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THE OXIDATION OF TIRON BY SUPEROXIDE ANION

KINETICS OF THE REACTION IN AQUEOUS SOLUTION AND IN CHLOROPLASTS

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SUMMARY

The rate of reaction between superoxide anion ($O_2^{\cdot-}$) and 1,2-dihydroxybenzene-3,5-disulfonic acid (tiron) was measured with pulse radiolysis-generated $O_2^{\cdot-}$. A kinetic spectrophotometric method utilizing competition between *p*-benzoquinone and tiron for $O_2^{\cdot-}$ was employed. In this system, the known rate of reduction of *p*-benzoquinone was compared with the rate of oxidation of tiron to the semiquinone. From the concentration dependence of the rate of tiron oxidation, the absolute second order rate constant for the reaction was determined to be 5×10^8 $M^{-1} \cdot s^{-1}$. Ascorbate reduced $O_2^{\cdot-}$ to hydrogen peroxide with a rate constant of 10^8 $M^{-1} \cdot s^{-1}$ as determined by the same method. The tiron semiquinone may be used as an indicator free radical for the formation of superoxide anion in biological systems because of the rapid rate of oxidation of the catechol by $O_2^{\cdot-}$ compared to the rate of $O_2^{\cdot-}$ formation in most enzymatic systems.

Tiron oxidation was used to follow the formation of superoxide anion in swollen chloroplasts. The chloroplasts photochemically reduced molecular oxygen which was further reduced to hydrogen peroxide by tiron. Tiron oxidation specifically required $O_2^{\cdot-}$ since O_2 was consumed in the reaction and tiron did not reduce the P_{700} cation radical or other components of Photosystem I under anaerobic conditions.

INTRODUCTION

Although tiron is known to react with $O_2^{\cdot-}$ generated in photochemical and enzymatic systems [1], the rate of reaction between $O_2^{\cdot-}$ and tiron has not been previously reported. The rate and specificity of the univalent oxidation of tiron must be

Abbreviations: Sodium 1,2-dihydroxybenzene-3,5-disulfonate, tiron; superoxide anion, $O_2^{\cdot-}$; the system I reaction center chlorophyll *a*, P_{700}; tiron semiquinone, $(TH)^{\cdot}$.

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known in order to establish that tiron may be used as a probe for $O_2^{\cdot-}$ formation in biological systems.

Chloroplasts and chloroplast fragments photochemically reduce oxygen to $O_2^{\cdot-}$ [2, 3]. This species may oxidize electron donors such as ascorbate or dismutate to oxygen and hydrogen peroxide. The latter reaction is catalyzed by several superoxide dismutases which are present in chloroplasts and thylakoid membranes [4].

MATERIALS AND METHODS

Preparation of chloroplasts. Chloroplasts were prepared from wheat leaf blades and stored at −80 °C in 0.5 M sucrose as described previously [5]. Thawed aliquots were swollen by dilution with 2 vol. of 0.05 M sodium phosphate buffer, pH 7. Final chlorophyll concentration was 0.8 mg per ml of sample. Total superoxide dismutase activity in the preparations was determined with a spectrophotometric procedure [6].

ESR Spectroscopy. ESR spectra were recorded with a Varian E-3 spectrometer. Thin (0.2 mm) quartz, aqueous sample cells were illuminated in the slotted spectrometer cavity with white light at an intensity of 2×10^5 ergs $\cdot$ cm$^{-2}\cdot$ s^{-1}. Rates of tiron semiquinone production were monitored by holding the magnetic field at a constant value and recording the height of the first derivative ESR signal as a function of time. ESR samples were made anaerobic by repeated evacuation of thylakoid preparations in Thunberg cuvettes followed by flushing with nitrogen. The ESR cells were also flushed with O_2-free nitrogen before filling with the anaerobic reaction mixture.

Pulse radiolysis system. The rates of reaction between $O_2^{\cdot-}$ and ascorbate, and $O_2^{\cdot-}$ and tiron were determined with pulse radiolysis-produced $O_2^{\cdot-}$. The radiolysis studies were carried out using single 50 ns pulses of 3 MeV electrons from a Van der Graaf generator. The average dose per pulse absorbed in the irradiation cell was 0.2–2 krad. The details of the optical and electronic apparatus are similar to those described elsewhere [7]. Solutions, saturated with oxygen, were prepared immediately before the pulse radiolysis experiments in sealed syringes and admitted to the irradiation cell and back-flushed periodically during the irradiation by a remote-controlled flow system [8]. Oscilloscope traces of the transient species were digitised and the absolute bimolecular rate constants determined by computer analysis.

