PURIFICATION AND CHARACTERIZATION OF TWO FORMS OF Cu/Zn-SUPEROXIDE DISMUTASE FROM BOVINE ERYTHROCYTES BY PREPARATIVE ISOELECTRIC FOCUSING

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Two forms of Cu/Zn-superoxide dismutase with isoelectric points (pI) 6·3 and 5·2 were isolated from bovine erythrocytes by preparative isolectric focusing. Both forms show a relative molecular weight of 32 000 daltons corresponding to the value reported for the monomer molecule. From spectral analysis the maximum in the ultraviolet region of the spectrum (276 nm) is identical for both forms of superoxide dismutase whereas the maxima in the visible region are different (for the pI 5·2 form the maximum lies at 405 nm and for the pI 6·3 form at 695 nm). The different migration of the enzymatically active zones toward the anode during electrophoresis in alkaline media corresponds to their different isoelectric points.

Superoxide dismutase (E.C. 1.15.1.1) catalyzes[1] the dismutation of free superoxide anion radicals ($O_2^{-\bullet}$) to hydrogen peroxide and oxygen according to the reaction

$$O_2^{-\bullet} + O_2^{-\bullet} \rightarrow H_2O_2 + O_2 .$$

So far the presence of three metalloprotein types showing superoxide dismutase activity has been detected in biological material. The first group of enzymes contains copper and zinc (the Cu/Zn enzyme) and was found in the cytosole and mitochondria of eukaryotic cells[1,2]. A manganese-containing superoxide dismutase was detected in bacteria yet also in the mitochondria of eukaryotes[3,4]. The third group of enzymes containing iron was found in prokaryotes only[5]. Superoxide dismutase protects the organism against the toxic action of active oxygen metabolites[6,7], mainly against $O_2^{-\bullet}$. Opinions have been voiced[8,9] that also the development of certain diseases is paralleled by changes of superoxide dismutase in tissues.

The aim of this study has been the characterization and purification of Cu/Zn-superoxide dismutase from bovine erythrocytes by preparative isoelectric focusing. The enzyme thus obtained was compared with the preparation isolated by chromatography on DEAE-cellulose[1].

EXPERIMENTAL

The superoxide dismutase for isoelectric focusing was isolated from an extract of bovine erythrocytes by chromatography on a column of DEAE-cellulose according to McCord and Fridovich[1]. The isoelectric focusing was performed in LKB Model 8102—440 ml apparatus (Produkter AB, Stockholm). The pH-gradient was developed with stock Ampholine solutions (40%) and stabilized by sucrose. The following solutions were prepared: Heavy anodic —0·8 ml of concentrated phosphoric acid, 56 ml of ice-cold water, and 48 g of sucrose (A.R.); light cathodic — 0·8 ml of ethylenediamine (98—99%) and 39·2 ml of ice-cold water; heavy focusing —7·5 ml of 40% Ampholine made up to 150 ml with water, 100 g of sucrose (final volume 215 ml); light focusing —2·5 ml of 40% Ampholine and 60 ml (*i.e.* 124·2 mg of protein or 8 016 U, respectively) of the superoxide dismutase preparation.

Electrofocusing. The lower electrode was the anode and the heavy focusing solution was poured into the column. The focusing solutions were mixed in a gradient mixing device which was connected with the focusing column. The flow rate during the filling of the column was 4 ml. . min^{-1}. After the focusing column had been filled up (Ampholine concentration 1%) the light cathodic solution was carefully layered onto the column which subsequently was connected with the power supply. The initial voltage was 500 V at a current of 12 mA and an output of 6 W. The drop of the current during the first 24 h was compensated by voltage in order to obtain a constant output. The current was checked during the subsequent period and the focusing was discontinued after the current value became constant. The necessary time was 45 h at 4—8°C. After the finishing of the separation the pH-gradient with the focused zones was let out and the individual fractions subjected to measurement of pH (in Metrohm pH-meter) at 25°C and absorbance (in Type Uvicord II LKB UV-monitor) at 280 nm. Fractions corresponding to one protein zone were pooled, dialyzed against deionized water, and characterized.

