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Age Dependence and Charge Isomers of Bovine Erythrocyte Cu_2Zn_2superoxide Dismutase

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Abstract

An improved simple and efficient method for the aqueous isolation of bovine erythrocyte Cu_2Zn_2-superoxide dismutase was devised and successfully employed. In the last purification step the preparative separation of charge isomers of this enzyme was performed. The properties of the isomers were examined using electrophoresis, isoelectric focusing and chromatofocusing. The isoelectric points of charge isomers **I** and **II** were 5.2 and 4.9, respectively. No significant differences of chemical and spectrometrical properties, including amino acid composition, XPS-spectroscopy and EPR-measurements, were seen. The chiroptical data at 261 nm differed markedly.

Aqueously isolated Cu_2Zn_2superoxide dismutase from the red blood cells of 1–2 year old cows had the same properties compared to the protein obtained from 12–15 year old species. For this reason, ageing of the erythrocyte was not attributed to an ageing of superoxide dismutase. Hydrogen peroxide was used as a model system for the generation of charge isomer **II** from charge isomer **I**. After treatment with hydrogen peroxide the band intensity at the position of charge isomer **II** increased dramatically, both during isoelectric focusing and/or gel electrophoresis. The data obtained from this species by CD-, EPR- and UV-spectroscopy are completely different from the respective parameters of the genuine charge isomer **II**. Therefore, it is concluded that this isomer is not generated by hydrogen peroxide inactivation of charge isomer **I** *in vivo*.

Introduction**

Among the many catalytically active metalloproteins Cu_2Zn_2 superoxide dismutase (EC 1.15.1.1.) is unique in its reactivity. The dismutation rate of the superoxide anion into hydrogen peroxide and molecular oxygen is near the diffusion control [1]. There is an intriguing phenomenon in that this enzyme is not only inhibited by one of the former reaction products, but is also completely deteriorated after a longer exposure to H_2O_2 [2]. Although there is some evidence on the cooperative action of catalase and superoxide dismutase [3], it has never been proven that the latter enzyme is physiologically important. For example, patients who are suffering from acatalasia display no symptoms of a metabolic disorder [4]. The biological function of Cu_2Zn_2-superoxide dismutase continues to be debated. Nevertheless, there are many biologically important sources of superoxide generation and the possible conversion into excited oxygen species [5]. This could explain the reduced oxygen tolerance found in mutants of *E. coli* with diminished superoxide dismutase activity [6]. The inactivation of Cu_2Zn_2-superoxide dismutase by hydrogen peroxide has been extensively studied [2, 7, 8]. It was suggested that a histidine residue in the active site may be involved in this inactivation [9]. Unfortunately, other data on the molecular mechanism of this inactivation are lacking.

The high stability *in vitro* is contrasted by the relatively fast inactivation *in vivo*. For example, isolated Cu_2Zn_2superoxide dismutase can be heated to 100 °C for five minutes. Unlike many other proteins which are deteriorated under these conditions, this protein survives extraordinary high concentrations of organic solvents. None of the presently known proteases are able to attack the native enzyme. Contrary to the other oxygen metabolizing proteins, including catalase and glutathione peroxidase, the highest loss of activity with regard to the age of the tissue is seen in the case of Cu_2Zn_2-superoxide dismutase [10]. Moreover, material with no enzymatic activity and which was crossreacting with antigens of SOD was detected in aged cells [11].

In comparing Cu_2Zn_2superoxide dismutase purified either by the aqueous method [12] or after treatment of the homogenate with chloroform/

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**Abbreviations: SOD, Cu_2Zn_2superoxide dismutase (EC 1.15.1.1); NBT, nitroblue tetrazolium.

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ethanol [13], the same electrophoretic pattern is obtained: a triplet with a slower migrating band of high intensity, and two faster migrating bands of lower intensity. This pattern, however, is strongly dependent on the employed electrophoretic conditions [14]. Upon treatment of Cu_2Zn_2superoxide dismutase with hydrogen peroxide an increase in the intensity of the weaker bands is seen with a concomitant decrease of the strong band. Therefore, the weak bands obtained with purified Cu_2Zn_2superoxide dismutase were attributed to hydrogen peroxide dependent ageing of the protein *in vivo* [8].

In this study the molecular properties of aqueously isolated Cu_2Zn_2superoxide dismutase are compared with those of the protein originally treated with organic solvents. The inactivation of the enzyme by hydrogen peroxide was re-examinated and an efficient purification method was developed, including the preparative separation of the charge isomers. The molecular mechanism of ageing of Cu_2Zn_2superoxide dismutase was examined using blood cells from both old and young cows.

