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DIAZONIUM MODIFICATION OF PHOTOSYSTEM I

A SPECIFIC EFFECT ON IRON-SULFUR CENTER B

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Modification of chloroplast membranes with diazonium benzene sulfonate (DABS) leads to a loss of Photosystem I-dependent ferredoxin reduction but not methyl viologen reduction. EPR studies of DABS-modified membranes show no inhibition of P-700$^+$ formation at cryogenic temperatures, but iron-sulfur Center A photoreduction is markedly inhibited. Iron-sulfur Center B photoreduction at physiological temperatures in DABS-modified membranes is also markedly inhibited and little Center B can be detected after dark chemical reduction. These results indicate DABS specifically modifies iron-sulfur Center B of the spinach chloroplast Photosystem I electron acceptor complex and that Center B is obligately required for the reduction of Center A at cryogenic temperatures. Possible electron transport pathways at physiological temperatures are also considered.

Introduction

Diazonium derivatives have been used extensively to study topological organization of electron carriers in biological energy-transducing membranes [1]. The most widely used compound of this group, *p*-diazonium benzene sulfonate (DABS), is a hydrophilic reagent that reacts rapidly with a variety of amino acid side-chains under mild conditions [2,3], making it an ideal marker for surface labeling of exposed membrane proteins. DABS modification has been applied to both mitochondrial [4–6] and chloroplast [7–10] membranes in an attempt to analyze the structural organization of specific electron transport proteins.

Dilley and co-workers have reported on the effect of DABS modification of chloroplast membranes on the activities of PS II [9] and PS I [10]. In the latter case, it was found that DABS appeared to interact specifically with an electron-transfer component in the PS I electron-acceptor complex required for reduction of chloroplast ferredoxin, while photoreduction of methyl viologen was not affected by this modification. No detailed study of this site of action of DABS was presented in this work and the present report was initiated in an attempt to define the nature of the modifications produced by DABS in PS I.

The PS I electron acceptor complex is known to contain several different acceptor molecules (for a recent review, see Ref. 11). Iron-sulfur Centers A and B were first identified using low-temperature EPR techniques [12], and an additional stable electron acceptor, known as Component X, was subsequently identified [13,14]. The chemical nature of Component X is not yet resolved. More recently, two additional intermediate electron

Abbreviations: DABS, diazonium benzene sulfonate; PSI, II, Photosystem I, II; DCIP, dichlorophenol indophenol.

acceptors have been proposed to function prior to Component X. These are known as A_0 and A_1 [15,16]. The detailed role of all these centers is not yet fully understood. For example, it is not known if the iron-sulfur centers function in a linear sequence or in parallel pathways [11]. Specific modification of one center would greatly aid in functional elucidation of the role of such centers.

The studies reported in the present work indicate DABS modification of PS I specifically affects one of the bound iron-sulfur centers of the primary electron-acceptor complex (Center B), and these results are discussed in terms of the functional roles of the different bound iron-sulfur centers of PS I.

Materials and Methods

Chloroplast isolation. Chloroplasts were isolated from freshly picked greenhouse-grown spinach leaves. Approx. 50 g deveined leaves were blended for 20 s at 4°C in a Waring blender in 150 ml solution comprising 300 mM sucrose/50 mM potassium phosphate buffer (pH 7.8)/20 mM NaCl. The slurry was filtered through filtering silk and the filtrate centrifuged for 1 min at $3000 \times g$. The pellet, which contained intact chloroplasts, was resuspended in 150 ml 50 mM potassium phosphate buffer (pH 7.2)/100 mM NaCl and centrifuged for 5 min at $35000 \times g$. The resulting pellet was resuspended in a small volume of the same solution at a chlorophyll concentration of 3–4 mg/ml. The chloroplast preparation was immediately used for DABS modification.

DABS modification of chloroplast membranes. Freshly prepared solutions of DABS were prepared as described by Tinberg et al. [17]. For some experiments, this stock solution was rapidly frozen to 77 K in small aliquots and these were stored at −20°C. Aliquots were then thawed when desired and used for modification studies. No differences between freshly prepared DABS solutions and frozen aliquots have been noted.

