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A Chloroplast Membrane Conformational Change Activated by Electron Transport Between the Region of Photosystem II and Plastoquinone

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Abstract

Various partial redox reactions involved in photosynthetic electron transport were studied in relation to the electron transport dependent incorporation of the water soluble chemical modifier, diazonium benzene sulfonic acid (DABS)* into chloroplast membranes. This electron transport dependent diazonium incorporation reflects a conformational change (unspecified at this time) in membrane components. The redox reaction(s) responsible for this conformational change was shown to be localized after the site of DCMU inhibition but before plastoquinone by the following evidence:

1. Electron transport from water to lipophilic "Class III" electron acceptors such as dimethyl benzoquinone and high concentrations of dibromothymoquinone potentiate the extra DABS binding to the membranes. These compounds are reduced prior to or at the plastoquinone site.

2. Electron transfer from water to silicomolybdate plus ferricyanide, a DCMU insensitive reaction, does not result in the incremental diazonium binding.

* Abbreviations: DCIP, 2,6-dichlorophenol-indophenol; DCMU, 3-(3-4-dichorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate; DMQ, 2,5-dimethyl-p-benzoquinone; DBMIB, 2-5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; CCCP, Carbonylcyanide 3-chlorophenylhydrazone; FCCP, carbonylcyanide 4-trifluoromethoxyphenylhydrazone.

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3. Photosystem I cyclic electron flow mediated by menadione (anaerobic), which requires participation of plastoquinone does not give the extra diazonium binding.

The exact redox step responsible for the conformational change is not known for certain, but there is a possibility that cytochrome b-559 may be involved. This is suggested by the observation that diazonium treatment of chloroplasts during illumination but not in darkness, causes the conversion of cytochrome b-559 from the high potential form to the low potential form.

Introduction

Various chloroplast membrane protein amino acid side groups react with diazonium benzene sulfonic acid (DABS) [1, 2, 3]. Interestingly, chloroplasts experiencing electron transport activity just prior to the addition of the diazonium reagent, show a two- to four-fold greater capacity to react with and covalently bond DABS [4, 5]. The incremental DABS binding is attended by inhibition of water oxidation [4, 5]. DABS reaction with chloroplasts in the dark does not result in such water oxidation inhibition, although the ferredoxin-$NADP^+$ reductase activity, and an electron transfer step between photosystem I and ferredoxin [6], as well as the phosphorylation coupling factor activity [2] are inhibited by DABS under dark conditions.

We have shown that cyclic electron transport supported by PMS does not potentiate the extra DABS binding nor the water oxidation inhibition, even though the usual proton uptake occurred [4]. Neither is electron transfer from water to photosystem II required for the extra DABS binding effect, since in Tris-washed chloroplasts that lacked water oxidation, electron donation from Manganese supported the extra DABS binding [5].

This phenomena is interesting in that it reveals a conformational change* different from that which attends PMS supported cyclic electron flow and H^+ accumulation [7]. The close association of the extra DABS binding effects with photosystem II and the recent work of Izawa and Good and colleagues [10, 11] showing ATP synthesis during electron flow between photosystem II and lipophilic (Class III) acceptors that accept electrons prior to plastoquinone, raises the possibility of a correlation between the extra DABS binding (a conformational change) and events associated with phosphorylation at Site II (terminology of Izawa and Good). The conformational change may be associated with the water oxidation mechanism.

* Regardless of whether DABS is very impermeant (as we assume based on work in erythrocyte [8] and mitochondria [9] membranes and by virtue of the diazonium ($N \triangleq N$) positive charge and the sulfonic acid negative charge) or somewhat permeant, the electron flow dependent extra DABS incorporation requires that some form of conformational change occur, permitting otherwise unavailable diazo-reactive groups to be exposed. Reference 5 presents data and discussion of this point.