Measuring $O_2^{\cdot-}$ reaction rates. Because the $O_2^{\cdot-}$ absorbance spectrum is weak in the ultraviolet region of the spectrum [9] it is difficult to measure $O_2^{\cdot-}$ reaction rates directly by following its decay. Two other methods for the determination of $O_2^{\cdot-}$ reaction rates are described in this paper: competition with *p*-benzoquinone [10], and build-up of solute free radicals.

In the competition method, $O_2^{\cdot-}$ is reacted with the solute s at a rate k_s[s] and with the competitive solute *p*-benzoquinone at a rate k_Q[Q]. The latter reaction forms a long-lived species $Q^{\cdot-}$ absorbing at 430 nm. In the absence of solute, for a given dose, the absorbance is A_0. When solute is added, a lower absorbance A is observed according to the following equation:

$$A_0/A = 1+k_s[s]/k_Q[Q] \quad (1)$$

RESULTS AND DISCUSSION

Pulse radiolysis studies

The radiolysis of aqueous solutions at neutral pH generates two principal re-

active species, hydrated electrons (e^-_{aq}) and hydroxyl free radicals (·OH), together with a small yield of hydrogen atoms (H·). In the presence of 0.1 M *t*-butanol the ·OH and some H· are scavenged to form unreactive *t*-butanol free radicals [11]:

$$\cdot OH + (CH_3)_3COH \rightarrow (CH_3)_2C^{\cdot}H_2COH \quad (2)$$

In oxygen-saturated solutions, the remaining reactive species, e^-_{aq} and H, react to form the superoxide radical anion:

$$e^-_{aq} + O_2 \rightarrow O_2^{\cdot-} \quad (3)$$

$$H^{\cdot} + O_2 \rightarrow HO_2^{\cdot} \quad (4)$$

$$HO_2^{\cdot} \rightleftharpoons O_2^{\cdot-} + H^+ \quad (5)$$

which is then free to react with tiron:

$$O_2^{\cdot-} + \text{tiron} \rightarrow HO_2^- + (TH)^{\cdot}. \quad (6)$$

The oxidized tiron semiquinone free radicals $(TH)^{\cdot}$ absorb weakly at 400 nm and the rate of build-up of this absorbance has been measured as a function of tiron concentration (Fig. 1). The $O_2^{\cdot-}$ reaction rate is first order in tiron concentration, and the absolute bimolecular rate constant calculated from the slope of the plot of Fig. 1 is $5 \times 10^8\ M^{-1} \cdot s^{-1}$.

This rate constant is difficult to measure precisely by direct observation of the build-up of $(TH)^{\cdot}$ because of the low extinction coefficient of the latter. Consequently a competition method was used. A fixed concentration of 50 μM *p*-benzoquinone (Q) was used as the competing solute. The rate constant for reaction of $O_2^{\cdot-}$ with *p*-benzoquinone was previously determined to be $9 \times 10^8\ M^{-1} \cdot s^{-1}$ by observing the build-up of *p*-benzoquinone radical anion (Q^-) absorption at 430 nm [12, 13].

$$O_2^{\cdot-} + Q \rightarrow O_2 + Q^- \quad (7)$$

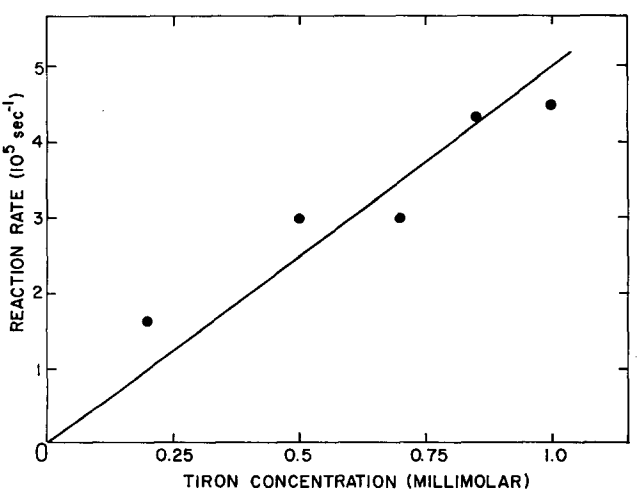

Fig. 1. Rate of oxidation of tiron by $O_2^{\cdot-}$ as a function of tiron concentration. The reaction was followed directly in neutral, oxygen-saturated, aqueous solutions containing 0.1 M *t*-butanol using pulse radiolysis, by monitoring the build up of absorbance at 400 nm attributed to the tiron semiquinone radical.

