EFFECT OF DEOXYCHOLATE ON OXYGEN EVOLUTION AND MANGANESE IN SPINACH CHLOROPLASTS

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1. Introduction

The importance of manganese for photosynthetic oxygen evolution is well documented. A number of treatments, which result in the release of bound manganese from chloroplasts, lead to the inactivation of O_2 evolution without affecting photosystem II-activated electron transport using artificial electron donors (review [1]). Agents such as Tris and hydroxylamine or gentle heating mobilize specifically ~2/3rds of the manganese bound in the chloroplast membrane [2–4]. This fraction of the photosystem II-associated manganese has been suggested to be directly involved in the water-splitting process. Considerable insight in the mechanism of O_2 evolution has been obtained from experiments which support a role for manganese in charge accumulation related to the oxidation of water [5,6]. The specific role of the tightly bound manganese of the smaller (1/3rd) pool has remained unclear, although this fraction has been suggested to function in photosystem II electron transport between the O_2-evolving site and the photosystem II reaction center [2,7].

Here, we demonstrate some effects of deoxycholate (DOC) on photosystem II electron transport in spinach chloroplasts. DOC inhibits O_2 evolution, but evidence is presented which suggests that the manganese of the O_2-evolving site remains in its native state. The loss of water-splitting activity may be due to the selective solubilization of a component between the O_2-evolving site and the photosystem II reaction center, possibly one related to the smaller manganese pool.

2. Materials and Methods

2.1. *Chloroplast preparation*

Spinach leaves were homogenized in a Waring blender in 400 mM sucrose, 50 mM Tricine (pH 7.2) and 10 mM NaCl. The homogenate was strained through 4 layers of 20 mesh nylon cloth. After centrifugation at 3000 × *g* for 3 min the chloroplasts were broken and washed twice in 50 mM Hepes (pH 7.3) and 10 mM NaCl and finally resuspended in Hepes buffer at ~5 mg chl/ml. The concentration of chlorophyll (chl) was determined as in [8]. The rate of electron transport of the freshly prepared class II chloroplasts in saturating white light was >100 μmol O_2 evolved . mg chl^{-1}. h^{-1}. The manganese content as determined by atomic absorption or by EPR measurements on acidified chloroplast samples [9] was typically ~1 mol Mn/70 mol chl.

2.2. *Activity measurements*

Oxygen evolution was measured polarografically at 25°C with a Clark electrode in a medium containing 30 mM phosphate (pH 6.5) and 10 mM NaCl with phenyl-*p*-benzoquinone (PPBQ) as electron acceptor. The rate of reduction of dichlorophenolindophenol (DCPIP) was followed spectrophotometrically at 590 nm in the same medium with water or 1,5-diphenylcarbazide (DPC) as donors. A 250 W tungsten source with 10 cm water and a Schott RG 665 filter was used to provide the excitation light. The photomultiplier was protected from the actinic light by a blue-filter combination.

2.3. *EPR measurements*

Room temperature EPR spectra were recorded in a Varian E-3 instrument at 9.5 GHz in quartz capillaries (i. d. 1 mm).

3. Results and discussion

When spinach chloroplasts are treated with 2% DOC, their ability to oxidize water is lost within a few minutes (fig.1A,B, table 1). However, the electron-transport chain around the photosystem II reaction center remains intact, since the electron transport from the artificial donor, DPC, to DCPIP is not inhibited (fig.1C). DOC appears to destroy the integrity of the initial segment of the photosystem II electron-transport chain which is known to be very sensitive to modification (see below). To gain additional information about the action of DOC on photosystem II, DOC-treated chloroplasts were examined by EPR. Manganese associated with photosystem II is known to be easily released from the chloroplast membrane as EPR-detectable Mn^{2+} by treatments which destroy O_2 evolution such as gentle heating, addition of Tris, hydroxylamine, some detergents, chaotropic agents and proteolytic digestion [1]. It is therefore somewhat surprising that almost no Mn^{2+} signal is seen in DOC-treated chloroplasts (fig.2). This is an indication that the manganese associated with the water-splitting site is still bound to the native sites. This idea is strongly supported by the observation that treatments, which specifically deactivates O_2 evolution in intact chloroplasts through labilization of manganese, release Mn^{2+} into the medium also in DOC-treated chloroplasts (fig.2).