The experiments reported here attempt to identify more precisely which redox step(s) are required to potentiate the conformational change detected by the extra DABS binding. We already know that the redox link driving the extra DABS binding is between the site of Manganese donation on the oxidizing side of photosystem II and somewhere between photosystem II and photosystem I [5]. Menadione catalyzed cyclic electron flow engages the redox links between (and including) plastoquinone and photosystem I, based on the inhibition of menadione cyclic phosphorylation by the plastoquinone antagonist dibromothymoquinone (DBMIB) [12, 13]. Electron flow from water to a point prior to plastoquinone is supported by the lipophilic, Class III, oxidants such as DBMIB (at high concentrations) and dimethylbenzoquinone (DMQ) [10, 11]. Based on new results of Girault and Galmiche [14], it is possible to obtain electron transfer from water to ferricyanide in the presence of silicotungstate and DCMU, i.e. electrons being accepted at a site prior to Q, the primary acceptor of photosystem II. Using these assays we will show that it is electron transfer from some point *after* the DCMU block but *before* plastoquinone that activates the electron flow dependent extra DABS binding, i.e. a conformational change.

Materials and Methods

Chloroplast isolation from spinach leaves was according to methods described previously [2]. The isolation medium contained 0.4 M Sorbitol, 20 mM Tricine-KOH (pH 7.8), 10 mM KCl, 3 mM $MgCl_2$, 3 mM ascorbate and bovine serum albumin (2 mg/ml final concentration). After isolation and centrifugation the chloroplasts (approximately equivalent to 4 mg Chl/ml) were resuspended in 0.1 M sucrose, 3 mM $MgCl_2$, 10 mM KCl, 20 mM Tricine-KOH buffer (pH 7.5). The diazotization of p-aminobenzene sulfonic acid to produce the DABS and the reaction of DABS with chloroplasts were as described previously [1]. Briefly, chloroplasts at a chlorophyll concentration equivalent to 100 μg Chl/ml were resuspended in the above sucrose buffer mixture and then reacted with 1.0 mM ^{35}S-DABS for 30 sec either in the dark or during illumination with heat-filtered white light of approximately 10^6 ergs/cm^2/sec intensity (variations as described in the Table legends). Either electron transport (oxygen evolution) or the extent of proton uptake was monitored in the chloroplast mixture during the 30 sec illumination period just prior to the addition of DABS to ascertain the electron or proton transfer rate at the time of the DABS addition.

For the experiments involving menadione cyclic electron transport, anaerobic conditions in the chloroplast reaction mixture were generated

by the addition of 20 mM glucose and 100 μg/ml of glucose oxidase. Anaerobic conditions were monitored with an oxygen electrode. The air-chloroplast mixture interface was flushed with nitrogen to prevent atmospheric oxygen equilibration into the chloroplast mixture during the reaction.

After reaction with ^{35}S-DABS the chloroplasts were washed twice with 0.1 M sucrose, 3 mM $MgCl_2$ and 20 mM Tricine-KOH (pH 7.5) to remove the non-covalently bound DABS from the membranes as well as to remove the co-factors present during the DABS treatment. Aliquots of chloroplasts were oxidized in liquid scintillation vials with 0.1 to 0.2 ml of 30% H_2O_2 for 2-3 hr at 70°C and then the radioactivity determined by liquid scintillation counting using a modified Bray's cocktail.

Dark oxidation and reduction of cytochrome b-559 was essentially as described by Cramer, Fan and Bohme [15]. High potential b-559 was defined by its hydroquinone reducibility while low potential b-559 being defined by its ascorbate but not hydroquinone reducibility [15]. Cytochrome b-559 was measured at 561 nm with 540 nm as the reference using an Aminco-Chance dual wavelength spectrophotometer.

Results and Discussion

Previously we reported that photosystem I cyclic electron flow catalyzed by PMS (in the presence of DCMU) did not potentiate the light induced binding of DABS to the membranes or the inhibition of oxygen evolution [5]. Since it is difficult to know with certainty what electron transfer components are involved in the PMS cyclic system we could not discount the possibility that some redox component(s) in the linear electron transport sequence located between the photosystems (and not common with the PMS system) was responsible for the increased DABS binding and proposed conformational change. To test this possibility we determined the effectiveness of the cyclic electron transport system catalyzed by menadione (2-methyl-1,4-napthoquinone) under anaerobic conditions in eliciting the increased DABS binding. The cyclic pathway mediated by menadione, in the presence of DCMU, has already been shown by Böhme *et al.* [12] to include plastoquinone, based on the sensitivity of the photoreaction to the plastoquinone antagonist DBMIB (which prevents the oxidation of reduced plastoquinone) [13]. The DBMIB sensitivity of this reaction is in contrast to the insensitivity of the PMS cyclic system to this inhibitor indicating the larger sequence of electron transport in the former system which is probably more closely related to the cyclic system found *in vivo* [16]. Table I shows that cyclic electron transport (under anaerobic conditions) mediated by menadione in the presence of DCMU did not result in the increased binding of DABS to the membranes. In these experiments the chloroplasts were

