DEACTIVATION OF SUPEROXIDE DISMUTASE ON EDTA-TREATED CHLOROPLASTS

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1. Introduction

The superoxide radical is involved in a variety of oxidative processes in biological systems [1]. It is obvious that nature had to provide protection against the potential toxicity of this radical. A variety of enzymes for its dismutation were detected in different biological systems (for bovine erythrocytes, yeast and *E. coli*, see [1] - for chloroplasts and algae, see [2,3]). In photosynthesis of green plants $O_2^{\cdot-}$ acts as an intermediate in the reduction sequence of photosystem I. This was inferred from ascorbate oxidation [4], from ascorbate mediated photophosphorylation [5] and from sulfite oxidation [6] by chloroplasts.

In the zig-zag arrangement of the electron transport chain in the chloroplast membrane the reducing site of system I is located at the outer side of the membrane [7–9]. There the formation of superoxide occurs. In the absence of Ferredoxin-NADP it is either formed directly by reduction of oxygen or mediated by artificial catalysts like viologens.

The reduction of oxygen to yield superoxide as the terminal product of the photosynthetic electron transport under these conditions can be monitored by pH-indicating dyes specific for the outer aqueous phase of thylakoids [7]. In contrast to terminal acceptors (e.g. dichlorphenolindophenol) which bind a proton on reduction, oxygen when reduced to superoxide should not bind a proton at a pH above 7 ($pK = 4.8$) [10]. According to Junge and Ausländer [7] flash excitation of chloroplasts should thus result in a net production of one proton per electron if superoxide is the terminal product.

In usual chloroplasts of the broken type this is not observed even if oxygen is the terminal acceptor via viologens [7]. It has been assumed that in this case oxygen is reduced either to H_2O_2 or to H_2O, both of which imply binding of 1 H^+/e. However, in chloroplasts treated with EDTA for removal of the coupling factor for photophosphorylation, CF_1 [11–13], indeed a net production of about 1 H^+/e is observed in the presence of viologens.

In this communication it is shown that the whole electron transport chain is active under these conditions as well as the sites of proton release into the internal phase. The net production of about 1 H^+/e is therefore attributable to the lack of proton binding at the reducing site of the light reaction at the outer side of the membrane. This net production is eliminated if superoxide dismutase is added. This suggests that EDTA-treated chloroplasts yield $O_2^{\cdot-}$ as the terminal reduced product. The remarkable stability of this radical might be due either to extraction or to deactivation of the intrinsic superoxide dismutase by EDTA-treatment.

That $O_2^{\cdot-}$ is a regular intermediate even in unmodified chloroplasts (viologens or oxygen as electron acceptor) is probable since the $O_2^{\cdot-}$ specific oxidation of hydroxylamine to nitrite [14] shows the same yield in EDTA-treated and in control chloroplasts.

2. Materials and methods

Isolated chloroplasts were prepared from greenhouse grown spinach leaves and they were extracted with 1 mM EDTA at 0.2–0.4 μM chlorophyll using the standard procedure [15] with alterations described in [13]. The pH-changes in the outer aqueous phase indicated by the dye cresol red [7] were measured

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in a rapid kinetic spectrophotometer after excitation with short saturating flashes [16]. For the measurements a 2 cm cuvette was used which contained 15 ml of the following reaction mixture: 10 mM KCl, 30 μM cresol red and 67 μM benzylviologen as artificial electron acceptor. The formation and determination of nitrite was assayed using the procedure of Elstner et al. [14,17]. Superoxide dismutase was purchased from Miles-Seravac.

3. Results and discussion

The transient pH-changes in the outer phase of a chloroplast suspension after flash excitation with benzylviologen (O_2) as acceptor are compared for control and EDTA-treated chloroplasts in fig.1. In control chloroplasts we observed the uptake of protons followed by a rather slow relaxation due to leakage of internally liberated protons into the external aqueous phase (upper trace in fig.1). In contrast to this we observed no proton uptake but a fast acidification after EDTA-treatment.

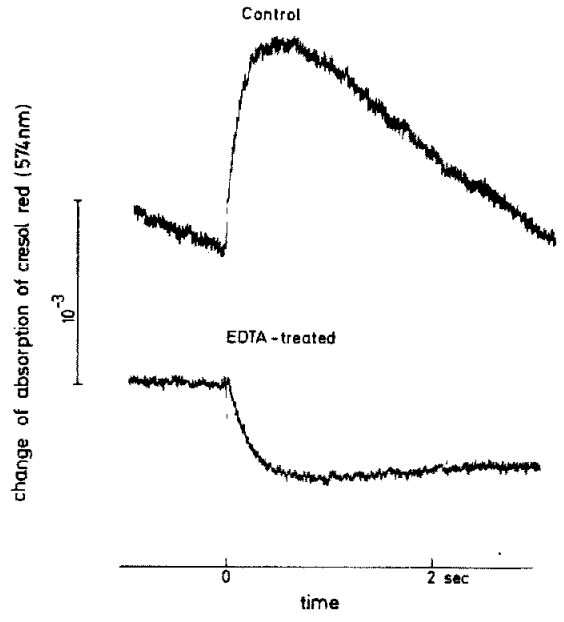

Fig.1. Absorption changes of the pH indicating dye cresol red monitored at 574 nm induced by a single turnover flash at t = 0. Upper trace: control chloroplasts; lower trace: EDTA-treated chloroplasts.