The activity of superoxide dismutase was determined by the spectrophotometric method of Kono[10] making use of $O_2^{-\cdot}$ generated during the autooxidation of hydroxylamine and of the nitrobluetetrazolium salt as an indicator of $O_2^{-\cdot}$ uptake. The specific activity of superoxide dismutase was expressed in units based on 1 mg of protein; one unit of enzyme activity was defined as the quantity of protein causing a 50% inhibiton of reduction of the nitrobluetetrazolium salt. The protein content was assayed by the biuret method using bovine serum albumin as a standard[11].

Physico-chemical characterization. The spectra were measured in a Beckman Model 25 Spectrophotometer both in the ultraviolet (200—400 nm) and visible (400—750 nm) region. The relative molecular weight of the enzyme was assayed by the method of gel filtration in a thin layer[12] of Sephadex G-150 Superfine (Pharmacia), 20 × 40 cm; standard proteins supplied as the MS-II Serva Kit were used for calibration. Disc electrophoresis in an alkaline medium (pH 8·9) was carried out according to Davis[13]. The proteins were detected both by staining with amido black and also specifically by assaying superoxide dismutase activity with photochemical riboflavin reduction as an $O_2^{-\cdot}$ source and with simultaneous reduction of the nitrobluetetrazolium salt to formazane.

RESULTS AND DISCUSSION

The isolation of the individual superoxide dismutase types from various animal and vegetal species by chromatographic methods has been described by numerous authors[1,4,15,16]. A comparison of the characteristics of the three superoxide dismutase types so far identified shows that the Cu/Zn-enzyme from the cytosole of eu-

karyotes is completely different from the two additional forms of the enzyme which are closely related. The purification of Cu/Zn-superoxide dismutase from the extract of bovine erythrocytes on a DEAE-cellulose column according to McCord and Fridovich[1] is shown in Fig. 1. The first peak (*I*) contains the majority (85%) of enzymatic activity. The characteristics of this preparation (Table I) are in agreement with the original recorded data[1].

TABLE I

Characterization of Cu/Zn-superoxide dismutase, obtained by DEAE-cellulose chromatography, by isoelectric focusing

DEAE zone	Total protein mg	Specific activity U/mg	Total activity units	Spectra nm	Relative mol.wt.[a] daltons
I	198·5	64·6	12 825	UV_{max} 276 VIS_{max} 700	33 000 ± 6 000 78 000 ± 3 000
Isoelectric zone					
III	16·8	40	1 920	UV_{max} 276 VIS_{max} 695	32 000 ± 5 000
IV	65·7	69	5 037	UV_{max} 276 VIS_{max} 405 565	32 500 ± 3 000

[a] Average ± standard deviation of six determinations.

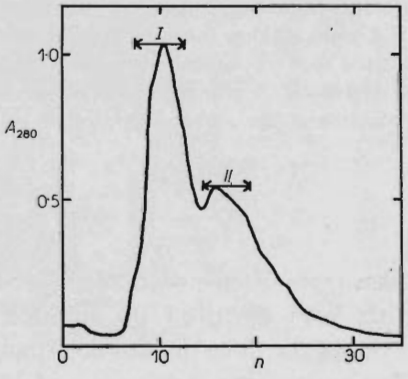

FIG. 1

Purification of Cu/Zn-superoxide dismutase from bovine erythrocyte extract on a DEAE-cellulose column, The column (2·5 × × 25 cm) was eluted by 2·5 mM phosphate buffer at pH 7·4, gradient of 0·0025—0·2M-KCl

Still another component was revealed by thin-layer gel filtration on Sephadex G-150; the molecular weight of this component is 78 000 daltons. A similar fraction has been detected in the cytosole fraction of chicken liver[14,15]. The occurrence of this component is being explained by the association of the enzyme molecules as a result of the formation of intermolecular disulfide bonds. Our value of molecular weight, *i.e.* 78 000 corresponds to that of the dimer since the value for the Cu/Zn-superoxide dismutase monomer reported by numerous authors is 31 000–33 000 daltons.

