Oligomerization States and Associations of Light-Harvesting Pigment–Protein Complexes of *Rhodobacter sphaeroides* As Analyzed by Lithium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis†

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ABSTRACT: The stability and migration behavior of bacteriochlorophyll *a*–carotenoid–protein complexes of *Rhodobacter* (formerly *Rhodopseudomonas*) *sphaeroides* in lithium dodecyl sulfate–polyacrylamide gel electrophoresis at 4 °C were examined. Yields of peripheral B800–850 and core B875 light-harvesting complexes of up to 57% and 15%, respectively, were obtained. Only B875 migrated ideally in gels of different acrylamide concentrations, and estimates of molecular weight suggested an $(\alpha\beta)_3$ structure. In mutant strain NF57, which lacks B875 and reaction centers, only bands in the position of higher B800–850 oligomers were observed. Strain M21, which lacks B800–850, yielded B875 and reaction center bands and an oligomeric series of B875 bands, which comigrated with bands of the wild type that contain the B800–850 and B875 complexes in various associations. It is proposed that these electrophoretic profiles reflect oligomerization states and associations of the pigment–protein complexes within the membrane. The sedimentation behavior of membranes of strains M21 and NF57, as well as ultrastructural studies of whole cells, suggested that B800–850 is essential for maturation of the developing intracytoplasmic membrane. Molar ratios of bacteriochlorophyll *a*–carotenoid estimated for the membranes of M21 and NF57 were 1.14 and 1.88 and were similar to those of the respective B875 and B800–850 complexes prepared from membranes of the wild type.

The intracytoplasmic membrane (ICM)[1] of the facultatively photoheterotrophic bacterium *Rhodobacter* (formerly *Rhodopseudomonas*) *sphaeroides* contains two light-harvesting pigment–protein complexes, designated B800–850 and B875 on the basis of their near-IR absorption maxima. B875 is associated with photochemical reaction centers in a constant molar ratio of ~30:1, while the amounts of B800–850 vary inversely with light intensity and can approach levels greater than 3 times those of B875 (Aagaard & Sistrom, 1972). Singlet–triplet annihilation studies (Monger & Parson, 1977) have suggested that B875, which functions as a core antenna, surrounds and interconnects reaction centers and that "lakes" of B800–850 are arranged peripherally to this core network. On the basis of recent low-temperature singlet–singlet annihilation data (Vos et al., 1986), this model has been refined such that B875 is now thought to surround and interconnect approximately four reaction centers with B800–850 arranged both peripherally and interspersed between these core units.

A detailed understanding of the structure and function of B800–850 and B875 requires the study of these complexes either isolated from the membrane or found in situ; the latter possibility arises from the availability of appropriate mutants (see below). It is possible to isolate B800–850 preparations after treatment of ICM vesicles (chromatophores) of *R. sphaeroides* with the detergents dodecyldimethylamine oxide and Triton X-100 (Clayton & Clayton, 1972; Sauer & Austin, 1978; Cogdell & Crofts, 1978). These preparations consist of α- and β-polypeptide subunits of M_r 5600 and 5850–6000, respectively, on the basis of amino acid sequences (Theiler et al., 1984).

Essentially homogeneous preparations of B875 have been obtained by an electrophoretic procedure in which *R. sphaeroides* chromatophores were treated with lithium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis in the presence of this detergent at 4 °C (Broglie et al., 1980). The isolated B875 apoprotein binds two molecules each of BChl and carotenoid, per α- and β-polypeptide subunit, which on the basis of amino acid sequences (Theiler et al., 1985) have molecular weights of 6800 and 5450, respectively. Besides B875, up to 10 additional discrete bands were observed. The band which migrated most rapidly consisted of reaction center pigments together with the reaction center L- and M-polypeptide subunits, while the bands of lowest mobility contained the majority of the light-harvesting pigments and consisted of essentially homogeneous B800–850.

The other bands observed within the gel migrated between the B800–850 and B875 bands and contained the two antennae in various associations. Fluorescence emission spectra (Hunter et al., 1981b) indicated that in several of these intermediate complexes, excitation energy was transferred from B800–850 to B875. Overall, these results suggested that (i) the B800–850 bands arise from large aggregates of this complex within the membrane, (ii) the intermediate complexes are derived from B800–850/B875 associations that exist in situ, and (iii) the B875 and reaction center complexes originate from the cores

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[1] Abbreviations: BChl, bacteriochlorophyll *a*; HPLC, high-performance liquid chromatography; ICM, intracytoplasmic membrane; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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of the photosynthetic units. This is also supported by the sequence in which the various complexes are assembled as characterized by lithium dodecyl sulfate electrophoresis (Hunter et al., 1982; Reilly et al., 1986).