The difference between the traces in fig.1 is due to two different effects:

(1) EDTA-treatment extracts the coupling factor (CF_1) and makes the membrane leaky for ions including protons as documented elsewhere [11,18].

(2) There is a change in the net production of protons summed up over the effects occurring at both sides of the membrane.

In this communication we pursue the second effect only.

In the light of results of other authors revealing that superoxide is an intermediate of the reduction sequence following light reaction I [4–6] which does not bind a proton on reduction at pH = 8 ($pK_{O_2^-} = 4.8$ [10]), we considered the possibility that EDTA-treatment might alter the reduction sequence after photosystem I. If the remainder of the electron transport chain was unaltered this according to [7] would lead to a net production of 1 H^+/e (one proton taken up from outside on plastquinone reduction, two released into the internal phase on oxidation of water and plastohydroquinone, respectively).

To test this hypothesis, superoxide dismutase was added to EDTA-treated chloroplasts to dismutate formed O_2^- to H_2O_2, inducing the binding of 1 H^+ per electron transferred to O_2. The result of an experiment analogous to the one in fig.1 (lower part) in the presence of superoxide dismutase is illustrated in fig.2. It is obvious that the net production of about 1 H^+/e is eliminated by addition of superoxide dis-

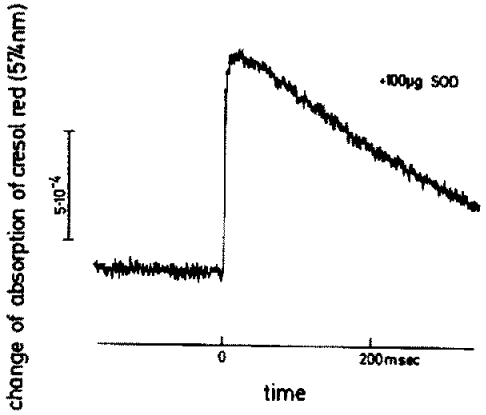

Fig.2. Kinetics of proton uptake indicated by cresol red at 574 nm on EDTA-treated chloroplasts after addition of 100 μg superoxide dismutase.

mutase. Kinetic details of the trace in fig.2 will be discussed below.

There is one problem not explained by our interpretation. Provided the non-protonated $O_2^{\cdot-}$ is the product of the reduction sequence at light reaction I, we should observe the uptake of 1 H^+/e at the outer side of the membrane connected with the reduction of plastoquinone. Measurements of plastoquinone at 265 nm in flash experiments [19] showed no alterations in the kinetics of reduction or oxidation of plastoquinone on extracted chloroplasts in comparison with control chloroplasts. Only the oxidation was accelerated, as expected, due to the uncoupling. Therefore the missing uptake of 1 H^+ at the outer side of the membrane is not due to an alteration in the sequence of the electron carriers at this site, but has still another unknown reason. Perhaps the proton consumed at the outer side is replaced by one which comes from the inner phase before it is noticed by the indicator.

The experiments suggest that EDTA-treatment eliminates an intrinsic superoxide dismutase of chloroplasts, which makes $O_2^{\cdot-}$ a rather stable end product of the electron transport. To corroborate this interpretation, another assay for $O_2^{\cdot-}$ was applied; the oxidation of hydroxylamine to nitrite [17].

Table 1 shows the rates of nitrite production for control and EDTA-treated chloroplasts, respectively, under continuous illumination. The strong inhibition by the superoxide dismutase demonstrates the involvement of the superoxide radical in all cases. Nevertheless we could not find a significant difference in the behaviour of EDTA-treated chloroplasts in comparison to the control. The reduced rate of nitrite production in EDTA-treated chloroplasts (benzylviologen as acceptor) was paralleled by a reduced rate for the electron transport. This is consistent with our earlier report that EDTA-treatment damages electron transport chains [18].

The results shown in table 1 suggest that both, control chloroplasts and EDTA-treated ones, form superoxide either by direct reduction of oxygen in absence of an artificial electron acceptor or by reduction of oxygen via benzylviologen. The pH-measurements, however, show that in control chloroplasts $O_2^{\cdot-}$ is only a shortlived (say $\tau_{1/2} < 10$ msec) intermediate, while in EDTA-treated ones it seems quite stable. Nevertheless, even in control chloroplasts the lifetime of $O_2^{\cdot-}$ seems to be long enough to give hydroxylamine more rapid access to it than the intrinsic superoxide dismutase of chloroplasts (see table 1 and [14]). Only if the superoxide dismutase is extrinsically added in excess it efficiently competes with hydroxylamine for the superoxide radical.