By disc electrophoresis at pH 8·9 of the preparation obtained by DEAE-cellulose chromatography the presence of four proteins zones was demonstrated of which three showing lower migration possessed superoxide dismutase activity. The multiplicity observed during electrophoresis of Cu/Zn-superoxide dismutase has repeatedly been described[8,14,19] yet always, however, for this type of enzyme only. The Mn- or Fe-enzymes behave as homogeneous proteins[16,20] under identical conditions. The cause of this phenomenon has not been elucidated. Some authors assume the presence of isoenzymes; it has also been postulated that the multiple forms of Cu/Zn-superoxide dismutase may result from the treatment of the enzyme[21,22].

The fact that two values of relative molecular weight were found for the Cu/Zn-enzyme obtained by chromatography on DEAE-cellulose indicates a difference in the size of the molecules. There is moreover a difference in net charge, as evidenced by the electrophoretic resolution into three zones. It follows from our data that the Cu/Zn-superoxide dismutase preparation purified chromatographically can be re-

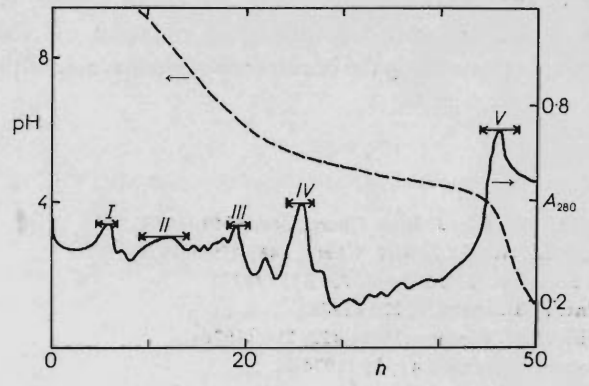

FIG. 2

Resolution of Cu/Zn-superoxide dismutase by isoelectric focusing. The conditions are described under Experimental

solved by isoelectric focusing into two catalytically active forms. Fig. 2 shows the pH-gradient with focused protein zones. The absorbance measurement at 280 nm demonstrated the presence of five protein zones ($I-V$); superoxide dismutase activity was found in zones *III* (pI 6·3) and *IV* (pI 5·2) only. Of the total enzyme activity applied to the electrofocusing column 86·8% was recovered; hence, the catalytic activity of the enzyme is not deteriorated any significantly during the focusing.

The isolation of two dismutase forms with distinct isoelectric point values showed that one of the causes of Cu/Zn-superoxide dismutase heterogeneity is the difference in the net charge of the molecules. The difference in migration of the enzymatically active zones toward the anode during electrophoresis in the alkaline medium corresponds to differences in isoelectric points. The zones identified during the electrophoretic separation of the individual enzyme forms are in agreement with the occurrence of zones obtained after chromatography of the starting preparation. Table I shows the values of the characteristics of the enzyme obtained by electrofocusing. A comparison with the data reported for the enzyme purified chromatographically demonstrates that the specific activity of the enzyme does not increase in the process of focusing in any of the separated forms of Cu/Zn-superoxide dismutase.

We found with both forms of the Cu/Zn-enzyme the relative molecular weight value reported[15,17,18] for the monomer molecule. The data of spectral analysis indicate that the maximum in the ultraviolet region of the spectrum is identical for both forms and at the same time identical with the wavelength value of the maximum of the Cu/Zn-superoxide dismutase preparation obtained chromatographically. If we compare the spectra in the visible region the difference in the position of the maxima of the pI 5·2 form becomes evident. The reason for this difference may be a different arrangement of the prostethic group of the enzyme including a difference in the net charge of the metal ions present in the active center. The identification of the two forms of Cu/Zn-superoxide dismutase obtained by isoelectric focusing supports the hypothesis postulating the occurrence of isoenzymes of this type of superoxide dismutase.

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