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Effect of proline on the production of singlet oxygen

Short Communication

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Summary. Molecular oxygen in electronic singlet state is a very powerful oxidant. Its damaging action in a variety of biological processes has been well recognized. Here we report the singlet oxygen quenching action of proline. Singlet oxygen ($^1O_2$) was produced photochemically by irradiating a solution of sensitiser and detected by following the formation of stable nitroxide radical yielded in the reaction of $^1O_2$ with the sterically hindered amine (2,2,6,6-tetramethylpiperidine, TEMP). Illumination of a sensitiser, toluidine blue led to a time dependent increase in singlet oxygen production as detected by the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) by EPR spectrometry. Interestingly, the production of TEMPO was completely abolished by the presence of proline at concentration as low as 20 mM. These results show that proline is a very effective singlet oxygen quencher. Other singlet oxygen generating photosensitizer like hematopophyrin and fluorescein also produced identical results with proline. Since proline is one of the important solutes which accumulate in many organisms when they are exposed to environmental stresses, it is likely that proline accumulation is related to the protection of these organisms against singlet oxygen production during stress conditions. A possible mechanism of singlet oxygen quenching by proline is discussed.

Keywords: Amino acids – Proline – Singlet oxygen – EPR

Abbreviations: $^1O_2$: singlet oxygen, TEMP: 2,2,6,6-tetramethylpiperidine, TEMPO: of 2,2,6,6-tetramethylpiperidine-1-oxyl.

Introduction

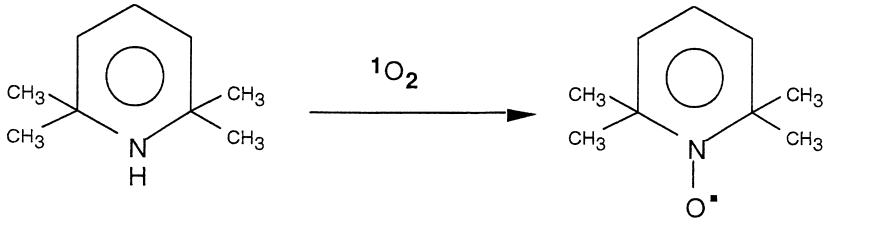
Singlet oxygen ($^1O_2$) is one of the active oxygen species, which plays a central role in oxidative damage of biological system (Kalai et al., 1998). The forma-

tion of $^1O_2$ in different organisms under various stressful conditions such as drought (Price et al., 1989; Khanna-Chopra et al., 1998), salt (Alia et al., 1993), low temperature and freezing (McKersie et al., 1993; Park et al., 1998), nutrient limitations (Cakmak and Marschner, 1992), heavy metal toxicity (Alia et al., 1995), air pollutants (Tanaka et al., 1998), herbicides (Holland et al., 1994), UV-exposure (Saradhi et al., 1995) etc., has been well reported. Another frequently reported response in many organisms under various stresses is the accumulation of proline. In spite of being the subject of intensive research, the actual reason behind proline accumulation remains controversial (Alia and Saradhi, 1993). It is generally believed that proline plays an important role in osmoregulation, acting as carbon and nitrogen source, protection of enzyme denaturation, regulation of cytosolic acidity, maintaining NAD/NADH ratio etc. (see Alia and Saradhi, 1993). Previously, it was also shown that proline can reduce the high light intensity promoted free radical generation from isolated thylakoids (Alia et al., 1991) and further, it was proposed that one of the adaptive role of proline, in plants exposed to stress, is to reduce free radical generation (Alia et al., 1993; 1997). In the present studies we utilized spin trapping EPR spectroscopy for analysing the singlet oxygen quenching action of proline. Our results show that proline is very effective in reducing the production of $^1O_2$.

Results and discussion

Singlet oxygen, which is a strong electrophile, can oxidise 2,2,6,6-tetramethylpiperidine (TEMP) (a sterically hindered amine) to a stable N-oxyl radical, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Aurich, 1982), which is paramagnetic and can be detected by EPR spectroscopy (Lion et al., 1976) (Scheme 1). This sensitive method of detecting $^1O_2$ enables us to determine the $^1O_2$ quenching action of proline.

In the present study $^1O_2$ was produced photochemically by irradiating the solution of toluidine blue (a sensitiser) and detected by following the



TEMP
(diamagnetic amine)

TEMPO
(paramagnetic N-oxyl radical)

Scheme 1

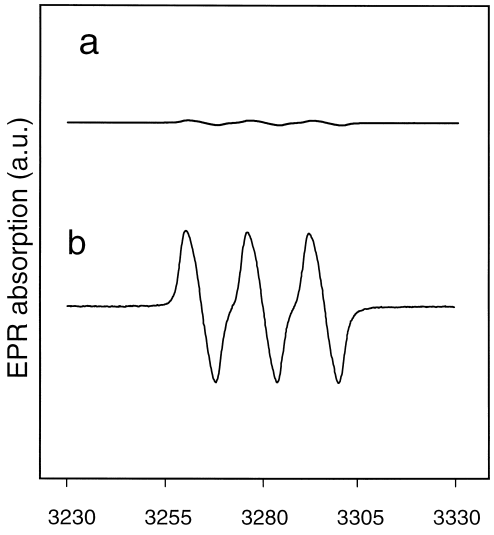


Fig. 1. EPR spectra of nitroxide radical (TEMPO) formed due to reaction of sterically hindered amine (TEMP) with singlet oxygen ($^1O_2$) generated by photoirradiation of toluidine blue. The sample contained 1 mM toluidine blue and 10 mM TEMP was irradiated to white light (1,200μE m^{-2}sec^{-1}) for 20 min and EPR spectra was recorded with X-band EPR spectrometer at modulation amplitude 1 Gauss; modulation frequency 100 KHz; microwave power 15 mW; temperature 25°C. (**a**) in the dark; (**b**) after illumination

