SUPEROXIDE-DEPENDENT PRODUCTION OF HYDROXYL RADICAL CATALYZED BY IRON–EDTA COMPLEX

Joe M. McCORD and Eugene D. DAY, Jr
Department of Biochemistry, University of South Alabama, Mobile, AL 36688 and *Departments of Biochemistry and Medicine, Duke University Medical Center, Durham, NC, USA*

Received 29 November 1977

1. Introduction

Shortly after the discovery of superoxide dismutase [1,2] Beauchamp and Fridovich employed the enzyme as a tool to study the mechanism of ethylene production from methional as catalyzed by xanthine oxidase plus xanthine [3]. They concluded that the species which attacked methional was most likely hydroxyl radical generated secondarily from a reaction between superoxide and hydrogen peroxide:

$$O_2^{\overline{\cdot}} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\cdot} \qquad (1)$$

Their proposal was supported by the following observations: (i) ethylene production could be inhibited by superoxide dismutase, (ii) by catalase, or (iii) by scavengers of hydroxyl radical such as ethanol and benzoate; and (iv) a lag in ethylene production was found to be due to the time required for hydrogen peroxide to accumulate to a reactive concentration. Reaction 1 was the simplest explanation accommodating all the observed facts, and the reaction had been proposed many years earlier by Haber and Weiss during their studies of the catalytic decomposition of hydrogen peroxide by iron salts [4].

In the last seven years the reaction has become known in the biological literature as the Haber-Weiss reaction, and has been invoked by many investigators to explain diverse biological phenomena which are characterized by the same inhibitory criteria described by Beauchamp and Fridovich and outlined above. On the other hand, attempts to demonstrate directly the simple biomolecular reaction shown as reaction 1 have met with uniform failure [5–7], leading to the conclusion that, although reaction 1 may indicate the stoichiometry of the overall process, it cannot be construed as the reaction mechanism.

Under chemically well-defined conditions, it has in fact been shown that reaction 1 does not proceed at any appreciable rate. Koppenol, however, has shown the reaction to be quite feasible thermodynamically [8], even if the oxygen molecule which appears as a product is in the electronically excited $^1\Delta_gO_2$ singlet state, as Kellogg and Fridovich have suggested [9].

The stoichiometry of reaction 1 can be achieved by summing two half reactions with catalytic intercession by a metal ion:

$$O_2^{\overline{\cdot}} + M^{n+} \longrightarrow O_2 + M^{(n-1)+} \qquad (2)$$

$$M^{(n-1)+} + H_2O_2 \longrightarrow M^{n+} + OH^- + OH^{\cdot} \qquad (3)$$

$$O_2^{\overline{\cdot}} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\cdot} \qquad (1)$$

Reactions analogous to (2) and (3) have been suggested or indirectly supported by many investigators [4,10, 11]. Because of the increasing interest and now obvious importance of superoxide and hydroxyl radicals in biological processes, we have examined the possibility that reaction 1 may be catalyzed by chelated iron ions, finding that this is indeed the case. We have utilized tryptophan as our indicator molecule because of its spectral properties and its reasonably well-characterized reaction with hydroxyl radical [12].

2. Materials and methods

Xanthine oxidase (Grade III), xanthine, transferrin, mannitol, catalase (beef liver, 2X crystallized), and L-tryptophan were obtained from Sigma Chemical Co. (St Louis, MO). Ferrous sulfate was obtained from Baker and Adamson (Morristown, NJ), and ethylenedinitrilotetra-acetic acid disodium salt (EDTA) from Matheson, Coleman, and Bell (Norwood, OH). Superoxide dismutase was prepared from bovine liver by a minor modification of the method in [2]. Hydrogen peroxide was obtained from Mallinckrodt Inc. (St Louis, MO). The rate of superoxide production by xanthine oxidase was determined by the rate of cytochrome *c* (Sigma, Type III) reduction [2]. All solutions were buffered at pH 7.4 with potassium phosphate. Tryptophan disappearance was monitored at 278 nm assuming a molar extinction coefficient of 5000 [13]. This wavelength, which is quite near the absorbance maximum for tryptophan, is an isosbestic point for the conversion of xanthine into uric acid. Hence the ultraviolet-absorbing substrate and product of the xanthine oxidase reaction did not interfere with the observance of small changes in tryptophan concentration. All reactions were carried out at 25°C in 1 cm quartz cuvettes. All solutions utilized deionized water, but no special precautions were taken to remove trace metals from other reagents.

3. Results

Tryptophan was exposed to a superoxide-generating system in the presence of varying concentrations of Fe–EDTA complex (fig.1). In the absence of added iron, no significant decrease in tryptophan absorbance was apparent. The rate of tryptophan disappearance increased linearly with Fe–EDTA concentration up to about 30 μM iron.