The labeling procedure used was based on the results of Selman et al. [10]. Chloroplast membranes at a concentration of 1 mg/ml in 50 mM potassium phosphate buffer (pH 7.2)/100 mM NaCl were illuminated at 4°C with red light (Corning 2-64 filter) for 30 s. The sample was gently mixed during this period. DABS was then added to give a final concentration of 2 mM and illumination was continued for another 1 min. At this time, the chloroplasts were diluted with approx. 40 ml phosphate NaCl buffer and the chloroplasts pelleted by centrifugation at $35000 \times g$ for 5 min. The resulting pellet was washed two additional times to remove excess DABS by suspension in the above buffer, followed by centrifugation. The final pellet was resuspended in a small amount of 300 mM sucrose/50 mM potassium phosphate buffer (pH 7.8)/20 mM NaCl/1 mM EDTA at a chlorophyll concentration of 4–5 mg/ml. The inclusion of EDTA was required to remove manganese EPR signals that appeared after DABS treatment. The chloroplast membranes were frozen rapidly at 77 K in small aliquots and stored at −20°C. Control membranes were treated in an identical manner except that the DABS treatment was omitted.

Assays of photochemical activity. Assays of PS I activity were done by measuring oxygen uptake in a Rank O_2 electrode. Reaction mixtures in a volume of 2.0 ml contained 100 mM Tricine-KOH (pH 7.2), 1 μM DCMU/7.5 mM NH_4Cl/10 mM sodium ascorbate/ 0.1 mM dichlorophenolindophenol (DCIP)/1 mM NaN_3/50 μg chloroplast membranes, and when present, 25 μM methyl viologen or 100 μg chloroplast ferredoxin. Samples were illuminated with saturating orange light of intensity of $\approx 5 \cdot 10^5$ erg/cm^2 per s at 20°C.

EPR spectroscopy. EPR analyses of chloroplast membranes were done in an X-band modified JEOL spectrometer operating with 100 kHz field modulation. Chloroplast samples, at a chlorophyll concentration of 3–5 mg/ml, were dark-adapted at 4°C for 10 min prior to freezing to 77 K in calibrated 3 mm i.d. quartz tubes. EPR spectra were recorded at temperatures indicated in figure legends with spectrometer settings given in appropriate figures. Illumination at cryogenic temperatures was done using light from a 150 W tungsten-halogen source that pased through a red filter (Corning 2-64). Illumination at room temperature was done using a 100 W incandescent source. Redox potentiometry in conjunction with EPR analysis was done as previously described [18] using an anaerobic titration cell, and potentials were monitored with a platinum electrode.

Results

PS I electron transport activity. The experimental results of Fig. 1 show the PS I-dependent O_2 uptake in the presence of an autoxidizable electron-acceptor and the electron-donor system, reduced DCIP. In untreated chloroplasts, O_2 uptake is stimulated approx. 7-fold by the addition of methyl viologen and approx. 3-fold by the addition of ferredoxin. In DABS-modified membranes, rates of methyl viologen reduction were comparable to control rates while a substantial inhibition of ferredoxin reduction occurred. With anthraquinone sulfonate as electron acceptor, results were similar to those with methyl viologen in that DABS treatment produced no inhibition. In a series of experiments, ferredoxin reduction was generally inhibited 80–90% after DABS treatment in the light. These results agree with those reported by Selman et al. [10] except that these workers used NADP photoreduction in the presence of endogenous ferredoxin and ferredoxin-NADP reductase as a measure of ferredoxin reduction, while methyl viologen reduction was measured on the basis of O_2 uptake. Also in agreement with these workers, ferredoxin reduction showed a more pronounced inhibition when DABS treatment was done under illumination as opposed to in the dark. These results demonstrate that after DABS modification, PS I can still function to transfer electrons to low-potential electron acceptors but that electron transfer to the physiological electron acceptor, ferredoxin, was specifically altered.

EPR studies of PS I. In order to study the site of action of DABS action in PS I, EPR studies of reaction center components were carried out at cryogenic temperatures in control and DABS-labeled membranes.

As shown in Fig. 2, the reaction-center chlorophyll of PS I, P-700, undergoes photooxidation at cryogenic temperatures and $P\text{-}700^+$ is characterized by a light-minus-dark EPR signal with a g-value of 20026. The results of a similar experiment with DABS-modified membranes are shown in Fig. 3. In the labeled membranes, $P\text{-}700^+$ formation can also be observed at cryogenic temperatures and the extent of the photoinduced free-radical signal is comparable to that in control membranes when corrected for differences in chlorophyll concentration between these two samples. It should also be noted that no difference in the $P\text{-}700^+$ signal was observed either during or after illumination at 30 K in either case, indicating $P\text{-}700^+$ formation to be irreversible in both samples.

