SUPEROXIDE ANION INVOLVEMENT IN NBT REDUCTION CATALYZED BY NADPH-CYTOCHROME *P*-450 REDUCTASE: A PITFALL

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Received 9 February 1978

1. Introduction

Superoxide anion (O_2^-) is produced either by univalent reduction of molecular oxygen (O_2) or by univalent oxidation of hydrogen peroxide (H_2O_2) [1]. In biological systems, O_2^- is usually produced from O_2 by the oxidation of reduced flavin [2], iron sulfur protein [3] or hemoprotein [4]. Because of the fast spontaneous dismutation of O_2^- into H_2O_2 [1], chemical methods for detection and measurement of O_2^- must be indirect. One, routinely used, relies on the ability of O_2^- to reduce the nitro-blue tetrazolium dye (NBT) [5] into insoluble formazan. NBT can also be reduced by diaphorase reactions (without involvement of O_2^-). Superoxide dismutase (SOD), an enzyme that strongly enhances the dismutation rate of O_2^-, and thus inhibits the O_2^--mediated NBT reduction, is used in order to distinguish O_2^--mediated NBT reduction from that resulting from diaphorase reactions [6]. Using this methodology it has been suggested that in liver microsomes, the reduction of NBT catalyzed by NADPH cytochrome *P*-450 reductase was, partially, O_2^--mediated, since it was inhibited by SOD [7]. Investigations about the validity of this interpretation and, that of the general use of SOD-inhibitable reduction of NBT as a measurement of O_2^-, is our purpose in the following report.

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2. Materials and methods

2.1. *Preparation of enzymes*

NADPH-cytochrome *P*-450 reductase was purified from liver microsomes of phenobarbital-induced rats (40 mg/kg/day over 5 days) as in [8]. Beforehand, the final preparation containing the purified enzyme, 20% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol and 0.1% sodium deoxycholate, in 0.3 M phosphate buffer (pH 7.7), was loaded on to a Sephadex G-75 column, and eluted from the gel with 0.05 M phosphate buffer (pH 7.7). The fractions containing NADPH *P*-450 reductase activity were pooled and concentrated by ultrafiltration with an Amicon XM-50 membrane. The concentrated preparation contained 0.4 mg protein/ml. No cytochrome *P*-450, cytochrome b_5 or NADH cytochrome b_5 reductase could be detected in the purified enzyme preparation.

Superoxide dismutase was obtained from Sigma Chemical Co. (St Louis, Mo) and was dialyzed overnight against 0.05 M phosphate buffer (pH 7.4). The enzyme did not exhibit catalase activity.

2.2. *Assays of enzymatic activities*

Reduction of NBT and cytochrome *c* were measured at 25°C through the increase in A_{530} and A_{550}, respectively, on a Zeiss PM QII spectrophotometer. Extinction coefficients used were 18.5×10^{-3} M^{-1} cm^{-1} for NBT and 15.5×10^{-3} M^{-1} cm^{-1} for cytochrome *c*. The assay medium, in final vol. 1 ml was as follows: 0.05 M phosphate buffer (pH 7.7), 40 μM NBT or cytochrome *c*, 0.16 mM NADPH and 0.25 μg protein. Under these conditions, the reduction rate of cytochrome *c* was 46.8 μmoles/min/mg

Elsevier/North-Holland Biomedical Press

protein, and the reduction rate of NBT was 36.1 μmoles/min/mg protein. NADPH oxidation rate was measured by the decrease in A_{340} using an extinction coefficient of 6.22×10^{-3} M^{-1} cm^{-1}. NADPH oxidation rate was measured by the decrease in A_{340} using an extinction coefficient of 6.22×10^{-3} M^{-1} cm^{-1}. NADPH oxidation was measured in the presence of 40 μM NBT and 0.16 mM NADPH as above. Oxidation rate of NADPH was 36.2 μmoles/min/mg protein.

2.3. *Measurement of oxygen consumption*

Oxygen consumption was measured by the conventional polarographic method using a Gilson oxygraph equipped with a Yellow Springs Clark electrode. Experimental conditions used are specified in the fig.1 legend.

2.4. *Protein estimation*

Protein estimation was as in [9] using crystalline serum albumin as a standard.

2.5. *Chemicals*

NBT and ferricytochrome *c* were obtained from Sigma Chemical Co. (St Louis, Mo). NADPH was obtained from Boehringer (Mannheim).

