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Determination of Manganese Superoxide Dismutase Activity By Direct Spectrophotometry

BJØRN J. BOLANN[1], ARILD TANGERÅS[2] and RUNE J. ULVIK[1]*

[1]*Institute of Clinical Biology, Section of Biochemistry, and* [2]*Institute of Biochemistry and Molecular Biology, University of Bergen, N-5021 Bergen, Norway*

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A method to determine Mn-superoxide dismutase activity by measuring directly the rate of decay of $O_2^{\cdot-}$ in a spectrophotometer, is described. Decay of $O_2^{\cdot-}$ generated by KO_2 at pH 9.5, was monitored as the fall in absorbance (A_{250nm}–A_{360nm}). Mn-superoxide dismutase was determined as the activity of cyanide-resistant superoxide dismutase, calculated from the rate of $O_2^{\cdot-}$ dismutation. Mn-superoxide dismutase could be determined in the presence of a 700 times higher Cu,Zn-superoxide dismutase activity. The alkaline pH did not cause analytical problems. The assay was used to measure both Mn- and Cu,Zn-superoxide dismutase activity in mitochondrial preparations. The assay had a detection limit of 2.8 ng/ml when Mn-superoxide dismutase from E. coli was used, and the between-day CV was 5.8%. The assay is an alternative to indirect methods for detecting superoxide dismutase activity.

Keywords: Superoxide dismutase, Manganese, Spectrophotometry

Abbreviations: SOD, Superoxide dismutase; DTPA, Diethylenetriaminepentaacetic acid

INTRODUCTION

The rapid decay of $O_2^{\cdot-}$ at physiological pH represents an analytical challenge when determining superoxide dismutase activity (SOD; EC 1.15.1.1). The lack of proper methods like pulse radiolysis or stopped-flow techniques probably explains the common use of indirect spectrophotometric assays. However, the indirect methods are sensitive to disturbances, and attempts to improve their analytical quality have not been satisfactory.[1,2] Immunological methods are not always relevant since they do not measure the enzyme *activity*.[3]

Recently we described a spectrophotometric method which directly measures the decay of $O_2^{\cdot-}$ in an aqueous solution at pH 9.5.[4] The assay was designed to determine Cu,Zn-SOD activity which is fairly stable at alkaline pH.[5–7] However, the role of Mn-SOD in biology and medicine is gaining interest.[8] In the present work we have modified the assay to differentiate between the Cu,Zn- and Mn-SOD isoenzymes and even allow determination of Mn-SOD activity in the presence of a large excess of Cu,Zn-SOD.

*Corresponding author: Tel.: + 47 5597 3149. Fax: + 47 5597 3115.

MATERIALS AND METHODS

Chemicals

Cu,Zn-SOD (from bovine erythrocytes), Mn-SOD (from *E. coli*), and catalase (EC 1.11.1.6) (from bovine liver) were from Sigma Chemical Co. (St. Louis, MO, USA). The enzymes were passed through a Sephadex G-100 column prior to use. SDS-polyacrylamid gel electrophoresis confirmed purity of the two SOD isoenzyme preparations without any mutual contamination.[7] Catalase contained no SOD-activity and its activity was defined according to[9]. Protein was determined as in[10].

KCN was from E. Merck, Darmstadt, Germany and KO_2 from Fluka AG, Buchs, Switzerland. The reactive KO_2 required careful handling.[4] Other chemicals were of the highest purity commercially available. All glassware was washed with acid to remove contaminating transition metals.

Preparation of Mitochondria and Assay of Marker Enzyme Activities

Rat liver mitochondria ("Fraction 1"), mitoplasts and the cytosol fraction were isolated as previously described.[11] To make "Fraction 2", lysosomal and peroxisomal contamination in "Fraction 1" was decreased by a digitonin treatment similar to that used in mitoplast preparation, except that only 100 μM of digitonin was used. Marker enzyme activities were measured as previously described.[12]

