MANGANESE LEVELS ASSOCIATED WITH INSIDE-OUT THYLAKOID MEMBRANES IN RELATION TO OXYGEN EVOLUTION

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1. Introduction

Manganese seems to be a key component of the photosynthetic O_2 evolving apparatus. This conclusion stems from a number of observations including Mn deficiency studies with algal cultures and experiments involving various washing procedures of isolated thylakoid membranes [1,2]. A dogma has been established from these observations that there exists a mangano-protein responsible for the binding and subsequent oxidation of water to molecular oxygen. The water-binding protein is thought to contain either 2 or 4 Mn atoms in its catalytic centre. This general idea has been used to interpret experiments using NMR and EPR spectroscopy [3,4]. Although the concept of a mangano-protein being involved in the water oxidation process is intuitively attractive, the evidence for its existence is poor [5]. Claims that a 65 000-M_r protein, containing 2 Mn atoms, is intimately involved in O_2 evolution and can be readily isolated from thylakoid membranes [6] have not been substantiated [7]. One of the most important indirect observations which support the idea that a mangano-protein is involved in O_2 evolution is the effect of washing isolated thylakoid membranes with alkaline Tris (hydroxymethyl) amino methane (Tris) buffer. Such a treatment usually removes ~2/3rd of the total Mn content of the membranes and inhibits photosynthetic O_2 evolution [1,2]. From this result it has been concluded that the Tris-removable Mn is derived from the water-binding protein and that this protein contains 4 Mn atoms [1]. No functional role has been suggested for the firmly bound Mn not removed by the Tris wash. The idea that alkaline Tris washing inhibits O_2 evolution because it removes Mn atoms from the water binding protein is widely assumed and, indeed, this washing procedure is often used as a test of reliability that a spectroscopic signal is indicative of Mn functionally active in the water splitting process [8]. However, there are doubts to the importance of Tris-removable Mn in O_2 evolution which stem from [9–11]: After alkaline Tris washing of spinach thylakoids, O_2 evolution could be restored by treatments such as 'dark reactivation' involving simply addition of reduced dichlorophenol indophenol (DCIP) or hydroquinone (HQ) to Tris-washed membranes [9]. Under the best conditions, ~70% of the original O_2 evolving capacity could be restored even though the Mn content of the membranes had been significantly reduced [9,10]. Such a result is inconsistent with the view that Tris washing removes functionally active Mn from the water splitting protein. An explanation for this inconsistency has come from [12,13]. Using EPR and atomic absorption spectroscopy to detect Mn^{2+} and total Mn, respectively, it was concluded that Tris washing removed only 10% of the total Mn and that 60% of the Mn pool was released into the intrathylakoid space as a consequence of the treatment [12]. Further, the dark reactivation procedure may be brought about simply by a reinsertion of this free Mn trapped within the intrathylakoid space into the membrane-bound protein responsible for water oxidation [13]. Here we show, using the dark reactivation procedure, that it is possible to restore O_2 evolution from Tris-treated inside-out thylakoid membranes. Our results indicate that the Tris-removable Mn is not necessary for photosynthetic O_2 evolution and that if a mangano-protein is involved in the water oxidation process it must be associated with the firmly bound Mn pool not removed by alkaline Tris treatment.

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2. Materials and methods

Chloroplast thylakoid membranes were prepared from peas (Feltham First) as in [14]. Membranes were washed twice in an EDTA solution (0.1 M sorbitol, 2 mM EDTA (pH 7.9–8.0) with Tris) for 5 and then 20 min to remove loosely bound manganese. These membranes were then used directly for Tris-washing or for preparation of inside-out vesicles as in [15]. To facilitate adequate membrane stacking, a necessary prerequisite for formation of inverted vesicles and particularly necessary after EDTA washing, samples were incubated for 30 min at 0°C in a high salt medium (0.1 M sodium phosphate, 50 mM NaCl (pH 7.5) with NaH_2PO_4) before passage through the Yeda Press.

Tris-treatment: Whole thylakoid membranes and inverted vesicles were resuspended from pellets in 0.6 M Tris–HCl (pH 8.4) at 0°C and incubated for 20 min under room illumination. To stop the reaction samples were diluted 10 times in a phosphate buffer (0.1 M sucrose, 5 mM NaCl, 10 mM Na_2HPO_4 (pH 7.5) with NaH_2PO_4) and recovered by centrifugation (5 min at 2000 × *g* for thylakoids and 30 min at 35 000 × *g* for vesicles). For dark reactivation, pellets were rinsed over with phosphate buffer and resuspended in the same medium containing 2 mM hydroquinone and 2 mM ascorbate and incubated for 20 min at 0°C in the dark. The reaction was stopped by 10-fold dilution in phosphate buffer and samples were retrieved by centrifugation. Control samples were subjected to the same washing procedures using the phosphate buffer. Samples for Mn determinations were resuspended in phosphate buffer (made with deionised–distilled water) and made to 1% HNO_3 before measurement by flameless atomic absorption spectroscopy. Rates of O_2 evolution were measured at 20°C with a Rank O_2 electrode illuminated with saturating white light. Samples were suspended in the above phosphate buffer and the electron acceptor was 0.5 mM benzoquinone. The membranes were uncoupled as a consequence of the EDTA washing.