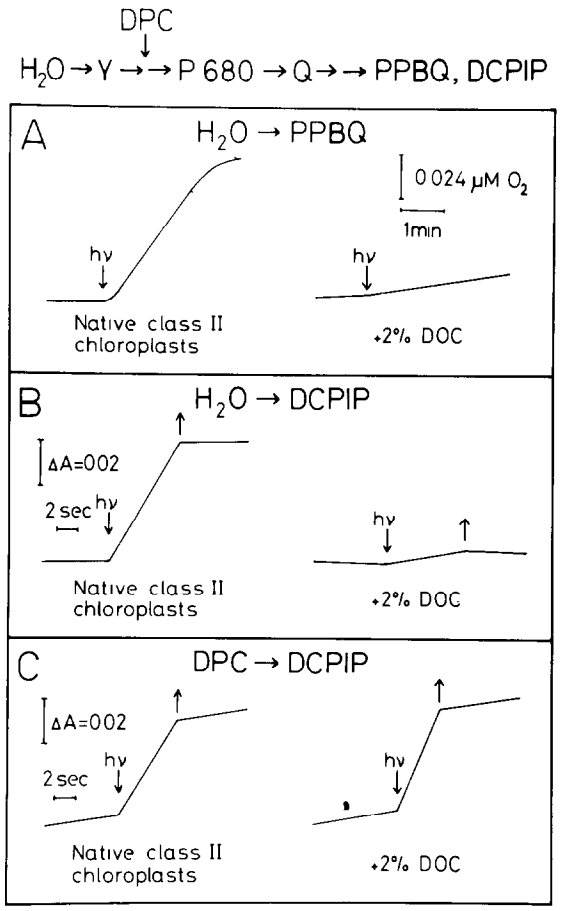

Fig.1. Effect of DOC on photosystem II electron transport. The concentrations of reactants were: (A) 30 μg chl/ml, 12 mM phenyl-*p*-benzoquinone; (B) 30 μg chl/ml, 25 μM dichlorophenolindophenol; (C) as in (B) but with 50 μM diphenylcarbazide as electron donor. Other conditions as in section 2. A schematic description of the relevant part of photosystem II is included in the figure.

When DOC-treated chloroplasts were subjected to centrifugation for 1 h at 40 000 × *g*, a dark-green pellet containing almost all chlorophyll (~98%) and a faintly green supernatant were obtained. After acidification with H_2SO_4 (pH 1) to release all bound manganese [9], EPR spectra of the two fractions were recorded to determine their manganese content. In a number of such experiments, invariably 70–75% of the total manganese was found in the pellet fraction. Atomic absoption analysis of the manganese content of the pellet and the supernatant fractions closely agreed with the EPR measurements. Neither of the two fractions showed any significant Mn^{2+} signal in EPR before acidification. This indicates that the metal is firmly bound in both fractions. Treatment with Tris, hydroxylamine or gentle heating produced the typical Mn^{2+} EPR signal in the pellet fraction where-

Table 1
Summary of photosystem II activity measurements

Relative PS II activity:	$H_2O \rightarrow PPBQ$	$H_2O \rightarrow DCPIP$	$DPC \rightarrow DCPIP$
Class II chloroplasts	100	100	100
+ 2% DOC	<10	<10	130

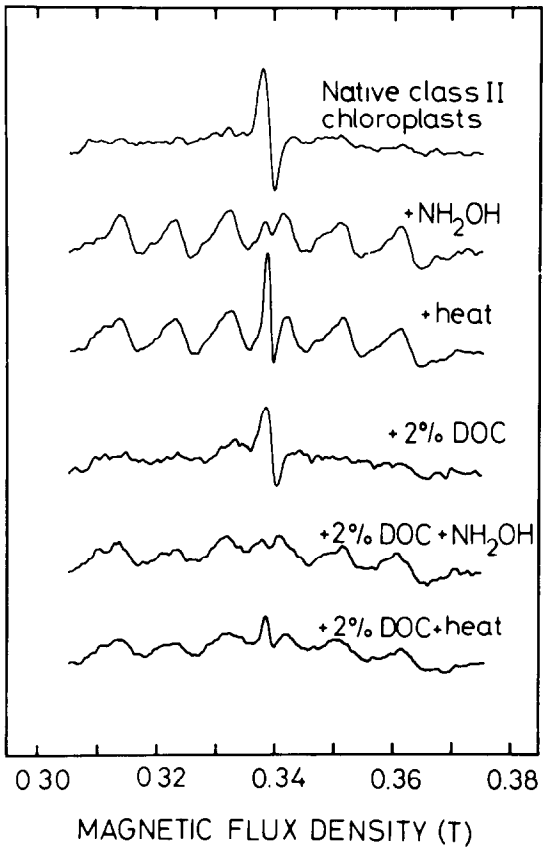

Fig.2. Room temperature EPR spectra showing the effect of DOC on the properties of chloroplasts: 5 mg chl/ml; 20 mM NH_2OH. For the heat treatment the samples were warmed at 55°C for 3 min. EPR operating conditions: microwave power, 10 mW; modulation amplitude, 1 mT; time constant, 1 s; scan rate, 50 mT/min.