A more detailed electrophoretic analysis of the pigment–protein complexes of *R. sphaeroides* is presented here. Membranes of *R. sphaeroides* mutant strains that lack either B800–850 or B875 and reaction centers (Ashby et al., 1987; C. N. Hunter, submitted for publication) were also examined. The results have provided an assessment of the stability and oligomerization states of the complexes in these gels. In addition, their pigment composition has been examined further. Possible implications of these findings for the structural organization and development of the ICM are discussed.

Materials and Methods

R. sphaeroides wild-type strain NCIB 8253 was grown photoheterotrophically at 30 °C under various light intensities as described by Holmes et al. (1980). Mutants M21 and NF57 were isolated following mutagenesis of strain 8253 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Ashby et al., 1987; C. N. Hunter, submitted for publication). Mutant M21 is capable of photosynthetic growth but lacks the B800–850 complex while mutant NF57 lacks the B875 and reaction center proteins. The transfer of B800–850 (*puc*) genes to strain M21 restored the B800–850 spectrum (Ashby et al., 1987), while photosynthetic growth was restored to strain NF57 with a plasmid containing the reaction center H subunit (*puh*) gene but not with the *puf* gene cluster coding for B875 and the reaction center L and M subunits (C. N. Hunter, submitted for publication). To induce formation of BChl-containing membranes, strain NF57 and, where indicated, M21 and wild type were grown semiaerobically for 24–48 h at 200 rpm on a gyratory shaker in flasks filled to 80% volume. Pigmented membrane fractions were prepared by direct rate–zone sedimentation of French pressure cell extracts on sucrose density gradients essentially as described by Niederman et al. (1979).

Lithium dodecyl sulfate–polyacrylamide gel electrophoresis was performed at 4 °C as described by Broglie et al. (1980). This procedure maintains pigment–protein interactions, and it has been suggested from coelectrophoresis studies (Hunter et al., 1982; Pennoyer, 1984) that no pigment exchange occurs and that the isolated complexes arise from pigment–protein associations that exist within the membrane. In some experiments, the complexes were purified by a second lithium dodecyl sulfate electrophoresis on a separating gel prepared with a 7.5–15% acrylamide gradient as described by Broglie et al. (1980). For assessment of polypeptide composition, doubly purified complexes were solubilized at 60 °C for 10 min in 2.0% (w/v) sodium dodecyl sulfate as described by Broglie and Niederman (1979) and subjected to polyacrylamide gel electrophoresis in a Bio-Rad minigel apparatus using the high-molarity Tris buffer system of Fling and Gregerson (1986). The resolving gel was formed with an exponential gradient of 8–18% acrylamide in 0.75 M Tris buffer, pH 8.85, as described by Smith and Bell (1986) except that glycerol was omitted. The stacking gel was prepared by using 4% acrylamide, and 0.1% sodium dodecyl sulfate was present in both resolving and stacking gel buffers. The upper reservoir buffer consisted of 0.05 M Tris, 0.37 M glycine, pH 8.3, and 0.1% sodium dodecyl sulfate.

The mobilities of the pigment–protein complexes in lithium dodecyl sulfate–polyacrylamide gel electrophoresis were assessed by Ferguson plot analysis (Ferguson, 1964; Frank & Rodbard, 1975) which is based upon the linear relationship given by $\log R_f = \log Y_0 - K_R T$ where R_f is the mobility of the protein species relative to that of the bromphenol blue dye front as measured by scanning the gels at 375 nm, Y_0 is the free electrophoretic mobility (y intercept), K_R is the retardation coefficient (slope), and T is the gel concentration (percent w/v) of acrylamide plus *N*,*N'*-methylenebis(acrylamide); these were maintained in a (w/w) ratio of 30:0.8. Gels were prepared at different T values, and the migration of the pigment–protein complexes was compared with that of soluble protein standards of known molecular weight and ideal electrophoretic behavior.

Apparent molecular weight values for the pigment–protein complexes were estimated from plots of R_f vs $\log M_r$ obtained with the protein standards at appropriate values of T. These plots fit a sigmoidal function, essentially as described by Frank and Rodbard (1975), in which $R_f = a/[1 + (M_r/10^b)^{1/c}]$ where a = the maximal R_f, $b = \log M_r$ at R_f 0.5 that of a, and c is related to slope. Very similar apparent molecular weight values were obtained by fitting these plots by polynomial least-squares regression with a polynomial of degree 2.

Near-IR absorption spectra were obtained at 295 K on a Johnson Research Foundation DBS-3 spectrophotometer equipped with a silicon photodiode detector; spectra of carotenoid bands were also obtained on a Perkin-Elmer Lambda 3B spectrophotometer. BChl and carotenoid were extracted and their concentrations determined by procedures described by Broglie et al. (1980), using the extinction coefficients for carotenoids and corrections for spectral crossover of Cohen-Bazire et al. (1957). Carotenoids were also separated in paper chromatography (Foppen, 1971) and by TLC (Cogdell et al., 1976) and HPLC (Wright & Shearer, 1984) procedures. About 15% conversion of spheroidene to spheroidenone usually occurred during the extraction procedure of Cohen-Bazire et al. (1957), and it was necessary to correct for recoveries (~73%) to obtain total carotenoid concentrations by the TLC procedure.