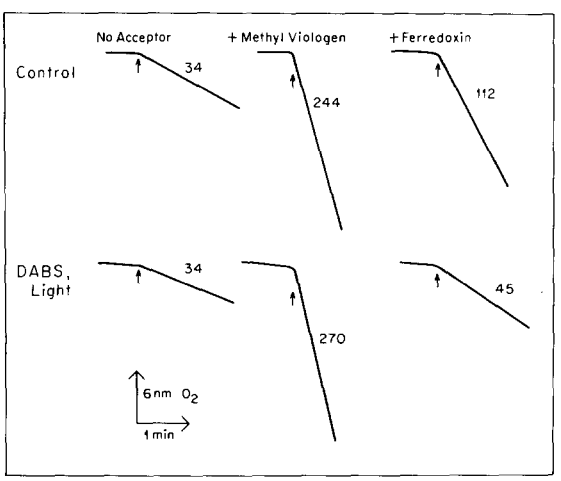

Fig. 1. PS I electron transport in DABS-modified chloroplasts. Reaction mixtures, described in Materials and Methods, were used for measurement of O_2 uptake in the absence of electron acceptor or in the presence of ferredoxin or methyl viologen. Rates next to each recorder trace are μmol O_2 taken up per mg chlorophyll per h.

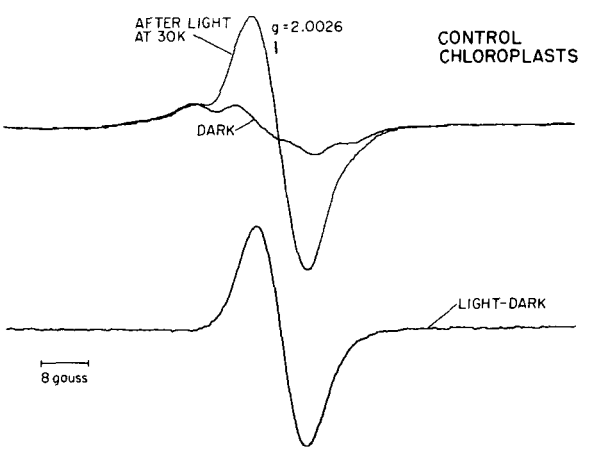

Fig. 2. P-700 photooxidation at 30 K in control chloroplast membranes. Chloroplasts (2.3 mg/ml) were incubated at 4°C in the dark in the presence of 10 mM ascorbate for 10 min prior to freezing to 77 K. After running a dark spectrum the sample was illuminated at 30 K for 30 s with red light. EPR conditions: field, 3285 ± 50 G; microwave power, 0.02 mW; modulation amplitude, 2.5 G; temperature, 30 K.

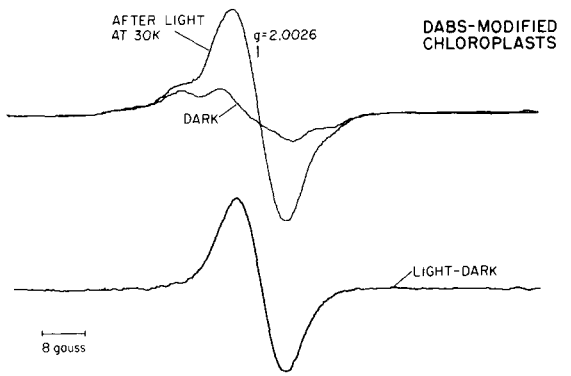

Fig. 3. P-700 photooxidation at 30 K in DABS-modified chloroplast membranes. DABS-modified chloroplasts (2.6 mg/ml) were treated as described in Fig. 2. EPR conditions were the same as in Fig. 2.

