A CONVENIENT METHOD FOR DETECTING THE SUPEROXIDE ION FROM SINGLET OXYGEN REACTIONS OF BIOLOGICAL SYSTEMS: SUPEROXIDE FORMATION FROM HYDROGENATED NICOTINAMIDE ADENINE DINUCLEOTIDE AND 5-HYDROXYTRYPTOPHAN†

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Summary

A new method for detecting the superoxide ion from singlet oxygen reactions of biological substrates in aqueous solutions is described. The method employs a water-soluble singlet oxygen source and a superoxide-detecting reagent such as a combination of *p*-nitrotetrazolium blue and superoxide dismutase. The generation of the superoxide ion from oxidation of hydrogenated nicotinamide adenine dinucleotide and 5-hydroxytryptophan with singlet oxygen was confirmed by this method. It was also suggested that biological substrates with oxidation potentials less than 0.5 V measured with respect to a saturated calomel electrode readily undergo single-electron transfer to singlet oxygen, leading to the formation of the superoxide ion.

1. Introduction

Recent studies have implicated that the superoxide ion ($O_2^{\cdot-}$) plays a key role in a variety of chemical processes which have biological significance [1, 2]. Thus, there is much current interest in uncovering chemical processes which could give rise to $O_2^{\cdot-}$ *in vivo*. We previously reported that substrates with oxidation potentials less than about 0.5 V measured with respect to a saturated calomel electrode (SCE) are capable of undergoing single-electron transfer to singlet oxygen ($^1O_2$) to generate $O_2^{\cdot-}$ preferentially in polar aqueous solvents [3]. There are many electron-rich substrates with oxidation potentials less than about 0.5 V (SCE) in biological systems such as mitochondrial components [4]. In fact, Peters and Rodgers [5] have recently succeeded in detecting the formation of $O_2^{\cdot-}$ in the reaction of hydrogenated

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nicotinamide adenine dinucleotide phosphate (NAD(P)H), one of the readily oxidizable mitochondrial components, with $^1O_2$ by monitoring the reduction of benzoquinone in a laser flash experiment. However, a more convenient and reliable method for detecting $O_2^{\cdot-}$ without using laser flash techniques is highly desirable to study $^1O_2$ reactions of biological systems. To avoid complications in the formation of $O_2^{\cdot-}$ which may arise with the intervention of photoexcited sensitizers, we used a newly developed water-soluble chemical $^1O_2$ source rather than a photochemical method for the oxidation of biological substrates in aqueous systems [3, 6]. By employing this water-soluble $^1O_2$ source and a combination of *p*-nitrotetrazolium blue (NBT) and superoxide dismutase (SOD) as a detecting agent, we were able to detect $O_2^{\cdot-}$ from the $^1O_2$ reaction of biologically important substrates such as hydrogenated nicotinamide adenine dinucleotide (NADH) and 5-hydroxy-L-tryptophan (5-OH-TRP) in aqueous media. This method utilizing a water-soluble $^1O_2$ source may be widely applicable to the detection of $O_2^{\cdot-}$ in the reaction of more complicated biological systems with $^1O_2$.

2. Experimental details

2.1. Apparatus

UV spectra were recorded with a Shimazu UV-200 spectrophotometer. Cyclic voltammograms were recorded with a Yanagimoto P-1000 in Britten–Robinson buffer (pH 7.5). All potentials are measured in volts with reference to an SCE.

2.2. Materials

SOD (type I, 3000 units), ferricytochrome c (type III), NADH, 5-OH-TRP, tryptophol and melatonin were purchased from Sigma. NBT and L-tryptophan (TRP) were purchased from Wako Chemicals. The other chemicals were commercially available and used without further purification. The water-soluble $^1O_2$ source **1** was prepared as described previously [3]. Doubly distilled water was used in all cases.

