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# Biomimetic and Bioorganic Chemistry II

Editors: F. Vögtle, E. Weber

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With 32 Figures and 26 Tables

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Biomimetic and Bioorganic Chemistry

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# Molecular and Functional Aspects of Superoxide Dismutases

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## Abbreviations

CD — Circular dichroism
CM — Carboxymethyl
Cmc — S-carboxymethylcysteine
DDC — Diethyldithiocarbamat
DEAE — Diethylaminoethyl
EDTA — Ethylendiamine tetraacetic acid
ENDOR — Electron-nuclear double resonance
EPR — Electron paramagnetic resonance
EXAFS — Extended X-ray absorption fine structure
HEPES — N-(2-hydroxyethyl)piperazin-N′-2-ethanesulphonic acid
NADH — Reduced nicotinamide adenine dinucleotide
NBT — Nitroblue tetrazolium chloride
NMR — Nuclear magnetic resonance
SOD — Superoxide dismutase
TEMED — Tetramethylethylendiamine
XPS — X-ray photoelectron spectroscopy

Superoxide dismutases are ubiquitously distributed in all biological systems including prokaryotic, eukaryotic and plant cells. These proteins contain either copper and zinc or manganese or iron in the active centre, respectively. During the catalytic activity, the superoxide anion is converted into dioxygen and hydrogen peroxide. $Cu_2Zn_2$superoxide dismutase is one of the most intensively studied metalloproteins. The primary structure of many of these proteins has been elucidated. A convincing structure-function correlation was deduced from biophysical and chemical data. The influence of the protein backbone on the reactivity of the metal was thoroughly studied. Of special interest was the catalytic centre of $Cu_2Zn_2$superoxide dismutase. The molecular architecture of the phylogenetically older iron and manganese enzymes is far less understood. At present, the clinical and biochemical aspects of reactive oxygen species enjoy a marked interest, as they are thought to be responsible for the oxygen derived cell-damage. This may reflect the overwhelming number of different assay systems described for the evaluation of superoxide dismutase activity. The most suitable and convenient assays are briefly discussed. The model chemistry for superoxide dismutases of low molecular mass complexes of transition metals is reviewed. Emphasis is placed on the biological significance of superoxide dismutase active copper ligands, frequently used as antiinflammatory drugs. The interaction of reactive oxygen species with superoxide dismutases and the biochemical and clinical relevance of this enzyme are critically summarized.

## 1 Introduction

Biochemically seen the periodic table of elements can be divided into four sections. Firstly, there are the group I and group II elements. They include typical metals of low ionization potentials for the first and the second electrons, respectively. Their polarizing power is low and they are known to participate neither in redox reactions nor in the formation of biopolymers. Together with the anions of group VII (halides), they are involved in maintaining the ionic strength in cellular and extracellular fluids. Electrophysiological reactions in nervous excitation are controlled. Magnesium and calcium form weakly stable complexes with nucleic acids and proteins, however, in general the tendency for complex formation of the group I and II elements is low.

Section two of the periodic table includes group IV up to group VI. The most prominent members are carbon, nitrogen, oxygen, sulphur and phosphorus. They contribute to the fundamental structural components of biopolymers. With regard to our aerobic atmosphere, oxygen plays a dual role in this event. Oxygen is incorporated into proteins, and, at the same time is required for energy supply. Due to its radical character and the possible formation of highly toxic intermediates (OH·), some protective systems against reactive oxygen species had developped during evolution [1].

The third section of the periodic table comprises transition metals or the d-group elements, which are involved in many biological defence mechanisms against toxic radicals. As in chemical catalysis the same metals are active in biochemical systems. Surprisingly these redox mediators are involved in both, the generation and the removal of reactive oxygen species.

Zinc having a filled d shell and two 4 s electrons reacts like a Lewis acid of considerable polarizing power. Electrons are withdrawn from substrates with the consequence that a nucleophilic attack is facilitated. The elements with partially filled d shells and multiple oxidation states include Sc, Ti, V, Cr, Mn, Fe, Co, Ni and Cu. Hence, they are good candidates to actively participate in redox reactions. Attributable to d-d transitions most of the complexes of these species including many metalloproteins are coloured. This facilitates investigations of their properties by electron absorption spectrometry.

A fourth group in the periodic table of the elements can be assigned to the toxic elements Cd, Hg, Ag and Tl as well as the rare earths for which a biological function has not yet been detected. The radioactive actinides are not suitable for biological processes.

