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SUPEROXIDE MEDIATED CHLOROPHYLL ALLOMERIZATION IN A DIMETHYL SULPHOXIDE-WATER MIXTURE

MARK N. MERZLYAK‡, VADIM A. KOVRIZHNIKH and
KIRILL N. TIMOFEEV§

Department of Cell Physiology & Immunology and §Department of Biophysics,
Faculty of Biology, Moscow State University, 119899 GSP Moscow W-234, USSR

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The interaction of chlorophyll *a* with superoxide anion was studied in an alkaline DMSO-water system. It was found that $O_2^{\cdot-}$, directly or via $HO_2^{\cdot}$, produces the chlorophyll enolate-ion (Molish's intermediate) that is oxidized to Mg-chlorine(s). The allomerization reaction was found to be inhibited by superoxide dismutase. A possible participation of oxygen radicals in chlorophyll degradation in plants is discussed.

KEY WORDS: Chlorophyll degradation, allomerization, superoxide radical.

INTRODUCTION

The molecular mechanisms of chlorophyll breakdown in plant leaves during seasonal senescence and under conditions of oxidative stresses are not well known, largely due to the instability and/or rapid turnover of the intermediates involved in the catabolic pathways of the pigments.[3] However, under some circumstances, both *in vivo*[5,6,9] and *in vitro*,[14] the production of chlorophyll oxidation products (so-called allomers) containing oxygen in cyclopentanone ring V was reported. The primary reaction involved in the formation of the products was suggested to be mediated by oxygen radicals.[8,9-10] Previously, with electrochemically generated superoxide anion we showed that the allomerization of chlorophylls *a* and *b* in acetonitrile is initiated by proton abstraction from carbon C-10 in the cyclopentanone ring.[10,11] Aprotic organic solvents may resemble the hydrophobic interior of biological membranes and proton abstraction is the dominant reaction in aprotic media.[1,2] However, some lines of evidence suggest that most superoxide in chloroplast thylakoids is generated in reactions coupled to reduction activity of the primary acceptor of photosystem I and of the oxygenase function of ribulose bisphosphate carboxylase.[6,12,13] Therefore, it is likely that any oxygen radical attack that is responsible for chlorophyll degradation occurs in the water phase. In this context it is important to know if superoxide-dependent chlorophyll allomerization occurs in proton-containing media. To elucidate this question, in the present paper we have studied the interaction of chlorophyll *a* with superoxide anion in DMSO-water mixture.

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‡To whom correspondence should be addressed.

MATERIALS AND METHODS

Chlorophyll *a* was purified from pea (*Pisum sativum* L.) leaves using TLC as described previously.[7]

Superoxide generation was performed in alkaline DMSO according to Hyland and Auclair.[4] The reaction was initiated by adding 0.55 M H_2O and NaOH (final concentration 4 mM) to deuterated DMSO-d_6. The production of superoxide radicals was followed by EPR measurements at liquid nitrogen temperature[4] or routinely photometrically by reduction of 50 μM nitro blue tetrazolium recorded at 560 nm. The reaction between $O_2^{\cdot-}$ and chlorophyll *a* was started by mixing equal volumes of their solutions in DMSO in the absence or in the presence of superoxide dismutase (final concentration 100 units/ml). Inactivation of the superoxide dismutase activity was carried out by heating at 90°C until complete loss of activity was achieved.

Absorption and EPR spectra were recorded on a Beckman-35 spectrophotometer and on a RE-1307 (USSR) EPR spectrometer, respectively. The superoxide concentration in the solutions was calculated from comparison with TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl) EPR spectra.

Deuterated DMSO-d_6 (purity $> 99.5\%$) and bovine Cu, Zn-superoxide dismutase (3300 units/mg) were obtained from Merck (Darmstadt, Germany) and Sigma Chem. Co (St Louis, USA), respectively. Water was purified by the Millipore Q system.

