THE SUPEROXIDE DISMUTASE ACTIVITY OF HUMAN ERYTHROCUPREIN

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1. Introduction

The superoxide dismutase activity of bovine erythrocuprein has usually been determined by a relatively complex procedure in which the protein competes with cytochrome *c* or nitroblue tetrazolium for the superoxide anion (O_2^-) produced either enzymatically [1–9] or photochemically [10] in low concentrations. Recently, however, the technique of pulse radiolysis has been used for the assay [11,12] as it is a convenient method of producing, virtually instantaneously, large concentrations of O_2^- [13–15]. The turnover of the substrate can then be followed directly using the absorption band of O_2^-, which has an extinction coefficient of 2000 M^{-1} cm^{-1} at 245 nm. In this paper we wish to describe some of the physical and chemical characteristics of the superoxide dismutase activity of human erythrocuprein as studied by pulse radiolysis.

2. Materials and methods

Human erythrocuprein was prepared from outdated human blood as described previously [16]. The pulse radiolysis equipment [17] and the determination of superoxide dismutase activity by pulse radiolysis [11] have been detailed elsewhere. Assays were performed in 2 mM pyrophosphate buffer, pH 9.1, saturated with oxygen prior to irradiation. Half of the superoxide anion is formed by the reaction of hydrated electrons, produced by the irradiation, with oxygen [18]:

$$e^-_{aq} + O_2 \rightarrow O_2^- .$$

The presence of 0.08 M "Analar" ethanol in the assay mixture served to remove H and OH radicals, which were ultimately converted to the extra yield of O_2^- [13].

By thoroughly removing all traces of oxygen before irradiation the hydrated electrons are available for reaction with the enzyme. The absorption at 690 nm was used to follow valency changes in the enzyme-bound copper atoms induced by reaction with O_2^-, H_2O_2 or hydrated electrons. After such reactions, the activity of the protein was measured within 2 min. During this time interval negligible reoxidation from the atmosphere occurred. EPR spectra were recorded on a Varian 4502-14 spectrometer equipped with 100 KHz field modulation with a Varian multipurpose cavity, and variable temperature accessory.

3. Results and discussion

Human erythrocuprein was shown to catalyse the dismutation of superoxide anion, as does the corresponding bovine protein.

$$O_2^- + O_2^- \xrightarrow[\text{Enzyme}]{2H^+} H_2O_2 + O_2$$

Table 1 shows that the dismutase activity is essentially independent of pH over a wide range and that ac-

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Table 1

pH	Enzyme concentration (μM)	$k \times 10^{-9}$ (M^{-1} sec^{-1})
5.7	2.0	1.6
7.5	2.0	1.4
9.2	2.0	1.5
10.5	2.0	1.2
9.1	0.4	1.6
9.1	2.0	1.4
9.1	8.5	1.4

The effect of pH and enzyme concentration on superoxide anion turnover, as measured by the bimolecular reaction rate constant, k.

tivity is directly proportional to the protein concentration over the twenty-fold range tested. The activity was also found to be unaffected by buffering the protein overnight within the range pH 4.8–10.0. At pH 3.0, however, this treatment reduced the activity by 40%. Incubating the protein (pH 9.1) for 2 min at temperatures up to 50° produced no loss of activity but a 36% loss was found following a 2 min treatment at 62°.

The turnover velocity for 0.4 μM protein increased linearly with superoxide concentration up to the maximum attainable concentration of 200 μM. In common with the bovine enzyme [11] the reaction shows no evidence of a Michaelis complex, since a Lineweaver–Burke plot (fig. 1) produces a straight line passing through the origin. The second order rate constant for the reaction of O_2^- with the enzyme (k), as determined under turnover conditions, is calculated from this data to be 1.5×10^9 M^{-1} sec^{-1} at about 25°, which is very similar to the value reported for the bovine protein [11]. Enzyme activity was suppressed in the presence of potassium cyanide (fig. 2), 16 μM and 1000 μM CN^- producing 50% and 100% inhibition, respectively, of 2 μM enzyme.

