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Quantification of superoxide radical production in thylakoid membrane using cyclic hydroxylamines

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ABSTRACT

Applicability of two lipophilic cyclic hydroxylamines (CHAs), CM-H and TMT-H, and two hydrophilic CHAs, CAT1-H and DCP-H, for detection of superoxide anion radical ($O_2^{\bullet -}$) produced by the thylakoid photosynthetic electron transfer chain (PETC) of higher plants under illumination has been studied. ESR spectrometry was applied for detection of the nitroxide radical originating due to CHAs oxidation by $O_2^{\bullet -}$. CHAs and corresponding nitroxide radicals were shown to be involved in side reactions with PETC which could cause miscalculation of $O_2^{\bullet -}$ production rate. Lipophilic CM-H was oxidized by PETC components, reducing the oxidized donor of Photosystem I, $P_{700}{}^{+}$, while at the same concentration another lipophilic CHA, TMT-H, did not reduce $P_{700}{}^{+}$. The nitroxide radical was able to accept electrons from components of the photosynthetic chain. Electrostatic interaction of stable cation CAT1-H with the membrane surface was suggested. Water-soluble superoxide dismutase (SOD) was added in order to suppress the reaction of CHA with $O_2^{\bullet -}$ outside the membrane. SOD almost completely inhibited light-induced accumulation of DCP•, nitroxide radical derivative of hydrophilic DCP-H, in contrast to TMT• accumulation. Based on the results showing that change in the thylakoid lumen pH and volume had minor effect on TMT• accumulation, the reaction of TMT-H with $O_2^{\bullet -}$ in the lumen was excluded. Addition of TMT-H to thylakoid suspension in the presence of SOD resulted in the increase in light-induced O_2 uptake rate, that argued in favor of TMT-H ability to detect $O_2^{\bullet -}$ produced within the membrane core. Thus, hydrophilic DCP-H and lipophilic TMT-H were shown to be usable for detection of $O_2^{\bullet -}$ produced outside and within thylakoid membranes.

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1. Introduction

Reactive Oxygen Species (ROS), namely singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•), can damage biomolecules and inhibit enzymes [1–3]. At the same time, H_2O_2 has been recognized as universal signaling agent in living organisms [4–7]. The important role of ROS in cellular metabolism demands investigation of ROS production mechanisms *in vivo*. Superoxide radical is the primary product in reactions coupled with oxygen reduction. In higher plants, $O_2^{\bullet -}$ is produced in plasma membranes [8], peroxisomes [9], mitochondria [10], and chloroplasts [11]. In the green cells of higher plants, chloroplasts are the main source of ROS in the light [12], and $O_2^{\bullet -}$ is produced there as the primary product of one-electron reduction of O_2 molecule by components of the photosynthetic electron transfer chain (PETC) in the course of so-called Mehler reaction [13–16].

The midpoint redox potential (E_m) of $O_2/O_2^{\bullet -}$ is equal to −160 mV in H_2O and close to −600 mV in dimethylformamide [17,18]. Many components of PETC have been proposed as O_2 reducing agents, including some components of Photosystem II (PSII) [19], plastosemiquinone of the plastoquinone pool [20],

Abbreviations: AscH, ascorbate; CAT1-H and CAT1•, 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium and its nitroxide radical derivative; CHA, cyclic hydroxylamine; Chl, chlorophyll; CM-H and CM•, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine and its nitroxide radical derivative; cyt. b_6/f, cytochrome b_6/f complex; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCP-H and DCP•, 1-hydroxy-2,2,5,5-tetramethylpyrrolidine-3,4-dicarboxylic acid and its nitroxide radical derivative; ESR, electron spin resonance; ET, electron transfer; PAM, pulse amplitude modulation; PETC, photosynthetic electron transfer chain; PSI and PSII, Photosystem I and Photosystem II, respectively; ROS, reactive oxygen species; SOD, superoxide dismutase; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TMT-H and TMT•, N-(1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-2-methylpropanamide and its nitroxide radical derivative