Accompanying P-700$^+$ formation at cryogenic temperatures under mildly reducing conditions (in the presence of ascorbate) is the photoreduction of a bound iron-sulfur center (Center A), characterized by EPR g values at 2.05, 1.94 and 1.86. As shown in Fig. 4, control membranes show a photoreduction of Center A as indicated by the appearance of EPR g values at 1.94 and 1.86. When the DABS-modified membranes are examined for the extent of Center A photoreduction, a significant difference over control membranes is observed (compare Figs. 5 and 4). As shown in Fig. 5, the extent of Center A photoreduction is markedly inhibited in DABS-modified membranes and in this particular experiment, based on equivalent chlorophyll concentrations. Center A photoreduction was inhibited approx. 75 percent even though P700$^+$ formation was 80% of that of the control sample. Thus, DABS-modified membranes are still able to photooxidize P-700 at cryogenic temperatures, but the electron released from P-700 does not go to Center A.

The results of Fig. 5 also document that no photoreduction of iron-sulfur Center B has occurred in the DABS-modified membrane. This center, with EPR g values of 2.05, 1.92 and 1.89, can be photoreduced at cryogenic temperatures under conditions where Center A is reduced in the dark prior to illumination at cryogenic temperatures [19] but the absence of the specific Center B g values after illumination at cryogenic temperatures indicates the absence of Center A photoreduction is not the result of an accumulation of reduced Center B.

Another possible electron acceptor in DABS-

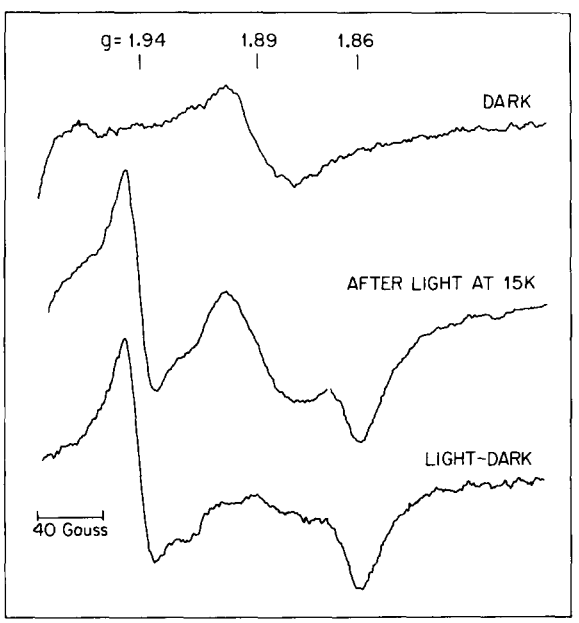

Fig. 4. Iron-sulfur center photoreduction at 15 K in control chloroplast membranes. Control chloroplasts (3.5 mg/ml) were treated as described in Fig. 2. EPR conditions: field setting, 3400 ± 250 G; microwave power, 5 mW; modulation amplitude, 10 G; temperature, 15 K.

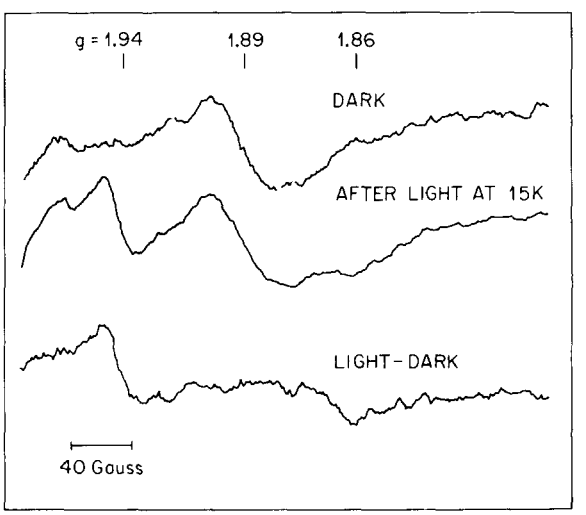

Fig. 5. Iron-sulfur center photoreduction at 15 K in DABS-modified chloroplasts. DABS-modified chloroplasts (4.5 mg/ml) were treated as described in Fig. 2. EPR conditions were as in Fig. 4.

modified membranes could be the EPR-detectable component denoted X. This carrier, with a characteristic EPR g value at approx. 1.75, has been proposed as the first stable electron acceptor in PS I [13,14]. The EPR signal of X is difficult to detect, requiring high power and extreme low temperatures (approx. 5–10 K) [20]. Under the conditions of the present EPR studies, it has not been possible to detect a photoreduction of X after illumination of DABS-modified membranes at cryogenic temperatures. However, because of the technical difficulties in detecting this signal, this negative result cannot be taken as conclusive evidence that X is not photoreduced in the modified membranes. If X acts as the electron acceptor under these conditions, its photoreduction must be irreversible, since $P\text{-}700^+$ formation is irreversible. In untreated membranes poised at low redox potentials where a photoreduction of X has been observed, a reversible back-reaction between $P\text{-}700^+$ and X^- has been observed at cryogenic temperatures [14,21].