RESULTS

Hydroxyl ions abstract protons from the activated methyl group of DMSO to form a methyl sulphinyl carbanion which can univalently reduce molecular oxygen. The superoxide radical produced as a result of the reactions has a long lifetime in an alkaline solution.[1,2,4] As expected, addition of water and NaOH to anhydrous deuterated DMSO brought about the appearance of an EPR signal with $g_{\parallel} = 2.0892$ and $g_{\perp} = 2.0072$ (not shown) identical to that of superoxide.[4] The maximal $O_2^{\cdot-}$ concentration (about 400 μM) was observed 30 min after initiation of the reaction.

Immediately after the addition of superoxide (final concentration about 200 μM) to a chlorophyll *a* solution in DMSO considerable changes of the pigment absorption spectrum, both in the Soret band and in the red region, were observed (Figure 1A). Simultaneously, an intermediate with absorption maxima at 476 and 517 nm was produced. The product is spectrally identical to Molish's intermediate (enolate-ion of chlorophyll β-keto ester) formed as a result of proton abstraction from carbon C-10.[10,11] A few minutes later the intermediate disappeared and the end product(s) (isobestic points at 438 and 555 nm) of the interaction was produced.

The absorption spectra of the final product(s) formed (Figure 1A, curves 8 and 16) with shorter wavelength maxima and low ratio of extinction at the Soret to red bands (compare with initial chlorophyll absorption spectrum) correspond to Mg-chlorine. The conversion of Molish's intermediates into chlorines as a result of its oxidation with molecular oxygen leading to cleavage of cyclopentanone ring is a well known reaction in alkaline alcohol solutions (see[10,11]). Thus, it appears that the oxidative reaction takes place in DMSO system.

The pattern of the spectral changes (Figure 1A) closely resembles those found during interaction of chlorophylls with superoxide in acetonitrile[10,11] and indicates

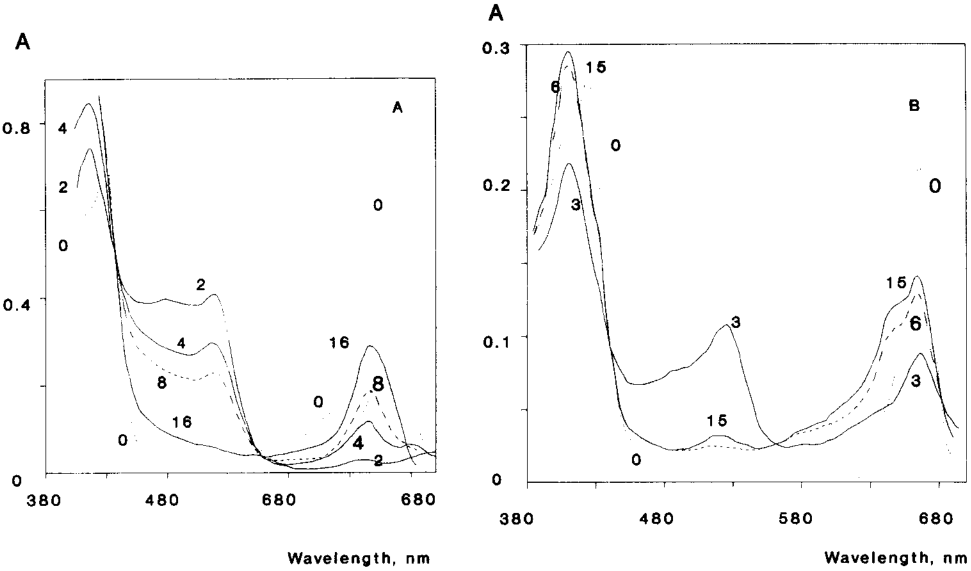

FIGURE 1 Absorption spectra of chlorophyll *a* during its interaction with superoxide anion in DMSO (A) and in DMSO-water (9 : 1) mixture (B). Spectra were recorded at time (in minutes) indicated. Cell pathlength 1.0 cm. O, initial chlorophyll absorption spectra.