2.3. Procedure

2.3.1. Participation of $^1O_2$ in the oxidation of hydrogenated nicotinamide adenine dinucleotide

Solutions containing 1 (2.5 mM) and NADH (0.4 mM) with and without a $^1O_2$ quencher were prepared in 0.26 mM phosphate buffer (pH 7.5). The sample solutions were shaken at 35 °C for 1.5 h. The disappearance of NADH was monitored at 340 nm with a UV spectrophotometer. A comparison of the oxidation rate of NADH in phosphate buffer at pH 7.5 and that at pD 7.5 was carried out under identical conditions using **1** (1.1 mM) and NADH (0.1 mM).

2.3.2. Detection of $O_2^{\cdot-}$

$O_2^{\cdot-}$ was detected by assay utilizing a combination of (i) NBT and SOD or (ii) ferricytochrome c and SOD. A typical procedure for the detection of $O_2^{\cdot-}$ is as follows. Solutions containing **1**, the substrate and NBT were prepared in phosphate buffer at the appropriate pH in the presence or absence of SOD (140 units). The sample solutions were shaken at 35 ± 0.1 °C. After a fixed time of incubation, a constant volume of dimethylformamide (DMF) was added to dissolve the formazan. The amount of formazan was measured at 560 nm with a UV spectrophotometer. The amount of $O_2^{\cdot-}$ was calculated from the difference between the absorbance in the absence and that in the presence of SOD, on the basis of the known stoichiometry of the reduction of NBT by $O_2^{\cdot-}$ [7, 8]. When a combination of ferricytochrome c and SOD was used as a detecting reagent, the formation of ferrocytochrome c was measured at 550 nm by UV spectroscopy. In this case, the absorbance was corrected by subtracting the initial value from that observed, since the ferricytochrome c used contains a small amount of the reduced form.

2.3.3. Control experiments for the generation of $O_2^{\cdot-}$

Solutions containing **1**, the substrate and NBT with and without SOD (140 units) were prepared in 0.26 mM phosphate buffer at the appropriate pH. An appropriate amount of additive was added to the reaction mixture before incubation. After incubation at 35 °C, a constant volume of DMF was added; the amount of formazan was measured at 560 nm by UV spectrophotometry.

2.3.4. Measurement of oxidation potentials

Half-wave oxidation potentials were measured by cyclic voltammetry in 40 mM Britten–Robinson buffer (pH 7.5) in a cell containing a glassy carbon electrode, a platinum auxiliary electrode and an SCE reference electrode at 25 °C. To remove dissolved oxygen, purified nitrogen was passed through the sample solution for 20 min before analysis. The oxidation potentials were measured under a stream of nitrogen. In all cases, half-peak oxidation potentials were employed as the half-wave oxidation potentials $E_{1/2}$.

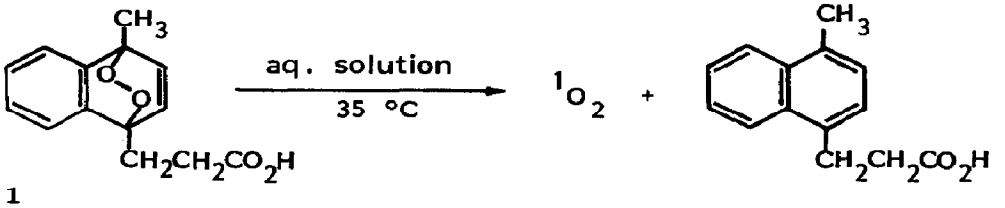
3. Results and discussion

3.1. Reaction of hydrogenated nicotinamide adenine dinucleotide

There has been much current controversy about the mechanism of the oxidation of NAD(P)H with $^1O_2$. Frenkel *et al.* [9] favoured one-electron oxidation with concomitant formation of $O_2^{\cdot-}$ which was detected by the reduction of ferricytochrome c. However, the haematoporphyrin sensitizer used has been shown to produce $O_2^{\cdot-}$ directly on photolysis [10]. In contrast, Bodaness and Chan [11] favoured a two-electron process consistent with the detection of H_2O_2 as a reaction product. It has also been suggested on thermodynamic grounds that single-electron transfer from NADH to $^1O_2$