Materials and Methods

DEAE-Cellulose DE-52 was obtained from Whatman, Maidstone. Apart from HA-Ultrogel which was provided by LKB Producter, Stockholm, all other gels were from Deutsche Pharmacia, Freiburg. Nitroblue tetrazolium chloride, Visking dialysis tubing, xanthine and Servalyt Precotes were purchased from Serva, Heidelberg. Xanthine oxidase was from Boehringer, Mannheim. Membrane filters were obtained from Amicon Corp., Oosterhout. Methionine sulfoxide was from Sigma, Munich. All other employed reagents were of analytical grade and from Merck, Darmstadt. Deionized water was additionally distilled over quartz. For routine isolations, deionized water proved to be sufficient.

Analytical Procedures

Metals were quantified on a Perkin Elmer 400 atomic absorption spectrophotometer equipped with a graphite furnace. Circular dichroism was measured on a JASCO J-20 A spectropolarimeter and electron paramagnetic resonance X-band spectra were run on a Varian E-109 spectrometer at 77 K. X-ray photoelectron spectroscopy (XPS) was performed on a Leybold LHS 10 apparatus at 123 K and 10^{-5} Pa. Ultraviolet and visible electron absorption spectra were recorded on a Beckman 25 spectrometer.

Superoxide dismutase activity was assayed using the nitroblue tetrazolium chloride method [15]. Polyacrylamide electrophoresis and isoelectric focusing were performed as in [16]. Protein was assayed using the microbiuret method [18]. Standards were bovine serum albumin as well as Cu_2Zn_2superoxide dismutase. Amino acid residues were calculated after 24–48 h hydrolysis with 6 N HCl at 110 °C on a Kontron Liquimat III amino acid analyzer [17]. Heat denaturation was followed at 77 °C in a sealed 1 ml flask in 15 min intervals for 2 h. The protein concentration was 3 mg/ml.

Hydrogen peroxide treatment was performed by adding a 50-fold molar excess of H_2O_2 per active center of superoxide dismutase (1 mg/ml protein was dissolved in 10 mM Tris/HCl buffer pH 7.1). This concentration was sufficient to cause a 50% loss of copper. Other concentrations of hydrogen peroxide were also employed, including molar excesses of 8, 15, 30, 60, 120 and 1200 per active center. The effects observed were similar to those obtained with a ratio of 50:1. Contrary to former investigations [2, 9], all reactions were performed at a physiological pH. Thus, considerable higher concentrations of hydrogen peroxide became necessary. Prior to dialysis against catalase in 10 mM Tris buffer pH 7.8 for 16 h at 4 °C, the mixture was incubated for one hour at room temperature. Suprapure hydrogen peroxide was used throughout. The concentration was estimated by titration with $KMnO_4$ [2].

Isolation of Cu_2Zn_2superoxide Dismutase

The isolation of superoxide dismutase using $CHCl_3$/EtOH treatment of haemolysate was performed as in [19]. The aqueous isolation of this enzyme was accomplished in the following manner: 6 l of citrated bovine blood were centrifuged and the remaining 2500 ml of erythrocytes were washed three times with 0.9% NaCl. The cells were lysed by adding one volume of water, and were dialyzed overnight against water. After removal of the membranes at 10 000 × g, the lysate was chromatographed on DEAE-Sephacel (8 × 25 cm). The gel was washed with 5 l of 10 mM Tris/acetate pH 6.8 and the bound non-haemoglobin proteins were eluted with a sharp gradient from 0–1 M NaCl. The eluate was concentrated 1:10 by membrane filtration under nitrogen pressure. Gel filtration on Sephadex G-75 yielded a copper-containing fraction of $M_r \sim 32\,000$. This fraction was further purified using chromatography on DE-52 and/or CM-Sephadex and/or phenylsepharose and/or hydroxyapatite. Column dimensions for DE-52 or CM-Sephadex were 2.5 × 15 cm. For phenylsepharose or hydroxyapatite, columns sizes of 2.5 × 30 cm proved convenient. Using CM-Sephadex or hydroxyapatite the crude SOD-fraction was passed through the column in the gel filtration buffer (pH 6.0). Phenylsepharose chromatography was performed after addition of solid potassium phosphate to a final concentration of 500 mM. DE-52

chromatography was followed by a gradient elution with a flat NaCl gradient from 0–200 mM.

Usually one or two of the four possible chromatographic steps were sufficient. The final purification, including the separation of the charge isomers, was accomplished by preparative chromatofocusing. A linear gradient of pH 5.9–4.1 (Polybuffer 1:10 diluted) was used. The fractions of homogeneous Cu_2Zn_2superoxide dismutase were desalted on Sephadex G-75 and lyophilized.