For electron microscopy, cells were fixed for 10 min at 25 °C in a solution of 1% (v/v) glutaraldehyde and 1% OsO_4 in 0.05 M sodium phosphate buffer, pH 6.8 (Kiss et al., 1987), followed by thorough rinsing with buffer, postfixation for 60 min with 1% OsO_4 prepared in phosphate buffer, and a distilled H_2O rinse. This was followed by dehydration through an ethanol series and absolute acetone and infiltration and embedding in Epon. Samples were sectioned with a Sorvall MT2-B ultramicrotome and poststained with 2% aqueous uranyl acetate and lead citrate. Electron micrographs were obtained with a Philips 300 electron microscope.

Results

Isolation and Characterization of Membranes from Mutant Strains. The nature and purity of membrane fractions are important factors in the separation of pigment–protein complexes in lithium dodecyl sulfate–polyacrylamide gel electrophoresis. Therefore, the rate–zone sedimentation profile of pigmented membranes from the mutant strains was determined (Figure 1). In strain NF57, as in the wild-type parent strain, photosynthetic pigments were observed mainly in ICM-derived chromatophores and in an upper pigmented band; the latter fraction is thought to arise in part from membrane sites in early stages of ICM development (Hunter et al., 1982; Bowyer et al., 1985; Reilly et al., 1986). In contrast, in strain M21, most of the photopigment was present in the upper band. These results suggest that sufficient B800–850 must be assembled within the growing membrane before the ICM has developed to a morphological stage that gives rise to discrete chromatophore vesicles when the cells are disrupted.

In order to relate the sedimentation behavior of the membranes to the morphology of internal membranes within the

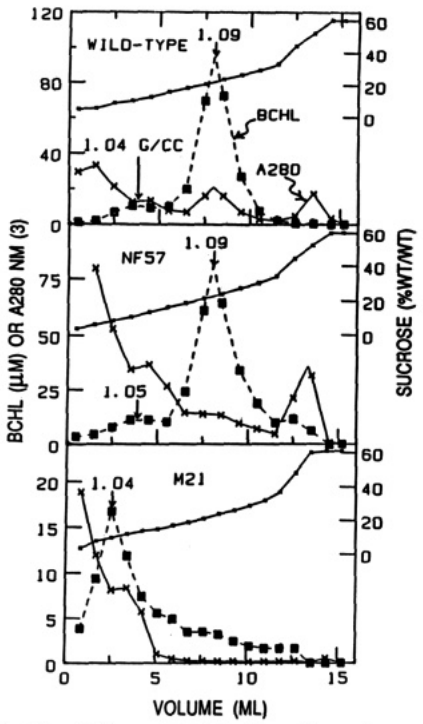

FIGURE 1: Profile of pigment distribution after rate–zone sedimentation. Cell-free extracts were layered on a 5–35% (w/w) sucrose gradient prepared over a 1.3-mL cushion of 60% sucrose and centrifuged at 4 °C for 105 min in a Beckman SW40Ti rotor at 40000 rpm. The density of each gradient fraction was determined on a refractometer; values are shown for pigmented fractions (top to bottom): upper pigmented band, chromatophores. Some pigment was also observed with the cell envelope at 60% sucrose interface. The mutant strains were grown semiaerobically.

cell, thin sections of whole cells were examined in an electron microscope (Figure 2). Vesicular ICM, typical of that in the wild type, was observed in mutant NF57, whereas in M21, the internal membranes were mainly tubular. These large tubular structures were sometimes observed running between two M21 cells that appeared to be arrested in cell division (not shown). The observed ultrastructural differences suggest that, in the absence of B800–850, morphogenesis of internal membranes is incomplete and is arrested at a tubular stage.

With regard to the chemical composition of the isolated and purified membrane fractions, phospholipid:protein ratios were found to be dependent upon the conditions and state of growth. The phospholipid:protein ratios (w/w) of the upper pigmented bands from the mutant strains NF57 and M21 were consistent with values determined previously for wild type (Reilly et al., 1986) and were up to 2-fold higher than for wild-type chromatophores.

Stability and Distribution of Pigment–Protein Complexes in Lithium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The effects of different lithium dodecyl sulfate levels in the solubilization buffer on the yields of the complexes were examined (Figure 3). With chromatophores from *R. sphaeroides* wild type, marked differences in the profiles of detergent-mediated release and stability of the light-harvesting and reaction center proteins were observed. The yields of B800–850 increased with increasing detergent concentration while those of B875 and reaction center were maximal over a narrow range of lower concentrations. For studies with chromatophores from the wild type, a lithium dodecyl sulfate:BChl ratio of 20:1 (w/w) was typically used.