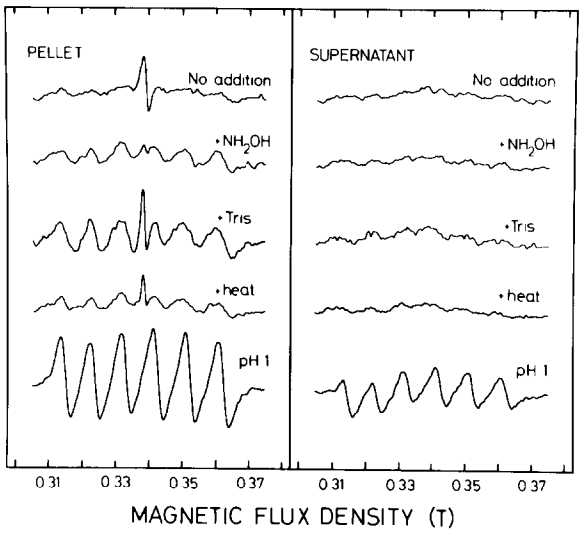

Fig.3. Room temperature EPR spectra of pellet and supernatant fractions after centrifugation of DOC-treated (2%) chloroplasts. The fractions were adjusted to the same volume before samples were drawn for the EPR measurements. For the Tris experiment, 2 M Tris–H_2SO_4 (pH 8.0) was added to 0.7 M final conc. The spectra were corrected for dilution. Other conditions as in fig.2.

as negligable effects were seen in the supernatant (fig.3).

When the pellet, obtained after DOC treatment and centrifugation, was resuspended in Tris buffer (0.8 M, pH 8.0) and recentrifugated, the released EPR-visible manganese partitioned between the new pellet and the supernatant. This shows that the membranes still have enough vesicular structure to retain some of the released manganese although the permeability has increased considerably compared to membranes unexposed to DOC [4]. About 50% of the manganese in the DOC pellet was found in the supernatant. Of the manganese still associated with the pelleted membrane fraction, only 35% was detectable by EPR before acidification. However, the amount of liberated manganese could be considerably higher than this. Chloroplasts are known to contain numerous unspecific sites which rebind manganese released from the native sites and abolish the typical EPR signal [4]. The rebinding is responsible for the rather weak signal after, for example, heat treatment of chloroplasts although ~2/3rds of the manganese has been released. The sites are inactivated at low pH (fig.2,3). The rebinding must be taken into consideration when the total amount of manganese, liberated by Tris treatment of the DOC pellet, is estimated. Thus, it is rather unlikely that this fraction contains much tightly bound, i.e., Tris-insensitive, manganese.

Manganese in chloroplasts is known to exist in two different environments: Labile manganese, comprising ~2/3rds of the total content and associated with O_2 evolution, and tightly bound manganese (~1/3rd) with unclear function. The latter type is not released by Tris, hydroxylamine or heating but may be removed by drastic treatments which completely destroy the structure of the chloroplast membrane, such as denaturation at extreme pH. Extraction of the tightly bound manganese [2] eliminated the fraction (20%) of the original ability of chloroplasts to oxidize ascorbate or *p*-phenylenediamine that remained after treatment with Tris or hydroxylamine. However, the oxidation

of artificial donors such as hydroxylamine or 1,5-diphenylcarbazide by photosystem II was unaffected by the virtually complete extraction of manganese [2,7]. This shows that the tightly bound manganese, at least under certain conditions, can function as an electron carrier in photosystem II, conceivably at an early stage in the electron-transport chain.

The properties and the relative amounts of manganese in the two fractions obtained after centrifugation of the DOC-treated chloroplasts are characteristic for the above two types of manganese. It is tempting to suggest that the supernatant fraction with Tris-resistant manganese represents the smaller pool of normally tightly bound metal with the manganese maybe in its original state, whereas most or all the manganese in the DOC-pellet is associated with the larger pool and the O_2-evolving site. The solubilization by DOC of a manganese-containing factor with vital function may explain the abolished O_2 evolution.

Finally, it should be pointed out that the loss of water-splitting activity after DOC treatment may be the result of the extraction or inactivation of components other than such associated with manganese. Preliminary EPR investigations show that one component with function possibly associated with photosystem II, cytochrome b_{559}, remains bound in the photosynthetic membrane but with EPR properties characteristic of the low-potential form ($g_z = 2.92, g_y = 2.24$) [10,11]. Although conversion of cytochrome b_{559} from high-potential to low-potential sometimes follows from treatments which eliminate O_2 evolution [12], this transition is probably not responsible for the inhibition. On the contrary, a number of studies have indicated the absence of correlation between high-potential cytochrome b_{559} and O_2 evolution (see [1]).

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