formation TEMPO, in the reaction of $^1O_2$ with TEMP. A minor EPR signal characterises nitroxide free radical (TEMPO) was already observed before reaction due to impurity in the commercial product (Aldrich) but its concentration does not exceed 0.3%. EPR spectra of nitroxide radical (TEMPO) formed due to reaction of TEMP with $^1O_2$ generated by toluidine blue is shown in Fig. 1. No $^1O_2$ formation was detected in dark (Fig. 1a). However, after illuminating toluidine blue to white light for 20 min, a signal of the paramagnetic N-oxyl radical (TEMPO) was observed, which indicates generation of $^1O_2$ upon illumination of toluidine blue (Fig. 1b). In the presence of azide (100 mM), a well-known $^1O_2$ quencher, the production of TEMPO was inhibited (data not shown), suggesting that $^1O_2$ is implicated in increasing TEMPO signal. Figure 2 shows the production of TEMPO against the duration of light exposure. An increase in the TEMPO signal was observed with time. Interestingly, when 20 mM proline was added to the reaction mixture, before illumination, the time dependent production of TEMPO was completely inhibited, which reflects the complete inhibition of $^1O_2$ production (Fig. 2). Illumination of other sensitisers (fluorescein or haematoporphyrin) also led to an increase in $^1O_2$ production with time and, the production of $^1O_2$ was completely abolished by the presence of proline at a concentration as low as 20 mM (data not shown). Therefore, these results show clearly that proline is a very effective in reducing the level of $^1O_2$. It is difficult to give the exact mechanism of quenching of $^1O_2$ by proline. It is

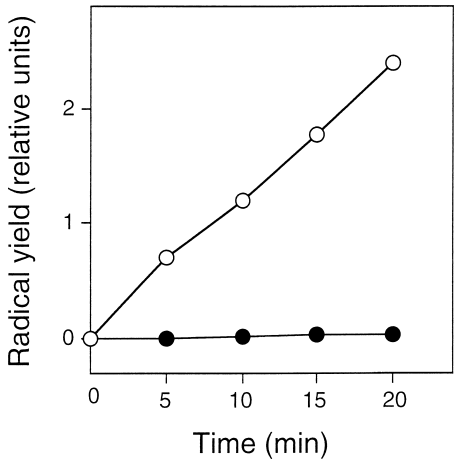


Fig. 2. Effect of proline on singlet oxygen production. Singlet oxygen ($^1O_2$) were generated through photoirradiation of toluidine blue and were detected by the formation of TEMPO due to reaction of TEMP with $^1O_2$. The sample contained 1 mM toluidine blue and 10 mM TEMP was irradiated to white light (1,200μE m^{-2}sec^{-1}) for 0, 5, 10, 15 or 20 min in the absence (○) or the presence of 20 mM proline (●) and EPR spectra was recorded as described in legend of Fig. 1. Results are average of 3 independent experiments

known that many amine compounds (A) react with $^1O_2$ by forming reversibly a charge-transfer complex (Lissi et al., 1993) as shown in eqn [1]. Since $^1O_2$ is a strong electron acceptor, the complex formation is controlled by the ionization potential of the amine. This means that amines, which easily provide negative charge, are also good $^1O_2$ quenchers. As a cyclic secondary amine, proline has a low ionization potential and can therefore act as a quencher. There are two possibilities how the initial charge-transfer complex can quench: Either chemically [eqn 2] by forming products (as superoxide or peroxide) or physically (inter-system crossing via spin-orbit coupling) [eqn III]. Proline can be reacted via both channels.

$$^1O_2 + A \quad \Leftrightarrow \quad {}^1[O_2{}^{\delta-} \leftarrow A^{\delta+}] \qquad [1]$$

$$^1[O_2{}^{\delta-} \leftarrow A^{\delta+}] \quad \rightarrow \quad \text{products} \qquad [2]$$

$$^1[O_2{}^{\delta-} \leftarrow A^{\delta+}] \quad \rightarrow \quad {}^3[O_2{}^{\delta-} \leftarrow A^{\delta+}] \quad \rightarrow \quad {}^3O_2 + A \qquad [3]$$

Schuessler and Schilling (1984) have earlier proposed that proline residues in polypeptide chain are the site of oxygen radical-mediated cleavage of polypeptide chain. Here our results suggest that free proline can efficiently react with $^1O_2$. Since the level of free proline in many living organisms increases upto 50 fold under various stressful conditions, it is likely that proline accumulation is related to protect these organisms against $^1O_2$ induced damages. We can not rule out the reaction of proline with other active oxygen species specially hydroxyl radical ($OH^{\bullet}$). Experiments are presently in progress in this direction.

Materials and methods

Proline was purchased from Sigma Chemical company. Toluidine blue, 2,2,6,6-tetramethylpiperidine (TEMP) and 2,2,6,6-tetramethylpiperidine-l-oxyl (TEMPO) were purchased from Aldrich Chemical Company. Solutions of toluidine blue, TEMP and TEMPO were prepared in ethanol.

For generating singlet oxygen, the photochemical experiment were performed in ethanol (Lion et al., 1976). A reaction mixture containing 1 mM toluidine blue and 10 mM TEMP was irradiated to white light (1,200 μE/m^2/sec) for various time intervals. Proline at various concentrations was added to the reaction mixture before illumination. After illumination an aliquot (50 μl) were taken in capillaries and measured immediately by EPR. EPR spectra was recorded at room temperature with X-band EPR spectrometer (Varian E-9) operating at 9.2 GHz, with 100 kHz field modulation and 0.5 mW microwave power.

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