Nearly all metals mentioned before are found in the organism. The most prominent transition metals in biology include iron, zinc and copper. Manganese, cobalt and nickel are less abundant. In oxygen metabolism Fe, Cu and Mn are involved. Sometimes they act antagonistically or display co-operative effects. The antagonism is observed in inflammation. Iron proteins like cytochrome P-450 or myeloperoxidase aggravate the metabolic disorder by the generation of reactive oxygen species or hypochlorous acid. By way of contrast, copper proteins or low molecular weight copper chelates can be curative [2, 3]. The co-operative effect of both, copper and iron is seen in cytochrome c oxidase [4].

Iron forms very weak complexes with chelating agents, that produce no strong ligand fields. Amine complexes are very unstable. The porphyrin complexes are exempted where iron is coordinated to four nitrogens. The haem-iron can undergo a multiplicity of reactions including changes in valence and spin state. Haemoglobin is a typical example in which binding of oxygen results in changes of the spin state [5]. Of course, these changes can also be seen in other iron proteins like dioxygenases [6] or iron-sulphur clusters [7].

Copper is markedly different in its reactivity compared to iron. In porphyrins copper is totally buried. Due to the rigid structure of the porphyrins, where no twisting of the ligands is possible no reactivity with oxygen is seen. Hence, copper porphyrin-complexes display no superoxide dismutase activity [8]. In biological systems copper is essentially coordinated to nitrogen or sulphur. In octahedral Jahn-Teller distorted Cu(II) complexes a tetragonal distortion of the octahedron, usually an extension

corresponding to the lengthening of the two bonds on either moiety side of the equatorial plane coordination is seen. The rate of exchange of the two ligands on the perpendicular axis is enhanced [9]. Therefore, it is not surprising that copper is an excellent reactant for fast exchange reactions. In comparing the exchange rates for water in the sixth coordination site of the aquo-complexes of different transition metals, it becomes clear, why iron, manganese and copper are exclusively bound in superoxide dismuting proteins [9, 10] (Table 1).

**Table 1.** Exchange rate of water in metal aquo-complexes [10]
$[M(H_2O)_6] + H_2O^{18} \rightarrow [M(H_2O)_5(H_2O^{18})] + H_2O$

| Aquo-complex | $k_1$ ($s^{-1}$) |
|---|---|
| $Cr^{3+}$ | $5 \times 10^{-7}$ |
| $Al^{3+}$ | $10^0$ |
| $Fe^{3+}$ | $3 \times 10^3$ |
| $Mg^{2+}$ | $> 10^4$ |
| $Ni^{2+}$ | $3 \times 10^4$ |
| $Co^{2+}$ | $10^6$ |
| $Fe^{2+}$ | $3 \times 10^6$ |
| $Mn^{2+}$ | $3 \times 10^7$ |
| $Cu^{2+}$ | $8 \times 10^9$ |

The $Cu_2Zn_2$superoxide dismutase has dismutation rates for the superoxide ion which are near the diffusion control ($2.0 \pm 0.5 \times 10^9$ $M^{-1}s^{-1}$) even at alkaline pH-values. The reaction catalyzed can be summarized as follows:

$$2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

The properties of superoxide dismutases (SOD's) have been extensively reviewed [11–18]. Currently, it seems attractive to work on the biological activity of superoxide dismutases, whereas the chemical aspects are sometimes disregarded. However, devoid of a founded knowledge of the biophysical parameters of these enzymes, the catalytic action of the superoxide dismutases could never have been understood. Thus, a solid structure function correlation is essential.

## 2 Isolation of Erythrocyte $Cu_2Zn_2$Superoxide Dismutase

Apart from laboratory scale preparations bovine erythrocytes have become the most convenient source for $Cu_2Zn_2$superoxide dismutase for all kinds of applications including pharmaceutical, technical or routine purposes. Red blood cells are easily obtainable in large quantities and the costs are low or nearly nill. Special preparation techniques and mincing of cell particles can be omitted. Therefore, the isolation techniques are simple and convenient. It was the merit of Mann and Keilin who

successfully performed the first isolation of $Cu_2Zn_2$superoxide dismutase from red blood cells of ox [19]. They actually prepared a zinc-free protein and called it haemocuprein.

In the sixties an improvement was achieved by the introduction of new methods for precipitation of the haemoglobin including heavy metal or ethanol treatment [20, 21]. Some years later two preparation methods were established which are still currently used for routine isolations. The first was the aqueous isolation using batch absorption of nonhaemoglobin proteins to DEAE-cellulose gels [22]. The second was the precipitation of haemoglobin with chloroform/ethanol, the so called Tsuschihashi procedure [23]. Recently a new method was developed making use of the high thermal stability of vertebrate $Cu_2Zn_2$superoxide dismutases [24].