$^1O_2 + NADH \longrightarrow O_2^{\cdot-} + NAD^{\cdot} + H^+$

is energetically feasible, while remaining unaffected by ground state triplet oxygen [12]. Peters and Rodgers [5] reported the detection of $O_2^{\cdot-}$ from NAD(P)H and $^1O_2$ in a laser flash photolysis and estimated that the extent of reactive quenching leading to $O_2^{\cdot-}$ is nearly 60% of the total quenching rate in acetonitrile–water. All the previous experiments utilize photochemical methods as the source of $^1O_2$. To see whether or not $O_2^{\cdot-}$ is produced by single-electron transfer from NADH to $^1O_2$ under physiological conditions, we examined the oxidation of NADH with $^1O_2$ generated from the water-soluble endoperoxide 1 [3]:



1 (1)

$^1O_2 + NADH \longrightarrow O_2^{\cdot-} + NAD^{\cdot} + H^+$ (2)

Incubation of NADH (0.4 mM) and 1 (2.5 mM) in phosphate buffer (pH 7.5) at 35 °C resulted in a smooth disappearance of NADH. Addition of NaN_3 (15 mM) and 1,4-diazabicyclo[2.2.2]octane (DABCO) (15 mM) to the reaction system inhibited 80% and 87% of the oxidation of NADH respectively. The rate of oxidation was accelerated by a factor of 6.5 by changing from H_2O to D_2O. These results confirmed the participation of $^1O_2$ in the oxidation of NADH, consistent with the previous findings [5, 9, 11].

Attempts were then made to detect $O_2^{\cdot-}$ by using a combination of NBT and SOD as a detecting reagent. Reduction of NBT was observed when a solution of NADH (0.56 mM), 1 (2.5 mM) and NBT (1 mM) in phosphate buffer (pH 7.5) was incubated at 35 °C. The amount of formazan formed was suppressed by the addition of SOD during or before incubation as indicated in Fig. 1. From the known stoichiometry of the NBT reduction by $O_2^{\cdot-}$ [7] and the amount of NBT reduction inhibited by SOD, the yield of $O_2^{\cdot-}$ was calculated on the basis of the amount of NADH initially used in the 2 h reaction. The results are listed in Table 1. The control experiments shown in Table 1 demonstrate that $O_2^{\cdot-}$ is formed directly from the reaction of NADH with $^1O_2$. Thus, $^1O_2$ quenchers such as NaN_3 and DABCO inhibit the formation of $O_2^{\cdot-}$. Addition of $OH^{\cdot}$ scavengers, *e.g.* isopropanol and ethanol, did not affect the formation of $O_2^{\cdot-}$. In addition, formation of $O_2^{\cdot-}$ from the autoxidation of NADH was negligibly slow. A bimolecular reaction between 1 and NADH may also be ruled out, since the rate of decomposition of 1 was not affected by the addition of NADH. It was also confirmed that

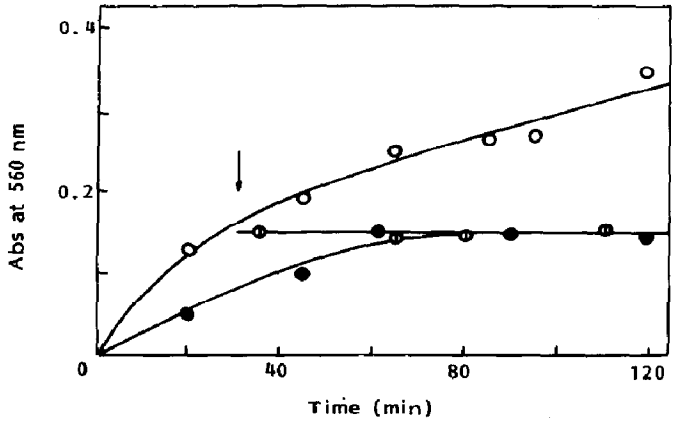


Fig. 1. Reduction of NBT during incubation of the endoperoxide **1** and NADH in the presence (●, ⊕) and absence (○) of SOD. SOD (280 units) was added at the point indicated by the arrow (⊕) or before incubation (●) ([NADH] = 0.56 mM; [1] = 2.5 mM; [NBT] = 1 mM; phosphate buffer (pH 7.5); 35 °C).