illuminated for 30 sec with menadione, as indicated in the table and legend, prior to the addition of DABS and for 30 sec thereafter. The extent of active proton accumulation by the chloroplasts was monitored during the illumination period prior to the DABS addition to determine the degree of activation of the membranes. Similar extents of proton uptake occurred in the menadione system ± DCMU (Table I) indicating that the observed lack of light induced binding in the presence of DCMU was not caused by insufficient electron transport. The DCMU insensitivity for menadione catalyzed cyclic phosphorylation has already been established [12]. The addition of DBMIB to the chloroplast reaction completely inhibited proton accumulation, as was expected [12], and resulted in the same low level of DABS binding found in the light + DCMU and dark treatments, further showing no relationship in this system between the cyclic electron flow (reflected in proton uptake) and the light-induced DABS incorporation. These experiments indicate that photosystem I cyclic electron flow which includes plastoquinone and subsequent redox components does not potentiate the increased

TABLE I. Incorporation of DABS into chloroplast membranes during light activation of menadione-mediated cyclic electron flow

Additions to assay	Illumination conditions	Relative extent of proton uptake prior to DABS addition (% of control)	^{35}S-DABS incorporation (cpm x 10^{-5}/mg Chl)
Menadione	light	100*	21.9
Menadione + DCMU	light	90	7.4
Menadione + DCMU + DBMIB	light	<2	6.8
Menadione ± DCMU or DBMIB	dark	–	6.4

Chloroplast isolation and reaction with DABS and generation of anaerobic conditions were as described in the Materials and Methods. Chloroplasts equivalent to 0.5 mg Chl/ml were suspended in 100 mM KCl, 5 mM $MgCl_2$ (final pH 7.5) for the DABS reaction. All reactions were first illuminated under anaerobic conditions for 1 min prior to the addition of DCMU and DBMIB in order to fully reduce the menadione [12]. Following the first cycle of illumination, the inhibitors were added to the reaction mixture and the mixtures were re-illuminated for 30 sec with white light (10^6 ergs/cm^2/sec) prior to the addition of 1 mM ^{35}S-DABS and for 30 sec thereafter. The extent of the light-induced proton accumulation was monitored under these conditions (i.e. during the 30 sec illumination prior to DABS addition) to ascertain the degree of activation of the systems. Dark treated chloroplasts were illuminated for 1 min and then DABS was added after a following 30 sec dark period. The concentrations of Menadione, DCMU and DBMIB were 40 μM, 25 μM and 12 μM respectively. The specific activity of the DABS was approximately 3.054×10^7 cpm/μmole.

*Approximately 0.5 μmole H^+ per mg Chl.

DABS binding. The redox step(s) responsible for the light-induced DABS binding is therefore prior to plastoquinone but after the manganese (and other alternate photosystem II donors) donation site. If DCMU was not added to the reaction, there was a three-fold increase in DABS binding (first line, Table I). This is probably due to a photosystem II functioning, and the same effect was found for the PMS ± DCMU experiments [4].

Recently it has been reported that in the presence of silicotungstate, ferricyanide can accept electrons at a site after the photosystem II reaction centre and prior to the site of DCMU inhibition [14]. The DCMU insensitivity for this ferricyanide reduction with concomitant oxygen evolution has also been shown to occur in the presence of silicomolybdate (Giaquinta *et al,* in press). We used this DCMU insensitive electron transport pathway to determine if the events involved in the light-induced DABS binding were associated with or dependent on activation of the photosystem II reaction centre (which is presumably unimpaired in this reaction) [14]. Table II demonstrates the extent of DABS binding during this electron transport sequence as well as the rate of oxygen evolution in the chloroplasts just prior to the addition of DABS. Noncyclic electron transport utilizing both photosystems (water → ferricyanide) results in three times the amount of DABS incorporated into the membranes compared to dark-treated conditions. The presence of DCMU in the water → ferricyanide assay inhibits electron flow greater than 95% and prevents the extra DABS binding. When silicomolybdate is included in the chloroplast mixture in