## 2.1 Precipitation with Organic Solvents

$Cu_2Zn_2$superoxide dismutases are proteins which are of remarkable stability in the presence of organic solvents [25]. They survive concentrations of these chemicals where many other proteins are deteriorated. In the early days of biochemistry Tsuschihashi discovered that undesired haemoglobin can be readily precipitated from haemolysate by treatment with chloroform/ethanol, leaving some particular proteins dissolved [26]. Based on the resistance of $Cu_2Zn_2$superoxide dismutase against organic solvents, McCord and Fridovich applied this method to the isolation of the former enzyme [23]. Some years later improved techniques with regard to gels and buffer systems were devised [27, 28].

The procedure includes the precipitation of haemoglobin with a mixture of 0.25 volume of ethanol and 0.15 volume of chloroform. After centrifugation the superoxide dismutase is found in the supernatant. Further purification is accomplished upon precipitation of contaminating proteins by the addition of solid $K_2HPO_4$ (300 g per litre). After this treatment two liquid phases are obtained. Superoxide dismutase is present in the upper phase. It is precipitated by the addition of 0.75 volume of cold acetone and washed once with 0,75% acetone. A final chromatography on DE-23 (DEAE cellulose) followed by gradient elution yields homogeneous $Cu_2Zn_2$superoxide dismutase. Separation of minor contaminants and desalting is performed on Sephadex G-75 with distilled water.

Attributable to its fast performance and high convenience this procedure is usually employed for routine isolations. Unfortunately, the use of organic solvents bears some disadvantages because they are noxious. Moreover, by treatment of haemolysate with chloroform/ethanol a considerable coprecipitation and/or denaturation of superoxide dismutase is seen.

## 2.2 Aqueous Chromatography

The isolation technique based on sequential aqueous chromatography was introduced by Stansell and Deutsch [22]. They used batch absorption of the nonhaemoglobin proteins on DEAE cellulose for the removal of haemoglobin. The overall procedure was very laborious and time consuming. Therefore, a more convenient method shorter in time was developed [29]. The yield of purified SOD was the same as obtained with the Tsuschihashi method. Compared to the latter technique the different operation

procedures were much more susceptible to erroneous results. This may explain why the aqueous isolation is not frequently used. Recently, an improved aqueous isolation was devised and successfully employed [30, 31]. A significantly increased yield was obtained and in the last purification step, the charge isomers of $Cu_2Zn_2$superoxide dismutase were separated on a preparative scale.

Crude haemolysate, obtained from citrated bovine blood (pH 6.8) is chromatographed on DEAE-Sephacel and the bound nonhaemoglobin proteins are eluted with a sharp NaCl gradient (0–1 M). The copper containing fractions are concentrated by membrane filtration under nitrogen pressure (1 : 10). Gel filtration on Sephadex G-75 yields one single copper containing protein band near $M_r \sim 32{,}000$. It can be exclusively assigned to $Cu_2Zn_2$superoxide dismutase [30]. It should be emphasized that neither high nor low molecular weight copper chelates can be detected after both, membrane filtration and gel filtration (Fig. 1).