Results

The aqueous isolation of erythrocyte Cu_2Zn_2-superoxide dismutase described by Stansell and Deutsch [12] resulted in a final yield of 25% of the originally present enzyme. Another disadvantage is the laborious isolation procedure. Charge isomers of the Cu_2Zn_2superoxide dismutase remained unseparated. Employing the present isolation technique these disadvantages can be overcome. Compared to the established method of McCord and Fridovich [13] the aqueous purification will still require nearly twice the time. A typical aqueous isolation is summarized in Table I.

The major advantage of our improved aqueous method is the separation of the charge isomers of Cu_2Zn_2superoxide dismutase with a concomitant purification of this enzyme. However, for routine isolations the method described in [16] is more suitable. Unlike the precipitation of haemoglobin by chloroform/ethanol, the thermal denaturation of haemoglobin, being the principle of this procedure, is even more rapid and efficient. It should be emphasized that the final step *i.e.* chromatofocusing, could also be appended onto other isolation techniques (for example refs. 13, 16) utilizing fewer column steps. However, the aim of this study was to scrutinize the exact amount of the charge isomers originally present and to obtain a maximum final yield. The suitability of the aqueous isolation within other purification methods will be discussed below.

In the following sections the properties of Cu_2Zn_2-superoxide dismutase employing focusing methods are described. In electrophoresis a three band pattern is seen. The bands are numbered I, II and III, respectively. In contrast, only two charge isomers **I** and **II** are resolved during chromatofocusing or electrofocusing. In the course of polyacrylamide electrophoresis the same three-band pattern is observed using both aqueously-isolated Cu_2Zn_2-superoxide dismutase and the enzyme originally treated with organic solvents. Likewise, the two-band pattern is obtained during isoelectric focusing or chromatofocusing with both proteins.

The doublet in isoelectric focusing, as well as the three-band pattern in gel electrophoresis, were noticed in the initial steps of the purification as well as in the finally purified material. Thus, the possible loss of a labile third component during the isolation can be excluded. The faster migrating band during chromatofocusing (charge isomer **I**), known to carry roughly 80% of the isolated protein, shows a doublet on polyacrylamide electrophoresis. This refers to the well known electrophoretic pattern of earlier studies [20]. Charge isomer **II** corresponds to the third electrophoretic band reported in more recent work [21]. Intriguingly, this electrophoretic variant displays no splitting during gel electrophoresis. This rather complex situation is demonstrated in Fig. 1.

The isoelectric points of charge isomers **I** and **II** were 5.2 and 4.9, respectively. These values are in good accordance with those previously described for the chloroform/ethanol treated enzyme [21]. The pattern obtained with the aqueously isolated enzyme is the same as the pattern of the protein treated with organic solvents. However, the intensity of charge isomer **II** during isoelectric focusing is significantly lower using the latter protein. Probably some portion of this isomer has precipitated in the course of solvent treatment.

TABLE I. Aqueous Isolation of Bovine Erythrocyte Cu_2Zn_2 superoxide Dismutase.

Fraction	Volume (ml)	Copper (μg)	% of total copper	Protein (mg)	Specific copper content (μg/mg)	Specific activity[a] SOD (U/mg)
Diluted hemolysate	2900	520	100	–	–	1.0[b]
Eluate of DEAE-Sephacel	250	440	85	3700	0.1	86
Concentrate after membrane filtration	55	410	80	3700	0.1	80
Eluate of Sephadex G-75	325	390	75	650	0.6	300
Eluate of CM-Sephadex	400	370	70	490	0.8	430
Eluate of Phenyl-Sepharose	500	340	70	250	1.4	700
Chromatofocusing charge isomer **I**, desalted	170	210	40	70	3.2	1700
Chromatofocusing charge isomer **II**, desalted	80	50	10	20	3.0	1600

[a]Nitrobluetetrazolium chloride assay (11). Note the specific activity in this assay is only half to that in the cytochrome c assay (9). 1700 U/mg refers to 3400 U/mg in the latter test. [b]Protein concentration estimated.