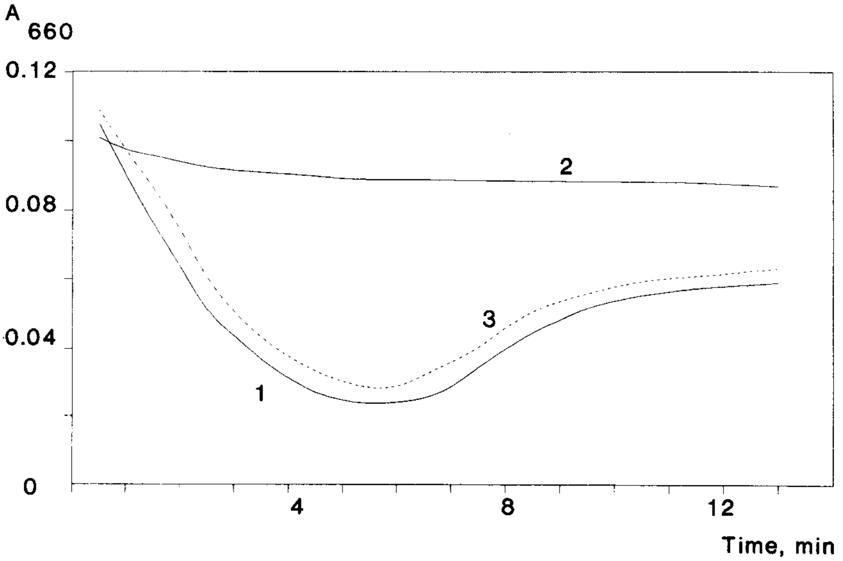

FIGURE 2 Time course of absorption changes at 660 nm during chlorophyll *a* interaction with superoxide anion in DMSO-water (9 : 1) mixture and in the presence of native (2) or inactivated (3) superoxide dismutase.

that in DMSO solution O_2^- is also able to deprotonate carbon C-10 of chlorophyll cyclopentanone ring V inducing the allomerization of the pigment.

Similar events were also observed when the water concentration in the reaction medium was increased to 10% (Figure 1B and 2). The absorption maxima of Molish's intermediate were located at 480 and 522 nm (Figure 1B). The shifts of the maxima are attributed to higher polarity of the system as compared to DMSO with low water concentration. Judging from remnant absorption of the native chlorophyll *a* in the red and Soret bands, the allomerization was not complete in this case. This effect is probably related to an increased solvation of OH^- ions as H_2O concentration increases, leading to lower levels of O_2^- production.[2,4] The conversion of the intermediate into chlorines occurred more rapidly as compared to the DMSO system with low water concentration (cf. Figure 1A and B).

The time-course change of absorption at 660 nm (Figure 2, line 1) reflects the production of Molish's intermediate and its subsequent conversion into chlorines (decrease and increase in optical density, respectively). As can be seen, superoxide-dependent chlorophyll allomerization in water/DMSO was almost completely inhibited by addition of superoxide dismutase (final concentration 100 units/ml). However, the effects were not observed with heat-inactivated enzyme (Figure 2).

DISCUSSION

The data presented indicate that superoxide is able to induce allomerization of chlorophyll *a* in DMSO-water systems. The patterns of the associated spectral changes and the final absorption spectra suggest that the end allomerized products of transformation in both systems are 10-hydroxy- and lactone derivatives of chlorophyll, produced as a result of Molish's intermediate oxidation with molecular oxygen (see also[10,11] and references therein).

Superoxide dismutase is able to catalyze superoxide dismutation in DMSO containing low water concentrations.[4] Inhibition of chlorophyll *a* enolate-ion formation in water/DMSO by superoxide dismutase clearly indicates the involvement of the superoxide anion in the allomerization. It is very likely that production of the intermediate in the system is related to proton abstraction from carbon C-10 of cyclopentanone ring V, as was previously found in acetonitrile.[10,11] On the other hand, it is probable that chlorophyll transformation observed is also brought about by the hydroperoxide radical ($HO_2^{\cdot}$) that was formed concomitantly in DMSO systems.[1,2,4] This stronger (than superoxide) oxidant is probably capable of abstracting a hydrogen atom at C-10, as was suggested.[10,11]

Thus, the data obtained suggest superoxide-dependent chlorophyll allomerization in water-containing media and protective effects of superoxide dismutase on the oxygen radical-mediated oxidative degradation of the pigments in green plants.

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