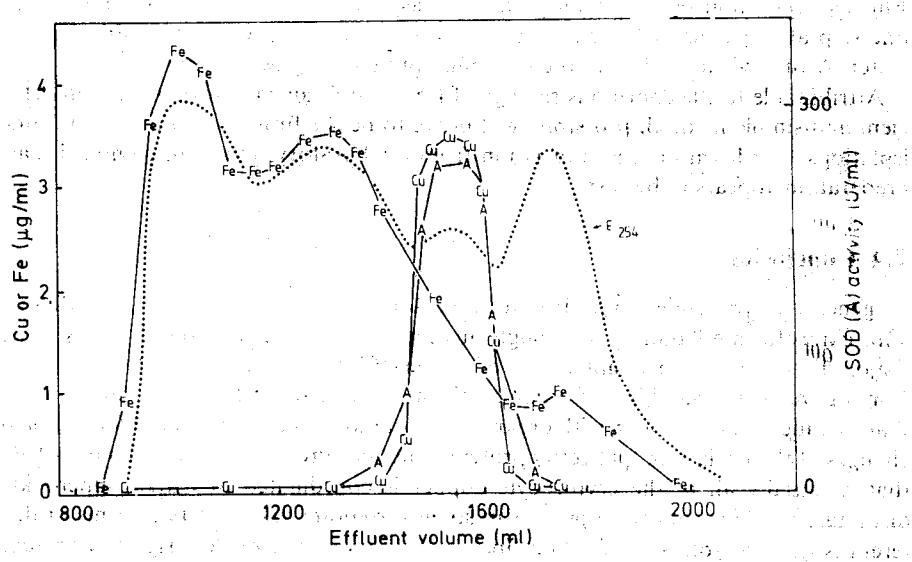


**Fig. 1.** Aqueous isolation of bovine erythrocyte $Cu_2Zn_2$superoxide dismutase. Gelfiltration of the DEAE-eluate on Sephadex G-75. The fractions are assayed for Cu, Fe, superoxide dismutase activity (NBT-assay) and electronic absorption ($A_{254}$). The observed peaks are assigned to (from left to right): catalase, haemoglobin, $Cu_2Zn_2$superoxide dismutase and cytochromes b,c

The crude superoxide dismutase is further purified using chromatography on DE-23 (DEAE-cellulose) and/or CM-Sephadex C-50 and/or phenylsepharose and/or hydroxyapatite. Usually one or two of the four possible chromatographic steps are sufficient. A final purification including the preparative separation of the charge isomers is accomplished by chromatofocusing. A linear gradient from pH 5.9 to pH 4.1 is applied (Polybuffer from Pharmacia 1 : 10 diluted). The two charge isomers are desalted on Sephadex G-75 in distilled water.

## 2.3 Heating of the Haemolysate

A completely different approach was made to modify the general isolation technique. Based on the thermal stability of $Cu_2Zn_2$superoxide dismutase partial heat-deterioration of the haemolysate was employed [24]. Subsequent chromatography of the supernatant on DEAE-Sephacel and Sephadex G-75 yielded an electrophoretically homogeneous protein within less than five days.

Bovine red blood cells are diluted with two volumes of tap water and the lysate is dialyzed for 14 h to a conductivity of 2 mS. After heating to 70–80 °C for 10–15 min under rigorous stirring, the precipitate is separated by centrifugation. Roughly 90% of the haemoglobin are removed by this procedure, whereas 60–70% of the superoxide dismutase are recovered. Upon chromatography on DEAE-Sephacel the residual haemoglobin passes completely the ion exchanger. The bound nonhaemoglobin proteins are eluted with a linear NaCl gradient (0–200 mM) and freeze dried. Further separation of contaminant proteins as well as desalting is accomplished in one step after passage through a Sephadex G-75 column equilibrated with distilled water. A final yield of 50% of the originally present enzyme is achieved.

Attributable to the enormous savings of time and financial efforts this highly efficient and simple method, probably, will prove to be the future technique for routine isolations. Furthermore, the translation from a laboratory-scale into technical-scale preparation appears to be easy.

## 2.4 Conclusion

In general $Cu_2Zn_2$superoxide dismutase is isolated after treating the haemolysate with chloroform/ethanol [23]. Although this method is rather expedient, it has some major disadvantages. Roughly 25% of the erythrocyte SOD are recovered, only. For routine work, handling of organic solvents can be hazardous. It is not unlikely that treatment of the protein with organic compounds results in minor conformational changes of the tertiary or quarterny protein structure. Previously it was demonstrated that essentially all of the erythrocyte copper is coordinated in $Cu_2Zn_2$superoxide dismutase [30]. This enzyme is present in some vertebrate tissues in two forms of different isoelectric points [32–34]. At a final yield of only 25% of the originally present enzyme, it is by no means clear, whether parts of form I or form II are lost or the loss of SOD is assigned to the statistical average of both forms during preparation.

Therefore, the aqueous isolation developed by Stansell and Deutsch [22] was applied and improved, leading to a final yield of 50% of originally present enzyme [30, 31]. It was demonstrated, that during the isolation either isoelectric variants are simultaneously lost. Although the aqueous isolation was substantially improved, it was still more time consuming than the method using organic solvents. Moreover, this procedure has another fundamental drawback. Haemoproteins and their degradation products are not readily removed by this technique.

Especially the latter species is known to act as a possible source of radical reactions. Thus, a gradual degradation of the protein in the course of the isolation process could not be fully excluded. However, the molecular properties of purified $Cu_2Zn_2$superoxide dismutase obtained by the aqueous isolation are identical to those of the chloroform/ethanol treated protein [30, 31].