This iron-catalyzed, superoxide- and hydrogen peroxide-dependent destruction of tryptophan showed all the characteristics common to the numerous examples which have been described in recent years and attributed to the so-called 'Haber-Weiss reaction'. That is, the disappearance of tryptophan was superoxide dependent (fig.2) and totally inhibitable by superoxide dismutase (4 units/ml). In the absence of added hydrogen peroxide a lag of several minutes was

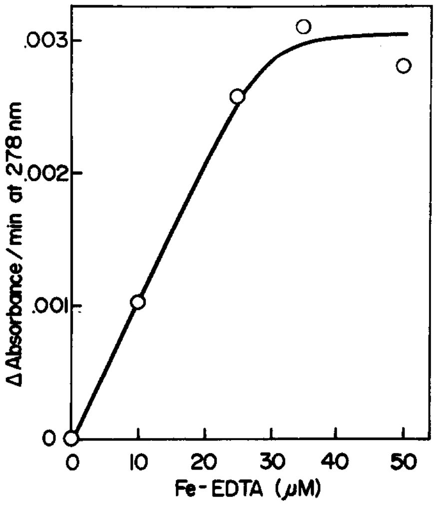

Fig.1. Haber-Weiss catalysis by iron–EDTA complex. Reaction mixtures consisted of 1.1 ml 50 mM potassium phosphate buffer, pH 7.4, containing 45 μM xanthine, 90 μM tryptophan and 0.005 units xanthine oxidase. Iron–EDTA was added at the concentrations indicated. Superoxide was produced at a rate of 5.3 nmol/min.

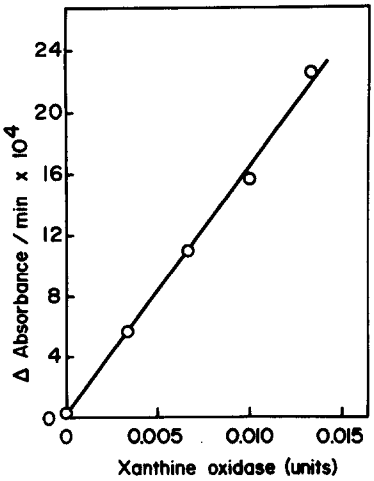

Fig.2. Dependence of the Haber-Weiss process on rate of superoxide production. Reaction mixtures consisted of 2 ml 50 mM potassium phosphate buffer, pH 7.4, containing 50 μM xanthine, 0.1 mM tryptophan. 5 μM iron–EDTA complex, 10 μM hydrogen peroxide and the indicated amounts of xanthine oxidase. Absorbance was monitored at 278 nm.

observed, which could be eliminated by the addition of 10 μM hydrogen peroxide. The destruction of tryptophan could be inhibited by catalase or by scavengers of hydroxyl radical such as mannitol and ethanol. A comparison of rate constants for the reaction of the $OH^\cdot$ with tryptophan and ethanol [12,14] predicts that a concentration of ethanol, 7.1-times that of tryptophan, would produce 50% inhibition. We found half-maximal inhibition at a concentration ratio of 8.7, in good agreement with the predicted value.

Copper–EDTA complex, substituted for the iron complex, failed to bring about the production of hydroxyl radical in an otherwise complete system, in agreement with the conclusions of [11]. Iron chelated by the plasma protein transferrin, however, did catalyze the process at least as efficiently as iron chelated by EDTA.

In attempt to elucidate the mechanism of the iron-catalyzed Haber-Weiss reaction, we examined the inhibition produced by superoxide dismutase in the presence of two different concentrations of Fe–EDTA. When these data were plotted on double-reciprocal coordinates (fig.3) a competitive relationship between these two components of the system became apparent, suggesting that both react with superoxide radical.

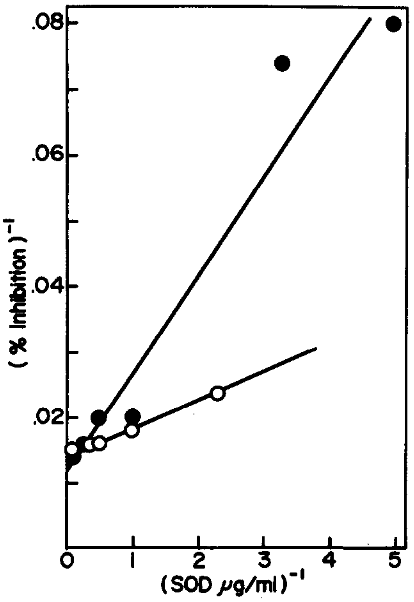

Fig.3. Competition between superoxide dismutase and iron–EDTA. Conditions were as in fig.1, except that the iron–EDTA concentration was fixed at 25 μM (○) or 50 μM (●). Superoxide dismutase (SOD) concentration was varied as shown.