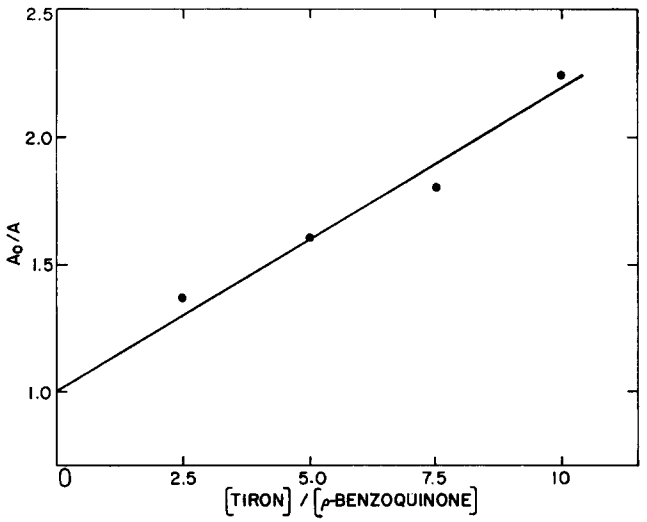

Fig. 2. Competition kinetic plot showing the decrease in *p*-benzoquinone radical anion (BQ^-) absorbance at 430 nm (A) upon adding increasing concentrations of tiron. Oxygen-saturated, neutral, aqueous solutions containing 50 μM benzoquinone, 0.1 M *t*-butanol and tiron was pulse irradiated, and the Q^- absorbance measured 1 μs after the pulse. The dose per pulse was maintained constant at 1.2 krad.

For a fixed dose (i.e. fixed $O_2^{\cdot-}$ concentration), addition of tiron up to the solubility limit results in a decrease in Q^- absorption in accord with Eqn 1. The contribution of the oxidized tiron-radical absorption at this wavelength is not significant. A typical competition plot is shown in Fig. 2 giving a rate constant of $1.5 \times 10^8\ M^{-1} \cdot s^{-1}$, which is somewhat lower than that obtained by the direct method. This may be due to some oxidation of the tiron by *p*-benzoquinone during the pulse radiolysis experiment.

Oxidation of tiron by $O_2^{\cdot-}$ in swollen chloroplasts

When chloroplast preparations containing 2 mM tiron were illuminated at 25 °C, the ESR signal of the tiron semiquinone attained maximal intensity within a few seconds. This signal showed the narrow 4-line hyperfine structure of the tiron radical [1] and was distinguished from other possible ESR signals in the sample such as that of P_{700} or endogenous quinones, since the latter signals are much broader. Indeed, spectrometer conditions (see legend, Fig. 3) were selected so that the endogenous signals did not interfere with the determination of changes in the tiron semiquinone signal [5]. Methyl viologen was added in order to facilitate reduction of molecular oxygen by reduced ferredoxins of Photosystem I [4].

Fig. 3 illustrates the kinetic course of the oxidation of tiron by $O_2^{\cdot-}$ in illuminated chloroplasts. Under the experimental conditions, the magnitude of the first derivative of microwave absorbance is proportional to $(TH)^{\cdot}$ concentration and this quantity was recorded as a function of time. On illumination, the signal rapidly increases with a half time of about 0.6 s, a value close to the time-resolution capability of the experimental system. Cessation of illumination resulted in a fall in the observed signal. This process could be repeated several times. Alternatively, if the illumination

