Free Rad. Res. Comms., Vols. 12-13, pp. 5-10
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COMPARISON BETWEEN DIFFERENT ASSAYS FOR SUPEROXIDE DISMUTASE-LIKE ACTIVITY

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The direct and indirect methods for assaying the superoxide dismutase activity of a compound are compared. With the use of a direct method, the mechanism of the catalysis of O_2^- dismutation by the tested compound can be determined, while with the indirect method it cannot, and this may lead to misinterpretation of the results. Assuming that the catalysis occurs via the 'ping-pong' mechanism, both the direct and indirect methods are limited to the determination of values of $k_{cat} > 10^5 M^{-1} s^{-1}$ and $k_{cat} > 3 \times 10^6 M^{-1} s^{-1}$, respectively. Moreover, many side reactions may occur with the indirect method which may interfere with the measurements. Nevertheless, the indirect method approximates better the *in vivo* conditions than the direct method, and a tested compound that has high SOD activity using a direct method and low SOD activity using an indirect method, will most probably be a poor SOD mimic *in vivo*.

KEY WORDS: SOD, O_2^-, dismutation, catalysis, assay.

INTRODUCTION

Since the discovery of superoxide dismutase (SOD) in 1969 by McCord and Fridovich,[1] studies of the reactions of oxygen radicals *in vivo* and *in vitro* as well as the biological role of SOD are of great interest. It is assumed that SOD protects the cells from uncontrolled and damaging reactions of O_2^- through catalyzing its dismutation to molecular oxygen and hydrogen peroxide.

O_2^- has been implicated as a major factor in radiation damage, inflammation, tumor promotion, reperfusion injury and in many other systems.[2-5] In many of these cases SOD has been shown to exert a protective effect. The presence of copper, iron or manganese in the active site of the enzyme focused the attention on the use of these metals and their low molecular weight chelates as catalysts of O_2^- dismutation. A stable, non toxic, low molecular weight complex, which catalyzes O_2^- dismutation efficiently, may be able to substitute SOD, and it would have the advantages of being capable to cross cell membranes and being inexpensive.

Thus, it is important to have a well defined assay for determining the SOD-like activity of low molecular weight metal complexes, a method which can compare the activity of the native enzyme to that of the tested compound at conditions similar to the *in vivo* systems.

DISCUSSION

In a direct assay for determining the SOD-like activity, O_2^- is generated with initially high concentrations ($> 1\ \mu M$) and the decay of its absorbance is followed in the UV

region ($\varepsilon_{245} = 2350\,M^{-1}cm^{-1}$[6]) in the absence and in the presence of a tested compound. With this method it is easy to discriminate between a catalytic and a non catalytic compound by making sure that the initial concentration of O_2^- is in excess over that of the tested compound. Thus, the order and the rate of the reaction can be studied at various conditions, and the mechanism of O_2^- dismutation catalyzed by the compound can be determined.

With an indirect assay O_2^- is generated chemically or enzymatically with a constant flux in the presence of a detector molecule (D), which scavenges the radical.

$$O_2^- + D \xrightarrow{k_D} O_2 + D^- \tag{1}$$

The yield of the detector product ($[D^-]_0$) or the initial rate of its formation (V_0) is followed.

$$d[D^-]/dt = k_D[D][O_2^-] \tag{2}$$

$$-d[O_2^-]/dt = \text{flux} - k_D[D][O_2^-] \tag{3}$$

Assuming the steady state approximation for $[O_2^-]$, rate equation (4) is obtained:

$$d[D^-]/dt = V_0 = \text{flux} \tag{4}$$

where

$$[D^-]_0 = V_0 \times t \tag{5}$$

In the presence of a compound which competes with the detector molecule for O_2^-, the detector product yield ($[D^-]_c$) or the initial rate of its formation (V_c) will decrease. With the use of an indirect method the mechanism of the catalysis cannot be determined and this may lead to misinterpretation of the results.