Membranes isolated from the mutant strains required higher lithium dodecyl sulfate:BChl ratios for optimal solubilization (Figure 4). With chromatophores of NF57, intermediate

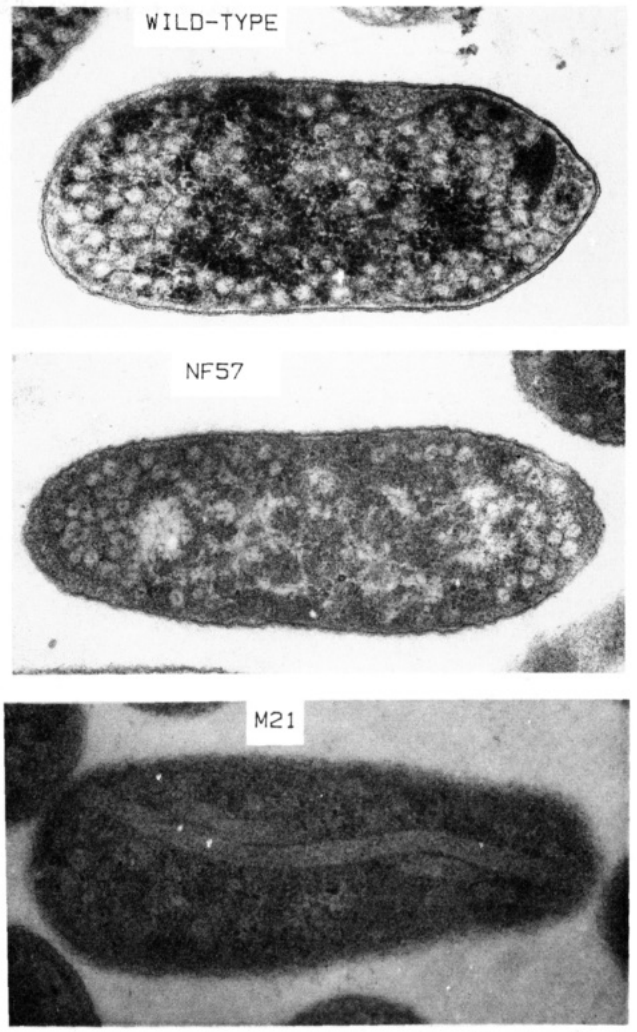

FIGURE 2: Electron micrographs of a thin section of semiaerobically grown cells. Magnification 50400×.

complexes were not observed, and virtually all pigment was present in B800–850 bands I–III, which migrated to essentially the same positions as those observed for the wild type. In contrast to NF57, with the pigmented membranes of M21, the B800–850 triad of bands was absent; the reaction center and two B875 bands were clearly resolved, but the majority of the B875 was present in a series of higher aggregates which banded in the positions of the intermediate complexes. With gels of wild-type membranes, subtraction of the B800–850 content from the intermediate complexes revealed a B875 distribution similar to that of M21 (not shown). It is noteworthy that when membranes of strains NF57 and M21 were mixed together and subjected to lithium dodecyl sulfate electrophoresis, a composite banding pattern was observed. Absorption spectra confirmed that the respective B800–850 and B875 bands were not intermixed and that intermediate complexes were not formed (not shown).

With chromatophores isolated from wild-type cells grown at a moderate light intensity (11000 lx), the combined yield of B800–850 and B875 complexes from bands within the gel approached 60%. Of the B800–850, >50% was present in the major bands (Table I); these were essentially homogeneous on the basis of absorption spectra at 295 K. Most of the remaining B800–850 was present in intermediate complexes 2–4. These intermediate complexes contained ~20% of the B875 with 15% present in the most homogeneous B875 bands (bands 5–8). In such B875 bands, a purity level of ~90% on a protein basis can be achieved by a second lithium dodecyl

Table I: Recovery of Pigment–Protein Complexes in Lithium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis[a]

band	identity	B800–850[b] (nmol)	B875[b] (nmol)	B800–850/B875	recovery (%) B800–850	recovery (%) B875
	initial preparation	703	622	1.13	100.0	100.0
1	B800–850[c]	403	14.2	28.4	57.3	2.3
2–4	intermediate complexes	89.3	128	0.70	12.7	20.6
5–8	B875	21.4	92.4	0.23	3.0	14.9
9	reaction center				(0.6)[d]	
10	free pigment[e]				nd[f]	
	excluded material	51.2	16.7	3.06	7.3	2.7
	total	565	251	2.25	80.4	40.4

[a] Chromatophore preparation from wild-type strain NCIB 8253 grown at 11 000 lx. [b] Molar levels of B800–850 and B875 calculated by using extinction coefficients of Clayton and Clayton (1981) after computer-assisted correction for crossover of near-IR absorption bands. [c] Major B800–850 bands. [d] Recovery as percent total pigment–proteins. [e] Material at gel front; absorbance spectra showed maximum near 775 nm and shoulders near 740 and 690 nm, and carotenoid peaks shifted ~14 nm to the blue. [f] Not determined; recoveries of radioactivity in free pigment zone at gel front were 14% and 38%, respectively, with chromatophores prepared from δ-aminolevulinate-requiring mutant H-5 (Lascelles & Altshuler, 1969) grown in the presence of δ-[3,5-^{3}H]aminolevulinate at 1800 and 5400 lx.

