A method for detecting superoxide anion in presence of peroxidases

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Summary. Cytochrome c, used as a scavenger for superoxide anion in some oxidase-like reactions of horseradish peroxidase, diminishes the conversion of the starting products to the oxidized forms. This behavior can be used as a tool for determining superoxide in the presence of peroxidases and hydrogen peroxide.

In a system containing a peroxidase, traditional kinetic methods[1] of detecting superoxide anion, such as the rate of reduction of ferricytochrome c, have the following drawback; one of the products of dismutation of $O_2^{\cdot-}$, namely hydrogen peroxide, is scavenged by peroxidase itself, and can thus react as a strong oxidant with the species present in the system, including ferrocytochrome. During a study of the N-dealkylation of tertiary amines catalyzed by horseradish peroxidase (HRP) we used stoichiometric rather than kinetic evidence in order to substantiate the presence of superoxide anion[2]. Here we report the results obtained after using the same method for the oxidase-like reaction of HRP with NADH, dihydroxyfumaric acid (DHF), and 3-indoleacetic acid (IAA)[3]. The enzyme catalyzes the oxidation of these compounds by molecular oxygen. The oxidation is accelerated by minor amounts of hydrogen peroxide, but still operates in its absence. HRP oxidase-like reactions with NADH, DHF, and IAA were followed in control experiments and in the presence of increasing concentrations of ferricytochrome c. The conversion of the substrates could be followed spectrophotometrically at 325 nm; at this wavelength, reduction of ferricytochrome does not interfere. The conversion of the substrates to the oxidized forms was kept below 100% by using suitable concentrations of both substrate and enzyme. The change in optical density (ΔOD) at 325 nm could be taken as a measure of the extent of the conversion. Final values of OD were attained within 30 min. They were recorded at those times when the change in OD slowed down sharply. After this, OD might vary slightly, but at a rate that could be explained by autooxidation of the substrate without any intervention of the enzyme. Incompleteness of the reaction in the presence of the enzyme can be accounted for by either enzyme inactivation or termination of the free radical chain involved in the reaction.

Materials and method. NADH and HRP were purchased from Boehringer-Mannheim (both grade II). IAA and DHF were prepared by standard methods[4,5]. Reactions were performed in acetate buffer 0.05 M at pH 5.0 and 37 °C. OD variations were measured on a Hitachi-Perkin-Elmer 124 spectrophotometer.

Results and discussion. A mechanism has been proposed for the oxidase-like HRP reaction, involving a free radical chain[3] (fig. 1). HRP is oxidized to compound E_1 either directly by oxygen or by traces of hydroperoxide adducts from substrate and oxygen. An electron transfer reaction occurs between E_1 and substrate AH_2 to give a radical species $AH^{\cdot}$ and compound E_2, which performs the same electron transfer with another AH_2 molecule yielding HRP. The radical species $AH^{\cdot}$ is responsible for oxygen activation.

Two main pathways are possible: a) an electron transfer between $AH^{\cdot}$ and molecular oxygen to give the oxidized product A and superoxide anion. This dismutates to give oxygen and hydrogen peroxide which re-enters the catalytic cycle; b) a σ bond formation between $AH^{\cdot}$ and O_2 to give a hydroperoxyl radical which can propagate the reaction, for instance by abstracting a hydrogen atom from another AH_2 molecule. It has been shown that superoxide dismutase strongly inhibits the oxidation of NADH and DHF, but not of IAA[3]. Thus it was suggested that NADH and DHF produce superoxide anion (pathway a), whereas IAA was said to yield a hydroperoxyl radical (pathway b).