We checked the pool size of $O_2^{\cdot-}$ which can be piled-up under continuous illumination of EDTA-chloroplasts. For this we excited these chloroplasts with a sequence of flashes and monitored the acidification of the medium, due to the lack of proton bind-

Table 1

	NO_2^- formed (μM/μM Chl·h)	NO_2^- formed + 100 μg superoxide dismutase (μM/μM Chl·h)
Control chloroplasts:		
+ benzylviologen	5.75	0.24
without acceptor	1.74	0.18
EDTA-treated chloroplasts:		
+ benzylviologen	3.55	0.22
without acceptor	2.1	0.18

Formation of nitrite from hydroxylamine on control and EDTA-treated chloroplasts. The reaction mixture contained in 3 ml: 80 μmol of tricine pH 8, 0.5 μmol of hydroxylamine and 0.1 μmol of chlorophyll. If indicated 25 nmol of benzylviologen. To have uncoupled conditions in both cases 5 μmol of NH_4Cl were added to the control experiments. The chloroplasts were illuminated for 5 min. Afterwards they were precipitated by heat and the nitrite was assayed in the supernatant by the method of Elstner and Heupel [17].

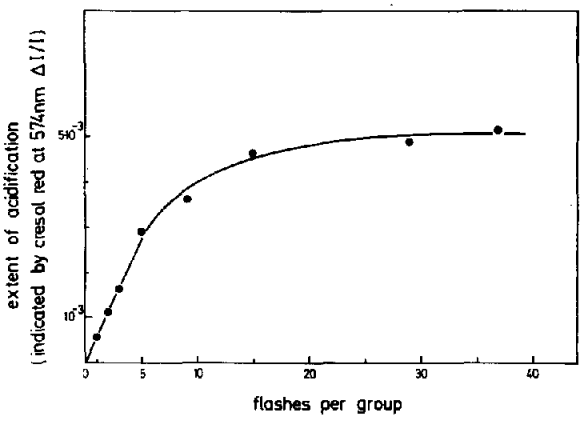

Fig.3. Acidification of the outer phase on EDTA-treated chloroplasts indicated by cresol red at 574 nm after excitation of photosynthesis by flash groups in dependence of the number of flashes per group (for 1 flash see fig.1 lower trace). The flashes in the group had a distance of 100 msec.

ing during reduction of O_2 to $O_2^{\cdot-}$. As shown in fig.3 a series of flashes fired at a frequency of 10 cps increased up to 10 times the value after excitation with a single flash. This pool for 'acidification' which corresponds to $O_2^{\cdot-}$ almost fully decomposes with a half-time of 1.8 sec.

In the subsequent considerations this halftime is related to the reported kinetic parameters of the dismutation reaction: $O_2^{\cdot-} + O_2^{\cdot-} + 2\,H^+ \rightarrow H_2O_2 + O_2$ Let us assume that each electron transport chain produces one molecule superoxide in a single turnover flash. Since one chain comprises about 800 chlorophyll molecules in EDTA treated chloroplasts [18], this implies an $O_2^{\cdot-}$ concentration in our reaction mixture (10 μM Chl) of 10^{-8} M superoxide after a single turnover flash. The maximal pool size under flash group excitation was 10 times this value. Considering the initial decomposition rate at the time when the group is switched off: $4 \cdot 10^{-8}$ M sec^{-1}, this corresponds to a second order rate constant $k = 4 \cdot 10^6$ $M^{-1}\,sec^{-1}$. This is at variance from the dismutation constant in vitro: 10^2 M^{-1} sec^{-1} [10]. One possible explanation of this discrepancy may be our assumption of a homogeneous distribution of the superoxide in the cuvette. Probably $O_2^{\cdot-}$ is not free to distribute over the cuvette volume. Another possible explanation is that there are a few superoxide dismutase molecules left intact which speed the dismutation.

It was shown that our conditions $O_2^{\cdot-}$ is an intermediate of the reduction sequence on photosystem I. EDTA-treated chloroplasts differ from the control by the deactivation of the intrinsic superoxide dismutase which leads to $O_2^{\cdot-}$ as a relative stable endproduct of the electron transport chain. This effect of EDTA-treatment can be reversed by externally added superoxide dismutase.

Similar effects were reported by Lumsden and Hall [20] for Triton subchloroplast particles. They assumed that the metal ions responsible for the activity of the enzyme are complexed and removed by EDTA. They could restore the activity with 0.1 mM $MnSO_4$ or $CuSO_4$. However, Fridovich found that the activity of a mammalian superoxide dismutase is not affected by EDTA [1]. Perhaps the whole enzyme or its reactive site is removed by EDTA-treatment together with the coupling factor.

The following arguments favor that EDTA-treatment detaches several different proteins from chloroplast membranes. (1) Berzborn found that antibodies against the ferredoxin-NADP reductase have easier access to the membrane after treatment with EDTA [8]. This indicates the removal of a 'shield' from the membrane. (2) It was shown by Ausländer and Junge that the kinetics of the proton uptake at the outer side of the membrane are delayed by about 60 ms compaired with the kinetics of the related redox reactions [21]. They attributed the delay to a 'diffusion barrier' covering the dielectric core of the membrane. The fast proton uptake depicted in fig.2. (compared with fig.1 upper part – different time scale!) indicates that this barrier is at least partly removed after EDTA-treatment.

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