The mechanism of the catalytic dismutation of O_2^- by SOD as well as by many other metal complexes has been suggested to involve alternate reduction and oxidation of the metal by O_2^- in a 'ping-pong' type mechanism:[7-10]

$$M^{n+} + O_2^- \longrightarrow M^{(n-1)+} + O_2 \tag{6}$$

$$M^{(n-1)+} + O_2^- + 2H^+ \longrightarrow M^{n+} + H_2O_2 \tag{7}$$

$$\text{net: } 2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \tag{8}$$

When $[M^{n+}]_0 \ll [O_2^-]_0$ rate equation (9) is obtained:

$$-d[O_2^-]/dt = k_{cat}[M^{n+}]_0[O_2^-] \tag{9}$$

where

$$k_{cat} = 2k_6k_7/(k_6 + k_7).$$

According to this mechanism rate equation (10) is obtained for the formation of D^-,

$$V_c = V_0k_D[D]/(k_D[D] + k_{cat}[cat]_0) \tag{10}$$

which after rearrangement gives:

$$V_0/V_c = [D^-]_0/[D^-]_c = 1 + k_{cat}[cat]_0/k_D[D] \tag{11}$$

Thus, a plot of V_0/V_c or $[D^-]_0/[D^-]_c$ versus $[cat]_0$ should yield a straight line. If the compound catalyzes O_2^- dismutation via the 'ping-pong' mechanism, and no side reactions occur to interfere with equation (10), then one can determine the catalyst concentration that causes 50% inhibition in the detector product yield or in its initial rate of formation (I_{50}), where $k_{cat}I_{50} = k_D[D]$, and k_{cat} can be calculated.

However, if the tested compound catalyzes O_2^- dismutation via another mechanism, e.g., where the reduced compound is reoxidized by molecular oxygen rather than by O_2^-, equations (10) and (11) will not be valid.

$$M^{n+} + O_2^- \rightleftharpoons MO_2^{(n-1)+} \tag{12}$$

$$MO_2^{(n-1)+} \rightleftharpoons M^{(n-1)+} + O_2 \tag{13}$$

$$MO_2^{(n-1)+} + M^{(n-1)+} + 2H^+ \longrightarrow 2M^{n+} + H_2O_2 \tag{14}$$

$$\text{net: } 2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \tag{8}$$

When $[M^{n+}]_0 \ll [O_2^-]_0$, assuming that the back reactions (−12) and (−13) can be neglected and the steady state approximation for $[M^{(n-1)+}]$ and for $[MO_2^{(n-1)+}]$, rate equation (15) is obtained:

$$-d[O_2^-]/dt = k_{12}[M^{n+}][O_2^-] \tag{15}$$

where

$$[M^{n+}] = ([M^{n+}]_0 - k_{13}/k_{14})/(1 + k_{12}[O_2^-]/2k_{13})$$

Thus, by following the decay of O_2^- one is able to distinguish between both mechanisms. For the 'ping-pong' mechanism the decay of O_2^- is first order and the observed rate constant depends linearly on the initial concentration of the catalyst. Assuming that this mechanism operates with an indirect method one will get a linear dependence of V_0/V_c on $[cat]_0$. For the second mechanism suggested the same correlation occurs only if $k_{12}[O_2^-]/2k_{13} \ll 1$ and $k_{13}/k_{14} \ll [M^{n+}]_0$. Under these conditions rate equation (15) reduces to (16),

$$-d[O_2^-]/dt = k_{12}[M^{n+}]_0[O_2^-] \tag{16}$$

and

$$V_0/V_c = 1 + k_{12}[cat]_0/k_D[D] \tag{17}$$

In the case where $k_{12}[O_2^-]/2k_{13} \gg 1$, the rate of the decay of O_2^- will obey a zero order rate law,

$$-d[O_2^-]/dt = 2k_{13}([M^{n+}]_0 - k_{13}/k_{14}) \tag{18}$$

and hence rate equation (19) is obtained,

$$V_c = V_0 - 2k_{13}([M^{n+}]_0 - k_{13}/k_{14}) \tag{19}$$

where

$$I_{50} = V_0/4k_{13} - k_{13}/k_{14}.$$

Thus, a comparison between I_{50} of a tested compound to that of the native enzyme

will be meaningless unless the compound catalyzes O_2^- dismutation via the same mechanism expected for SOD.

Beside that the added compound could interfere with the O_2^- generating system, the compound itself may react with the detector product or its reduced form may react with the detector product or its reduced form may react with the detector molecule leading to misinterpretation of the results. Moreover, molecular oxygen, which is present in the solutions at relatively high concentration may compete with O_2^- for the reduced compound. Thus, if reaction (-6) cannot be neglected in the 'ping-pong' mechanism, rate equation (20) is obtained,