We observed that for NADH and IAA, an HRP concentration of 2 U/ml (25 °C, guaiacol and H_2O_2 as substrates) was low enough to give only incomplete conversion of the starting material to the oxidized products. In order to obtain incomplete oxidation of DHF, HRP concentrations as low as 0.25 U/ml were necessary. Figure 2 shows that cytochrome c strongly lowers the conversion of NADH and DHF to the oxidized forms. Moreover no appreciable reduction of ferricytochrome is observed at 550 nm. This behavior is similar to that observed in the HRP catalyzed oxidation of aromatic amines by hydrogen peroxide and is consistent with the hypothesis of the formation of superoxide. Thus (fig. 3) the presence of ferricytochrome c can lower the conversion both because it scavenges $O_2^{\cdot-}$, yielding ferrocytochrome, and because this latter scavenges H_2O_2 deriving from $O_2^{\cdot-}$ dismutation, yielding the ferric form. A completely reversed situation is found in the case of IAA oxidation, at low substrate concentrations. At concentrations as high as 6×10^{-4} M, the behavior of the system seems to invert. At this substrate concentration another reaction seems to prevail, as is shown by the vigorous reduction of ferricytochrome c appearing at 550 nm. Several hypothesis could account for this reduction, such as, for instance, the direct oxidation of IAA by ferricytochrome c; however, it is evident that the reduction cannot be explained by $O_2^{\cdot-}$ formation. If this were the case, partial dismutation of $O_2^{\cdot-}$ should provide hydrogen peroxide, whose presence is incompatible with the reduced form of cytochrome c. The release of superoxide anion should therefore be ruled out in the case of IAA oxidation catalyzed by HRP.

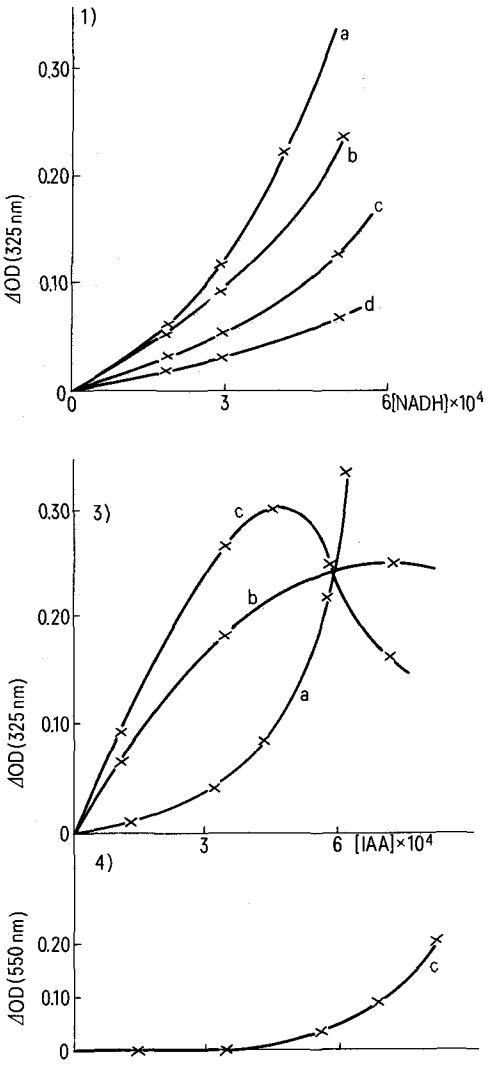
In conclusion, these measurements show that in the cases where superoxide is formed ferricytochrome reduces the conversion of substrates to the oxidized form, whereas no reduction is observed when superoxide is not produced. Thus the method can be used to detect superoxide anion in reactions catalyzed by peroxidases, in the presence of added or endogenous hydrogen peroxide without adding any substance, such as catalase, scavenging hydrogen peroxide itself.

$$\mathrm{HRP + ROOH \rightarrow E_1}$$
$$\mathrm{E_1 + AH_2 \rightarrow E_2 + AH^{\cdot}}$$
$$\mathrm{E_2 + AH_2 \rightarrow HRP + AH^{\cdot}}$$

a)
$$\mathrm{AH^{\cdot} + O_2 \rightarrow A + H^+ + O_2^{\cdot-}}$$
$$\mathrm{2\,O_2^{\cdot-} + 2\,H^+ \rightarrow O_2 + H_2O_2}$$
$$\mathrm{H_2O_2 + HRP \rightarrow E_1}$$

b)
$$\mathrm{AH^{\cdot} + O_2 \rightarrow AHOO^{\cdot}}$$
$$\mathrm{AHOO^{\cdot} + AH_2 \rightarrow AHOOH + AH^{\cdot}}$$
$$\mathrm{AHOOH + HRP \rightarrow E_1 + A}$$

Figure 1. The mechanism of the oxidase-like reaction of horseradish peroxidase.