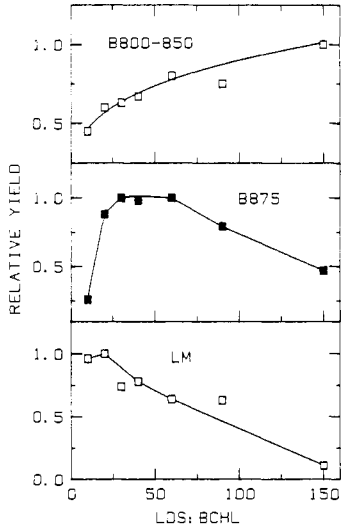

FIGURE 3: Yields of pigment–protein complexes after solubilization of chromatophores at 4 °C with different lithium dodecyl sulfate concentrations. Chromatophores (40 μg of BChl) were isolated from cells grown at 1800 lx, treated at the indicated lithium dodecyl sulfate:BChl (w/w) ratios, and subjected to lithium dodecyl sulfate electrophoresis at 4 °C on a gel prepared with 7.5% acrylamide. The B800–850, B875, and reaction center bands were excised from the gel, and concentrations were determined in situ from their absorbance at 850, 875, and 800 nm, respectively. The maximal yield for each complex was taken arbitrarily as 1.0. The actual yields as a function of amount of chromatophores applied to the gel are presented in Table I. LM, reaction center particle consisting of L- and M-polypeptide subunits (Broglie et al., 1980).

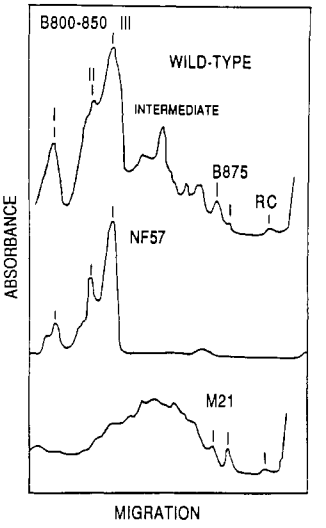

FIGURE 4: Lithium dodecyl sulfate gel electrophoresis of membranes from wild-type and mutant strains. Electrophoresis was performed at 4 °C on a gel prepared with 7.5% acrylamide that was 0.75 mm thick. For membrane solubilization, lithium dodecyl sulfate:BChl (w/w) ratios were as follows: wild type, 20:1; NF57, 30:1; M21, 40:1. Amounts of protein applied to the gel were the following: wild type, 0.71 mg; NF57, 0.75 mg; M21, 2.0 mg; each lane received 50 μg of BChl. The gel was xeroxed onto a transparency and scanned with a red filter in an Ortec Model 4310 scanning densitometer; a similar banding pattern was obtained when the wet gel was scanned at 375 nm. The material at the gel front was free pigment. Intermediate, complexes of intermediate migration between those of B800–850 and B875 containing various levels of these complexes. Note the absence of B875 and reaction center bands in strain NF57 and the lack of the typical B800–850 bands in M21. In denaturing sodium dodecyl sulfate gel electrophoresis, reaction center and B875 polypeptides were absent from NF57 membranes, and B800–850 polypeptides appeared to be absent from M21 (D. Farrelly and R. A. Niederman, unpublished experiments).

sulfate electrophoresis (Theiler et al., 1985; see also Figure 5); absorption and excitation spectra at 77 and 4 K indicated no apparent cross-contamination in these preparations (Kramer et al., 1984a) or in B800–850 preparations purified by two electrophoretic cycles (H. J. M. Kramer, J. D. Pennoyer, J. Amesz, and R. A. Niederman, unpublished experiments). Table I also demonstrates that ~7% of the B800–850 was excluded from the gel which apparently represents higher aggregation states of this complex (see below).

The yields of both light-harvesting complexes were greater with chromatophores from the cells grown at the higher light intensity than with those from cells grown at low illumination levels (1800 lx). In the latter preparations (B800–850:B875 molar ratio = 2.3), the total yield of pigment–protein complexes within the gel was ~35% with 25% of the B800–850 in the major bands and 8% of the B875 recoverable in the most B875-enriched bands. In addition, nearly 25% of the B800–850 existed as B800–850-enriched higher aggregates that failed to enter the gel. Solubilization at increased concentrations of lithium dodecyl sulfate resulted in decreased levels of these aggregates and increased levels of the major B800–850 bands. The phospholipid composition of this excluded material was similar to that of the major B800–850 complex (Pennoyer, 1984); this included an enrichment of ~6-fold in the minor phospholipid cardiolipin over that of chromatophores or bulk phospholipid at the gel front.

