CHLOROPLAST MANGANESE AND SUPEROXIDE

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SUMMARY Particles prepared from spinach chloroplast membranes with Triton X-100 inhibited the superoxide-mediated reduction of nitro-blue tetrazolium by riboflavin. This superoxide dismutase-like activity was of two kinds, one inactivated by heating and inhibited by H_2O_2 and the other insensitive to both of these treatments; both activities were destroyed by washing with concentrated Tris buffer or with EDTA. Attempts at reconstitution with transition metal ions suggested that two different forms of bound manganese may be responsible and it is proposed that the inhibition by H_2O_2 is indicative of three different oxidation states of particle-bound manganese. The possibility that the photosynthetic water-splitting system and superoxide dismutase have evolved from a single precursor is discussed.

INTRODUCTION It is well established that membrane-bound manganese plays an important, if undefined, role in photosynthetic oxygen evolution by plants and algae[1,2]. Currently, most studies of chloroplast Mn involve either treatments of whole chloroplasts which remove the Mn such as Tris-washing[3] or heating[4], or conditions necessary for reconstitution[5]. By such methods, evidence was found for two distinct pools of Mn[6] - one pool, removable by Tris, which can accept electrons from water and a second, tightly bound, pool which transfers electrons to the primary donor of the photoact. In illuminated chloroplast systems, it has been shown that Mn^{2+} can stimulate the oxygen uptake mediated by methyl viologen in a manner similar to ascorbate, which functions by the reduction of superoxide (O_2^-). However, no suggestion was made as to the possible fate of the resultant Mn^{3+}, an unstable ion in aqueous systems[8]. In similar experiments, Walker *et al*[9] observed an oxygen

Abbreviations: SOD - superoxide dismutase E.C. 1.15.1.1 ; SDS - sodium dodecyl sulphate ; NBT - nitro-blue tetrazolium

uptake in a dark period following illumination which they attributed to the reaction of Mn^{3+} with H_2O_2 thus: $2Mn^{3+} + H_2O_2 \longrightarrow 2Mn^{2+} + O_2 + 2H^+$.

Free Mn^{2+} has long been known to be a potent inhibitor of lipid peroxidation in biological membranes[10] and recently it was shown that 1mM Mn^{2+} inhibited the superoxide-mediated reduction of cytochrome c by xanthine oxidase[11]. Similarly, micromolar concentrations of Cu^{2+}, from boiled erythrocuprein (Cu/Zn SOD) or as $CuCl_2$, were found to inhibit lipid peroxidation[12]. From pulse radiolysis studies it was discovered that free Cu^{2+} could catalyse the dismutation of O_2^- at pH 7.5[13]; these workers also demonstrated that a number of complexes of Cu^{2+} with simple oligopeptides had 2nd order rate constants for O_2^- dismutation within the same order of magnitude as erythrocuprein and they commented that similar effects might be expected for other transition metal ions.

As mentioned in our previous paper[14], the chloroplast stroma contains a soluble Cu/Zn SOD ; Asada et al[15] had shown that about one third of this cyanide-sensitive SOD remains associated with the chloroplast lamellae after repeated hypotonic washes. Although intact chloroplast lamellae exhibit no SOD activity in the presence of cyanide (B. Halliwell - personal communication) we have presented evidence for a cyanide insensitive SOD-like activity associated with subchloroplast particles. A similar activity appeared to be present in a soluble form after treatment of lamellae with anionic detergents[14], but we have now shown that this was an artefact. However, in the present paper, we reaffirm that the SOD-like activity associated with subchloroplast particles, prepared using the non-ionic detergent Triton X-100, is more than a simple artefact, though its precise physiological significance remains to be clarified.

MATERIALS AND METHODS The Triton particles were prepared by a modification of the method of ref. 14. To a suspension of washed chloroplast lamellae at 2.5 mg chlorophyll/ml. was added an equal volume of 10% ($^v/_v$) Triton X-100 (Sigma) in 50 mM potassium phosphate pH 7.8, 1.0 M sucrose. After stirring for about

5 min, the suspension was centrifuged briefly at low speed and the supernatant stirred into 2 volumes of cold polypropylene glycol 2025 (BDH). The turbid suspension was layered over 50% ($^{W}/w$) sucrose and centrifuged in a swing-out rotor for 1 hr. at 30,000 x g. The pale-green pellet beneath the sucrose layer was resuspended in 50 mM potassium phosphate pH 7.8,20% ($^{W}/w$) sucrose and stored in liquid nitrogen until use. SOD was assayed by a photochemical method[16]. Gel electrophoresis in SDS was by the method of ref. 17.