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cytochrome b_6/f -complex (cyt. b_6/f) [21], electron transfer (ET) cofactors of Photosystem I (PSI) [22], and stromal protein ferredoxin [23]. PSI is believed to be the main site of O_2 reduction in PETC, with its terminal cofactors F_A/F_B [24] and phylloquinone [25] suggested to be involved in O_2 reduction, leading to $O_2^{\bullet -}$ appearance outside and within thylakoid membrane, respectively [26,27]. $O_2^{\bullet -}$ production within the membrane, where most of the PETC components are localized, was suggested based on a number of indirect data provided in [22–31], with the first direct evidence being reported in [26].

Although plenty of reagents for $O_2^{\bullet -}$ has been suggested, the reliable quantification of $O_2^{\bullet -}$ *in vivo* and in biological samples remains a challenge (for rev. see [32,33]). First of all, many reagents for $O_2^{\bullet -}$ are non-specific and react with other ROS, especially with $HO^{\bullet}$ and $^1O_2$, giving the same or hardly distinguishable products. Secondly, many reagents are not reactive with $O_2^{\bullet -}$ enough to efficiently compete with natural antioxidants and antioxidant enzymes, such as superoxide dismutase (SOD), and even with spontaneous dismutation of $O_2^{\bullet -}$. Thirdly, reagents for $O_2^{\bullet -}$ themselves can affect metabolic processes altering $O_2^{\bullet -}$ production or functioning of biological structures. The latter is especially crucial for assays with photosynthetic structures since a number of strong oxidants and reductants originate there in the light. The variety of $O_2^{\bullet -}$ detection methods includes photometric (cytochrome *c* [34], epinephrine [35], nitroblue tetrazolium [36], *etc.*), fluorescent (hydroethidine derivatives [37,38]) and ESR-based assays. The latter is assumed to be one of the most prominent approaches to detection of $O_2^{\bullet -}$ (see rev. [39–44]). The modern spin trapping (*e.g.* 5,5-dimethyl-pyrroline-*N*-oxide (DMPO) and its derivatives such as DEPMPO, EMPO, BMPO, *etc.*) ensures unambiguous identification of trapped oxygen-centered radicals since they produce radical-specific adducts. However, the rates of $O_2^{\bullet -}$ trapping are rather low, *e.g.* estimation of k_{DEPMPO} is within 0.5 and 90 M^{-1} s^{-1} at pH around 7.4 [41,44]. For partial compensation of that, large concentrations of spin traps (25–100 mM) are applied, although even such high concentrations of the trap could not provide efficient competition with spontaneous dismutation of $O_2^{\bullet -}$ at physiological pH values or, moreover, with SOD-driven dismutation. Furthermore, such increase in concentration could have undesirable side effects [42,45].

An alternative ESR assay of $O_2^{\bullet -}$ is based on redox transformation of cyclic hydroxylamines (CHAs) with transition to ESR-detectable form (for rev. see [33,46]; see Fig. 1). In contrast to spin traps, CHAs are characterized by high second-order rate constants of the reaction with $O_2^{\bullet -}$. This provides efficient competition with spontaneous dismutation of $O_2^{\bullet -}$ at weakly alkaline pH values enabling both quantification of $O_2^{\bullet -}$ production and avoidance of high concentrations of reagents. However, the nitroxide radical is not a product specific for $O_2^{\bullet -}$, therefore this approach requires SOD application for confirmation of $O_2^{\bullet -}$ being the CHA oxidizing agent. A plenty of CHAs is available commercially, varying in lipophilicity and charge. Previously we have successfully applied two CHAs, hydrophilic 1-hydroxy-2,2,5,5-tetramethylpyrrolidine-3,4-dicarboxylic acid (DCP-H) and lipophilic N-(1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-2-methylpropanamide (TMT-H) for detection of $O_2^{\bullet -}$ produced by photosynthetic membranes containing PETC (thylakoid membranes) [26].