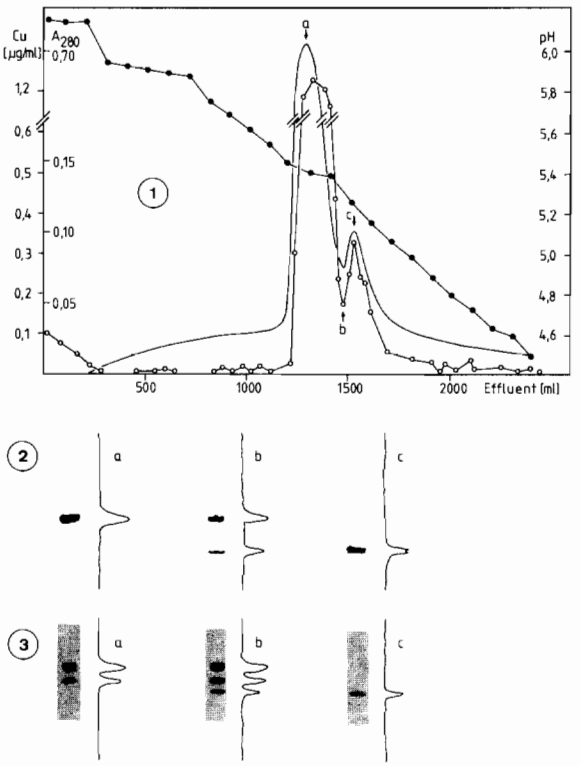

Fig. 1. Chromatofocusing, isoelectric focusing and polyacrylamide gel electrophoresis of aqueously isolated Cu_2Zn_2superoxide dismutase. In chromatofocusing ① and isoelectric focusing ② a two-band pattern is obtained due to the two charge isomers of the enzyme. In electrophoresis ③ charge isomer I is splitted in two bands whereas charge isomer II remains single-banded in isoelectric focusing and electrophoresis. Adjacent to the photographs of the respective gels the corresponding densiometrical scanning is depicted. a, b and c refer to the peaks obtained by chromatofocusing. Protein concentration in ② and ③ was 3 mg/ml. Staining was accomplished with Coomassie blue. Other conditions see [12]. In chromatofocusing ① the following symbols are used A_{280} (——), pH (•) and Cu (○).

The formation of methionine sulfoxide is known to inactivate reticulocyte lipoxygenase [22]. This mechanism was also suggested to account for the occurrence of the charge isomerism in Cu_2Zn_2-superoxide dismutase. All fractions of the purified enzyme obtained from either the aqueous isolation or from the precipitation of haemoglobin with organic solvents were assayed to monitor oxidized sulfur species. In the amino acid analyses no oxidized methionine was seen, neither in the untreated nor in the hydrogen peroxide treated samples, whereas unoxidized methionine was fully recovered. XPS-spectroscopy, also showed no oxidized sulfur. It is therefore concluded that the different mobilities of the charge isomers of Cu_2Zn_2superoxide dismutase cannot be assigned to the oxidation of sulfur (Fig. 2).

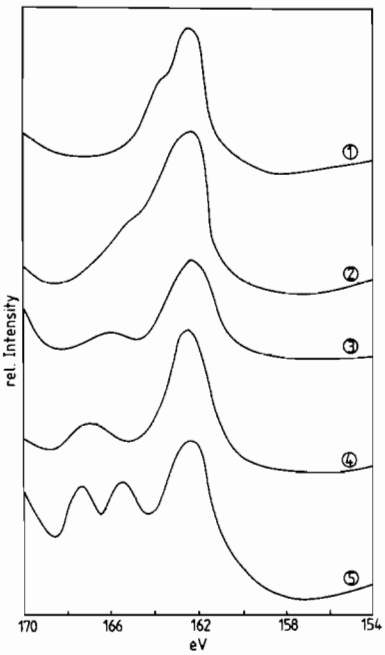

Fig. 2. Binding energy values of sulfur $2_{p1/2,3/2}$-levels of lyophilized Cu_2Zn_2superoxide dismutase. ① charge isomer I ② charge isomer II ③ superoxide dismutase isolated using the chloroform/ethanol treatment ④ superoxide dismutase treated with hydrogen peroxide ⑤ fully oxidized enzyme. It is seen that essentially no oxidized sulfur can be seen in all isolated proteins. Oxidized sulfur species are detected in the region from 164–168 eV. Temperature 123 °K, pressure 10^{-5} Pa.

Moreover, the amino acid composition of charge isomer I was essentially the same as of charge isomer II. The metal content (copper and zinc) is equivalent in both charge isomers. We consider that the differences in the charge cannot be attributed to molecular changes of the protein backbone.

There are some reports on the age-dependent deterioration of Cu_2Zn_2superoxide dismutase which was thought to be responsible for the observed cellular damages in aged cells [23, 24]. Furthermore, it was suggested that the multiplicity of electrophoretic bands is derived from one homogeneous species of superoxide dismutase which was exposed with hydrogen peroxide *in vivo* [8].

Superoxide dismutase from the red blood cells of 12–15 years old cows was isolated and the molecular properties were compared with those of the enzyme isolated from 1–2 year old animals. Although in whole blood a population of erythrocytes of variable age is present, changes in the properties of superoxide dismutase between old and young species should be detectable, if they exist, because the haematopoietic stem cells are also subjected to ageing. The copper content in erythrocytes can be used as an internal standard for the content of Cu_2Zn_2superoxide dismutase [25]. In

old cows the copper content is reduced by 20% compared to young species. This result is paralleled by the reduction of superoxide dismutase activity. An age-dependent deterioration of the native enzyme is a plausible explanation. However, a genuine age dependency of superoxide dismutase activity cannot be deduced from the preceding data alone. Lactating cows in general have lower copper levels in the blood than unstressed young species [25]. Further molecular studies are required.