3. Results

Table 1 shows that alkaline Tris treatment removed ~50% of the Mn associated with EDTA-washed isolated pea thylakoids. Concomitant with this loss of Mn was a significant reduction in the rate of O_2 evolution. The dark reactivation procedure caused an almost 4-fold stimulation of the inhibited O_2 evolution rate while at the same time slightly reducing the Mn level. We were unable to dark reactivate to 70% or 100% of the control as reported [9] or [13], respectively. However, we could obtain rates of 75% of the control by a light reactivation procedure which involved the incubation of illuminated Tris-treated membranes which had been subjected to dark reactivation in 0.1 M sorbitol, 2 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.05% bovine serum albumin and 0.5 mM 1,5-diphenyl carbozide at pH 7.5 for 20 min [11,16]. This light reactivation was associated with a slight decrease of the Mn level compared with the Tris-treated membranes [5,16].

The ability of the dark or light reactivation procedures to give O_2 evolution rates comparable with controls seemed to be dependent on the degree of the Tris-treatment. For example, in this series of experiments the reduced severity of the Tris inhibition of right-side-out vesicles (see table 2) allowed a dark reactivation to 71% of the control. Nevertheless, as with intact thylakoids, the alkaline Tris-treatment depleted both inside-out and right-side-out vesicles by ~50% of their total Mn (see table 2). O_2 evolution from inside-out vesicles responded to the dark reactivation proce-

Table 1
Effect of Tris-washing and dark-reactivation on activity and manganese levels of pea chloroplast thylakoids

Sample	% O_2 evolution ± SD (16 samples)	Mn/400 chl ± SD (6 samples)	Mn/200 chl *b* ± SD (6 samples)
Chloroplasts (EDTA-washed)	100[a]	5.70 ± 0.32	10.72 ± 0.60
Tris-washed	16 ± 9	3.02 ± 0.25	5.51 ± 0.46
Dark-reactivated	59 ± 9	2.90 ± 0.27	5.12 ± 0.48

[a] Av. control rate of O_2 evolution was 415 μequiv. . mg chl^{-1} . h^{-1}

Table 2
Effect of Tris-treatment and dark-reactivation on the activity and manganese levels of inside-out and right-side-out vesicles

Sample	% O_2 evolution ± SD (3 samples)	Mn/400 chl ± SD (4 samples)	Mn/200 chl *b* ± SD (5 samples)
Inside-out			
Control[a]	100	6.46 ± 1.67	12.88 ± 0.27
Tris-washed	23 ± 5	3.44 ± 0.44	5.96 ± 0.34
Dark-reactivated	57 ± 9	2.61 ± 0.64	3.77 ± 0.19
Right-side-out			
Control[b]	100	6.08 ± 0.99	12.25 ± 0.72
Tris-washed	37 ± 5	3.39 ± 0.61	5.48 ± 0.25
Dark-reactivated	71 ± 7	2.42 ± 0.66	3.17 ± 0.20

Control rate: [a] inside-out vesicles, 196 μequiv. . mg chl^{-1} . h^{-1}; [b] right-side-out vesicles, 236 μequiv. . mg chl^{-1} . h^{-1}

dure to an extent equivalent to that found with intact membranes. Inside-out and right-side-out vesicles both lost further Mn during the dark reactivation process.

4. Discussion

Although the dark reactivation procedure was unable to give O_2 evolution rates equal to those of the controls, significant increases were achieved with all 3 types of Tris-treated membranes. The stimulation of the inhibited O_2 evolution occurred even though significant proportions of the total Mn had been removed by the Tris washing. Because there was no obvious difference in response of inside-out vesicles and normal thylakoids to the reactivation procedure, it can be concluded that the stimulation of Tris-inhibited O_2 evolution is not due to the rebinding of Mn trapped in the intrathylakoid space to an inner membrane site as advocated in [13]. These results support the suggestion [5,16] that if Mn is involved as a catalytic centre for the water oxidation process then the Mn which functions in this capacity is not that removed by alkaline Tris-treatment as often assumed [1,4]. The tightly bound Mn is equivalent to ~3 Mn atoms/400 chl (both *a* and *b*) molecules or 4–5 Mn atoms/200 chl *b* molecules. Chlorophyll *b* may be a better marker for the PS2 reaction centre concentration since the levels of total chlorophyll will vary depending on the stoichiometric ratio of PS2 and PS1 [17]. Taking 200 chl *b* molecules/PS2 reaction centre gives 4 or 5 Mn/trap if all the firmly bound Mn is directly associated with PS2 photochemical activity as argued from recent NMR and EPR studies [18,19].

Our results emphasise that it is unlikely that the inhibition of O_2 evolution by Tris washing is due to removal of Mn from the catalytic centre of the protein directly responsible for binding water and its subsequent oxidation. Rather the Tris effect seems to involve some other perturbation of reactions on the oxidising side of PS2 which can be overcome in the absence of Mn [21].

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