RESULTS The red colour produced in assays of the "soluble preparation" of ref. 14 is due to cholate while SDS, at > 50 μg/ml in the assay, reduces the ΔA560 to 30% of the control. Acetone extraction of the "soluble preparation" showed that it contained enough residual SDS to account for its activity. Simple salts of the four metal ions known to occur in SOD were tested for their effect on the assay. All except Zn^{2+}, which does not undergo redox reactions, inhibited NBT reduction, with 50% of control level at 0.12μM Cu^{2+}, 0.82μM Mn^{2+} and 40μM Fe^{3+} ; cf. ~1nM for erythrocuprein-Cu^{2+} [16]. As we have routinely used 4μM riboflavin in this assay and the reaction rate increases linearly from 2 to 5μM riboflavin, inhibition by complex formation with riboflavin is difficult to rationalise. 3.3μM Mn^{2+}, which totally suppressed NBT reduction, did not alter the amount of H_2O_2 produced in the assay with NBT omitted.

Triton particles If particles were subjected to SDS gel electrophoresis, multiple green bands were visible, just behind the free carotene and chlorophyll bands. Staining for protein with Coomassie blue confirmed the absence of the high molecular weight chlorophyll-protein band characteristic of photosystem I particles. The dithionite (reduced) minus ferricyanide (oxidized) spectrum, recorded at 77°K, exhibited a single band at 557 nm indicative of the presence of cytochrome b 559.

The inhibitory effect of the particles on the SOD assay was of two kinds and these could be differentiated by heating at 85°C or by addition of H_2O_2. A fairly constant proportion (about 60%) of the activity was inactivated by

heating for 3 min. at 85°C; the remainder was insensitive to prolonged heating, and if the particles were rapidly centrifuged down after heating, remained in the supernatant and was probably due to Mn^{2+} or Cu^{2+}, both of which are released from chloroplasts on detergent treatment. Neither activity was impaired by heating for 15 min. at 50°C. This is in contrast to oxygen evolution in whole chloroplasts, but an increased resistance of Mn in subchloroplast particles to removal by heating has been reported[18]. Unexpectedly, it was found that added H_2O_2 had no effect on the assay, even at concentration of 2M - it is difficult to reconcile this with the accepted mechanism of this reaction[16]. In the presence of 1mM KCN, to inhibit any catalase or peroxidase activity, H_2O_2 appeared to suppress the activity of the particles and increase NBT reduction (Fig. 1). That this was an inhibition rather than an inactivation was shown by incubating particles with 1mM KCN and 24 mM H_2O_2 for 2 hrs at 25°C. The particles were then centrifuged out, washed and assayed and the activity was found to be the same as a control sample incubated with KCN alone. This is the opposite situation to that found for erythrocuprein which is not inhibited by H_2O_2 but is inactivated by the

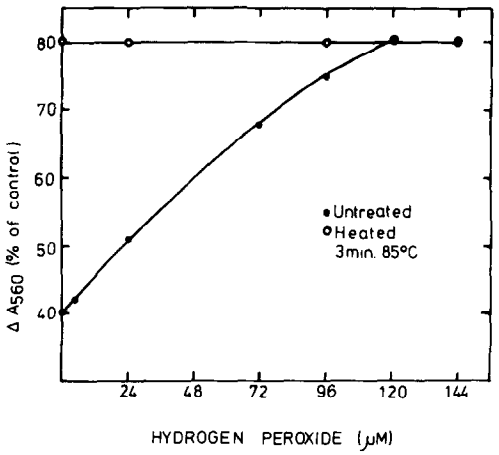


Figure 1. Effect of hydrogen peroxide on SOD activity of Triton subchloroplast particles (175μg protein). All assays in presence of 1mM KCN.

destruction of a histidine residue at the active site[19]. It was noted that inhibition by H_2O_2 did not reduce the activity of the particles below the level which was heat-insensitive.

The effects of a number of reagents on the activity of the particles were investigated by incubating the particles in each reagent for 1 hr. at 4°C then centrifuging down and washing with buffer before assaying. A number of potentially informative treatments (e.g. hydroxylamine) were rendered unusable by absorption of the reagent by the particles with resulting ambiguities in the assay. Although Mg^{2+} at 0.2M has been reported to remove Mn from chloroplast photosystem II[20], it had no effect on the particles; however both 1.0M Tris-Cl pH 8.4 and 2mM EDTA produced complete inactivation (Fig. 2). Inactivation by EDTA suggested that metal ions could be responsible for the activity of the particles and attempts were made to reactivate Tris-and EDTA-washed particles by incubation with 0.1mM $MnSO_4$ or $CuSO_4$ followed by careful washing and assaying. Both metal ions restored a SOD-like activity to the particles. Cu^{2+} - reconstituted particles were insensitive to H_2O_2 but Mn^{2+} - reconstituted particles, although only 20% as active as Cu^{2+}-reconstituted