TABLE 1

Effect of additives on the formation of $O_2^{-\cdot}$ in the reaction of hydrogenated nicotinamide adenine dinucleotide with $^1O_2$ in phosphate buffer at pH 7.5 (2 h)

System[a]	*Additive*	*Yield of* $O_2^{-\cdot}$ [b] (%)
NADH, 1, NBT	—	6.3
	DABCO (22 mM)	2.7
	NaN_3 (22 mM)	2.7
	Isopropanol (10 mM)	6.0
	Ethanol (10 mM)	6.2
NADH, NBT	H_2O_2 (7 mM)	0
	O_2 bubbling	1.1

[a] [NADH] = 0.56 mM; [1] = 2.5 mM; [NBT] = 1 mM.
[b] Calculated from the difference between the formazan formation in the absence and that in the presence of SOD. The yield is based on the amount of NADH initially used.

H_2O_2 is not responsible for the formation of $O_2^{-\cdot}$. A similar result was obtained when ferricytochrome c was used in place of NBT.

All the above results strongly suggest that $O_2^{-\cdot}$ is formed via single-electron transfer from NADH to $^1O_2$ according to eqn. (2). However, we were unable to detect the counterpart of the single-electron transfer, namely NAD· (λ_{max} = 400 nm) [13], within the time scale of our experiments. It has been well established that NAD· once formed is very rapidly ($k = 2 \times 10^9$ M^{-1} s^{-1}) removed by reaction with ground state triplet oxygen to generate $O_2^{-\cdot}$ [13]:

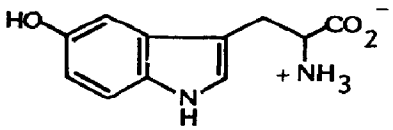
$$NAD\cdot + O_2 \xrightarrow{k = 2\times10^9\ M^{-1}\ s^{-1}} NAD^+ + O_2^{-\cdot} \qquad (3)$$

Thus, the amount of $O_2^{\cdot-}$ shown in Table 1 corresponds to the total amount of $O_2^{\cdot-}$ formed by both reactions. We are not confident that the amount of $O_2^{\cdot-}$ trapped by NBT accurately corresponds to the extent of single-electron transfer from NADH to $^1O_2$, owing to the inherent complex redox reactions involving NBT or ferricytochrome c [8]. Nevertheless, our preliminary results demonstrate that the generation of $O_2^{\cdot-}$ from the oxidation of NADH ($E_{1/2}$ = 0.056 V (SCE)) with chemically generated $^1O_2$ is a viable process under physiological conditions.

3.2. Reaction of 5-hydroxytryptophan

The generation of $O_2^{\cdot-}$ and H_2O_2 from photo-oxidation of TRP has been a subject of current interest in view of the phototoxicity induced by irradiation of biological systems in the presence of TRP [14]. The formation of $O_2^{\cdot-}$ was reported during the near-UV (300 - 375 nm) photo-oxidation of TRP [15]. The possibility of single-electron transfer from TRP to $^1O_2$ has also been suggested [16]. However, our recent work has demonstrated that single-electron transfer from TRP to $^1O_2$ leading to $O_2^{\cdot-}$ formation cannot be observed even in aqueous solution at alkaline pH [17].