The pattern during electrophoresis as well as in the course of isoelectric focusing remains unchanged using the enzymes of both old and young cows. Likewise the distribution of the charge isomers is identical in both species. Charge isomer **I** carries 80% (I.P. 5.2) and charge isomer **II** (I.P. 4.9) contains 20% of the protein. There is strong evidence that charge isomer **II** is not derived from charge isomer **I** by ageing. If this were the case an increase of charge isomer **II** in old animals should be detectable. Neither in XPS-spectroscopy nor in amino acid analyses was oxidized sulfur seen in the protein of old animals.

Circular dichroism measurements of two charge isomers of erythrocyte Cu_2Zn_2superoxide dismutase obtained by aqueous isolation from old and young cows were performed. Regardless of age the chiroptical data of charge isomers **I** and **II** differed markedly. The θ values of charge isomer **I** from both young and old animals were 20 000 deg cm^2/dmol at 261 nm and -6000 deg cm^2/dmol at 208 nm. These values were the same as described for the enzyme originally treated with organic solvents [19]. Although charge isomer **II** showed the same lineshape as charge isomer **I**, the θ-value at 261 nm was only half to that of the latter species, namely 10 000 deg cm^2/dmol. However, the value at 208 nm remained unchanged. It is suggested that the geometry of the chromophore at 261 nm is distorted to some extent. No bleaching of this chromophore was seen in ultraviolet electron absorption spectroscopy, and the specific enzymic activity remained unchanged (Fig. 3).

To elucidate possible age-dependent differences in the stability of charge isomers, heating to 77 °C was performed. Subjecting the freshly prepared aqueous proteins to this procedure produced an unusual behaviour. The specific enzymic activity increased dramatically, followed by a decrease after 60–120 min (Fig. 4).

This phenomenon is not observed when employing proteins which were stored as lyophilized powders for three months at room temperature. Regardless of age and isoelectric point, neither oxidized sulfur nor loss of histidine was detected. Electrophoresis of the stored freeze-dried protein resulted in less distinct, multiple bands. This was not seen using protein held in Tris or phosphate

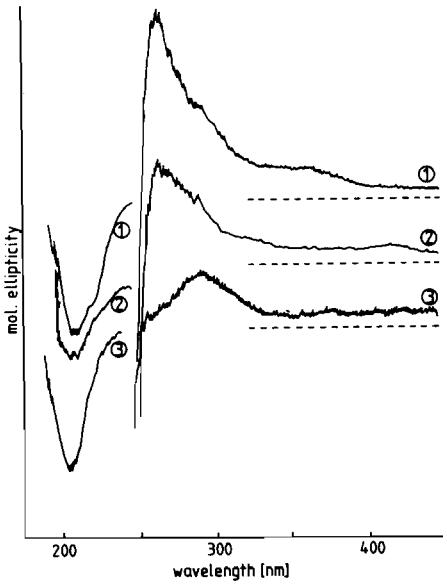

Fig. 3. Circular dichroism of charge isomer **I** ① charge isomer **II** ② and hydrogen peroxide treated charge isomer **I** ③ of Cu_2Zn_2superoxide dismutase. The zero ellipticities for each of the three curves are depicted as dotted lines. The θ-value at 261 nm of charge isomer **I** is twice as high to that of charge isomer **II**. Treating charge isomer **I** with hydrogen peroxide a new Cotton band is seen at 295 nm. Protein concentration was 3 mg/ml. The light path was 1 cm.

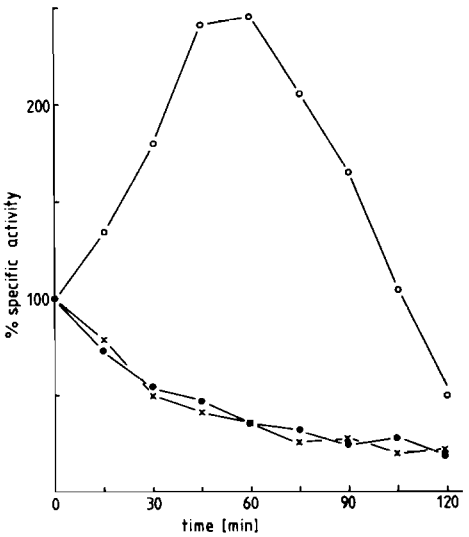

Fig. 4. Heat denaturation of Cu_2Zn_2superoxide dismutase prepared by solvent treatment (•), aqueously isolated enzyme (o) and aqueously isolated enzyme stored for three months as lyophilized powder at room temperature (X). When freshly prepared, all fractions of aqueously superoxide dismutase show the same behaviour as (o) regardless of age of the animal and of charge isomerism. The temperature was 77 °C. Cytochrome c reductase assay [10]. The protein concentration was 3 mg/ml.

buffer at 4 °C for the same period. With respect to unusual enzymic properties, a similar result was obtained earlier where Cu_2Zn_2superoxide dismutase was titrated to pH 11 for five minutes [26]. After readjustment to pH 7.8 a dramatic increase of the enzymic activity up to 300% was seen.

