

## Catalytic activity of superoxide dismutase: A method based on its concentration-dependent constant decrease in rate of autoxidation of pyrogallol

A blue copper protein was isolated in 1938 by Mann and Keilin<sup>1</sup> from erythrocytes and liver and later from many animal tissues, prompting the names hemocuprein, erythrocuprein, cerebropcuprein and cytocuprein. Discovery of its catalytic activity of dismutating two molecules of superoxide ( $O_2^{\cdot-}$ ) to  $H_2O_2 + O_2$  in 1969 by McCord and Fridovich<sup>2</sup>, led to the rechristening this protein as superoxide dismutase (SOD). Since then it became iconic in studies on oxygen radicals and their toxicity. Inhibition by SOD protein, of the reduction of cytochrome *c* by superoxide generated by xanthine oxidase reaction, remains the best method of its assay<sup>2</sup>. Indeed inhibition by this protein of a reaction is equated to the presence and participation of superoxide, sometimes resorting to chain amplification<sup>3</sup> of presumed traces.

Is the function of this protein, originally Keilin's erythropoetin, only dismutation of superoxide to  $H_2O_2$ ? Over three decades the concept did not permit thinking of alternatives. A plea made 25 years ago by Fee<sup>4</sup> that 'alternative biological functions for these proteins should seriously be considered' for SOD, sadly received little attention. SOD is proposed to protect cells from oxidative stress by removing superoxide, despite the fact that the product of its reaction,  $H_2O_2$ , is a stronger oxidant. By reducing bound iron, superoxide releases free  $Fe^{2+}$  and can purportedly lead to cellular damage through hydroxyl radicals formed in the presence of  $H_2O_2$ , but the same reduction can be done more effectively by other abundant cellular reductants. Metal complexes can dismutate the tiny amounts of superoxide produced, obviating the need for an enzyme. Occurrence of SOD protein in unusually large quantities in some plant seeds (1–2% of protein) and in *Mycobacterium lepreum* (10% of protein), and in some anaerobic cells with no obvious relationship to oxidative metabolism, remains an enigma. Two recent findings compel rethinking on other functions of SOD: (i) Action of SOD in the replicative lifespan of yeast cells 'involves function(s) other than superoxide scavenging'<sup>5</sup>; (ii) Addition of SOD pro-

tein to HepG2 cells decreases cholesterol synthesis and activity of the regulatory enzyme, HMG-CoA reductase<sup>6</sup>.

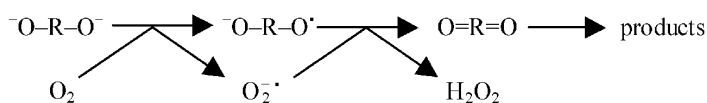
A clue for an alternative function comes from the assay procedure of SOD based on its inhibition of autoxidation of catechol compounds such as adrenaline<sup>7</sup>, 6-hydroxydopamine<sup>8</sup> or pyrogallol<sup>9</sup>. Their phenolate anions in alkaline medium are oxidized to quinone products accompanied by consumption of oxygen. The autoxidation reaction can be represented as a transfer of two electrons from oxy-anions to molecular oxygen in two steps (Scheme 1).

Attempts made to explain inhibition of this alkali-promoted chemical reaction by SOD protein relied on its well-known dismutation function. We can see from Scheme 1 that removal of  $O_2^{\cdot-}$  by dismutation to  $(1/2)H_2O_2 + (1/2)O_2$  by SOD should always produce 50% decrease in the rate of oxygen consumption. However, inhibition obtained experimentally increased with increase in concentration of SOD, nearly abolishing oxygen consumption at high concentrations. To fit with the theory, superoxide from another source (e.g. xanthine oxidase) was assumed to be the primary oxidant in a purported chain reaction<sup>7</sup> broken instantly by SOD. Then the concentration of SOD required for termination of autoxidation will depend on the initiating source, but experimentally no relationship was found. And it is well known that autoxidation of pyrogallol in alkaline medium requires no external superoxide source.

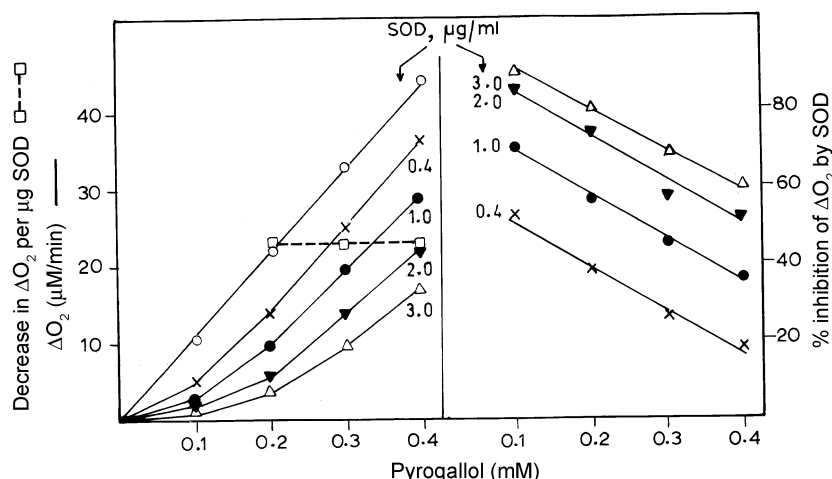
Using a catechol substrate, this chemical reaction can be followed by measuring oxygen consumed or the quinone product by specific absorbance. Addition of excess catalase released back into the medium up to 50% of consumed oxygen indicating its conversion to  $H_2O_2$ . The reaction continued at a decreased rate in the presence of SOD. Extrapolating such

data for the amount of protein required for 50% inhibition at a fixed substrate concentration formed the basis of quantitation of SOD<sup>7-9</sup>. Increased catechol (pyrogallol) concentrations increased rates of autoxidation and also concentrations of SOD required for 50% inhibition. In other words, higher amount of SOD protein is required to obtain 50% inhibition of higher rate of autoxidation.