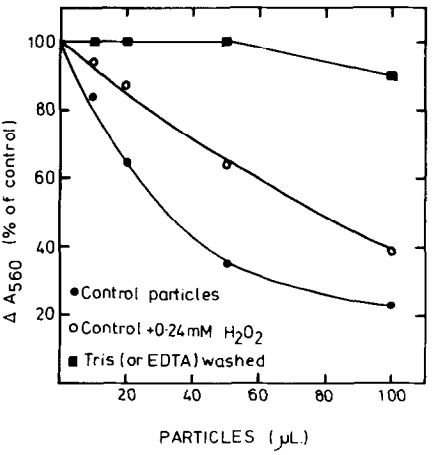


Figure 2. Effect of washing with Tris-Cl (or with EDTA) on SOD activity of Triton subchloroplast particles. All assays in presence of 1mM KCN.

particles, did exhibit sensitivity to H_2O_2 which is not shown by free Mn^{2+} (Fig. 3). Surprisingly, incubation with 1.0mM Zn^{2+} in addition to 0.1mM Mn^{2+} greatly increased the recovery of activity (Fig. 3). As Zn^{2+} alone had no effect it may act by competing with Mn^{2+} for unreactive binding sites, making Mn^{2+} more available to the active sites. It became apparent that the Mn^{2+}- reconstituted particles were not to be equated with untreated particles - on heating at 85°C the reconstituted particles remained as a fine suspension and did not clump together as the untreated ones did; more significantly, the SOD-like activity, including the peroxide-sensitive part, was completely unaffected by heating.

DISCUSSION The discovery that certain Mn-containing proteins are superoxide dismutases[21,22,23], suggested the possibility that chloroplast Mn might exhibit a similar activity[24], so that the assay system for this enzyme might be used as a probe of the state of chloroplast Mn. If it is assumed that bound Mn is responsible for the SOD-like activity of the Triton-subchloroplast particles, it appears that it is no longer in the "native" site, since it can be displaced by EDTA but not by Mg^{2+}; nor is it in the site described by Gross[25]

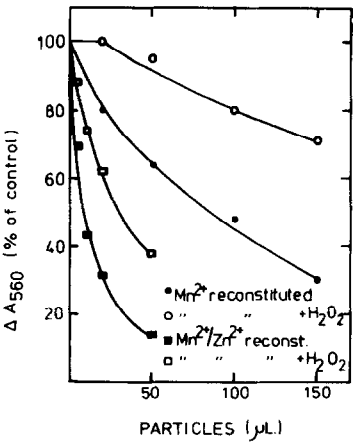
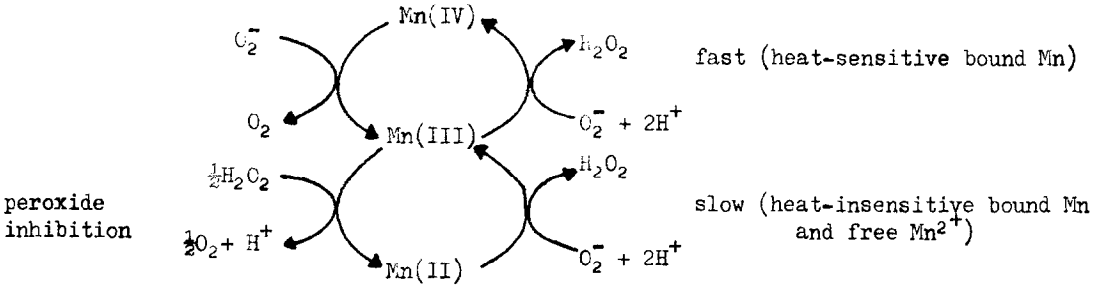


Figure 3. Reconstitution of SOD-like activity by incubation of particles with $MnSO_4$. All assays in presence of 1mM KCN± 0.24mM H_2O_2.

as being present in whole lamellae, since neither Mg^{2+} nor Zn^{2+} appear to bind competitively with Mn^{2+}.

The peroxide inhibition might be explained in terms of three different redox states of bound Mn with the Mn (IV)/Mn(III) couple catalysing the removal of O_2^- more effectively than the Mn(III)/Mn(II) couple, as proposed for the E.coli enzyme by Pick et al[26] e.g.



Such higher oxidation states of manganese have been implicated in the water-splitting reaction of chloroplasts[27].

Some of this work may be of relevance to the problem of how the ancestors of to-day's blue-green algae were able to develop a water-splitting capability with comcomitant O_2 evolution when they, in common with all other life forms at that time,had not previously been exposed to toxic O_2 concentrations[28]. One possible explanation is that a single primitive manganoprotein or peptide carried out both the functions of O_2^- dismutation and water-splitting, with a subsequent independent evolution of the two functions. Possibly in support of this hypothesis is the recent isolation of a Mn-containing "Hill-factor" peptide from the blue-green alga Phormidium luridum[29], which appears to act near the water-splitting site on the lamellae. This factor may be a retained primitive characteristic and it would be of interest to determine whether it has SOD activity comparable to that reported for simple Cu^{2+} complexes[13]. It has been pointed out[30] that, under the primitive reducing atmosphere, copper would have been largely insoluble while the sea-water concentration of Mn^{2+} would have been very high - thus simple Mn complexes, but not copper ones, would have been available.

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