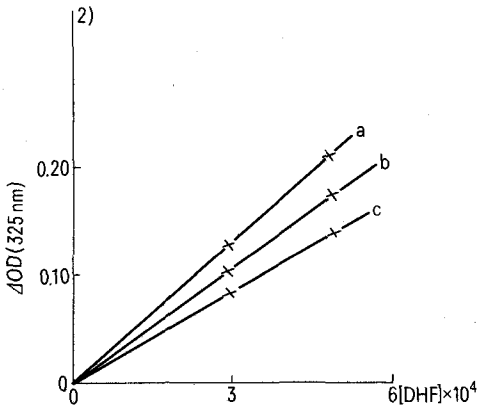


Figure 2. Conversion of NADH (1), DHF (2), and IAA (3) to the corresponding oxidized products in presence of HRP and oxygen, measured as the difference of optical density (ΔOD) at 325 nm at varying concentrations of ferricytochrome c. For NADH oxidation, the concentrations of ferricytochrome are: a, 0; b, 10^{-6} M; c, 2×10^{-6} M; d, 3×10^{-6} M; for DHF oxidation: a, 0; b, 5×10^{-6} M; c, 10^{-5} M; for IAA oxidation: a, 0; b, 10^{-6} M; c, 3×10^{-6} M. Reduction of ferricytochrome in presence of IAA is also shown (4).

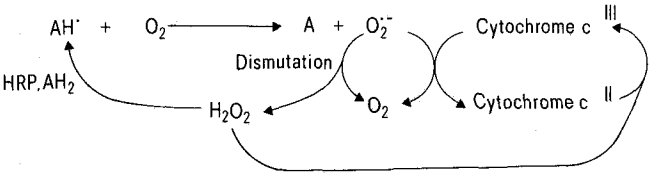


Figure 3. The role of ferricytochrome c in a oxidase-like reaction of HRP proceeding via superoxide formation.

1 Fridovich, I., Acc. chem. Res. *5* (1972) 321.
2 Galliani, G., and Rindone, B., J. chem. Soc. Perkin II *1980,* 1.
3 Yamazaki, I., in: Molecular Mechanisms of Oxygen Activation, p. 535. Ed. O. Hayaishi. Academic Press, New York 1974.
4 Vogel, A.I., in: A Textbook of Practical Organic Chemistry, p. 1013. Longmans, London 1962.
5 Neuberg, C., Biochem. Z. *71* (1951) 112.

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Announcement

France

EUCHEM conference on 'Organic approaches to biochemical problems'

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Organizing committee: D. Arigoni, Zürich, A. Marquet, Paris, and V. Ullrich, Konstanz. - The purpose of this conference is to illustrate, with various topics, the contribution of organic chemistry concepts and methods for the elucidation of biochemical problems. It will emphasize the important questions which are presently being investigated and the developing trends.
For information, contact Prof. A. Marquet, Laboratoire de Chimie Organique Biologique, Université P. et M. Curie, 4, Place Jussieu, F-75231 Paris Cedex 05/ France.

Correction

G. Bynke, R. Håkanson and *J. Hörig:* Ocular responses evoked by capsaicin and prostaglandin E_2 are inhibited by a substance P antagonist, Experientia *39* (1983) 996-998. The *summary* should read:
Application of capsaicin or prostaglandin E_2 to the rabbit eye resulted in miosis and breakdown of the blood-aqueous barrier, manifested in aqueous flare. Pretreatment with the neuronal blocker tetrodotoxin or the substance P antagonist (D-Pro^2, D-Trp^{7,9})-SP_{1-11} greatly reduced the ocular responses to capsaicin and prostaglandin E_2. The results suggest a role for neuronal substance P in the ocular response to injury.