Product of extracellular-superoxide dismutase catalysis

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Extracellular-superoxide dismutase is a tetrameric enzyme containing four copper atoms. It has previously been shown to catalyse the decay of the superoxide radical, but the resulting product was not determined. In a xanthine oxidase-xanthine system in which about 30% of the electron flux resulted in superoxide radical formation, accumulation of hydrogen peroxide was determined. Catalysis of superoxide radical decay by extracellular-superoxide dismutase was found to result in hydrogen peroxide formation. The catalysed reaction is thus identical to those of previously investigated superoxide dismutases. Human manganese superoxide dismutase was also found to dismute the superoxide radical to hydrogen peroxide and water.

Superoxide dismutase *Superoxide radical* *Oxigen radical* *Hydrogen peroxide* *Plasma*

1. INTRODUCTION

Superoxide dismutases (EC 1.15.1.1) catalyse the dismutation of 2 superoxide radicals to oxygen and hydrogen peroxide, $2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$. In eukaryotes, two intracellular superoxide dismutase isoenzymes are generally found, the CuZn superoxide dismutase [1] and the Mn superoxide dismutase [2]. The formation of H_2O_2 as a product of the reaction catalysed by CuZn superoxide dismutase has been described [1]. Recently, a third isoenzyme catalyzing the decay of O_2^- was described, extracellular (EC)-superoxide dismutase. This enzyme is a tetrameric glycoprotein which appears to possess four Cu atoms and possibly also four Zn atoms [3]. It can be demonstrated in a number of tissues, but at much lower concentrations than CuZn superoxide dismutase and Mn superoxide dismutase [4,5]. In the extracellular fluids it is the major superoxide dismutase, and a number of circumstances indicate that it is synthesized for a function in the extracellular space [4–6].

No analysis of the product of the reaction catalysed by this enzyme has been performed so far. EC-superoxide dismutase, which contains four transition metal ions, might conceivably handle the more complicated task of forming water; e.g., according to the formula: $4O_2^- + 4H^+ \longrightarrow 3O_2 + 2H_2O$. The present paper reports the analysis of the product of EC-superoxide dismutase catalysis. In addition, the products formed by human CuZn superoxide dismutase and Mn superoxide dismutase were determined.

2. MATERIALS AND METHODS

Human EC-superoxide dismutase [3] and CuZn superoxide dismutase [7] were prepared as described. Human Mn superoxide dismutase was isolated following the procedure of McCord et al. [8]. Xanthine oxidase from cow milk was obtained from Boehringer Mannheim. Horseradish peroxidase type C (= IIIb) [9] was a kind gift from Drs K.G. Paul and P.I. Olsson, Department of Physiological Chemistry, Umeå University. Oxidized horse heart cytochrome *c* type III was obtained from Sigma Chemical Company. To ensure complete oxidation, the preparation was treated with ferricyanide and was thereafter subjected to gel filtration. Dicarboxidine, γ,γ^1-4,4^1-diamino-3,3^1-diphenyldioxydibutyric acid, was obtained from Kabi-Vitrum (Stockholm).

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2.1. *Accumulation of H_2O_2 during xanthine oxidase activity*

Xanthine (0.1 mM) was added to xanthine oxidase in air-equilibrated 50 mM sodium phosphate buffer (pH 7.40) with 0.25 mM diethylenetriaminepentaacetic acid at 25°C, in a cuvette, and absorbance at 440 nm was recorded. After 2 min, horseradish peroxidase (40 nM) and dicarboxidine (0.2 mM) were added and accumulated H_2O_2 was estimated from the immediate rise in A_{440}. The molar absorption of H_2O_2 as determined with peroxidase-dicarboxidine is 12500 $M^{-1} \cdot cm^{-1}$.

2.2. *Rate of O_2^- production by the xanthine oxidase–xanthine system as determined by ferricytochrome* c *reduction*

Cytochrome *c* (30 μM) was added to xanthine (0.1 mM) and xanthine oxidase in 50 mM sodium phosphate buffer (pH 7.40) with 0.25 mM diethylenetriaminepentaacetic acid. The rate of O_2^- formation was calculated from the rate of cytochrome *c* reduction employing a difference in molar absorption between the reduced and oxidized form of 21100 $M^{-1} \cdot cm^{-1}$ [10].