Charge isomers **I** and **II** of aqueously isolated Cu_2Zn_2superoxide dismutase display the previously-described transient rise in enzymic activity during heat treatment. Both charge isomers reacted identically and no age dependency was seen. This observed phenomenon was absent in lyophilized samples. The lineshapes and intensities of the EPR spectra employing the charge isomers isolated by the aqueous procedure from old and young cows were identical to those of the enzyme prepared by treatment with organic solvents [25]. The same spectra were recorded using lyophilisates and samples after the first chromatographic step in the course of aqueous isolation.

In the search for model reactions generating either the charge isomers or ageing of Cu_2Zn_2superoxide dismutase, treatment with hydrogen peroxide seemed appropriate. Although there are earlier reports dealing with this problem [2, 7–9, 27], many questions remained unsolved. Most of the investigations were performed at alkaline pH-values. Therefore, the equilibrium of the reaction:

$$H_2O_2 \rightleftharpoons H^+ + HO_2^-$$

is pushed to the right due to a pK_a of 11.6. The anion of peroxide is much less stable than the protonated form. These highly instable anions can be stabilized by addition of a proton or electrophils like histidine. Sinet and Garber investigated hydrogen peroxide treatment or the inactivation of Cu_2Zn_2-superoxide dismutase under physiological conditions [7]. They found that both hydrogen peroxide and superoxide are required for the inactivation of superoxide dismutase. After addition of hydrogen peroxide, both compounds are present near the active center due to a partial reversed reaction of the equilibrium:

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

In our study the crucial point was whether or not ageing and occurrence of charge isomerism can be attributed to possible hydrogen peroxide denaturation *in vivo*. Although Cu_2Zn_2superoxide dismutase of highest purity was employed for these reactions, a slight catalase activity at pH 7.1 was detectable. Thus, concentrations of hydrogen peroxide up to a 50 molar excess were used per active centre. This led to a 50% loss of copper after dialysis. Usually 40% of the enzymatic activity originally present was retained. It was demonstrated that at alkaline pH the inactivation was followed by a loss of histidine in the active center [9]. Likewise an arginine residue in the catalytic funnel is affected [27]. At pH 7.1 the loss of histidine using amino acid analysis was confirmed. A detectable oxidation of sulfur was seen during XPS-spectroscopy. Recently, oxidation of methionine-sulfur following the H_2O_2 treatment *in vivo* was reported in red blood cells SOD of rats [28].

Circular dichroism measurements revealed the complete disappearance of the Cotton band at 261 nm and a new extremum at 295 nm was seen (θ_{295} = 10 000 deg cm^2/dmol). The θ-value at 208 nm remained unchanged. Concomitant with the changes in circular dichroism the electronic absorption profile in the ultraviolet region showed the well known bleaching of the absorption band at 260 nm. EPR spectroscopy revealed a linesharpening in the $g_\perp$-region due to axial geometry of the copper binding site (Table II). Reaction of Cu_2Zn_2superoxide dismutase with H_2O_2 results in a reduction of the major portion of Cu(II) to Cu(I) and a considerable loss of this metal due to the weaker binding of Cu(I), paralleled by structural changes of the active center.

Upon incubation of the protein with hydrogen peroxide a multiplicity of bands is seen in electrophoresis [8]. Portions of band I are transformed into band II and III. In isoelectric focusing a similar result is obtained. The intensity of charge isomer **I** is decreased, whereas the intensity of charge isomer **II** is increased and a new charge isomer **III** is generated with an isoelectric point of 4.7. Apart from a loss of histidine amino acid analyses revealed no differences between charge isomers **I** and **II** and H_2O_2-treated protein. These analyses were nearly identical with those described in [2]. In Table II, the physicochemical parameters of the charge isomers and H_2O_2 treated protein are compared with those of the chloroform/ethanol treated enzyme.

