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Research article

Inhibition of spinach chloroplast F_0F_1 by an Fe^{2+}/ascorbate/H_2O_2 system

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Abstract

Plant chloroplasts are particularly threatened by free radical attack. We incubated purified soluble spinach chloroplast F_0F_1 (CF_0F_1, EC 3.6.3.34) with an Fe^{2+}/H_2O_2/ascorbate system, and about 60% inactivation of the ATPase activity was reached after 60 min. Inactivation was not prevented by omission of H_2O_2, by addition of catalase or superoxide dismutase, nor by the scavengers mannitol, DMSO, or BHT. No evidence for enzyme fragmentation or oligomerization was detected by SDS–PAGE. The chloroplast ATP synthase is resistant to attack by the reactive oxygen species commonly found at the chloroplast level. DTT in the medium completely prevented the inhibition, and its addition after the inhibition partially recovered the activity of the enzyme. CF_0F_1 thiol residues were lost upon oxidation. The rate of thiol modification was faster than the rate of enzyme inactivation, suggesting that the thiol residues accounting for the inhibition may be hindered. Enzyme previously oxidized by iodobenzoate was not further inhibited by the oxidative system. The production of ascorbyl radical was identified by EPR and is possibly related to CF_0F_1 inactivation. It is thus suggested that the ascorbyl radical, which accumulates under plant stress, might regulate CF_0F_1.

Keywords: ATP synthase; Chloroplasts; Oxidative stress; Free radicals; ATPase; Ascorbate

1. Introduction

The ability of plants to transform light in metabolic energy through photosynthesis and the release of molecular oxygen is paralleled by the disadvantage of the production of huge amounts of reactive oxygen species capable of damaging proteins and biological membranes [5]. Plant chloroplasts are particularly biased for oxidative damage because the light harvesting systems and electron transfer chains within the thylakoid membranes coexist with high oxygen concentrations [5,37]. Evidence about in vitro and in situ oxidative damage of several chloroplast proteins by peroxide, superoxide and hydroxyl radicals is available [3,49]. The mechanisms that protect plant cells against injury by ROS include different enzymatic (Cu/Zn-, Fe- or MgSOD, APX, CAT) and non-enzymatic (ascorbate, glutathione, tocopherols, carotenoids) systems, all involved in ROS dismutation and/or neutralization [3,4,24]. Regardless of all defenses available, damage to proteins and membranes as a consequence of oxygenic photosynthesis is inevitable. On the other hand, under optimal growth conditions, ROS are produced within plants at a very controlled level, and play an important role in mediating responses to pathogens [3] and signaling processes besides cell death [32].

Abbreviations: ROS, reactive oxygen species; IBZ, iodobenzoate; SOD, superoxide dismutase; APX, ascorbate peroxidase; CAT, catalase; CF_0F_1, chloroplast ATP-synthase; NEM, *N*-ethylmaleimide; PSI and PSII, photosystems I and II.

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Photosystem II proteins are amongst the most affected by oxidative damage in chloroplasts [31] and their repair mechanisms involve a sophisticated, well-regulated, selective turnover of damaged proteins, mostly of the D1 protein [31,36]. Although one of the culminating steps in photosynthesis is the formation of ATP by the chloroplast ATP-synthase (CF_0F_1) [30,35], no information is available about the susceptibility of this protein to oxidative damage by ROS or reactive radicals.

It is well known that during light-driven ATP synthesis, the chloroplast ATP-synthase undergoes regulation by conformational changes induced by the electrochemical proton gradient formed across the thylakoid membrane, and by the redox state of a disulfide bridge present at the gamma subunit [30]. The enzyme is maintained in its optimal, reduced state, by the concerted action of thioredoxins and glutathione [19,30]. CF_0F_1 is formed by nine different types of subunits, with a stoichiometry of α_3, β_3, γ, δ, ε (CF_1 part) and I_1, II_1, III_{10-15}, IV_1 (CF_0 part). β subunits are responsible for the reversible synthesis of ATP, the intercalated α subunits are both regulatory and structural, γ works as a clockwork gear, determining which β subunit will alternately synthesize, release or rebind nucleotides, and the ε subunit has many regulatory roles [12,38,41]. The membrane-inserted subunits I and II appear to be mainly structural, while the set of subunits III form a ring that together with subunit IV constitute the proton channel [22,38,40,42,43]. Therefore, oxidative damage to any of the subunits might impair ATP-synthesis, thereby contributing to cell death and senescence of the leaves. In this work we looked for the vulnerability of CF_0F_1 to oxidative damage, and the effects on the structure and ATPase activity of the purified CF_0F_1. We found that the enzyme is unusually resistant to attack by peroxide, superoxide or hydroxyl radicals, but is sensitive to reversible oxidation of Cys-groups by an Fe^{2+}-ascorbate system. Ascorbyl radical, whose formation was formerly described in situ for spinach leaves [4,29], appeared to be directly involved with inactivation of this ATPase in vitro.