2.3. *H_2O_2 and O_2^- production by xanthine oxidase as determined in an oxygen electrode*

The relative rates of H_2O_2 and O_2^- production by the xanthine oxidase at pH 7.4 was determined in a Clark oxygen electrode, employing oxidized cytochrome *c*. The cytochrome *c* will oxidize O_2^- back to oxygen, thereby reducing the rate of oxygen consumption in proportion to the electron flux in the xanthine oxidase that forms O_2^- instead of H_2O_2. Xanthine oxidase was added to 50 mM sodium phosphate buffer (pH 7.4) containing 0.25 mM diethylenetriaminepentaacetic acid and 0.1 mM xanthine, equilibrated with air at 25°C. The basal rate of oxygen consumption was 15 μM/min; 50 μM oxidized cytochrome *c* reduced the rate by 25%.

3. RESULTS

3.1. *H_2O_2 and O_2^- production by the xanthine oxidase–xanthine system*

The relative rates of H_2O_2 and O_2^- production were determined in two ways. As determined from the cytochrome *c*-induced reduction of oxygen consumption by a xanthine oxidase–xanthine system in an oxygen electrode, 25% of the electron flux resulted in O_2^- formation. A comparison between the rate of cytochrome *c* reduction by xanthine oxidase–xanthine and the accumulation of H_2O_2 in the xanthine oxidase–xanthine system indicated that 31% of the electron flux resulted in O_2^- formation. We have no explanation for the difference, but the results are in reasonable agreement with previous estimates [11].

3.2. *Product analysis of superoxide dismutase action*

As seen in table 1, there was no significant difference between the amounts of H_2O_2 accumulated during xanthine oxidase action in the presence of the three superoxide dismutase isoenzymes and in the absence of added enzyme. Spontaneous dismutation and dismutation catalysed by the superoxide dismutases therefore appears to give the same amount of H_2O_2. Given the relative rate of O_2^- formation in the system as described above,

Table 1

Effect of superoxide dismutase isoenzymes on the amount of hydrogen peroxide produced by xanthine oxidase

	Hydrogen peroxide formed
Blank (no addition) (n = 5)	100 ± 1.8% (SD)
Human CuZn superoxide dismutase, 2 μg/ml (n = 5)	100.4 ± 1.2% (SD)
Human Mn superoxide dismutase, 2 μg/ml (n = 6)	100.5 ± 0.3% (SD)
Human EC-superoxide dismutase, 1.7 μg/ml (n = 5)	101.6 ± 1.6% (SD)

Superoxide dismutase isoenzymes were added to a buffer containing 100 μM xanthine. The reaction was initiated by addition of xanthine oxidase. After 2 min, horseradish peroxidase and dicarboxidine were added to determine H_2O_2 formed, as described in section 2. The results are presented as the percentage of the amount formed in the absence of superoxide dismutase (blank). 100% corresponds to 4.3 μM H_2O_2

a significant formation of water or other products would easily have been detected.

4. DISCUSSION

Formation of H_2O_2 as a result of spontaneous and CuZn superoxide dismutase-catalysed O_2^- degradation has been demonstrated in [1]. The product of EC-superoxide dismutase action has not been investigated before. Neither have the tetrameric mammalian Mn superoxide dismutases been investigated in this respect. No difference in H_2O_2 formation between spontaneous dismutation and dismutation catalysed by human EC-superoxide dismutase, Mn superoxide dismutase and CuZn superoxide dismutase could be detected. The results establish H_2O_2 as the product of human EC-superoxide dismutase and human Mn superoxide dismutase action.

The H_2O_2 resulting from EC-superoxide dismutase action is of special interest. Whereas catalase and glutathione peroxidase can dispose of H_2O_2 formed in tissues, there is very little of such protection in the extracellular space. The catalase activity is negligible [12] and the small amounts of glutathione peroxidase [13] will be inefficient since very little reduced glutathione is available [14]. This fact may be one of the reasons why the EC-superoxide dismutase activity is kept low in the extracellular space [5,6]. Given the membrane permeability of O_2^- in some cell types [15], extracellular superoxide dismutase will tend to shift towards the extracellular space the formation of H_2O_2 from intracellularly and extracellularly produced O_2^-. H_2O_2 permeates membranes, which means that extracellularly produced H_2O_2 can be disposed of by erythrocytes and other catalase- and glutathione peroxidase-rich cell types. However, there are large differences between tissues in the content of catalase and glutathione peroxidase [16]. This protective mechanism will therefore be less efficient in the interstitial space in tissues with a low content of the H_2O_2 scavenging enzymes. Ceruloplasmin is an inefficient O_2^- scavenger ([17], submitted), but given its high concentration it will contribute to the disposal of O_2^- in the extracellular space (submitted). In this context it is interesting to note that this scavenging does not lead to the formation of H_2O_2 [17].

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