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Free Radical Biology & Medicine 41 (2006) 937–941

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Original Contribution

Kinetic properties of Cu,Zn-superoxide dismutase as a function of metal content—Order restored

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Received 24 April 2006; revised 21 May 2006; accepted 24 May 2006

Abstract

In a recent publication (Michel et al. *Arch. Biochem. Biophys*. **439**:234–240; 2005) the authors argued that the catalytic rate constant, k_{cat}, for wild-type Cu,Zn-superoxide dismutase (Cu,Zn-SOD), determined previously by pulse radiolysis, was overestimated due to contamination with excess copper. They reported that addition of 0.1 mM EDTA to a sample that already contained excess copper did not remove spurious activity, which is incompatible with well-known stability constants of copper complexes and contradicts previous observations. In the present study we verified that the addition of EDTA eliminates completely the effect of excess copper on the decomposition rate of $O_2^{\bullet -}$ in the presence of Cu,Zn-SOD. We determined that $k_{cat} = (2.82 \pm 0.02) \times 10^9\ M^{-1}\ s^{-1}$ at low ionic strength ($2 < I < 15$ mM) and $(1.30 \pm 0.02) \times 10^9\ M^{-1}\ s^{-1}$ in the presence of 50 mM phosphate at pH 7.8 ($I \approx 150$ mM), which are about twice higher than those reported by Michel et al. We also determined k_{cat} by the cytochrome *c* assay and demonstrated the correlation between these direct and indirect assays. The phenotypic deficits imposed by deletion of SODs, and the oxygen dependence of these deficits, have repeatedly demonstrated that the several SODs do in fact, as well as is theory, provide an important protection against that facet of oxidative stress imposed by $O_2^{\bullet -}$.

Keywords: Superoxide; Superoxide dismutase; SOD activity; Radiolysis; Cytochrome *c*; Ionic strength

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Introduction

Recently, it has been argued by Michel et al. [1] that the catalytic rate constants, k_{cat}, for wild-type Cu,Zn-superoxide dismutase (Cu,Zn-SOD), previously determined by pulse radiolysis [2–8], appear to have been overestimated due to contamination with excess copper. Michel et al. [1] reported that the addition of 0.1 mM EDTA to a sample that already contained excess copper did not remove spurious activity, and determined that $k_{cat} = (1.5 \pm 0.2) \times 10^9\ M^{-1}\ s^{-1}$ at $I = 15$ mM. This result was ascribed to the formation of a stable complex between copper and phosphate, the latter being usually at least 10-fold higher than EDTA concentrations, and to nonspecific binding of copper to charged surface residues of SOD [1]. However, this observation is incompatible with the well-known stability constants [9,10], and also contradicts previous observations, where addition of EDTA eliminates completely the effect of excess copper [2].

McCord and Fridovich [11] defined the specific activity of SOD in units per milligram, where 1 unit is the amount in milligrams of the protein that inhibits 50% of the rate of the reduction of 10 μM cytochrome *c* by superoxide (generated by xanthine/xanthine oxidase) in a 3.0-ml reaction volume containing 0.1 mM EDTA and 50 mM phosphate buffer (PB) at pH 7.8. The first direct determination of k_{cat} for Cu,Zn-SOD by pulse radiolysis yielded a value of $2.3 \times 10^9\ M^{-1}\ s^{-1}$, which was independent of the presence of EDTA and the pH in the range 4.8–9.5 [2]. Later it was shown that k_{cat} decreased with increasing the ionic strength (I) [6,12–14], and therefore it is important to note that the first determination was done at $I < 20$ mM. The literature values of k_{cat} for Cu,Zn-SOD as determined by pulse

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Abbreviations: Cu,Zn-SOD, Cu,Zn-superoxide dismutase; PB, phosphate buffer; cyt, cytochrome.

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doi:10.1016/j.freeradbiomed.2006.05.026

radiolysis in the presence of 0.1 mM EDTA and low ionic strengths vary between 1.8×10^9 and 3.7×10^9 M^{-1} s^{-1} [2–8]. In those studies the concentration of the enzyme was determined by its absorbency at 258 or 680 nm [11]. Klug et al. [2] measured a value of 2.3×10^9 M^{-1} s^{-1} for Cu,Zn-SOD having a specific activity of 3300 units/mg (private communication). Since then, the specific activity of the enzyme has achieved 4000 units/mg [15], 5000 units/mg [16], and even 7000 units/mg (Sigma catalogue). In fact, the specific activity of the Sigma products varies between 2500 and 7000 units/mg, and consequently k_{cat} can vary between 1.7×10^9 and 4.9×10^9 M^{-1} s^{-1}.

In the present study we determined k_{cat} by pulse radiolysis and the specific activity by the cytochrome *c* assay of three different commercial products of Cu,Zn-SOD. The correlation between these direct and indirect assays is demonstrated. We also verified the earlier observation showing that addition of EDTA eliminates the effect of excess copper on the decomposition rate of $O_2^{\bullet-}$ in the presence of Cu,Zn-SOD.