Another experimental technique to study the PS I electron acceptor complex involves illumination of samples at physiological temperatures in the presence of different electron donors [22]. This procedure has been used to observe the EPR signals of early PS I electron acceptors [15,16,23–25] as well as those of X and Centers A and B [20,26]. As shown in Fig. 6, when membranes are treated with sodium dithionite at an alkaline pH and illuminated with strong light during freezing, the photoreduction of Centers A and B occurs (a g-value shift from 1.86 to 1.89 for Center A is observed under these illumination conditions). However, when DABS-modified membranes are treated in a similar manner, the resulting low temperature EPR signal originates primarily from Center A with g values at 2.05, 1.94 and 1.86, while there is little reduction of Center B as evidenced by the absence of prominent signals at 1.92 and 1.89. The amount of Center A photoreduced at physiological temperatures in DABS-modified membranes is approximately the same as that in control membranes.

Photoreduction at physiological temperatures has also been done using ascorbate plus dichlorophenol indophenol (DCIP) as the electron donor at pH 7.2. The results of this experiment are

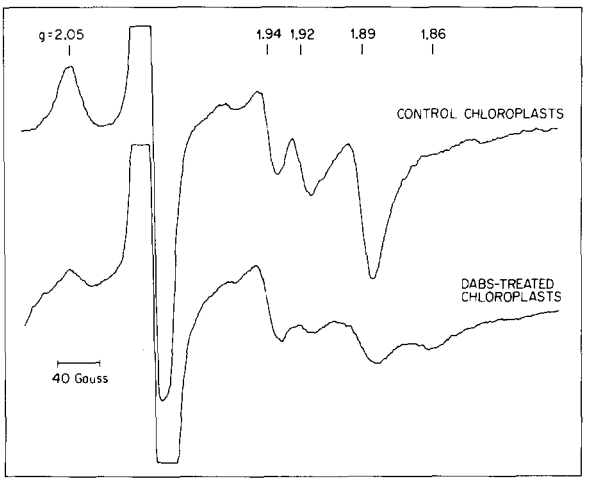

Fig. 6. Iron-sulfur center photoreduction in control and DABS-modified chloroplast membranes illuminated at 300 K. Control and DABS-modified chloroplasts (3.5 mg/ml) were incubated in 0.1 M glycine buffer (pH 11.0) in the presence of 3 mg sodium dithionite at 4°C for 10 min under N_2. Samples were illuminated for 30 s with white light prior to freezing to 77 K in the light (approx. 1 min freezing time). EPR conditions were as in Fig. 4.

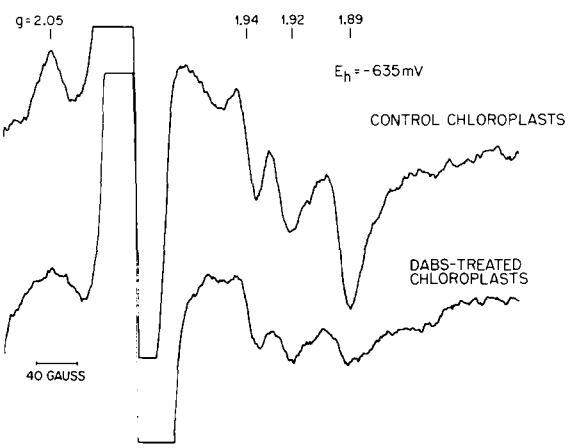

Fig. 7. Dark chemical reduction of iron-sulfur centers in control and DABS-modified chloroplast membranes. Control or DABS-modified membranes (3.5 mg/ml) were in a reaction mixture containing 0.1 M glycine buffer (pH 11), 50 μM methyl viologen and 50 μM triquat. The sample was in an anaerobic redox-titration cell and the redox potential was adjusted to −632 mV by the addition of sodium dithionite. Samples were removed under anaerobic conditions and transferred to anaerobic EPR tubes prior to freezing to 77 K. All operations were done in darkness. EPR conditions were as in Fig. 4.

similar to those shown in Fig. 6 where Center A is photoreduced with little evidence for a concomitant photoreduction of Center B.