In order to avoid the disadvantages of the above mentioned two different isolation methods a more simple, rapid and efficient method was devised [24]. Based on the long known thermostability of $Cu_2Zn_2$superoxide dismutase [25] undesired haemoglobin is readily removed. One important advantage of this method is, that nearly no chemicals are required to yield homogeneous SOD within five days.

The biophysical characterization of the differently prepared enzymes is summarized in Table 2 [29,30].

**Table 2.** Physicochemical data of $Cu_2Zn_2$superoxide dismutases obtained by different isolation techniques. Aqueously isolated charge isomers I and II are compared with the enzymes which were isolated from haemolysate previously treated with chloroform/ethanol or heating to 75 °C for 15 minutes

| | Aqueously isolated | | $Cu_2Zn_2$SOD isolated by $CHCl_3$/EtOH treatment | Heat treatment of haemolysate |
|---|---|---|---|---|
| | charge isomer I | charge isomer II | | |
| Electron absorption | | | | |
| $\varepsilon_{259}$ [$mol^{-1}cm^{-1}$] | 9840 ± 50 | 9820 ± 50 | 9840 ± 50 | 9870 ± 50 |
| $\varepsilon_{680}$ | 313 ± 5 | 310 ± 5 | 315 ± 5 | 310 ± 5 |
| Circular dichroism | | | | |
| $\theta$ 208 $\left[\frac{deg \cdot cm^2}{dmol}\right]$ | −6000 | −5950 | −6000 | −6100 |
| $\theta$ 261 | 20000 | 10000 | 20000 | 19400 |
| EPR spectroscopy | | | | |
| $A_{\|\|}$ $cm^{-1}$ | 0.014 | 0.013 | 0.014 | 0.013 |
| $g_\perp$ | 2.062 | 2.060 | 2.062 | 2.063 |
| $g_{\|\|}$ | 2.263 | 2.262 | 2.263 | 2.261 |
| X-ray photoelectron spectroscopy | | No differences | | |
| Amino acid analyses | | No differences | | |
| Electrophoreses | Two-bands | One-band | Three-bands | Three-bands |
| Isoelectric focusing | One-band | One-band | Two-bands | Two-bands |
| Specific SOD activity [U/mg] (Nitroblue tetrazoliumchloride assays) | 1700 | 1600 | 1700 | 1700 |
| Copper (µg/mg]protein | 3.6 | 3.4 | 3.7 | 3.6 |
| Zinc [µg/mg]protein | 4.1 | 4.0 | 4.2 | 4.1 |

Essentially no profound changes are seen. Isolated superoxide dismutase employing chloroform/ethanol treatment had a slightly different electrophoretic pattern compared to the aqueously isolated enzyme. The quantity of charge isomer II was diminished to a minor extent.

When isolating superoxide dismutases from other tissues than erythrocytes the methods explained here are normally not suitable. For the purification from solid sources like liver a lot of other techniques are described [35]. Moreover, Mn-SOD and Fe-SOD are neither resistant to organic solvents nor to heat treatment. Therefore, mild conditions are required for the isolation of these enzymes. The techniques used

for the purification of these SOD's are markedly different from those applied for the isolation of erythrocyte $Cu_2Zn_2$SOD's [35, 36].

An intriguing phenomenon was observed, when the purified bovine erythrocyte $Cu_2Zn_2$superoxide dismutases, obtained by the different isolation techniques were compared. Thermal stability measurements revealed that the purified SOD's are much less heat resistant compared to the enzymes in the homogenates. Therefore, heat deterioration of the isolated protein must not be connected with the isolation technique described in Chapter 2.3. When freshly aqueously isolated $Cu_2Zn_2$superoxide dismutase was heated to 77 °C a transient and marked increase of the specific enzymic activity is seen.