Discussion

In general the so called Tsuchihashi procedure [13] proved to be convenient to isolate Cu_2Zn_2-superoxide dismutase from the red blood cell. The aqueous purification as developed by Stansell and Deutsch is laborious and is not used frequently. Although the former isolation method is rapid, it has some major disadvantages. The first is the low yield: only 25% of any superoxide dismutase originally present is recovered. Therefore, it is by no means clear whether portions of a more labile component or an unspecific part of the overall superoxide dismutase fraction is lost during purification. This question is important with regard to the occurrence of charge isomers in this enzyme. Obviously, activity staining of haemolysate yields nearly the same pattern as the purified material. However,

TABLE II. Physicochemical Data of Cu_2Zn_2superoxide Dismutase Charge Isomers. Charge Isomer I was Treated with Hydrogen Peroxide at pH 7.4. The Same Variables are Given for the Enzyme Prepared by the $CHCl_3$/EtOH Method.

Variable	Aqueously isolated		Cu_2Zn_2 SOD isolated by $CHCl_3$/EtOH-treatment	Hydrogen peroxide treated charge isomer I
	Charge isomer I	Charge isomer II		
Electron absorption ϵ_{259} (mol^{-1} cm^{-1})	9840 ± 50	9820 ± 50	9840 ± 50	7500 ± 50
Circular dichroism				
θ 208	−6000	−6000	−6000	−6000
θ 261 ($\frac{deg\ cm^2}{dmol}$)	20000	10000	20000	–
θ 295	–	–	–	10000
EPR				
$g_\perp$	2.062	2.061	2.062	2.052
$g_\parallel$	2.263	2.262	2.263	2.262
$A_\parallel$ (cm^{-1})	0.014	0.013	0.014	0.013
XPS-spectroscopy		no oxidized sulfur		detectable oxidation of sulfur
Amino acid analysis		no loss of histidine		loss of histidine
Electrophoresis	two-band	one-band	three-band	four-band
Isoelectric focusing	one-band	one-band	two-band	three-band
Superoxide dismutase activity (U/mg) NBT assay (see Table I)	1700	1600	1700	600
Copper content (μg/mg protein)	3.6	3.4	3.7	1.9

this is only a qualitative approach to the problem. In addition possible conformational or other unknown changes in the protein backbone generated by treatment with organic solvents cannot be excluded.

The present results demonstrate that roughly 50% of originally present Cu_2Zn_2superoxide dismutase is obtained by the aqueous isolation. Electrophoresis, isoelectric focusing and EPR-measurements throughout the isolation procedure revealed that the loss of superoxide dismutase during purification is not derived from a loss of a labile component, *i.e.* a possible new electromorph, but from the statistical loss of the overall superoxide dismutase fraction.

In comparing the molecular aspects of aqueously isolated and organic solvent treated enzyme, some biophysical data were collected. Apart from heat denaturation and some minor differences in the electrophoretic pattern, the purified proteins from either isolation are indistinguishable. Chloroform/ethanol treated enzyme has the same conformational properties as the aqueously isolated enzyme. Due to its rapidity and efficiency the best isolation method for erythrocyte Cu_2Zn_2superoxide dismutase is the method by heat treatment of the haemolysate [16]. The quality of the isolated protein obtained by the latter method is equal to that of the enzyme isolated by the two former methods. Moreover, this method requires fewer chemicals.

Special emphasis was placed on the occurrence of charge isomerism in purified Cu_2Zn_2superoxide dismutase. Two different isomers are found in the chloroform/ethanol treated as well as in the aqueously isolated enzyme during chromatofocusing. After treatment with hydrogen peroxide a third isomer is detected. The appearance of a third isoelectric variant could be assigned to conformational changes of the protein backbone with a concomitant loss of copper.

The conditions used for hydrogen peroxide treatment in our experiments seem to be extreme compared to other studies. However, it should be noticed that at neutral pH-values H_2O_2 is much less aggressive than at alkaline pH. Moreover, the same physicochemical properties (though less pronounced) are seen at lower hydrogen peroxide concentrations. The loss of activity was markedly higher at increasing H_2O_2 concentrations and did not parallel the loss of copper.

It was suggested that oxidation of methionine-sulfur leading to methionine sulfoxide may cause charge differences in superoxide dismutase. Although human Cu_2Zn_2superoxide dismutase contains no methionine, the same three-band electrophoretic pattern is seen, identical to that of the bovine enzyme which is known to contain one methionine per subunit [29]. Moreover, an oxidation of methionine was detected neither by XPS-spectroscopy nor by amino acid analyses. Of course, XPS-spectroscopy is

limited to a qualitative approach to this problem. When standards of oxidized SOD were used, oxidized sulfur species could be detected easily.