Assay to Measure SOD Activity

The sample to be tested for total SOD activity was added to 3 ml of a medium consisting of:[4] 50 mM of 2-amino-2-methyl-1-propanol HCl, pH 9.5, 0.2 mM of DTPA, and 0.35 U/ml of catalase. The incubations were performed in a thermostatically controlled Hewlett-Packard HP 8450A diode array spectrophotometer at 5°C. The outside of the cuvet was flushed with N_2 to prevent it from becoming misted. $O_2^{\bullet-}$ was generated by dissolving about 100 mg of KO_2 in 12.5 ml of 50 mM NaOH with 0.5 mM of DTPA on ice. After about 30 seconds, 7.5–15 μl was transferred to the incubation medium and the declining concentration of $O_2^{\bullet-}$ was monitored as the decrease in the difference in absorbance (ΔA) at two wavelengths (A_{250nm}–A_{360nm}). The rate of $O_2^{\bullet-}$ decay was expressed as the apparent pseudo-1st order rate constant, calculated from the decay of $O_2^{\bullet-}$ from 16 to 4 μM. The SOD activity of the material tested was determined as the rate of decay of $O_2^{\bullet-}$ which exceeded that of the spontaneous dismutation. One unit (U) of SOD was defined as the amount required to achieve a pseudo-1. order rate constant of 0.1 s^{-1} in a volume of 1 ml.

The specific activity of Mn-SOD was determined by adding 10 mM of KCN to inhibit the activity of Cu,Zn-SOD. Catalase was omitted. In the text the "blank" refers to the $O_2^{\bullet-}$-containing incubation medium without addition of SOD.

RESULTS

Decay of $O_2^{\bullet-}$ Catalyzed by Mn-SOD

The rate of $O_2^{\bullet-}$ decay increased linearly with increasing amounts of Mn-SOD both in the absence (not shown) and presence of KCN (Figure 1). As a control, corresponding concentrations of albumin had no effect on the $O_2^{\bullet-}$ decay (not shown).

KCN concentrations above 1 mM inhibited Cu,Zn-SOD about 98.5%, while Mn-SOD was inhibited <10% even in 10 mM of KCN. Since KCN had no adverse effect on the Mn-SOD activity, and Cu,Zn-SOD is inhibited competitively by CN^-,[13] we used 10 mM KCN although 2–5 mM has been used by others.[2,14]

To study if the low KCN-resistant activity of Cu,Zn-SOD might disturb the measurement of

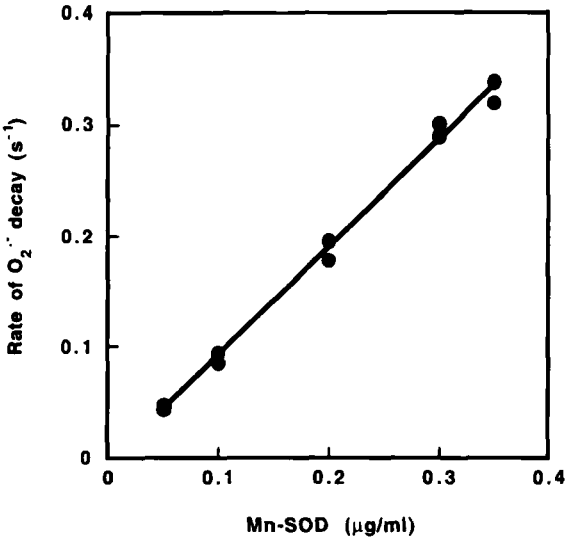

FIGURE 1 Effect of Mn-SOD on the decay of $O_2^{\cdot-}$. The rate of $O_2^{\cdot-}$ decay was calculated as described in Materials and Methods, and was corrected for the spontaneous dismutation. The results shown are single experiments. The correlation between the amount of Mn-SOD present and the rate of $O_2^{\cdot-}$ decay was $r^2 > 0.99$.