Although it was possible to separate pigment–protein complexes after a single lithium dodecyl sulfate gel electrophoresis, denaturing sodium dodecyl sulfate electrophoresis revealed contaminating polypeptides; most of these were removed from the intact complexes by a second lithium dodecyl sulfate electrophoresis on a gradient gel. Figure 5 shows that increasing levels of B875 protein concomitant with decreased B800–850 protein levels were present in those intermediate complexes of higher electrophoretic mobility which correlated

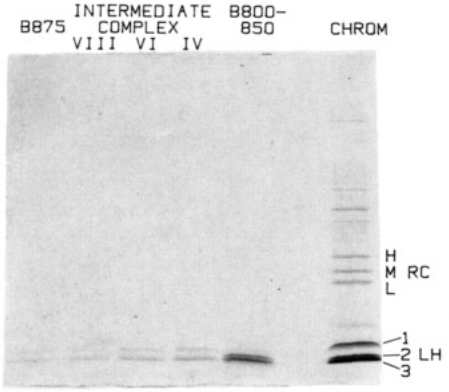

FIGURE 5: Polypeptide composition of doubly purified pigment–protein complexes isolated from chromatophores of wild-type cells grown at 1800 lx. Two cycles of lithium dodecyl sulfate electrophoresis were performed as described by Broglie et al. (1980) except that complexes eluted from the first gel in 10 mM Tris buffer, pH 8.0, were concentrated and transferred to 62.5 mM Tris buffer, pH 6.8, containing 20% (v/v) glycerol and applied to a second gel. Excised, doubly purified complexes were eluted, concentrated, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a minigel apparatus at 25 °C and stained with Coomassie brilliant blue. CHROM, chromatophores subjected directly to sodium dodecyl sulfate electrophoresis; B800–850, B800–850 band III; B875, B875 band I; RC-H, -M, and -L, reaction center polypeptide subunits migrating with apparent molecular weights near 28 000, 24 000, and 21 000; LH-1 and -2, α-polypeptide subunits of B875 and B800–850, respectively; LH-3, a composite band formed by β-polypeptides of B875 and B800–850 (LH-1, -2, and -3 migrate with apparent molecular weights near 12 000, 10 000, and 8000, respectively). Note higher levels of LH-3, relative to other LH bands, in chromatophores and in intermediate complexes IV–VIII. Silver staining of the gel revealed low levels of impurities of higher molecular weight, but the LH-2 band was stained poorly.

with their B875/B800–850 contents as observed in near-IR absorption spectra (Broglie et al., 1980). The various complexes, including the intermediate complexes, migrated to the same relative positions in the second lithium dodecyl sulfate gel, suggesting that impurities from the first gel did not affect electrophoretic mobility. Futhermore, addition of a cytoplasmic membrane fraction to chromatophores had no effect upon the profile of pigmented complexes (not shown), and the electrophoretic mobility of the reaction center LM band was unaffected when wild-type chromatophores were mixed with those of a mutant lacking reaction centers (Pennoyer, 1984).

Apparent Sizes of Pigment–Protein Complexes in Lithium Dodecyl Sulfate Gels. The sizes and aggregation states of the pigment–protein complexes were assessed from their mobility of gels prepared with different acrylamide concentrations. Lithium dodecyl sulfate treated chromatophores and protein standards of known molecular weight were applied to gels prepared with five acrylamide concentrations (T) ranging from 5% to 13%. By plotting the logarithm of R_f vs T (Banker & Cotman, 1972), it was possible to assess both the migration behavior and relative sizes of the migrating species. This was especially pertinent because of the demonstration that pigment–protein complexes isolated from chloroplasts migrate anomalously in lithium dodecyl sulfate–polyacrylamide gel electrophoresis (Delepelaire & Chua, 1981).

In plots of mobility vs gel concentration (not shown),[2] the intercept at $T = 0$ (Y_0) was essentially the same for each of the soluble protein standards, consistent with ideal migration during electrophoresis at 4 °C. Of the pigment–protein complexes, only the B875 band of higher mobility (B875-II) migrated with ideal electrophoretic behavior as indicated by a Y_0 comparable to that of the standards. Elevated Y_0 values were obtained with B875 band I, the LM particle, and with each of the B800–850 bands, indicating that they migrated anomalously. Using Ferguson plots for the reaction center (Williams, 1986) or gradient gels of appropriate acrylamide concentrations for light-harvesting proteins (Picorel et al., 1983), it has been possible to obtain apparent molecular weight values in reasonable agreement with molecular weights derived from amino acid sequences, even though anomalous migration has been observed with these apoproteins. Although it is not known whether the intact complexes and the denatured apoprotein components bind dodecyl sulfate in a similar manner, the method for determination of the oligomerization states of the various bands in the lithium dodecyl sulfate gels uses actual protomer molecular weights.