The differences which are seen with Cu_2Zn_2-superoxide dismutase in both electrophoresis and isoelectric focusing are confusing. Care must be taken to interpret these results, because the electrophoretic mobility of Cu_2Zn_2superoxide dismutase is different to the mobility on isoelectric focusing. The electrophoretic behaviour of superoxide dismutase depends strongly upon the conditions applied throughout electrophoresis [14]. It was suggested that the multiplicity of bands is caused by a loss of copper [30]. Chromatofocusing revealed no significant decrease of copper in the different charge isomers. However, it cannot be assumed that this occurs during electrophoresis. If this is the case, it only takes place with charge isomer I which shows a splitting during electrophoresis. With respect to metal-binding, charge isomer **II** seems to be more stable.

Upon treatment of Cu_2Zn_2superoxide dismutase with hydrogen peroxide a loss of copper is detected. Concomitant with this fact a multiplicity of bands appeared during electrophoresis. Therefore the occurrence of band II could eventually be attributed to a loss of copper. However, the similar pattern of apo- and holo-superoxide dismutase during electrophoresis is contrasted by the different antigenicity of these two species which is not seen with the charge isomers [21].

In Cu_2Zn_2superoxide dismutase which was isolated by organic solvent treatment of the haemolysate, bands II and III had a weaker intensity during electrophoresis than those of the aqueously-isolated enzyme. This result can be interpreted in two ways. Firstly, unlike the chloroform/ethanol method the aqueous isolation requires more time and may be more difficult for the protein, because of the longer exposure to oxygen. Hence, a generation of bands II and III from band I could be deduced. Another interpretation is that organic solvents precipitate a greater portion of the latter species compared to the aqueous isolation. The second interpretation seems to be more likely, because the high intensity of this band is already seen after the first chromatographic step.

In contrast to other studies [23, 24], no differences are found in the Cu_2Zn_2superoxide dismutases isolated from bovine species of variable age. Not necessarily related to age, a lower copper content in blood of old cows compared to young species is observed [24]. It is concluded that the ageing of the erythrocyte cannot be significantly related to the possible ageing of superoxide dismutase.

Treatment of the protein with hydrogen peroxide is only to a certain extent a mimic of the ageing of Cu_2Zn_2superoxide dismutase. The changes of the molecule detected by hydrogen peroxide *in vitro* do not correlate with the differences seen in the charge isomers. Furthermore, it is very unlikely that significant concentrations of hyrdogen peroxide are present in the erythrocytes. The concentration of haemoproteins is extremely high in these cells. Although they have rather low rate constants, they all are able to react with hydrogen peroxide. Additional scavenging of H_2O_2 is provided by catalase and glutathione peroxidase [31].

The molecular changes in Cu_2Zn_2superoxide dismutase during treatment with hydrogen peroxide can be assigned to a loss of histidine in the active center, even at a physiological pH. However, it is also well known that histidine alone can be oxidized very fast by hydrogen peroxide but is not attacked in a protein backbone [32]. It seems likely that the metals in the active center play a vital role in this inactivation [7, 9].

The multiplicity of bands with isolated superoxide dismutase during electrophoresis was thought to be caused by exposure of the protein with hydrogen peroxide *in vivo* [8]. To our present knowledge the solution of this problem cannot be so simple. Although the electrophoretic mobilities of native and hydrogen peroxide treated enzymes are similar, the different spectrophysical data should be taken into consideration. In circular dichroism the spectrum obtained from hydrogen peroxide treated protein is completely different from both charge isomer **I** and charge isomer **II**. Moreover, in contrast to H_2O_2 treated enzyme, electron absorption spectra of the charge isomers show no bleaching of the chromophore at 259 nm. The line shapes and the g-values in EPR spectroscopy of untreated Cu_2Zn_2superoxide dismutase are different from those obtained after hydrogen peroxide treatment.

The unusual behaviour of freshly prepared Cu_2Zn_2superoxide dismutase during heat denaturation is interesting. The catalysis of the dismutation of superoxide is facilitated by electrostatic interaction [33, 34]. It may be speculated that the electrostatic funnel direction towards the active center of the protein seems to be more susceptible to anions during heat denaturation. A higher catalytic activity would be the consequence. A reasonable explanation may be a partial unfolding of the protein. This phenomenon is not observed when the lyophilized protein is used. This could be due to a more rigid structure of the active center.

It may be concluded that the isolation method of McCord and Fridovich [13] is quite convenient compared to the aqueous isolation technique with regard to the quality of the purified material. Unfortunately, the time saving is offset by the substantially lower yield obtained by the former method. The yield of the isolation using organic solvents

was reported to be 60% [13]. However, it should be emphasized that this value refers to the supernatant after the treatment with chloroform/ethanol. The yield of originally present enzyme in the erythrocyte is only 25%. Ageing of the erythrocyte is not significantly assigned to the ageing of Cu_2Zn_2superoxide dismutase.

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