TABLE II. Incorporation of DABS during activation of photosystem II in the presence of silicomolybdate and DCMU

Additions to assay	Illumination conditions	Electron transport prior to DABS addition (μeq/hr/mg Chl)	^{35}S-DABS incorporation (cpm × 10^{-5}/mg Chl)
FeCy	light	544	19.1
FeCy	dark	—	6.4
FeCy + DCMU	light	20	5.8
FeCy + DCMU + SiMo	light	264	5.98
FeCy + DCMU + SiMo	dark	—	6.57

Chloroplasts equivalent to 0.5 mg Chl/ml were suspended in 0.1 M sucrose, 10 mM NaCl, 2 mM $MgCl_2$ and 20 mM Tricine-KOH buffer (pH 8.0) prior to DABS reaction. The chloroplast mixture was illuminated with white light for 15 sec prior to the addition of 1 mM ^{35}S-DABS and for 30 sec thereafter. Oxygen evolution was monitored with a Clarke-type electrode during the 15 sec illumination period prior to DABS addition. The concentrations of ferricyanide (FeCy), DCMU and silicomolybdate (SiMo) were 0.5 mM, 20 μM and 50 μM respectively.

addition to DCMU, electron transport (oxygen evolution) is substantially restored (50% of control) but no extra DABS binding occurs under this condition in which both the oxygen evolution apparatus and the reaction centre of photosystem II are operative. Therefore, activation of the photochemical steps prior to the site of DCMU inhibition does not give the DABS binding, suggesting that the region necessary for the light-induced DABS binding occurs after the primary acceptor, Q.

To determine the involvement of electron transport after the acceptor Q but prior to plastoquinone, chloroplasts were reacted with DABS during activation of the photosystem II partial reaction from water to Class III acceptors such as DMQ and high concentrations of DBMIB. These lipophilic electron acceptors have recently been shown to accept electrons after the primary acceptor, prior to or at the region of plastoquinone but not beyond [10]. Light activation of the electron transport sequence from water to the region of plastoquinone (high DBMIB concentration) potentiates the extra DABS binding to the chloroplast membranes as shown in Table III, line 3, and in Table IV. The increased incorporation of DABS is also found when DMQ is used as the acceptor in conjunction with low concentrations of DBMIB which prevents electron transfer beyond the level of plastoquinone (Table III, line 4). Addition of DCMU to both these photoreactions prior to the DABS treatment prevents the extra incorporation and results in the dark level of binding (Table III, line 5). Table III also shows the rates of

TABLE III. Incorporation of DABS into membranes during activation of photosystem II electron transport from water to DMQ and DBMIB

Additions to assay	Illumination conditions	Electron transport prior to DABS addition (μeq/hr/mg Chl)	^{35}S-DABS incorporation (cpm $\times 10^{-5}$/mg Chl)
1. FeCy	light	80	11.8
2. FeCy + DMQ	light	260	9.7
3. FeCy + 25 μM DBMIB	light	112	9.0
4. FeCy + DBMIB (2 μM) + DMQ	light	164	8.0
5. FeCy + DBMIB (2 μM) + DMQ + DCMU	light	<15	4.5
6. FeCy	dark	–	4.9
7. FeCy + DMQ	dark	–	5.4
8. FeCy + DCMU	dark	–	5.1

Chloroplasts (100 μg Chl/ml) were resuspended in 0.1 M sucrose, 10 mM KCl, 3 mM $MgCl_2$ and 40 mM Tricine–KOH (pH 7.7). The mixture was illuminated for 30 sec (as indicated in the table) prior to the addition of 1 mM DABS and for an additional 30 sec after the DABS addition. Oxygen evolution was monitored prior to the DABS addition. The concentration of FeCy was 0.5 mM, DCMU was 20 μM and DMQ was 0.2 mM.

electron transport in these partial reactions just prior to DABS reaction so as to show the level of electron flow obtained. Accompanying this increased binding, subsequent assays on DABS-treated chloroplasts showed electron flow from water to DMQ is inhibited compared to the dark or light + DCMU states consistant with our previous findings [5].

