, et al, 1985), but not with CuZn-SOD from all sources (bovine CuZn-SOD, Civalleri et al, 1982; ponyfish CuZn-SOD, Martin and Fridovich, 1981). In the absorption spectra of the two isomers no distinctive features were noted. Previously, the the absorbtion

spectra of hSOD I- and hSOD II-like components was found to differ. The hSOD I contained a unique contribution at 320-325nm that was not observed in hSOD II (Bannister et al, 1977; Briggs and Fee, 1978a and 1978b). The absence of a unique spectral feature in rhSOD I is in disagreement with these earlier findings. This may support the contention that the 320nm chromophore is an artifact (Briggs and Fee, 1978a and 1978b), and also suggests that the modification to the free cysteine residue responsible for this effect (Calabrese et al, 1975; Briggs and Fee, 1978a and 1978b) may not be associated with the differences in electrophoretic mobility observed for these hSOD isomers.

The possibility that one of these isomers represented either yeast CuZn-SOD or a yeast-human heterodimer was discounted by IEF. The isoelectric focusing pattern of rhSOD I showed similar quantities of the pI 5.2 and 5.35 isomers with a small amount of a pI 5.8 form while rhSOD II showed equal amounts of the pI 4.9 and 5.2 isomers. No bands different from that observed in the standard samples of rhSOD or hSOD was observed. Isoelectric focusing of hSOD I and hSOD II has not appeared in the literature. Bovine CuZn-SOD isomers which were isolated as two major peaks during chromatography and which differ in PAGE mobility, show single molecular forms with pIs of 4.9 and 5.2 by IEF (Civalleri et al, 1982). This suggests that the mechanisms by which these isomers are produced may be slightly different for the bovine and human CuZn-SODs.

The finding of multiple forms of rhSOD I and rhSOD II by IEF was unexpected as the IEF was run under native conditions. By native PAGE analysis

it appeared that rhSOD I and rhSOD II were both single forms. Multiple forms of CuZn-SOD were observed by IEF for both rhSOD I and rhSOD II. It is possible then, that the same mechanism gives rise to both of these two forms of the molecule. Several explanations can account for these findings. Basically, the two forms observed can arise either as a natural consequence of the protein's structure, or from various artifacts induced by the separation process itself.

It may be that the isomers observed by IEF were not resolved with the PAGE conditions used. The presence of multiple forms by IEF can be due to conformers. Here microheterogeneity would result from alternate and stable conformations of the protein, each with different surface charge properties. These forms may arise naturally (Drysdale and Righetti, 1972) or be induced by the electrofocusing procedure itself (Cann, 1979). The transition between alternate stable conformations in a protein can be pH-dependent (Talbot, 1975; Stimpson and Cann, 1977). Multiple banding patterns in IEF can also arise artifactually, mostly due to interactions of the protein with the carrier ampholytes responsible for the formation of the pH gradient. Isoelectric focusing bands may appear due to protein isomerization induced by the binding of ampholytes (Frater, 1970; Kaplan and Foster, 1971; Wallevik, 1973; Cann and Stimpson, 1977; Hare et al, 1988), or by a recombination, association or dissociation of multi-subunit proteins induced by the ampholytes (Cann et al, 1978). Ampholytes are also known to have metal-chelating properties and may induce the dissociation of cations like copper from proteins during focusing (Haglund, 1971). These possibilities can be addressed

only by rigorous experimentation involving focusing at a variety of ampholyte and protein concentrations and by eluting and rerunning the observed IEF bands. Such experiments were not attempted here.

SDS-PAGE of these samples revealed a complicated pattern. In contrast to the findings of other investigators, hSOD was found to be comprised of three differently sized subunits (20 kD, 21 kD, 22 kD). Typically hSOD shows a single band by SDS-PAGE with a molecular weight of 16-19 kD (Sugiura et al, 1981; Arai et al, 1986; Weselake et al, 1986a). No explanation for the presence of multiple CuZn-SOD subunits was found in the literature. A doublet pattern similar to the pattern observed here, is sometimes observed during SDS-PAGE of CuZn-SOD; this anomoly is thought to arise from mishandling of the enzyme or improper preparation of the reagents (W.H. Bannister, University of Malta, personal communication). Perhaps this pattern is due to an incomplete denaturation of enzyme before electrophoresis.

Previous investigations on the structure of these isomers from hSOD revealed a single monomer subunit for each isomer (Bannister et al, 1977). By SDS-PAGE, rhSOD I showed primarily a single species of monomer molecular weight 20 kD, and a small amount of the 21 kD subunit. rhSOD II showed an equal abundance of 21 kD and 22 kD forms. Samples of rhSOD showed all three forms with a preponderance of the 21 kD form. Similar results were also observed for samples of hSOD.