Experimental

Materials

All chemicals were of analytical grade and were used as received. Water for preparation of the solutions was purified using a Milli-Q purification system. SOD from bovine erythrocytes was purchased from Sigma (products from 1997 and 2005) and from Boehringer-Mannheim. Cytochrome *c* Type VI from horse heart was purchased from Sigma. The absorption spectrum of cytochrome *c* solution before and after the addition of a small crystal of $K_3Fe(CN)_6$ was identical, indicating that the commercial product did not contain the reduced form. The concentration of the oxidized form was determined after its reduction with solid $Na_2S_2O_4$ using $\Delta\varepsilon_{550} = 21{,}000$ M^{-1} cm^{-1} [17].

Methods

Pulse radiolysis experiments were carried out using a 5-MeV Varian 7715 linear accelerator (1.5 μs electron pulses, 200 mA current). A 200 W Xe lamp produced the analyzing light. All measurements were made at room temperature in a 4-cm Spectrosil cell using three light passes (optical path length 12.1 cm). The dose was 20 Gy per pulse, as determined from the absorption of the superoxide ion in oxygenated solutions containing 10 mM formate and 2 mM phosphate buffer at pH 6.9. At least three experiments were averaged to obtain the observed rate constant.

Steady-state γ-irradiation experiments were carried out at room temperature using a ^{137}Cs source. The dose rate was determined by the Fricke dosimeter to be 8.7 Gy min^{-1}.

Results

Radiolytic generation of $O_2^{\bullet-}$

$O_2^{\bullet-}$ was formed by irradiation of aerated or oxygenated solutions containing formate and PB at pH 6.9 or 7.8. Under such conditions, all of the primary radicals formed by the radiation (Eq. (1)) are converted into superoxide via Reactions (1)–(5). In Eq. (2) the values in parentheses are *G* values, defined as the number of species produced by 100 eV of energy absorbed (*G* = 1 equals 10^{-7} M Gy^{-1}).

$$H_2O \xrightarrow{\gamma} e^-_{aq}(2.6),\ {}^{\bullet}OH(2.7),\ H^{\bullet}(0.6),\ H_3O^+(2.6),\ H_2O_2(0.72) \qquad (1)$$

$$e^-_{aq} + O_2 \rightarrow O_2^{\bullet-} \quad k_2 = 1.9 \times 10^{10} M^{-1} s^{-1} [18] \qquad (2)$$

$$H^{\bullet} + O_2 \rightarrow HO_2^{\bullet} \quad k_3 = 1.2 \times 10^{10} M^{-1} s^{-1} [18] \qquad (3)$$

$${}^{\bullet}OH + HCO_2^- \rightarrow CO_2^{\bullet-} + H_2O \qquad (4)$$

$$k_4 = 3.2 \times 10^9 M^{-1} s^{-1} [18]$$

$$CO_2^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} + CO_2 \quad k_5 = 4.2 \times 10^9 M^{-1} s^{-1} [18] \qquad (5)$$

The SOD activity was determined directly by pulse radiolysis and indirectly using the cytochrome *c* assay. According to the direct method, $O_2^{\bullet-}$ was generated by pulse radiolysis and k_{cat} was determined by following the absorption decay of $O_2^{\bullet-}$ in the UV region in the presence of limiting concentrations of SOD. According to the indirect method, $O_2^{\bullet-}$ was generated by γ-radiolysis in the presence of cytochrome *c* and the reduction of cytochrome *c* was monitored at 550 nm in the absence and presence of SOD at a constant dose where $[O_2^{\bullet-}]_{total} < [\text{cytochrome } c]_o$.

Direct assay

Pulse irradiation of oxygenated solutions containing 10 mM formate, 0.1 mM EDTA, and 2 mM PB at pH 6.9 produced 12 μM $O_2^{\bullet-}$. The decay of $O_2^{\bullet-}$ was followed at 270 nm and obeyed second-order kinetics with $2k_{dis} = (1.5 \pm 0.2) \times 10^6$ M^{-1} s^{-1}. Under limiting concentrations of SOD, the decay of $O_2^{\bullet-}$ turned into a first-order kinetics and k_{obs} increased linearly upon increasing [SOD]. Furthermore, k_{obs} was unaffected by the number of pulses delivered into the solutions. The absolute value of k_{cat} depends on the correct determination of the concentration of SOD. We prepared stock solutions of 1 mg/ml of all commercial SOD products. The two products from Sigma had the same absorption at 258 ($OD_{258} = 0.303 \pm 0.007$), whereas that from Boehringer was lower, i.e., $OD_{258} = 0.245 \pm 0.005$. The dependence of k_{obs} on [SOD] (expressed in μg/ml and nM; using $\varepsilon_{258} = 10{,}300$ M^{-1} cm^{-1}) is shown in Figs. 1A and 1B, respectively. The concentration of SOD in Fig. 1A was calculated using MW 32.6 kDa, and from the slope of the lines in Figs. 1A and 1B we determined k_{cat} for the various SODs (Table 1). The results in Table 1 also demonstrate that k_{cat} for the Sigma products obtained from Figs. 1A and 1B is within experimental error the same, which indicates that Sigma provides a pure product. The difference in k_{cat} for SOD prepared by Boehringer is the same as the difference between