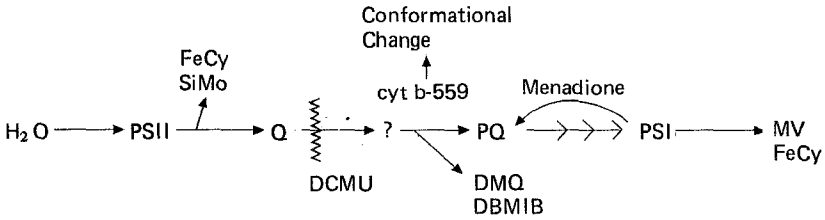
TABLE IV. Incorporation of DABS into membranes during activation of photosystem II electron transport from water to methylviologen and DBMIB

Additions to assay	Illumination conditions	Electron transport prior to DABS addition (μeq/hr/mg Chl)	^{35}S-DABS incorporation (cpm x 10^{-5}/mg Chl)
1. —	light	58	22
2. + 5 μM DBMIB	light	20	11.4
3. + 10 μM DBMIB	light	40	16.5
4. + 20 μM DBMIB	light	55	16.7
5. ± 20 μM DBMIB	dark	—	4.0

Reaction conditions as described in Table III. Methylviologen concentration was 0.5 mM in the reaction mixture.

The incremental binding of DABS to the membranes depends on light dependent transfer of reducing equivalents past the primary acceptor of photosystem II. Treatment of chloroplasts with DABS in the dark which were first reduced either by illumination under anaerobic conditions (no acceptors present) or with dithionite did not result in the extra binding (unpublished results) suggesting that it is not just the reduced state of the membrane *per se* that generates the binding but that electron flux through the membrane is an obligatory requirement.

We interpreted the light-induced increased binding of DABS to the membranes as resulting from a conformational change in the membrane components dependent on photosystem II electron transfer [5]. We previously indicated that this conformational change is not related to the well-characterized conformational changes (chloroplast shrinkage) monitored by light scattering and turbidity measurements which are dependent on massive proton accumulation. Neither the removal of coupling factor by EDTA treatment, nor the presence of uncouplers (nigericin, CCCP, FCCP, gramicidin) nor valinomycin prior to DABS treatment prevents the light-induced binding (ref. 5 and unpublished results) indicating that the proposed conformational change is not one synonomous with cyclic electron flow and concomitant H^+ uptake. We believe the increased binding is reflecting the rearrangement of various membrane components in response to photosystem II electron transfer through the aforementioned region. This is shown schematically on the following page.



It is known that cytochrome b-559 is closely associated with photosystem II, although its exact function and mode of interaction with other redox components is a controversial subject [17, 18]. Since some workers [15] believe cytochrome b-559 is an electron carrier between the primary acceptor (Q) of photosystem II and plastoquinone, the same region implicated in the DABS effects, we investigated the influence of light and dark DABS treatment on cytochrome b-559. One of the few parameters at our disposal is the high and low potential states that can occur with cytochrome b-559 [15, 19]. Table V and Fig. 1 show that control or dark DABS treated membranes are similar in that