The aim of the present study is to provide deeper insight into CHA applicability for $O_2^{\bullet -}$ detection in photosynthetic membranes and limitations of CHAs use for $O_2^{\bullet -}$ quantification. In this work, four CHAs (lipophilic TMT-H and 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CM-H), and hydrophilic DCP-H and 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium (CAT1-H); see Table 1) were tested. A further confirmation is obtained that $O_2^{\bullet -}$ is produced not only outside thylakoid membranes but also within the membrane core.

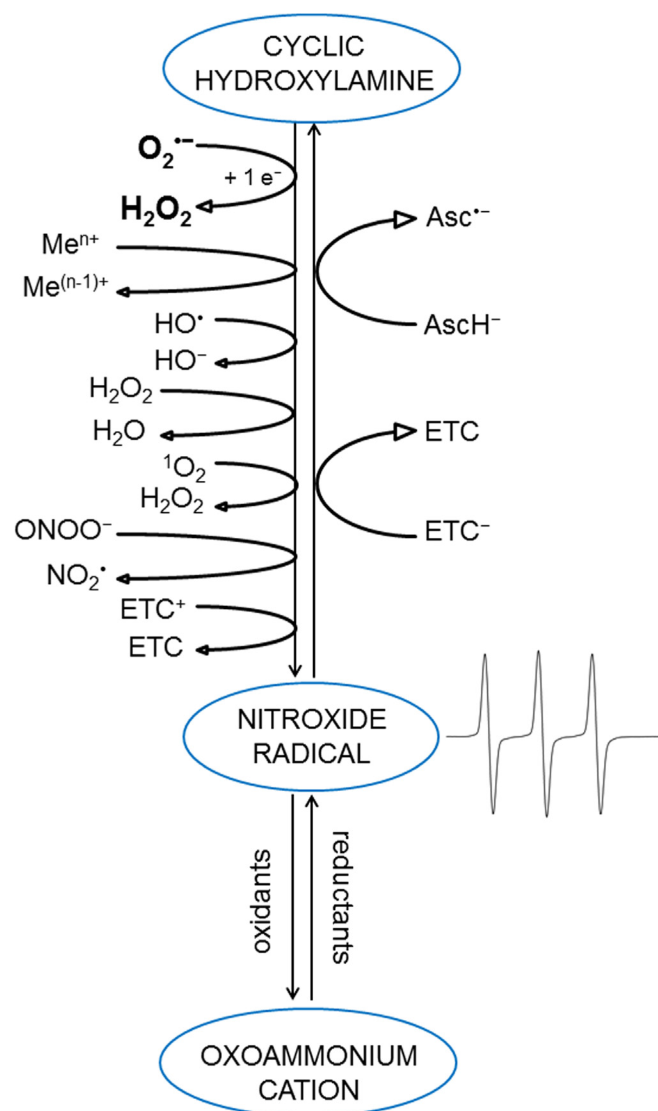

Fig. 1. Approximate scheme of cyclic hydroxylamine (CHA) redox transformations illustrating major possible reactions of CHA oxidation and nitroxide radical reduction in chloroplasts. ETC – electron transfer chain; Me^{n+} – transition metal ions. Reactions are presented in a qualitative manner.

2.. Materials and methods

2.1. Thylakoid preparation

Pea (*Pisum sativum* L.) plants were grown in a greenhouse. Thylakoids were isolated from the leaves as described earlier [50]. The final pellet was suspended in 1–2 ml of medium containing 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, and 50 mM HEPES–KOH (pH 7.6), put on ice, and used on the day of isolation. Chlorophyll (Chl) concentration was determined spectrophotometrically in 95% ethanol extracts [51]. The thylakoid preparations were free of superoxide dismutase, which was confirmed by two independent assays, namely inhibitory analysis performed as in [52] and immunodetection with antibodies specific for Cu/Zn-SOD [53] (not shown).

2.2. Measurement conditions

The reaction medium contained thylakoids as indicated in figure and table legends, 50 mM HEPES-KOH (pH 7.6), 20 mM NaCl, 5 mM $MgCl_2$, 0.1 M sucrose (if not stated otherwise), and 1 μM

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