Circular dichroism (CD) studies of rhSOD samples was undertaken to see

if any spectral feature or alteration in secondary structure was evident in the rhSOD isomers isolated. The spectra produced were similar to those observed before for hSOD (Bannister et al, 1972; Bannister et al, 1977), rhSOD produced in E. coli (Kajihara et al, 1988a), and bovine CuZn-SOD (Wood et al, 1971; Weser et al, 1971). Sample rhSOD II showed a negative Cotton band at 240nm not seen in rhSOD I or rhSOD. This spectral region has not previously been found to be informative. Chromophores at approximately 320nm (hSOD, Bannister et al, 1977) and 260nm (rhSOD produced in E. coli, Kajihara et al, 1988a; bovine CuZn-SOD, Gartner et al, 1985) have been shown to differ between PAGE isomers of CuZn-SOD before. No consistent differences in the 260nm region were noted here; the 320nm region was not probed in these experiments.

A variety of algorithms were used to determine protein secondary structure from the CD spectra and the method of Bolotina et al (1981) was found to give the lowest sum of squares error (best fit of experimental to predicted data). Using this technique, standard rhSOD showed 9.40% α-helix, 26.3% ß-sheet, 24.4% ß-turn, and 40.0% unordered structure. These values were similar to the secondary structures derived previously by CD for hSOD (4.5% α-helix, 54.8% ß-structure, and 40.7% unordered structure: Bannister et al, 1977) and bovine CuZn-SOD (10.1% α-helix, 44.5% ß-structure, and 45.3% unordered structure: Bannister et al, 1977; 1-4% α-helix, 40-49% ß-sheet, and 4-5% ß-turn: Brahms and Brahms, 1980) and are consistent with the secondary structure of the bovine enzyme determined by X-ray crystallography (3-8% α-helix, ca 45% ß-structure: Tainer et al, 1982).

Sample rhSOD I showed a similar structure of 6.65% α-helix, 25.5% ß-sheet, 25.4% ß-turn, and 42.6% unordered structure when analyzed by the method of Bolotina et al (1981) and sample rhSOD II was found to contain 7.40% α-helix, 23.9% ß-sheet, 24.4% ß-turn, 44.4% unordered structure. The differences, if any, in secondary structure among these different rhSODs were not significant. The secondary structure of hSOD I and hSOD II isomers was examined once before by CD (Bannister et al, 1977). In this study, when compared to standard hSOD, hSOD I showed slightly less α-helical (3.6%) and unordered structure (38.3%), and slightly more ß-structure (58.1%). The hSOD II showed slightly more α-helical (8.6%) and unordered structure (44.7%), and slightly less ß-structure (46.6%). The findings with the rhSOD isomers studied here corroborate a difference in CD spectral features between the two CuZn-SOD isomers, but do not support the earlier finding of a difference in the secondary structure of these two isomers (Bannister et al, 1977).

The finding of two major peaks of CuZn-SOD activity associated with electrophoretic variants in PAGE has been previously observed during the purification of CuZn-SOD from human RBCs (Bannister et al, 1977; Briggs and Fee, 1978a and 1978b) and of CuZn-SOD from other sources (bovine, Civalleri et al, 1982; Gartner et al, 1985; rat liver, Reiss and Gershon, 1976; kidney beans, Kono et al, 1979; Iris pseudoacorus, K. Fagerstedt, University of Helsinki, personal communication). The SOD I component is not seen when fresh blood or liver are used as starting materials; this component can be removed by successive

rechromatography (Bannister and Bannister, 1984). Here it was shown that rhSOD I had physiochemical properties distinct from that of the other major CuZn-SOD form, and most importantly, that rhSOD I showed a low specific activity for a pure CuZn-SOD species. Pure CuZn-SOD typically shows specific activities of 3000 U/mg in the cytochrome c assay (McCord and Fridovich, 1969). The presence of this SOD I isomer in CuZn-SODs from many species, points to a common mechanism for their formation. This may be due to hydrogen peroxide-induced metal-catalyzed oxidative damage to CuZn-SOD (Hodgson and Fridovich, 1975), already known to produce electrophoretic variants of the enzyme (Bartoz et al, 1981; Jewett, 1983; Mavelli et al, 9183; Gartner et al, 1985; Viglino et al, 1985; Jewett, 1986; Gartner et al, 1986; Davies, 1987; Jewett et al, 1989; Salo et al, 1990).

In conclusion, microheterogeneity in rhSOD produced in yeast was examined. Samples of rhSOD appeared in all respects identical to the natural hSOD. Notably, by IEF analysis the same three forms of the molecule were observed for either rhSOD or hSOD. The presence of yeast CuZn-SOD or a yeast-human CuZn-SOD heterodimer was not detected in samples of rhSOD. Two species of rhSOD were separated chromatographically which appeared homogeneous and differed in mobility by PAGE. These two CuZn-SOD isomers were found to differ in specific activity, absorption spectra, subunit composition, IEF patterns, and in the placement of chromophores and in secondary structure as determined by CD. These two rhSOD isomers share many properties with other CuZn-SOD isomers which have already been characterized, but show differences

too. The actual role, or cause for the formation of these isomers is not completely known, but considerable evidence in the literature suggests that similar changes observed in the properties of CuZn-SOD are part of the normal process of enzyme ageing and turnover in vivo induced by inactivation of the enzyme by hydrogen peroxide.

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