Table II: Apparent Sizes and Oligomerization States of Pigment–Protein Complexes As Estimated from Electrophoretic Migration

complex	T (%)	apparent M_r ($\times 10^{-3}$)[a]	oligomerization state[b]
B800–850			
I[c]	5, 7	251 ± 31 (248)	~14
II	5, 7	160 ± 6 (170)	~9
III	5, 7, 9	145 ± 6 (136)	~8
B875			
I	7, 9, 11, 13	54.6 ± 4.4 (52.6)	~4
II	7, 9, 11	48.0 ± 3.0 (48.0)	3.2
reaction center	9, 11, 13	44.7 ± 5.3 (43.8)	~1

[a] Obtained for complexes of wild-type strain NCIB 8253 from plots of R_f vs log M_r for protein standards at indicated acrylamide concentrations. Numbers in parentheses estimated from migration of complexes in gel prepared with a gradient of 5–15% acrylamide; standards fitted by least-squares regression with a polynomial of degree 2 (correlation coefficient 0.9998 and standard error of estimate 0.0046). [b] Monomer molecular weights for B800–850 and B875 were 17 400 and 15 200, respectively, on the basis of amino acid sequences of polypeptide subunits plus pigment constituents; B800–850 also included three molecules of phospholipid per complex (Radcliffe et al., 1984); this consisted of phosphatidylglycerol, 36%; phosphatidylcholine, 21%; phosphatidyl ethanolamine, 34%; and cardiolipin, 5% (Pennoyer, 1984). The molecular weight value of 72 850 used for the reaction center included sequence-derived molecular weights of L and M subunits and molecular weights of prosthetic groups (Williams, 1986). [c] Bands designated in order of increasing electrophoretic mobility (Figure 3); band III represents the major B800–850 band.

In Table II, apparent sizes of the pigment–protein complexes calculated from their electrophoretic migration are presented. Plots of R_f vs log M_r (not shown) for the protein standards fit well to the symmetrical sigmoidal function described under Materials and Methods. Because of the similarity between the electrophoretic behavior of B875 band II and that of the standards, a reliable estimate of the apparent molecular weight (48 000 ± 3000) of this band was obtained. This complex also yielded a molecular weight value of 47 300 from a plot of K_R vs M_r (Frank & Rodbard, 1975). The apparent molecular weight values obtained for the B800–850 and reaction center bands from plots of R_f vs M_r showed a systematic increase as the acrylamide concentration was increased, while apparent molecular weights obtained for B800–850-III, B875-I, and the LM particle (184 000, 70 000, and 89 000, respectively) from K_R vs M_r plots were widely disparate from those obtained from the R_f data presented in Table II. Thus, it was possible only to estimate the oligomerization state for B875 band II which appeared to migrate as a trimer of α- and β-polypeptides, $(\alpha\beta)_3$, containing six BChl and six carotenoid molecules.

Several of the intermediate complexes which contained both of the light-harvesting antennae migrated in gels of $T = 7.5\%$

[2] In these plots, points were fitted by linear regression analysis; correlation coefficients >0.988. B800–850 bands I and II deviated from linearity at the higher gel concentrations, and points at $T > 9\%$ were neglected in their fits.

Table III: Pigment Composition of Membrane and Pigment–Protein Preparations[a]

	BChl/total carotenoid (mol/mol)	spheroidene/ spheroidenone (mol/mol)
membranes		
wild-type[b]	1.54	6.1
NF57[c]	1.88	0.72
M21[b]	1.14	0.51
B800–850[d,e]	1.84 ± 0.19	6.4 ± 3.2
B875[d,e]	1.05 ± 0.12	1.04 ± 0.42
reaction center[d]	3.45	1.26
B800–850[f]	2.17	11.5

[a] Complexes eluted from gels and pigments extracted and determined as described by Broglie et al. (1980). [b] Chromatophores from wild-type strain 8253 and upper pigmented band from strain M21 grown at 1800 lx. [c] Grown semiaerobically; most spheroidene was oxidized to spheroidenone. [d] Prepared from strain 8253 by lithium dodecyl sulfate gel electrophoresis. [e] Mean ± SD for at least six preparations. [f] From Broglie (1980); prepared as described by Clayton and Clayton (1972).

with apparent molecular weights of 75 000–115 000. Bands in the positions of each of these intermediate complexes were observed for the B875 complex in mutant M21 which lacks B800–850, but not in mutant NF57 which contained only B800–850 (Figure 4).

