SUPEROXIDE FORMATION IN SPINACH CHLOROPLASTS: ELECTRON SPIN RESONANCE DETECTION BY SPIN TRAPPING*

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SUMMARY: The new technique of spin trapping has been applied to a biological system for the first time. The light induced generation of O_2^- by chloroplasts in the presence of oxygen has been shown by the production of the O_2^- adduct of the spin trap 5,5-dimethyl-1-pyrroline-1-oxide. The O_2^- adduct was detected by electron spin resonance spectroscopy. Methyl viologen enhanced the production of the O_2^- adduct thus providing support for the hypothesis that methyl viologen accepts electrons from the primary acceptor of photosystem I and subsequently reduces O_2 to O_2^-.

INTRODUCTION

Free radicals have been postulated as intermediates in biochemical reactions ever since the hypothesis of Michaelis (1). Indeed the technique of electron spin resonance (ESR) spectroscopy has allowed the detection of these intermediates in some cases. However, it is only in cases where the radicals are relatively long lived or can be produced very rapidly that detection by ESR is possible. For example, in the study of photosynthesis the ability to use intense flashes of light has aided in the detection of radical intermediates (2). Nevertheless, for most reactions of biological interest where free radicals may be involved as intermediates, the radical lifetimes are too short to allow detection by ESR.

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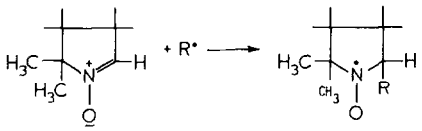
Fortunately a new technique has become available which should permit the study of systems with reactive radical intermediates. This method, known as spin trapping (3), involves the use of certain compounds (spin traps) which are very reactive with free radicals and produce a relatively long-lived free radical product (spin adduct) which can be studied by ESR. For example, the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) reacts with reactive free radicals R· as follows:



Both the β-proton hyperfine splitting and the nitrogen hyperfine splitting of the nitroxide spin adduct are very sensitive to the nature of R· (4). In favorable cases this information can be used to identify R·. In this report we present what we believe to be the first application of the spin trapping technique to a biological system.

The superoxide ion $O_2^{\cdot-}$ or its protonated form HO_2· has been postulated as an intermediate in many biological oxidation-reduction reactions. In particular it has been proposed that $O_2^{\cdot-}$ is photogenerated by spinach chloroplasts (5,6) (in the presence of ascorbate or low potential electron acceptors such as methyl viologen). Following on a study (7) where we characterized the spin adducts of HO_2· and ·OH with DMPO we are now able to detect directly the formation of $O_2^{\cdot-}$* in the chloroplast system.

MATERIALS AND METHODS

ESR spectra were obtained on a Varian E12 Electron Spin Resonance spectrometer. Illumination was accomplished either with a tungsten quartz-iodide lamp system described elsewhere (8) or a Spectra Physics Model Krypton Ion Laser operated at 647.1 nm.

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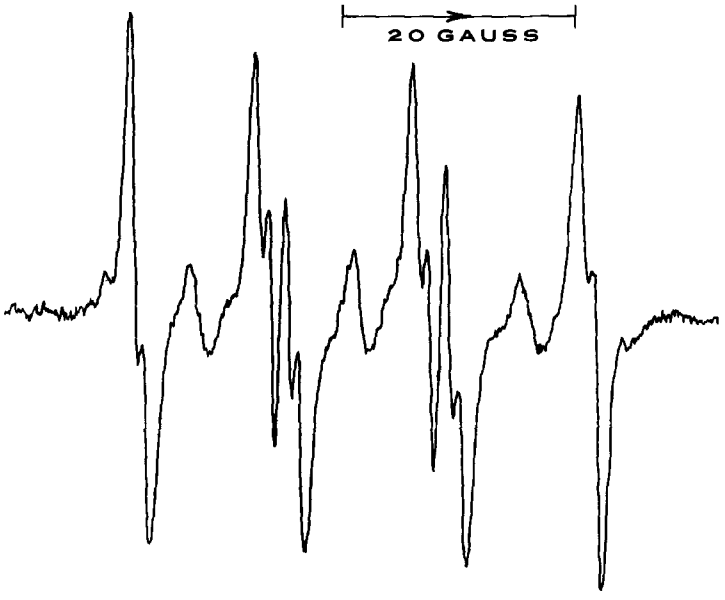
* It is still uncertain as to whether DMPO traps $O_2^{\cdot-}$ directly (followed by protonation) or traps HO_2· which is in equilibrium with $O_2^{\cdot-}$. In any event this point is not critical to the conclusions drawn here.