4. Discussion

While we believe that reaction I adequately describes the stoichiometry of the net Haber-Weiss phenomenon, the mechanism appears to be better represented by the following reactions:

$$O_2^{\cdot -} + Fe^{3+}\text{–EDTA} \longrightarrow O_2 + Fe^{2+}\text{–EDTA} \quad (4)$$

$$Fe^{2+}\text{–EDTA} + H_2O_2 \longrightarrow Fe^{3+}\text{–EDTA} + OH^- + OH^\cdot \quad (5)$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + OH^- + OH^\cdot \quad (1)$$

Another possibility might be the formation of a ternary complex consisting of Fe^{3+}–EDTA, H_2O_2 and $O_2^{\cdot -}$. It has been suggested that the term 'Haber-Weiss reaction' is inappropriate to describe the phenomenon under discussion, since reaction 1 (uncatalyzed) is kinetically so slow as to be negligible. Haber and Weiss, however, in the same oft-cited paper in which reaction 1 was proposed, also proposed reactions 4 and 5, so we see no problem in continuing to credit these early investigators with the reaction(s), especially in view of the now widespread usage. The reaction sequence we have observed cannot be adequately described as a 'Fenton-like' reaction, since our system clearly requires an outside source of superoxide radicals and proceeds at very low concentrations of hydrogen peroxide and iron.

The competitive relationship seen in fig.3 between Fe^{n+}–EDTA and superoxide dismutase is predicted by the suggested reaction sequence. Although superoxide is shown to react only with ferric iron in reaction 4, Halliwell has shown that it can also react with ferrous iron, oxidizing it to ferric [15]:

$$O_2^{\cdot -} + Fe^{2+}\text{–EDTA} + 2H^+ \longrightarrow H_2O_2 + Fe^{3+}\text{–EDTA} \quad (6)$$

In fact, Halliwel deduced that reaction 6 proceeds more favorably than reaction 4; i.e., the iron chelate functions via reactions 4 and 6 as a superoxide dismutase. Thus, superoxide and hydrogen peroxide compete for Fe^{2+}–EDTA, and the overall Haber-Weiss process becomes more efficient as H_2O_2 accumulates. In the presence of 5 μM iron, 10 μM H_2O_2, and a rate of superoxide production of 3.2 nmol/min, our observed overall efficiency was at least 7.5% (see fig.2). That is, for every superoxide radical produced at least 0.075 hydroxyl radicals were produced. (This calculated estimation of efficiency actually represents a lower limit, since it is assumed that the product of the reaction between tryptophan and $OH^{\cdot}$ results in a species with no absorption at 278 nm. The reaction products are multiple and almost certainly some contribute to the absorption at 278 nm [12]. Hence, we can only say the efficiency lies between 7.5% and 100%.) Under different conditions, slightly higher efficiencies were observed. These data yield an apparent rate constant for the net process (reaction 1) of about 1000 M^{-1} s^{-1}. This degree of efficiency is sufficient for biological consequences of considerable significance, as have indeed been claimed by many investigators. In particular, the Haber-Weiss process can be expected to occur when superoxide is generated in extracellular fluids devoid of catalase and superoxide dismutase, but containing the necessary catalyst (such as transferrin). These conditions are met in instances of the phagocyte-mediated inflammatory process [16,17].

References

[1] McCord, J. M. and Fridovich, I. (1968) J. Biol. Chem. 243, 5753–5760.
[2] McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[3] Beauchamp, C. and Fridovich, I. (1970) J. Biol. Chem. 245, 4641–4646.
[4] Haber, F. and Weiss, J. (1934) Proc. R. Soc. London Ser. A 147, 332–351.
[5] McClune, G. J. and Fee, J. A. (1976) FEBS Lett. 67, 294–298.
[6] Halliwell, B. (1976) FEBS Lett. 72, 8–10.
[7] George, P. (1947) Disc. Faraday Soc. 2, 196–205.
[8] Koppenol, W. H. (1976) Nature 262, 420–421.
[9] Kellogg, E. W. and Fridovich, I. (1975) J. Biol. Chem. 250, 8812–8817.
[10] Walling, C., Partch, R. E. and Weil, T. (1975) Proc. Natl. Acad. Sci. USA 72, 140–142.
[11] Van Hemmen, J. J. and Meuling, W. J. A. (1977) Arch. Biochem. Biophys. 182, 743–748.
[12] Armstrong, R. C. and Swallow, A. J. (1969) Radiat. Res. 40, 563–579.
[13] White, A., Handler, P. and Smith, E. (1973) in: Principles of Biochemistry, 5th edn, p. 92, McGraw-Hill, New York.
[14] Dorfman, L. M. and Adams, G. E. (1973) Nat. Stand. Ref. Data Ser. Nat. Bur. Stand. 46.
[15] Halliwell, B. (1975) FEBS Lett. 56, 34–38.
[16] McCord, J. M. (1974) Science 185, 529–531.
[17] Salin, M. L. and McCord, J. M. (1975) J. Clin. Invest. 56, 1319–1223.