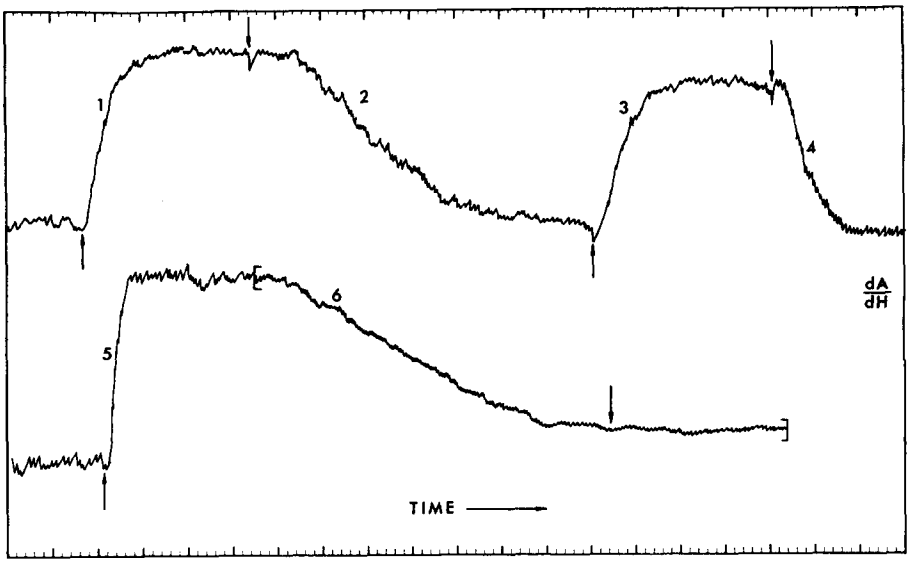

Fig. 3. Oxidation of tiron by chloroplast-generated $O_2^{\cdot-}$. Isolated chloroplasts suspended in 0.5 M sucrose were diluted with 2 parts of 0.05 M sodium phosphate buffer, pH 7 at 25 °C. Tiron 2 mM, methyl viologen 14 μM, and catalase 1 mg/ml, were added to give a final chlorophyll concentration of 0.8 mg chlorophyll per ml. The samples were introduced into flat, quartz ESR cells. Field strength was preset at a precise value corresponding to the maximum positive-going peak of the high-field hyperfine resonance of the tiron semiquinone radical. After temperature equilibration (25 °C) the illuminating lamp was turned on as indicated by the upward-pointing arrows and off at the downward-pointing arrows. ESR parameters were as follows: microwave power, 20 mW; nominal microwave frequency, 9.44 Ghertz; modulation amplitude, 1.0 gauss.

The speed of pen travel (x-axis) was varied as was the time constant of the recorder response according to the kinetic requirements of changes in the signal. These parameters are listed below:

Curve No.	Time constant (s)	$T_{\frac{1}{2}}$ (s)	Time per major x-axis division (s)
1	0.3	1.0	3.0
2	0.3	10.0	3.0
3	0.3	1.2	3.0
4	0.3	10.0	12
5	0.1	0.6	1.5
6	1.0	48.0	12

was left on, the signal eventually declined spontaneously on consumption of the oxygen in the sample. Reaeration of the sample by ejection from the ESR cell, followed by reintroduction into the cell, permitted the reversible light-dependent reaction to be repeated any number of times. Anaerobic reaction mixtures produced no $(TH)^{\cdot}$ signal on illumination.

Although potassium cyanide (2 mM) had no effect on the rate of oxidation of tiron as followed by ESR, considerable superoxide dismutase activity was present in the thylakoids as determined with a spectrophotometric procedure [6]. The amount of superoxide dismutase found in the preparations was equivalent to 60 μg of purified bovine erythrocyte superoxide dismutase per mg of chlorophyll. However, since some

of the superoxide dismutase is probably present as an iron enzyme [4], complete inhibition by KCN would not be expected since only copper-containing superoxide dismutase is sensitive to the inhibitor. The amount of KCN employed was insufficient to inhibit electron transport from water to methyl viologen.

CONCLUSION

From the pulse radiolysis studies it may be concluded that tiron reacts sufficiently rapidly with O_2^- to be considered as a diagnostic probe. For example, at concentrations of tiron utilized in biological studies (1–4 mM), the rate of this reaction would be $10^6\ s^{-1}$, corresponding to a half-time of a few microseconds for equimolar (2 mM) concentrations of tiron and O_2^-. This is far faster than the observed rate of oxidation of tiron in enzymatic or photochemical systems [1] or in the chloroplastic system of the present report. Hence the rate of formation of O_2^- in these systems must be rate limiting. The fact that tiron oxidation indicates that a very significant amount of O_2^- is produced in illuminated chloroplasts suggests incomplete dismutation of the oxygen radical by endogenous superoxide dismutase [5]. Since KCN had no demonstrable effect on oxygen-dependent tiron semiquinone formation, it is possible that in the presence of tiron, all the O_2^- which is formed reacts with the reagent. More detailed aspects of the use of tiron as an indicator probe for O_2^- in unswollen and subchloroplast fragments have been reported elsewhere [5].

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