The Occurrence of Superoxide Anion in the Reaction of Reduced Phenazine Methosulfate and Molecular Oxygen

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Received November 30, 1971

SUMMARY

The reduction of nitro blue tetrazolium (NitroBT) with NADH mediated by phenazine methosulfate (PMS) under aerobic conditions was inhibited upon addition of superoxide dismutase. This observation indicated the involvement of superoxide aninon radical (O_2^-) in the reduction of NitroBT, the radical being generated in the reoxidation of reduced PMS. Similarly, the reduction of NitroBT coupled to D-amino acid oxidase-PMS system under aerobic conditions was also inhibited by superoxide dismutase. A simple method for detecting superoxide dismutase is described.

INTRODUCTION

The univalent reduction of O_2 has been observed in a number of enzyme-catalyzed reactions (1-6). The earliest reports were those of McCord and Fridovich (1) and of Knowles *et al.* (2), who showed the formation of O_2^- in the xanthine oxidase reaction. Recently, superoxide dismutase, catalyzing dismutation of O_2^- to form O_2 and H_2O_2, has been reported (7). The determination of this enzyme was based on the inhibition of aerobic reduction of cytochrome c or NitroBT by xanthine oxidase (7,8). On the other hand, Ballou *et al.* (9) demonstrated generation of O_2^- in the reoxidation of photoreduced flavin in air and this system was used for the estimation of superoxide dismutase activity (8). Considering the structural similarity between flavin and PMS, we predicted that O_2^- might also occur in the reoxidation of reduced PMS with O_2. This prediction

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was confirmed by using superoxide dismutase in the present study, which provided a new method for generation of O_2^- using PMS and its application to the determination of superoxide dismutase activity.

MATERIALS AND METHODS

Superoxide dismutase from bovine erythrocytes was purified up to the step of acetone precipitation according to the method of McCord and Fridovich (7). Xanthine oxidase was a generous gift of Dr. Uozumi, Osaka University. Unit of superoxide dismutase activity was defined as the amount of the enzyme required to inhibit the rate of reduction of cytochrome c by 50% under the conditions described by McCord and Fridovich (7), except that the reaction was carried out at 20°. D-Amino acid oxidase was prepared as reported previously (10). The concentration of the enzyme was expressed in terms of the bound FAD. Cytochrome c, type III, was a product of Sigma. All reagents were of analytical grade.

The transmittance changes caused by the reduction of electron acceptors were measured in a Yanaco SPS-1 stopped-flow spectrophotometer connected to a pen-recorder. The details of the apparatus were described elsewhere (11). The reduction of NitroBT and cytochrome c was followed at 560 and 550 mμ, respectively. All measurements were performed in 0.017 M sodium pyrophosphate buffer, pH 8.3, and at 20°.

Disc gel electrophoresis was performed according to the method of Davis (12). The protein bands were stained using amido black.

RESULTS AND DISCUSSION

Non-enzymatic method for generation of O_2^- —— The reduction of NitroBT (50 μM) by NADH (78 μM) under aerobic conditions was negligible. The addition of PMS to this system provoked the reduction, and its rate increased linearly with increasing concentration of PMS added (0.14-8.7 μM), indicating that PMS was acting as an electron carrier in this system (Fig. 1). In order to test for the participation of O_2^- in this system, the effect of superoxide dismutase on the reduction of NitroBT by NADH-PMS system was examined. As shown in Fig.

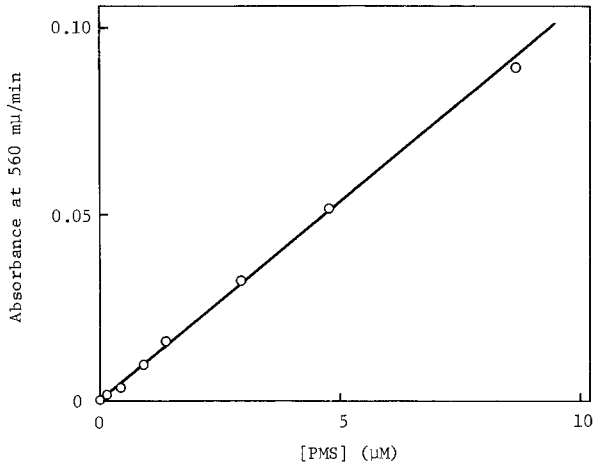

Fig. 1. The effect of PMS on the rate of reduction of NitroBT with NADH under aerobic conditions. The reaction mixtures contained 50 μM NitroBT, 78 μM NADH and PMS (concentrations indicated in figure). The reaction was carried out in 0.017 M sodium pyrophosphate buffer, pH 8.3 and at 20°. Rate of the reaction was obtained using the linear portion of absorbance increase at 560 mμ after the initial lag (see Fig. 2A).