The above-described experiments are all based on a photoreduction procedure during which the bound iron-sulfur centers of PS I are detected following illumination at either cryogenic or physiological temperatures. It is also possible to reduce Centers A and B in the dark by chemical redox titration at an alkaline pH [27,28], and the results of such a measurement with control and DABS-modified membranes are shown in Fig. 7. Both Centers A and B are almost fully reduced at a redox potential of -635 mV in control membranes, in agreement with the reported E_m values of these centers [27,28]. However, at a similar potential the DABS-modified membranes show only a substantial reduction of Center A and little reduced Center B.

Discussion

Modification of chloroplast membranes with the membrane-impermeant probe, DABS, leads to specific effects in the electron-acceptor complex of PS I. While methyl viologen reduction is unaffected, ferredoxin reduction is markedly inhibitied and this site of inhibition also results in inhibition of NADP reduction. This differential effect on soluble low-potential electron acceptors indicates the site of action of DABS is not on the electron donation side of the PS I reaction center but is specifically in the electron-acceptor complex.

EPR studies at cryogenic temperatures have localized the site of DABS action at iron-sulfur Center B. This center does not undergo appreciable photoreduction in samples illuminated at physiological temperatures and is significantly diminished in amount in samples poised at low enough redox potentials to reduce it chemically in the dark. The absence of Center B manifests itself in an inhibition of iron-sulfur Center A photoreduction at cryogenic temperatures. Under these conditions in untreated membranes, only a single electron is removed from P-700 to form $P\text{-}700^+$ in the light, and this electron passes though several electron carriers before reducing Center A. The results of the present study support a strict linear sequence of electron transfer in which Center B is reduced prior to Center A and is obligately required for Center A photoreduction at cryogenic temperatures. This result disagrees with a recent model proposed by Golbeck and Warden [29], based on an entirely different experimental approach. These workers have suggested spinach chloroplast Center A and B act as alternate electron acceptors from a common intermediate, presumably component X.

While the results obtained at cryogenic temperatures are consistent with a linear sequence of electron carriers, a similar conclusion at physiological temperatures is less definitive. Under the latter conditions, Center A can be photoreduced in samples deficient in Center B. However, the nature of the reactions at physiological temperatures differ in several important respects from those at cryogenic temperatures, since multiple turnovers of the reaction center can occur and molecular diffusion is not inhibited. Although there is no apparent mechanism by which a substoichiometric of Center B could interact with a larger amount of Center A, presumably located in different reaction centers, these experiments do not eliminate this possibility. The possible involvement of reactants related to the presence of dithionite which mediate electron transfer between reactions centers is another possibility that cannot yet be excluded. Alternatively, a pathway for Center A photoreduction might occur at physiological temperatures that is independent of Center B, and therefore the pathway of Centers A and B reduction at cryogenic and physiological temperatures might actually differ. Further experimental work at physiological temperatures is required to clarify these points.

Another important observation with DABS-modified membranes is the stable formation of $P\text{-}700^+$ in the absence of reduced Center A. Presumably, Component X^- has been formed as the reaction center partner in this reaction and while these two electron carriers have been reported to back react at cryogenic temperatures [14,21], this reaction is inhibited after DABS-modification. Direct attempts to measure X^- have not yet been successful in the DABS-modified membranes but further studies with DABS-modified enriched PS I preparations may provide direct evidence on the role of this component. An additional conclusion

from the present work is that methyl viologen can accept electrons directly from an electron acceptor, presumably X, in DABS-modified membrane while ferredoxin reduction requires both bound iron-sulfur centers in functional form in order to be reduced. This difference may be related to the relative impermeability of ferredoxin and a presumed specific binding site at or near the membrane surface.

These studies have provided an experimental tool for the specific modification of one electron carrier in the primary electron acceptor complex of spinach chloroplast PS I. In addition to providing evidence for a linear chain of electron carriers in this complex, DABS treatment may offer an approach for structural identification of the specifically labeled component, using radioactively labeled DABS to identify the modified portion of the reaction center.

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