5-OH-TRP has a lower oxidation potential ($E_{1/2}$ = 0.40 V (SCE)) than TRP ($E_{1/2}$ = 0.80 V (SCE)) and is expected to undergo single-electron transfer to $^1O_2$ to generate $O_2^{\cdot-}$:



5–OH–TRP

$$+ \ ^1O_2 \longrightarrow O_2^{\cdot-} + [\text{5-OH-TRP}^{\cdot+}] \qquad (4)$$

In fact, incubation of a solution of 5-OH-TRP (1 mM), NBT (2 mM) and 1 (2 mM) in phosphate buffer (pH 8.0) at 35 °C resulted in the smooth reduction of NBT. Addition of SOD to the reaction system inhibited the formation of formazan as shown in Fig. 2. Table 2 summarizes the effect of pH and additives on the yield of $O_2^{\cdot-}$. Under these conditions autoxidation of 5-OH-TRP gave only a low yield (0.4%) of $O_2^{\cdot-}$. The yield of $O_2^{\cdot-}$ increased with increasing pH, suggesting that the dissociation of the 5-hydroxyl group of 5-OH-TRP is involved in the formation of $O_2^{\cdot-}$. Addition of mannitol, an OH· scavenger, did not affect the yield of $O_2^{\cdot-}$, whereas DABCO and triethylamine, well-known $^1O_2$ quenchers, unexpectedly accelerated the formation of $O_2^{\cdot-}$. This is probably due to promotion of the dissociation process of the 5-hydroxyl group by these tertiary amines, although the exact reason for the anomalous behaviour of tertiary amines is by no means clear at present.

In contrast, the $^1O_2$ reaction of melatonin ($E_{1/2}$ = 0.72 V (SCE)) (Me ≡ methyl) and tryptophol ($E_{1/2}$ = 0.71 V (SCE))

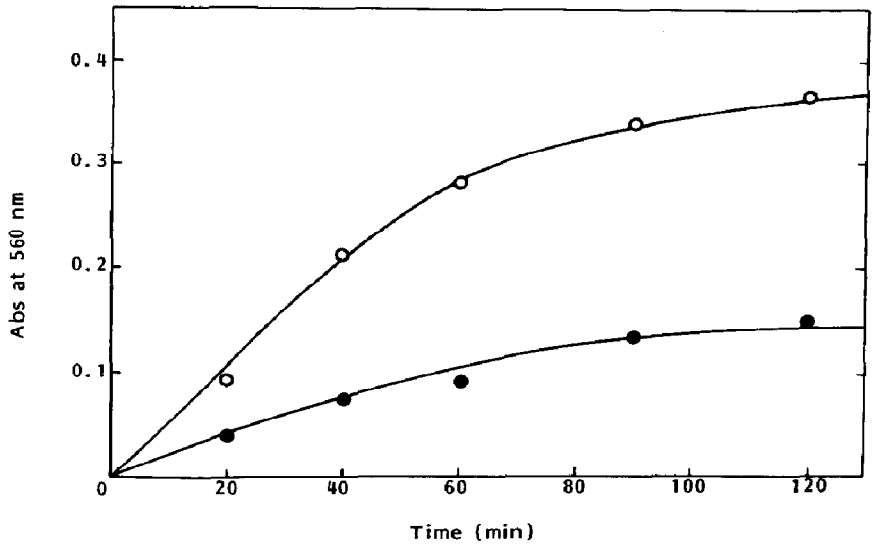


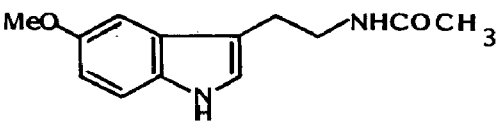
Fig. 2. Reduction of NBT during incubation of the endoperoxide **1** and 5-OH-TRP in the presence (●) and absence (○) of SOD. SOD (280 units) was added before incubation ([5-OH-TRP] = 1 mM; [**1**] = 2 mM; [NBT] = 2 mM; phosphate buffer (pH 8.0); 35 °C).