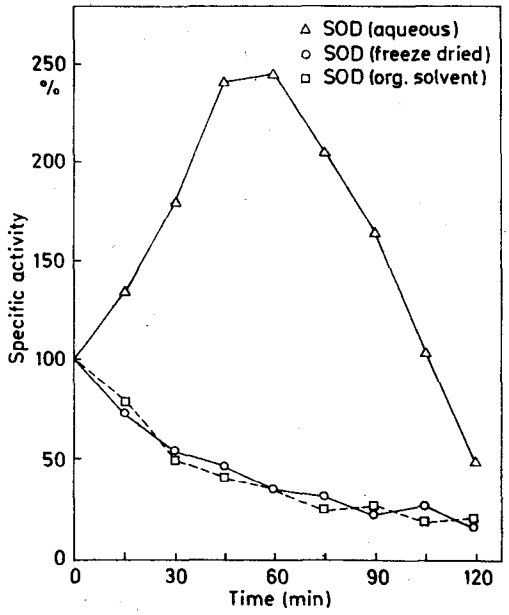


**Fig. 2.** Heat denaturation of purified bovine erythrocyte $Cu_2Zn_2$superoxide dismutase. (○) SOD prepared by treatment with chloroform/ethanol, (△) aqueously isolated enzyme and (□) the same enzyme stored for three months as lyophilized powder at room temperature. When freshly prepared, all fractions of aqueously isolated superoxide dismutase showed the same behaviour as (△) regardless of the age of the animal and the isoelectric point. SOD-activity was estimated using the cytochrome c assay [23]

This phenomenon is not observed using SOD which was previously treated with organic solvents or lyophilized enzyme from either source stored for three months. It was suggested, that the molecular architecture of the active center of aqueously isolated enzyme differs from that of the other species [31].

The molecular properties of the SOD's summarized in the next chapter are essentially all derived from data collected from the enzyme which was isolated employing the chloroform/ethanol method. Data from $Cu_2Zn_2$superoxide dismutases obtained by other isolation methods are awaited with great interest.

## 3 Molecular Properties of Superoxide Dismutases

Since the early work of Mann and Keilin a lot of structural investigations on superoxide dismutases were carried out. Little is known on the structural aspects of the phylogenetically older iron and manganese enzymes than on the properties of the $Cu_2Zn_2$-SOD. Currently, the copper-zinc-protein is one of the best known metalloproteins. Richardson and Richardson achieved the first breakthrough with the X-ray cristallographic analysis of bovine $Cu_2Zn_2$superoxide dismutase [37]. Sequencing of the amino acid structure of $Cu_2Zn_2$-SOD's from different sources and alternatively the iron and manganese enzymes revealed a high degree of sequence homology within the respective class of proteins [1]. It could be demonstrated that all amino acid residues involved in the catalytic reaction are conserved. Attributable to the founded knowlege on the structure and function of superoxide dismutases, they are excellent examples for understanding enzymatic catalysis. Moreover, the influence of the protein backbone on the catalytic effect of the metals can be comprehensively demonstrated.

### 3.1 Molecular Aspects of the $Cu_2Zn_2$ Enzyme

$Cu_2Zn_2$superoxide dismutase is an enzyme of intriguingly high stability in vitro. It can be heated to 100 °C for one minute without any detectable loss of activity and it survives pH-ranges from 2–12 [26]. Moreover, the enzymic activity survives the presence of ten molar urea and/or four per cent sodium dodecylsulphate. In a solution of six per cent guanidinium hydrochloride where most of the other proteins are deteriorated the denaturation of superoxide dismutase is reversible [38, 39].

The $Cu_2Zn_2$ enzymes can be obtained from many sources. Apart from two exceptions [40, 41] they are exclusively found in eucaryotic organisms in nearly all tissues [42]. Only the enzymes from higher species have a blocked N-terminal group. These enzymes are generally more stable. Whether this phenomenon can be ascribed to the acetylation or not is still open to discussion. Iron SOD's are exclusively prokaryotic, whereas manganese superoxide dismutases are present in procaryotic cells as well as in mitochrondria and the serum of vertebrates [43–45]. Their relative molecular mass is differing. Recently a high relative molecular mass copper containing enzyme of $M_r$ = 135,000 was deduced to be present in human lung [46]. $Cu_2Zn_2$ enzymes in general have a relative molecular mass around 32,000.

The $Cu_2Zn_2$superoxide dismutase of bovine erythrocytes is a homodimer of 31,300 daltons and contains one g atom of both, copper and zinc per subunit. SOD's from fungi, plants and vertebrates have remarkable homologies in the amino acid sequence [47]. The sequence of the bovine erythrocyte enzyme is summarized in Table 3 [48].

The sequence of human erythrocyte superoxide dismutase has been elucidated some years later [49, 50]. Bovine superoxide dismutase contains no tryptophane and very few aromatic and heteroaromatic amino acid residues, respectively. Eight histidines, one tyrosine and one phenylalanine are detected. The main portion of the protein is formed by neutral amino acids. Four sulphur containing amino acids (three cysteins and one methionine) are also present. Cysteine residues 55 and 144 are disulphide bridged [51], whereas one cysteine sulphhydryl group remains unbound. These data are contrasted by the properties of the human enzyme where 153 amino