Exposure to a ten-fold excess of hydrated electrons or hydrogen peroxide resulted in reduction of the divalent enzyme copper. The reduction was followed at 690 nm and it was found that, within 1 min, the absorbance was reduced by 82% with hydrogen peroxide and by 85% with hydrated electrons at this wavelength. The superoxide anion, however, produced only a 15% decrease in absorbance when reaching equilibrium

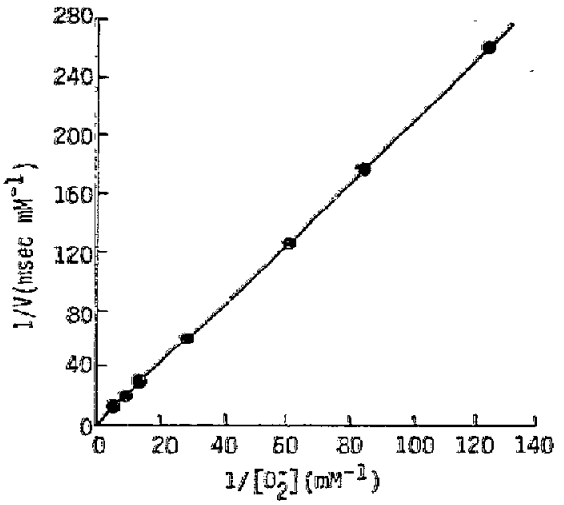

Fig. 1. Lineweaver–Burke plot of the enzyme catalysed reaction. Enzyme 0.4 μM.

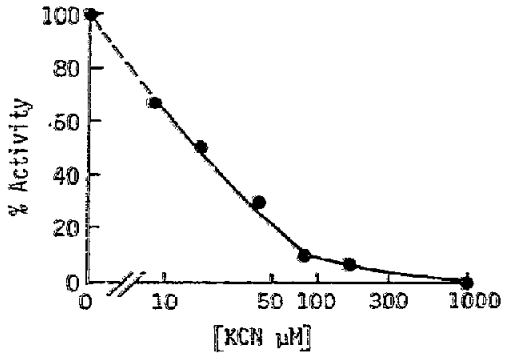

Fig. 2. Inhibition of enzyme activity by CN^- at pH 9.1. Enzyme was 2 μM and O_2^- 30 μM.

conditions during turnover. When protein which had been reduced by a brief pre-treatment with hydrogen peroxide or hydrated electrons was exposed to O_2^-, the superoxide was removed catalytically, but in this case the absorbance at 690 nm increased during turnover by an amount equivalent to 26% of the absorbance of the oxidised enzyme. The overall bimolecular rate constant for turnover of O_2^- by chemically reduced enzyme was 1.5×10^9 M^{-1} sec^{-1}. This value is exactly that found for turnover by untreated enzyme and indicates that the oxidation of the copper has no effect on the activity of the enzyme.

In this study, the enzymic properties of human erythrocuprein were found to be essentially identical

to those reported for the corresponding bovine protein [11,19]. A wide range of pH or temperature does not alter the activity of either enzyme significantly, whilst cyanide inhibits both proteins equally. The enzymes possess similar turnover velocities and lack a detectable Michaelis complex. In addition, the valency changes brought about in the enzyme copper by hydrogen peroxide [19–22] hydrated electrons and O_2^- [19,22] in the bovine enzyme are paralleled in the human enzyme. Other evidence that the differences between the human and bovine enzyme were minimal is that the EPR spectrum of the human protein used in this work (fig. 3) has shape and parameters very close both to those of the bovine protein [4] and those obtained for the human enzyme by Beinert (private communication; the value of all quoted in [23] and obtained in Beinert's laboratory is incorrect owing to a typographical error). Isolation of the human protein used in this work and the bovine enzyme [4] involves chloroform–ethanol treatment, whereas the preparation of the human enzyme used in [23] does not. The great similarity of the EPR and kinetic parameters for the human bovine enzymes and the near diffusion-controlled rates observed indicate, however, that the isolation procedures used for the enzymes do not affect significantly the catalytic properties. This is important in view of the possibility that the chloroform–ethanol treatment could modify some vital properties of the enzyme [24].

The results indicate that the dismutation of superoxide radicals is catalysed by virtually identical sites in the bovine and human enzymes. The chemical composition of the two proteins is already known to be very similar [16]. Thus the results of any mechanistic studies obtained with the bovine enzyme [19,22] should be applicable to the human enzyme. Further studies are in progress on the enzyme, including the effect of divalent metal ion substitution on the dismutase activity.

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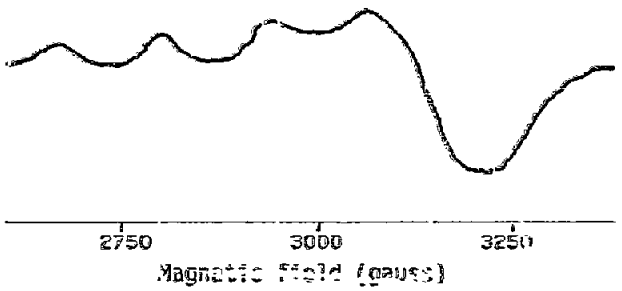

Fig. 3. The electron paramagnetic resonance spectrum of human erythrocuprein; 1.5% solution in water. Microwave frequency: 9.15 GHz, microwave power: 6 mW; modulation amplitude 10 gauss, temperature: −186°.

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