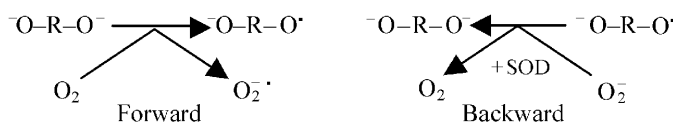
Data in Figure 1 show the expected linear increase in the rate of autoxidation with increased concentration of pyrogallol, used as the substrate (left panel). In the presence of increasing concentrations of SOD the rates of autoxidation decreased, but these values showed initial curvilinear lines that became linear and parallel to the control line at higher pyrogallol concentrations. Autoxidation of dopamine, noradrenaline and tea extract (mixture of polyphenols), and also with the assay of the corresponding quinone products, also gave the same curvilinear relationship between the SOD-inhibited rates and pyrogallol concentrations (unpublished data). This surprising kinetic behaviour of the nonlinear response of the rate of autoxidation in the presence of SOD revealed an important facet of SOD action. The extent of inhibition, denoted by a decrease in the rate of oxygen consumption ( $\Delta O_2$ ,  $\mu M/min$ ), remained the same for each concentration of SOD. However, the percentage inhibition values decreased as the rates increased at high concentrations of pyrogallol (Figure 1, right panel). This is consistent with the report of requirement of higher concentrations of SOD protein for 50% inhibition at higher concentrations of pyrogallol, also in a curvilinear fashion (see figure 2 of Marklund and Marklund<sup>9</sup>). Decrease in the rate of autoxidation that remained constant at high concentrations of pyrogallol represents the 'inhibitory' catalytic activity of SOD, given as units of



Scheme 1.



**Figure 1.** Nonlinear relationship of pyrogallol concentration and rate of oxygen consumption on autoxidation in the presence of SOD. The reaction mixture contained K-phosphate buffer (50 mM, pH 8.0), EDTA (1 mM) and increasing concentrations of pyrogallol (0.1–0.4 mM) and SOD (0.4–3.0  $\mu\text{g/ml}$ ; Cu,Zn-SOD from bovine erythrocytes, Sigma Chemical Co. USA). Aqueous solutions of pyrogallol (Merck AR reagent) are made fresh for each experiment and used within 2 h. Consumption of oxygen was recorded after adding pyrogallol in a Gilson 5/6H oxygraph fitted with a Clark electrode and the rates of oxygen consumption measured are given as  $\Delta\text{O}_2$ ,  $\mu\text{M/min}$  (Left panel). Values of percentage inhibition (Right panel). Dotted line indicates decrease in the rate of autoxidation by added SOD that remained constant at high concentrations of pyrogallol ( $\Delta\text{O}_2$ ,  $\mu\text{M/min}/\mu\text{g}$  SOD protein calculated for values of 0.4  $\mu\text{g}$  SOD added).



**Scheme 2.**

$\Delta\text{O}_2$  ( $\mu\text{M/min}$ ) per  $\mu\text{g}$  SOD protein, calculated for 0.4  $\mu\text{g/ml}$  of SOD added (Figure 1, broken line).

Crucial to the understanding of this phenomenon is our present finding that the action of added SOD is limited to 'constant inhibition' that depends only on its concentration, irrespective of the varying activity of autoxidation. In simple terms, this means that a fixed portion of autoxidation is nullified by SOD, saving both the phenolate and oxygen.

From Scheme 2 we can see that the forward reaction of the first one-electron transfer produces two oxygen radicals. Then it becomes self-evident that by catalysing backward dismutation of these

radicals, SOD protein can nullify autoxidation. Some evidence already exists that phenolate-O radical can react with SOD-Cu<sup>2+</sup> in a dismutation reaction<sup>10</sup>. In the absence of normally active SOD, decreased lifetime of some easily autoxidizable compounds may also be expected. An example is provided by protection of calcium-dependent phosphoprotein phosphatase activity of calcineurin by SOD from oxidative inactivation and this protection decreased with mutant proteins of SOD1 with low dismutation activity in familial amyotrophic lateral sclerosis<sup>11</sup>. The rate of autoxidation at pH 7.5 is about one-quarter of that at pH 8.0 and saving by SOD from losses under physiological

conditions will not be insignificant. This function of SOD can prolong life and action of catechol compounds, such as endogenous neurotransmitters and exogenous antioxidant compounds, and also autoxidizable proteins (e.g. calcineurin). This interpretation of SOD activity of nullifying autoxidation and a simple modification of the assay give a rate measurement of its catalysis, like all other enzymes.

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