TABLE 2

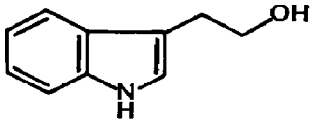
Effect of pH and various additives on the formation of $O_2^{-\cdot}$ in the reaction of 5-hydroxytryptophan with $^1O_2$ at 35 °C (2 h)

System	*Additive*	*Yield of* $O_2^{-\cdot}$ [a] (%)
5-OH-TRP, **1**, NBT	—	3.4
Phosphate buffer pH 8.0[b]	NaN_3 (5.6 mM)	0.2
	Triethylamine (2.0 mM)	5.4
	Triethylamine (5.7 mM)	8.9
	Triethylamine (10 mM)	13.0
	DABCO (5 mM)	5.7
	Mannitol (2 mM)	3.9
5-OH-TRP, **1**, NBT[b]		
Phosphate buffer pH 7.5	—	1.5
$NaHCO_3$ buffer pH 8.3	—	3.4
5-OH-TRP, **1**, cytochrome c[c]		
Phosphate buffer pH 8.0	—	1.1
5-OH-TRP, NBT[d]	O_2 bubbling	0.4
	Triethylamine (5.7 mM)	0.8
	O_2 bubbling	

[a] The yield is based on the amount of 5-OH-TRP initially used.
[b] [5-OH-TRP] = 1 mM; [**1**] = 2 mM; [NBT] = 2 mM.
[c] [5-OH-TRP] = 0.1 mM; [**1**] = 0.2 mM; [ferricytochrome c] = 0.2 mM.
[d] [5-OH-TRP] = 1 mM; [NBT] = 2 mM.



melatonin



tryptophol

under similar conditions did not produce $O_2^{\cdot-}$ at all. These observations imply that single-electron transfer giving rise to $O_2^{\cdot-}$ is possible for indoles with oxidation potentials less than about 0.5 V (SCE), in accordance with the previous results obtained with substituted *N,N*-dimethylanilines [3].

In summary, a combination of water-soluble chemical $^1O_2$ sources and $O_2^{\cdot-}$-detecting reagents can serve as a convenient and mechanistically less complicated method for detecting $O_2^{\cdot-}$ in $^1O_2$ reactions of biological systems.

References

1 I. Fridovich, *Annu. Rev. Biochem., 44* (1975) 147.
2 A. M. Michlson, J. M. McCord and I. Fridovich, *Superoxide and Superoxide Dismutases*, Academic Press, New York, 1977.
3 I. Saito, T. Matsuura and K. Inoue, *J. Am. Chem. Soc., 105* (1983) 3200.
4 G. Peters and M. A. J. Rodgers, *Biochem. Biophys. Res. Commun., 96* (1980) 770.
5 G. Peters and M. A. J. Rodgers, *Biochim. Biophys. Acta, 637* (1981) 43.
6 I. Saito, T. Matsuura and K. Inoue, *J. Am. Chem. Soc., 103* (1981) 188.
7 V. Rapp, W. C. Adams and R. W. Miller, *Can. J. Biochem., 51* (1973) 159.
8 B. H. Bielski, G. G. Shive and S. Bajuk, *J. Phys. Chem., 84* (1980) 830.
9 A. W. Frenkel, L. S. Jahnke and Z. J. Petryka, *Oxygen and Oxyradicals in Chemistry and Biology*, Academic Press, New York, to be published.
10 G. S. Cox and D. G. Whitten, *Chem. Phys. Lett., 67* (1980) 511.
11 R. S. Bodaness and P. C. Chan, *J. Biol. Chem., 252* (1977) 8554.
12 W. H. Koppenol, *Nature (London), 262* (1976) 420.
13 E. J. Land and A. J. Swallow, *Biochim. Biophys. Acta, 234* (1971) 34.
14 K. Inoue, T. Matsuura and I. Saito, *Photochem. Photobiol., 35* (1982) 133, and references cited therein.
15 J. P. McCormick and T. Thomason, *J. Am. Chem. Soc., 100* (1978) 312.
16 L. I. Grossweiner, *Curr. Top. Radiat. Res. Q., 11* (1976) 141.
17 K. Inoue, T. Matsuura and I. Saito, *Bull. Chem. Soc. Jpn., 55* (1982) 2959.