$$-d[O_2^-]/dt = k[M^{n+}]_0[O_2^-] \tag{20}$$

where

$$k = 2k_6k_7/(k_6 + k_7 + k_{-6}[O_2]/[O_2^-])$$

With a direct method $[O_2^-]_0 = 10\,\mu M$ and $[O_2]/[O_2^-] = 10$, and as for most metal complexes $k_{-6} \ll 10^7\,M^{-1}s^{-1}$, $k = k_{cat}$. With an indirect method where $[O_2^-]_{s.s} \leqslant 10^{-8}\,M$[1,11-13] and $[O_2]/[O_2^-] \geqslant 10^4$, $k < k_{cat}$, depending on k_{-6}. Under *in vivo* conditions the steady state concentration of O_2^- is even lower than that obtained with an indirect method and $[O_2]/[O_2^-] > 10^6$.[14] Therefore, if reaction (-6) cannot be neglected in an indirect system, and it is the cause for the decrease in the catalytic activity of the tested compound as compared to that determined in a direct system, the SOD activity of the compound *in vivo* will be even lower than that measured with an indirect assay.

Both direct and indirect methods are limited in the determination of the value of k_{cat}, assuming that the catalysis proceeds via the 'ping-pong' mechanism. Using a direct method, usually $1-10\,\mu M$ of O_2^- are generated, and therefore under catalytic conditions $[cat]_0 < 0.1-1\,\mu M$. In order to be able to observe any acceleration of O_2^- decay, the half life of O_2^- in the presence of the catalyst should be lower than in its absence where the radical dismutates spontaneously with $k_{dis} = 5 \times 10^5\,M^{-1}s^{-1}$ at physiological pH.[6] Thus,

$$\ln 2/k_{cat}[cat]_0 \ll 1/2[O_2^-]_0k_{dis} \tag{21}$$

and hence $k_{cat} > 10^5\,M^{-1}s^{-1}$, which is about four orders of magnitude lower than k_{cat} of the native enzyme.[7]

Using an indirect method, we usually have a flux of about $1\,\mu M\,O_2^-$/min, and if the measurements are taken within the first 5–10 minutes, then a total of about $10\,\mu M$ of O_2^- is generated. Therefore under catalytic conditions the concentration of the catalyst should be less than $1\,\mu M$ at the most. In order to be able to observe any change in V or $[D^-]$, $k_{cat}[cat]_0$ should be of the same order of magnitude as $k_D[D]$. Under the conditions usually used with the cytochrome c assay ($k_D = 2.6 \times 10^5\,M^{-1}s^{-1}$; $[D] = 10\,\mu M$)[1,11,12] and with the NBT assay ($k_D = 5.9 \times 10^4\,M^{-1}s^{-1}$; $[D] = 100\,\mu M$),[13] we get $k_{cat} > (3-5) \times 10^6\,M^{-1}s^{-1}$, which is still about three orders of magnitude lower than k_{cat} of SOD.[7]

With both direct and indirect methods, assuming that the catalysis occurs via the 'ping-pong' mechanism, the determination of k_{cat} is limited to values higher than 10^5 and $3 \times 10^6\,M^{-1}s^{-1}$, respectively, for the two methods. Thus both methods are satisfactory as catalysts with lower k_{cat} values will not be able to compete with the

spontaneous dismutation of O_2^-, and using high concentration of such catalysts only a scavenging reaction and not a catalytic one will take place. Therefore, a catalyst with a $k_{cat} < 10^6 M^{-1} s^{-1}$ is of no interest.

CONCLUSIONS

The direct method for assaying the SOD-like activity of a compound seems to be the most reliable and sensitive method for determining the mechanism and the 'turnover' rate constant of the catalysis. The indirect method can lead to misinterpretation of the results because the mechanism of the catalysis cannot be determined and because many side reactions can interfere with the measurements. Nevertheless, the indirect method approximates much better the *in vivo* conditions than a direct method. Under *in vivo* conditions the steady state concentration of O_2^- is even lower than that obtained with an indirect method, and there are many cell components at relatively high concentrations that can compete with O_2^- for the compound or for its reduced form. Thus, a compound that has high SOD activity in a direct system and low SOD activity in an indirect system will most probably be a poor SOD mimic *in vivo*. Therefore, with the use of the direct method the mechanism of the catalysis can be determined, while the use of an indirect method provides more information about the ability of the compound to mimic SOD *in vivo*.

Moreover, the native enzyme exerts almost the same catalytic activity with more than 20 direct and indirect methods described in the literature. This points towards the uniqueness of SOD as compared to its mimics as apparently no side reactions (oxidation or reduction) interfer with the 'ping-pong' mechanism. This suggests that searching for a good substitute for SOD to operate *in vivo* will not be an easy task.

Acknowledgements

This work was supported by the Council for Tobacco Research and by The Israel Academy of Sciences.

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Accepted by Prof. B. Halliwell