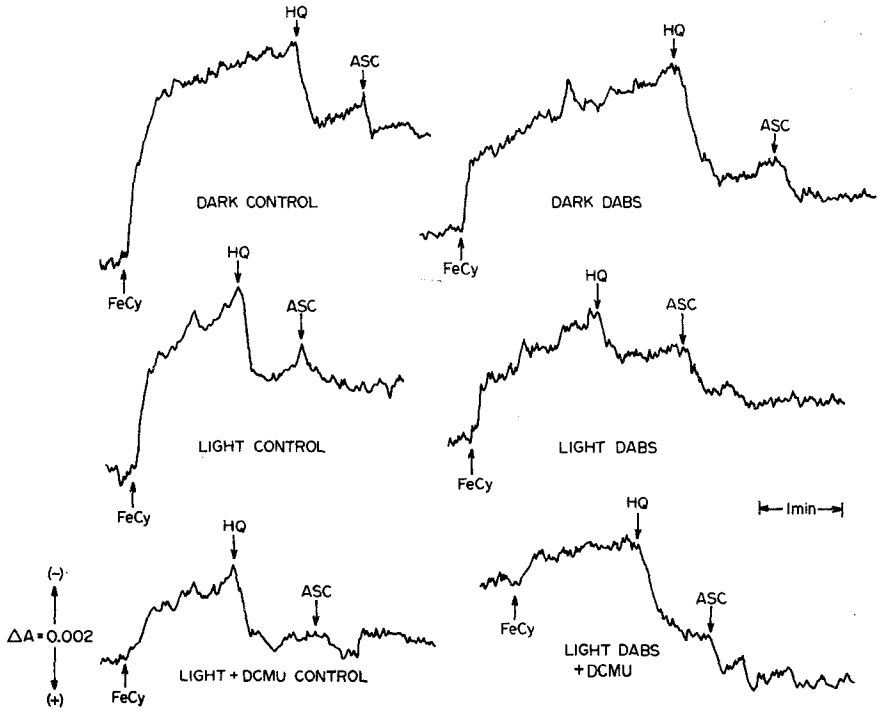


Figure 1. Dark oxidation and reduction of cytochrome b-559 in DABS treated chloroplasts. Methods described in Table V.

approximately 75% of the cytochrome b-559 exists in the high potential form (reducible by hydroquinone) whereas light plus DABS treatment causes a shift in cytochrome b-559 to 40-45% high potential and 55-60% low potential (reducible by ascorbate but not hydroquinone [15]). This high potential to low potential conversion of the cytochrome b-559 is not a consequence of uncoupling [15] since we have previously shown [2] that dark DABS treatment maximally uncouples electron transport.

TABLE V. Redox state of cytochrome b-559 after light and dark DABS treatment

	Redox state of b-559 (% of total)*	
Treatment	$b_{559_{HP}}$	$b_{559_{LP}}$
Control chloroplasts		
Dark	70-75%	25-30%
Light	75	25
Light + DCMU	80	20
DABS treated chloroplasts		
Dark	70-75	25-30
Light	40-45	55-60
Light + DCMU	70	30

The reaction medium contained in 3 ml, 25 mM Tricine-KOH (pH 7.8), 5 mM K_2HPO_4, 5 mM NaCl, 2 mM $MgCl_2$, 0.1 mM Methylviologen and chloroplasts equivalent to 80 μg Chl/ml. Dark oxidation of the chloroplasts was with 0.25 mM ferricyanide. Hydroquinone (1 mM) was used to reduce the high potential form of b-559 (HP) and ascorbate (2 mM) was used to reduce the low potential b-559 (LP). Chloroplasts were treated with or without DABS (± DCMU) in the light (2.2×10^5 ergs/cm^2/sec intensity) or dark for 1 min in the same cuvette used for the cytochrome determination. Dark oxidation and reduction of b-559 was then measured [15].

* Percent of total amplitude of reduction. Data obtained from Figure 1.

Chloroplasts illuminated in the presence of DCMU prior to DABS treatment in the light did not show the cytochrome b-559 high potential to b-559 low potential conversion. This treatment (Light + DCMU + DABS) also prevents the extra DABS incorporation and inhibition of Photosystem II.[5]

It is tempting to link this light + DABS cytochrome b-559 conversion to the postulated conformational change implicated by the extra DABS binding. Whether cytochrome b-559 redox function is normally coupled to conformation changes is not proven by these data, but the possibility is suggested. Chance and co-workers [20] have observed anomolous behaviour of mitochondrial b cytochromes and have hypothesized their dual role in electron transport and energy transduction. The DABS effect observed here on chloroplast membrane conformational changes and on cytochrome b-559 and the implication of this region of the electron transport chain in a phosphorylation site, site II of Izawa and colleagues

[10], suggests that cytochrome b-559 may be linked to membrane conformational changes necessary for energy transduction associated with photosystem II. This is only a working hypothesis since it cannot be ruled out that the potential shift of cytochrome b-559, induced by the DABS + light treatment, may be a side effect due to membrane changes that result from the extra diazo coupling promoted by electron transport.

Note added in proof: Recent experiments in this laboratory have shown that the electron transport sequence $H_2O \rightarrow$ silicomolybdate + FeCy + DCMU does not give internal acidification (assayed by NH_4^+ uptake), i.e. water protons apparently are not deposited inside under these conditions (SiMo does not induce leakiness to protons), nor does the extra DABS binding occur. Hence, it is possible that the conformational change alluded to in this manuscript is closely related to water oxidation.

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