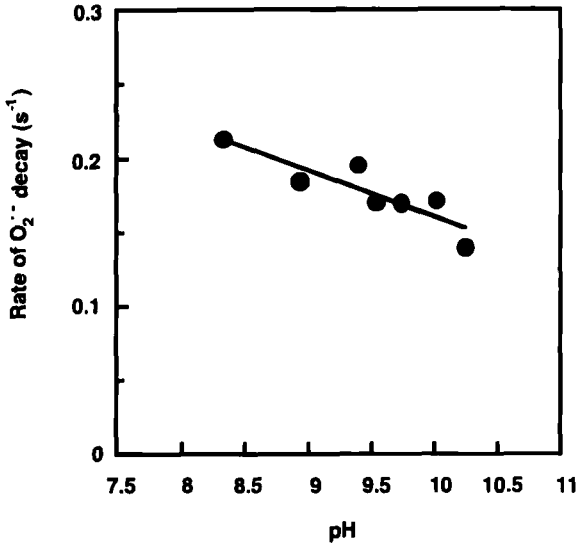

FIGURE 2 Effect of pH on Mn-SOD activity. Rate of $O_2^{\cdot-}$ decay (corrected for the spontaneous dismutation) in the presence of 150 ng/ml of Mn-SOD. The results are the mean of 2-3 measurements.

Mn-SOD in a mixture of both enzymes, KCN-resistant SOD activity was determined in samples with increasing ratios of Cu,Zn-SOD/Mn-SOD, up to 3300 U/U. The KCN-resistant SOD activity increased with increasing ratio, and at a Cu,Zn-SOD/Mn-SOD ratio of 700 U/U it was 15% higher than expected from the Mn-SOD present. At higher ratios the KCN-resistant SOD activity could no longer be interpreted as Mn-SOD.

Effect of pH

At a physiological pH the direct spectrophotometric assay cannot differentiate enzyme-induced rate changes from the high rate of spontaneous $O_2^{\bullet-}$ dismutation.[7] Therefore pH 9.5 was generally used. By changing pH step-wise from 9.5 towards 8.0, the relationship between pH and the Mn-SOD-catalyzed rate of decay of $O_2^{\bullet-}$ appeared as shown in Figure 2. The reaction was about 25% faster at pH 8.3 than at pH 9.5. In contrast, the spontaneous dismutation is about 30 times faster at pH 8 than at pH 9.5.[7]

Mn-SOD Activity Determined in Rat Liver Fractions

In eukaryotic cells Mn-SOD is found in the mitochondria, whereas Cu,Zn-SOD is found in the cytoplasm, lysosomes and possibly also in peroxisomes.[15–17] To demonstrate the capability of the assay to determine SOD in subcellular fractions, we measured total and KCN-resistant SOD activity in mitochondrial fractions contaminated to different degrees with Cu,Zn-SOD (Figure 3). No fraction had pure Mn-SOD activity. 9% of the total SOD activity of "Fraction 1" was Mn-SOD, while in mitoplasts the corresponding figure was 72%. In the cytosol the KCN-insensitive SOD activity was low, as expected.[15]

Precision and Sensitivity

The Mn-SOD assay had a within-day CV between single tests (n = 6) from 2 to 5%. When using the mean of 2 measurements of each sample, the between-day CV was 5.8% (13 days, mean activity: 9.02 U/μg of Mn-SOD). The detection limit,

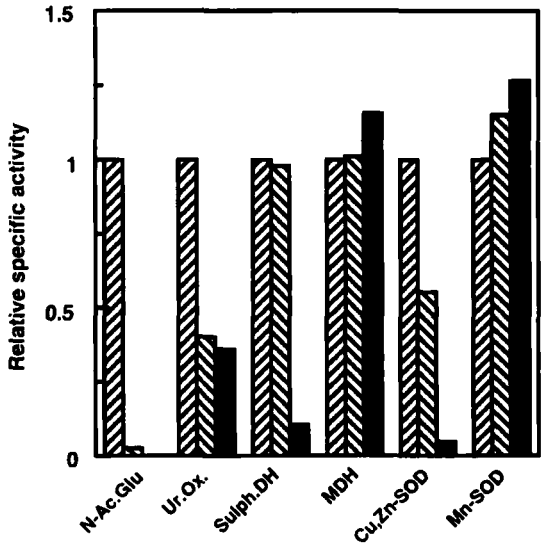

FIGURE 3 Distribution of Cu, Zn-SOD, Mn-SOD and marker enzymes in three mitochondrial preparations, ▨ " Fraction 1 "; ▧ "Fraction 2"; ■ , Mitoplasts. The activity of N-acetylglucosaminidase (N-Ac. Glu), urate oxidase (Ur. Ox.), sulphite dehydrogenase (Sulph. DH), malate dehydrogenase (MDH), Cu, Zn-SOD was measured in the three preparations and specific activity relative to that found in " Fraction 1 " is given. The results are the mean of 2-3 experiments, except the results for SOD which are the mean of 6 experiments (CV=15%).