3. Results and discussion

NADPH cytochrome *P*-450 reductase catalyzes the electron transfer from NADPH on various acceptors such as ferricytochrome *c*, NBT, ferricyanide and dichlorophenolindophenol [10]. Our purified enzyme reduced effectively both ferricytochrome *c* and NBT with a velocity in keeping with other reports [11]. Addition of SOD was followed by a strong decrease in NBT reduction rate and data shown in table 1 indicate that the inhibitory effect of SOD was quite similar over a large pH range. In contrast, and as previously reported with intact microsomes [7], cytochrome *c* reduction rate was not affected by SOD. On the other hand, absence of inhibition of NADPH oxidation rate by SOD indicates that the catalytic activity of the enzyme was not affected by SOD. Since O_2^- reacts with both NBT and ferricytochrome *c* and yields reduced NBT and ferrocytochrome *c* [6] the absence of inhibitory effect of SOD on cytochrome *c* reduction suggests that O_2^- could be

Table 1

Effect on SOD on NBT reduction, cytochrome *c* reduction and NADPH oxidation activities catalyzed by NADPH-cytochrome *P*-450 reductase

pH	NBT red.	Cyt. *c* red.	NADPH ox.
6.5	50	0	0
7.4	53.5	0	0
7.7	54.2	0	0
8.2	55.3	0	0

Measurements of enzymatic activities were carried out as in section 2. Values indicated are in terms of % inhibition of the reactions, induced by 20 μg SOD

a secondary product which depends on the presence of NBT. It is well known that the reductase catalyzes the reduction of electron acceptors by means of one electron transfer reaction [10], and the NBT reduction might have occurred as follows:

$$Enz_{ox.} + NADPH + H^+ \longrightarrow Enz_{red.} + NADP^+ \quad (1)$$

$$Enz_{red.} + 2\ NBT \longrightarrow NBT^{\cdot} + NBT^{\cdot} + Enz_{ox.} \quad (2)$$

$$NBT^{\cdot} + NBT^{\cdot} \longrightarrow NBT_{ox.} + NBT_{red.} \quad (3)$$

We anticipated that NBT free radical ($NBT^{\cdot}$) reacted with O_2 and produced O_2^- as secondary product as follows:

$$NBT^{\cdot} + O_2 \longleftrightarrow NBT_{ox.} + O_2^- \quad (4)$$

Since O_2^- undergoes a spontaneous dismutation according to reaction [5],

$$O_2^- + O_2^- + H^+ \longrightarrow H_2O_2 + O_2 \quad (5)$$

O_2-uptake should occur during NBT reduction. Addition of SOD, that strongly increases the dismutation rate of O_2^- would shift the reaction (4) equilibrium towards $NBT_{ox.}$ production from $NBT^{\cdot}$, and both inhibit NBT reduction rate and increase O_2-uptake. In order to ascertain this hypothesis, we measured O_2-consumption associated with NBT reduction in the presence and absence of SOD. Figure 1 shows the O_2-consumption when NADPH, NBT and SOD were successively added to the reac-

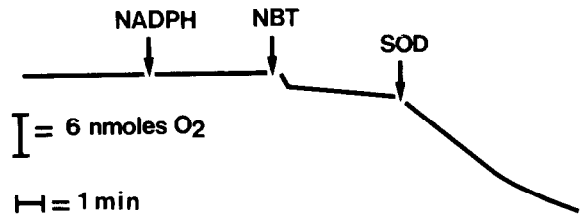

Fig.1. Oxygen consumption associated with NBT reduction. Oxygen consumption was carried out at 25°C in a cylindrical glass chamber (vol. 1 ml). Assay medium was composed of 0.05 M phosphate buffer (pH 7.4) and 0.4 μg reductase. Where indicated by the arrows, 0.16 mM NADPH, 40 μM NBT and 2 μg SOD were successively added. In the absence of NBT no oxygen consumption occurred with or without SOD. In the presence of both NBT and SOD, O_2-uptake was 4.21 nmol/min.

tion mixture. No O_2-uptake was detectable after the addition of NADPH alone or even in the presence of SOD, indicating that under these experimental conditions, the purified reductase does not act as an oxidase. Addition of NBT after that of NADPH was followed by a slight O_2 consumption. Further addition of SOD strongly increased the O_2-consumption. These results clearly indicate that the inhibitory effect of SOD on NBT reduction catalyzed by NADPH-cytochrome *P*-450 reductase is not in connection with a primary production of O_2^- by the enzyme since O_2 consumption was observed only in the presence of NBT and not in its absence.

To conclude, with our experimental conditions, NADPH *P*-450 reductase has not 'per se' an oxidase activity, but in fact, NBT being present, O_2^- is secondarily produced through a primary univalent reduction of this dye. This implies that in such reactions as may produce NBT^+, the use of SOD-inhibitable reduction of NBT is not suitable for the detection of a primary production of O_2^-, and even less for its measurement.

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