2. Materials and methods

2.1. Purification of CF_0F_1

CF_0F_1 ATPase (E.C. 3.6.3.34) was isolated from New Zealand spinach (*Tetragonia expansa*) chloroplasts according to Pick and Racker [39], as modified by Fromme et al. [14]. The enzyme was stored at a concentration of 3–5 mg ml^{-1} in buffer containing 30 mM Tris-succinate (pH 6.5), 2 mg ml^{-1} Triton X-100, 0.5 mM EDTA, 1 mg ml^{-1} asolectin and 900 mM sucrose. In most experiments, the latent, non-activated enzyme was pre-incubated in the oxidative medium and thereafter the activity of small samples was measured in the presence of 100 mM Na_2SO_3 as CF_0F_1 activator (see below).

2.2. Pre-incubation in oxidative conditions

In general, 0.6–1.0 mg ml^{-1} of CF_0F_1 were incubated for the period indicated in the legends or otherwise for 60 min under the oxidative conditions at 25 °C, in medium containing 50 mM Tris–Cl (pH 8.0), 50 μM Fe_2SO_4, 1.5 mM H_2O_2, 6 mM ascorbate, in absence of added Mg^{2+} or nucleotides. At the indicated times, samples were withdrawn and mixed with a 50-fold excess of the medium used for activity measurements.

2.3. ATPase activity of CF_0F_1

Samples (10–20 μl) samples were diluted to reach final concentrations of 0.02–0.05 mg ml^{-1} CF_0F_1 in a reaction medium containing 5 mM [γ-^{32}P]ATP (3×10^8 Bq mol^{-1}), 5 mM $MgCl_2$, 50 mM Tris–Cl (pH 8.0), 1 mM EDTA and 100 mM Na_2SO_3 as activator. The reactions were quenched after 3–5 min at 25 °C with one volume of 0.1 N HCl, followed by two volumes of activated charcoal suspended in 0.1 N HCl. After spinning for 5 min at 7000 rpm in an Eppendorf centrifuge, the radioactive [^{32}P]P_i in the supernatant was counted by liquid scintillation [17]. Preparations had a basal activity around 1.0 μmol mg^{-1} min^{-1} and were activated 10–15 fold by sulfite, but were insensitive to reduction with DTT. Oxidation with 2 mM iodosobenzoate for 10 min at pH 8.0 decreased the activity to 0.2–0.3 μmol mg^{-1} min^{-1} evidencing that the preparations were obtained in the reduced, non-activated state [10,11].

2.4. SDS–PAGE

SDS–PAGE was done according to Laemmli [23] in 12% polyacrylamide running gels, with 15 μg per lane, either with or without 0.5% mercaptoethanol in the sample buffer. The gels were subsequently revealed by silver-staining [2].

2.5. Assessment of –SH residues with DTNB

After different treatments, protein was precipitated with 1% TCA (final concentration) and centrifuged at 5000 rpm for 5 min. The pellet was washed twice with deionized water, and then dissolved in 1 ml of 1% SDS. Reaction was started by addition of 90 μM DTNB (250× molar excess over CF_0F_1) and carried for 20 min at room temperature. The modified thiol groups were assessed by measurement of $O.D._{412}$, assuming $\varepsilon = 13\ 600\ M^{-1}\ cm^{-1}$.

2.6. Identification of the ascorbyl radical

The sustained formation of the ascorbyl radical in the oxidizing medium was determined by EPR with a Brucker ER-200 Spectrometer, using 50 mM Tris–Cl (pH 8.0), 50 μM Fe_2SO_4, 1.5 mM H_2O_2, and 6 mM ascorbate.

2.7. Reagents

ATP (disodium salt), and asolectin were from Sigma. Other reagents were of analytical grade or better. [^{32}P]P_i (carrier-free) was from the Brazilian Institute of Nuclear and Energy