defined as blank + 3 SD, was 0.0254 U/ml, which corresponds to 2.82 ng/ml of Mn-SOD from E. coli. In rat liver homogenate the CV between single tests for Mn-SOD (n = 6) was about 15%. The activity of Cu,Zn-SOD from bovine erythrocytes was, on a weight basis, 29 times higher than that of Mn-SOD from *E. coli*.

DISCUSSION

We have shown that the activity of Mn-SOD can be determined with high sensitivity and precision by measuring directly the rate of decay of $O_2^{\bullet-}$ in a spectrophotometer. The detection limit of 2.8 ng/ml is of the same order as that of immunological methods.[3,14] In the indirect assays, the amount of Mn-SOD required to achieve a 50% inhibition of the rate of reduction of ferricytochrome C or nitroblue tetrazolium, is 50–167 ng/ml.[2,18]

Both Cu,Zn-SOD and Mn-SOD retain significant activity up to pH 10.[5–7,19] By extrapolating the curve in Figure 2, the activity appears to be about 50% higher at pH 7.0 than at pH 9.5, which is in good agreement with others.[19] Thus, the activity at pH 9.5 is sufficient to measure both Cu,Zn- and Mn-SOD activity.

The different sensitivity to KCN is utilised to separate Mn-SOD from Cu,Zn-SOD.[13,20] Catalase is inhibited by KCN,[21] but Mn-SOD is not inhibited by H_2O_2,[20] so catalase was not needed in the Mn-SOD assay. H_2O_2 generated by the dismutation of $O_2^{\bullet-}$, did not disturb the assay when the baseline of the $O_2^{\bullet-}$ decay curve was obtained as previously described.[4]

Fe-SOD may be cyanide-resistant but is inactivated by H_2O_2.[20] Its effect on the assay was not tested.

The advantages of using dual wavelength measurements and to cool the incubation medium have been discussed previously.[4] Using room temperature and/or single beam technique is possible, but may reduce the analytical precision.

At very high concentrations, the small KCN-insensitive fraction of Cu,Zn-SOD may cause an overestimation of the Mn-SOD activity. However, this error was ≤15% when the Cu,Zn-SOD/Mn-SOD ratio was below 700:1.

To determine Mn-SOD activity in samples which also contain Cu,Zn-SOD, the following procedure is recommended: (1) Measure total SOD activity. Dilute the specimen until the activity falls within the optimal range of measurement of 1–3 U/ml (final concentration).[4] (2) Repeat using the same dilution with 10 mM of KCN, as described in this work. (3) If KCN-resistant activity is low or absent, repeat with progressively higher concentrations of the specimen, until a KCN-resistant activity between 1 and 3 U/ml (final concentration) appears. (4) If the KCN-resistant activity found is more than 1/700 of the total activity, it can be considered as Mn-SOD activity.

The present assay may be particularly useful in tissue homogenates where redox active sub-

stances may disturb the indirect methods. Purification procedures which are recommended for indirect assays,[14] may inactivate SOD, particularly Mn-SOD,[22] and are not necessary with the present method. Moreover, cyanide may interfere with indirect SOD assays and complicate the differentiation between Cu,Zn- and Mn-SOD in these assays.[23]

The high Mn-SOD/Cu,Zn-SOD ratio found in the mitochondria, and the low ratio found in the cytosol, is in accordance with other studies.[15,24] The mitoplast preparation is the fraction which is least contaminated with lysosomal and peroxisomal marker enzymes (Figure 3), and both these organelles may contain Cu,Zn-SOD.[16,17,24] A similar effect of digitonin on the release of SOD activity in rat liver mitochondrial fractions has been reported by others.[24,25]

In *conclusion*, the direct spectrophotometric assay is a sensitive, precise and specific method for the determination of Mn-SOD activity. Furthermore, Mn-SOD activity can be determined in the presence of high Cu,Zn-SOD activity, and the equipment required is available in most laboratories.

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