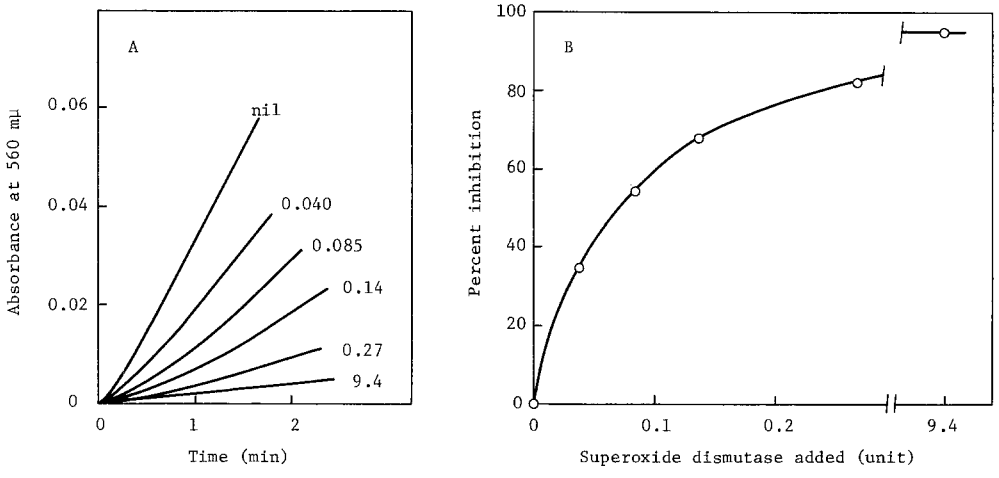

Fig. 2. Inhibition by superoxide dismutase of the reduction of NitroBT with NADH mediated by PMS under aerobic conditions.

A. The time course of the absorbance change at 560 mμ. The reaction mixtures contained 50 μM NitroBT, 78 μM NADH, 3.1 μM PMS and superoxide dismutase (unit indicated in figure). The reaction was carried out in 0.017 M sodium pyrophosphate buffer, pH 8.3 and at 20°.

B. A plot of percent inhibition against superoxide dismutase added. Rate of the reaction was obtained using the linear portion of the reaction trace after the initial lag.

2, superoxide dismutase inhibited the aerobic reduction of NitroBT; 50% of the maximum inhibition was achieved upon the addition of 0.064 unit. The maximum inhibition attained was 95%, indicating that 5% of the reduction of NitroBT was

due to its direct reaction with reduced PMS. Little or no inhibition by superoxide dismutase was observed when cytochrome c was used in place of NitroBT as a terminal electron acceptor. This indicated that the reduction of cytochrome c occurred through O_2^- independent pathway. This result was in accord with that of McCord and Fridovich (13) who were unable to observe inhibition by superoxide dismutase when the aerobic reduction of cytochrome c by ferredoxin-NADP reductase or xanthine dehydrogenase was mediated by PMS.

Detection of superoxide dismutase in acrylamide gels —— NADH-PMS-NitroBT system was used to locate superoxide dismutase activity. After electrophoresing about 0.01 unit of the enzyme, the gel cylinders were first immersed for 20 min in a solution containing 2.4 mM NitroBT and 28 μM PMS, followed by soaking for 1 hr in a solution of 150 μM NADH in 0.017 M sodium pyrophosphate buffer, pH 8.3. An achromatic zone was visible at the position corresponding to superoxide dismutase (indicated by protein staining). The whole procedure for the identification of superoxide dismutase should be performed in the dark to prevent the decomposition of PMS.

Generation of O_2^- coupled to the enzymatic reaction —— The occurrence of O_2^- in the oxidation of reduced PMS in air was also achieved by producing reduced PMS in coupling to an enzymatic reaction. Since the reduction of PMS by D-amino acid oxidase-D-arginine system** was established (14), this reaction system was adopted for the above purpose. The rate of aerobic reduction of NitroBT and cytochrome c in the presence of D-amino acid oxidase, D-arginine and PMS, was examined in the absence and presence of various amounts of superoxide dismutase. The results are summarized in Table I. Inhibition by superoxide dismutase of the reduction of NitroBT was remarkable and that of cytochrome c was marginal.

The results reported in the present paper indicated that the aerobic reduction of NitroBT was dependent on the concentration of PMS in either non-enzymatic NADH-PMS system or enzymatic D-amino acid oxidase-PMS system and that the amount of inhibition was dependent on the concentration of superoxide

** The aerobic reduction of cytochrome c in the presence of D-amino acid oxidase and D-arginine was not observed, indicating that the reoxidation of reduced D-amino acid oxidase with O_2 does not involve O_2^-.

TABLE I.

The effect of superoxide dismutase on the reduction of NitroBT and cytochrome c coupled to D-amino acid oxidase-PMS system under aerobic conditions.

Electron acceptor	Superoxide dismutase added (unit)	Percent inhibition
NitroBT[1]	0.07	47
	0.22	84
	3.8	96
Cytochrome c[2]	2.4	4

The reactions were carried out in 0.017 M sodium pyrophosphate buffer, pH 8.3 and at 20°.

[1]The reaction mixture contained 100 μM NitroBT, 28 μM PMS, 1.7 mM DL-arginine, 0.31 μM D-amino acid oxidase, 10 μM FAD and superoxide dismutase (unit indicated). Rate of the absorbance change at 560 mμ in the absence of superoxide dismutase was 0.0065 absorbance unit per min.

[2]The reaction mixture contained 12 μM cytochrome c, 33 μM PMS, 2 mM DL-arginine, 0.31 μM D-amino acid oxidase, 10 μM FAD and superoxide dismutase (unit indicated). Rate of the absorbance change at 550 mμ in the absence of superoxide dismutase was 0.044 absorbance unit per min.

dismutase added. These results indicate the reduction of NitroBT by O_2^- occurring in the reoxidation of reduced PMS with O_2. The maximum inhibition by superoxide dismutase achieved in NADH-PMS-NitroBT system was 95% and the same degree of inhibition was also found in D-amino acid oxidase-PMS-NitroBT system. When cytochrome c was used as electron acceptor, the inhibition was marginal in both systems. This emphasizes the importance of examining the reaction with different acceptors before ruling out the occurrence of O_2^- in various reactions in which it is postulated to occur.

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