Spinach chloroplasts were prepared as described elsewhere (9). Methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride) and sodium ascorbate were obtained from Sigma and used without further purification. DMPO was a gift from Dr. K.S. Chen.

RESULTS AND DISCUSSION

Illumination of a chloroplast suspension with red light ($\lambda > 650$ nm) in the presence of DMPO (10^{-1} to 10^{-2}M) results in the generation of the ESR spectrum shown in fig. 1. This spectrum can be analyzed in terms of the parameters: $a^N = 14.3$G, $a^H_\beta = 11.7$G, $a^H_\gamma = 1.25$G and $g = 2.0061$. These values are essentially identical to those found for the $HO_2\cdot$ spin adduct of DMPO (7) and thus we conclude that $HO_2\cdot$ or O_2^- radicals are photoproduced in this chloroplast system. The generation of the signal seen in fig. 1 requires the presence of oxygen as saturating the solution with pure oxygen rather than air greatly increases the signal amplitude whereas when dissolved oxygen is removed by extensive bubbling with nitrogen no ESR signal can be generated.

The ESR signal of the $HO_2\cdot$ spin adduct with DMPO is not as strong as might be expected if all O_2^- molecules were trapped. In this regard it is significant that



Allen and Hall (6) have reported that there are between 5 and 10 superoxide dismutase (SOD) molecules per electron transport chain. SOD causes $O_2^{\cdot-}$ to dismutate to O_2 and H_2O_2. There may in fact be a competition between SOD and DMPO for $O_2^{\cdot-}$ in this system accounting for the relatively low yield of the DMPO spin adduct.

It has been shown that illumination of chloroplasts in the presence of low potential acceptors such as methyl viologen, results in the oxidation of exogenous ascorbate with the simultaneous uptake of dissolved oxygen (10). Bohme and Trebst have proposed that the ascorbate-stimulated O_2 uptake by isolated chloroplasts may be explained by ascorbate effectively replacing water as the electron donor for photosystem II. However, Epel and Neumann (5) have proposed an alternative hypothesis which supposes that these observations reflect photochemistry in photosystem I (PSI) with $O_2^{\cdot-}$ as an intermediate. In their interpretation light drives the reduction of the primary electron acceptor of PSI which subsequently reduces MV. The $MV^{\cdot}$ anion radical then rapidly transfers its electron to O_2 forming $O_2^{\cdot-}$ which then reacts with ascorbate to yield H_2O_2.

Since Epel and Neumann's proposal involves $O_2^{\cdot-}$ formation, we tested their hypothesis by adding DMPO to a chloroplast suspension containing ~1 x 10^{-3}M MV. On illumination with red light a very large signal* from $HO_2\cdot$ ($O_2^{\cdot-}$) spin adduct of DMPO was observed. This signal was dependent on dissolved oxygen in the same manner as demonstrated before. These results are thus in agreement with the Epel and Neumann mechanism, i.e. that MV intercepts electrons from the primary acceptor of PSI leading to the production of $O_2^{\cdot-}$ from O_2.

Since ascorbate reduces these nitroxide spin adducts, it was not possible to carry out the experiment with chloroplasts containing ascorbate, MV and DMPO. Such a system would have shown if ascorbate can efficiently compete with DMPO for the $O_2^{\cdot-}$ generated.

CONCLUSIONS

We have used the technique of spin trapping to demonstrate the presence of the

* The signal amplitude was much greater (a factor of about 10) than obtained with chloroplasts alone under the same conditions.

O_2^- radical in illuminated chloroplasts in the presence of dissolved oxygen. MV stimulates the production of O_2^- in agreement with a proposal of Epel and Neumann

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