Carotenoid Composition of Isolated Light-Harvesting Complexes. Previous analyses of the pigment composition of the light-harvesting proteins isolated from *R. sphaeroides* have suggested that per complex of α- and β-polypeptide subunits, B800–850 contains 3 BChl and 1 (Cogdell & Crofts, 1978) to 1.5 (Hunter et al., 1981a; Radcliffe et al., 1984) carotenoid molecules, while values of 2 BChl and 2 carotenoid molecules have been reported for B875 (Broglie et al., 1980). From studies in membrane preparations, a 2:1 ratio of BChl to carotenoid was predicted for B800–850 (Sistrom, 1978). In addition, the carotenoid absorbance maxima for the isolated complexes differed; those of B875 were located at wavelengths ~5–6 nm shorter than those of B800–850 (Holmes et al., 1980). Isolated preparations of B875 were also observed to exhibit a more reddish color than B800–850 preparations.

R. sphaeroides wild-type strains contain two distinct spectral forms of carotenoid; these are red and yellow aliphatic carotenoids of the spheroidenone and spheroidene types, respectively (Cohen-Bazire et al., 1957). Marked differences were observed in the ratios of these carotenoid types in the isolated B875 and B800–850 complexes (Table III). The B875 preparations were enriched ~5–10-fold in spheroidenone in comparison with the B800–850 preparations; the spheroidenone content of B875 was also observed to be higher for membranes of mutant M21 which lacks B800–850 than for the wild type grown under the same conditions. Thus, B875 carotenoids may be more susceptible to direct oxidation in air or, alternatively, by an enzymatic mechanism which catalyzes the conversion of spheroidene to spheroidenone that may be associated with B875 domains within the membrane.

It is also noteworthy that the BChl:total carotenoid molar ratio was near 1.0 in isolated B875 preparations and approached 2.0 in the B800–850 complex isolated by the electrophoretic procedure (Table III). These data were confirmed in membranes of mutants M21 and NF57 where ratios similar to those of the respective B875 and B800–850 preparations were observed. The BChl:carotenoid molar ratio near 2.0 for B800–850 not only corroborates our previous observations but also provides further support for structural models for this complex that were based upon spectroscopic properties (Kramer et al., 1984b). Some losses of the B800 component occurred from B800–850 isolated in the presence of lithium dodecyl sulfate. Although this might account for the somewhat lower BChl levels observed in comparison to the B800–850 preparation isolated in the presence of dodecyldimethylamine oxide (Table III), the B800 BChl is not lost, since the B800 band can be reconstituted by treatment with the latter detergent (Clayton & Clayton, 1981; Kramer et al., 1984b).

Table IV: Carotenoid Absorbance Maxima in Membrane and Pigment–Protein Preparations from *R. sphaeroides* Mutant Strains

	carotenoid absorbance maxima (nm)
membranes	
Ga[a]	432, 458, 490
8253G[b]	430, 456, 487
NF57G[b]	430, 457, 489
M21G[b]	424, 454, 485
B800–850[c]	432, 458, 490
B875[c]	429, 452, 484

[a] Green mutant derived from wild-type strain 2.4.1 by Cohen-Bazire et al. (1957). [b] Green derivatives of respective strains grown semiaerobically; strains 8253G and M21G showed essentially the same respective carotenoid absorbance maxima when grown phototrophically. Paper chromatography and HPLC analysis indicated that these mutants contained a carotenoid composition similar to that of strain Ga (Scolnik et al., 1980a). [c] Prepared from strain Ga by lithium dodecyl sulfate gel electrophoresis.

The differences in the positions of the carotenoid absorbance maxima in the isolated B800–850 and B875 preparations were not due to the observed differences in carotenoid composition. This was shown with light-harvesting complexes isolated from *R. sphaeroides* Ga, a green strain which contains only a single spectral form of carotenoid of the neurosporene family (Table IV). The carotenoid absorbance maxima in the B875 preparations were at wavelengths 3–6 nm shorter than in the isolated B800–850 preparations. These maxima coincided closely with the in vivo absorbance maxima in membranes of green derivatives of the mutants which contain only a single light-harvesting complex (Table IV). These results, together with similar studies of Scolnik et al. (1980b) in mutant strains of *Rhodobacter capsulatus*, suggest that the carotenoid spectral differences are not artifacts of lithium dodecyl sulfate–polyacrylamide gel electrophoresis. Instead, these differences reside in the B800–850 and B875 apoproteins which provide distinct environments for carotenoid associations.

Discussion

The use of mutant strains which lack light-harvesting and reaction center complexes has provided a more complete understanding of the associations of these complexes as well as their apparent role in development and maturation of vesicular ICM. Ultrastructural and sedimentation analyses presented here suggest that mainly tubular internal membranes are formed in mutant M21. The possibility that this is due to the inability of this strain to form the B800–850 protein is supported further by studies of a partial revertant of strain M21 which produced low levels of B800–850 (J. N. Sturgis and R. A. Niederman, unpublished results). It was possible to isolate small amounts of chromatophores from the revertant cells in which B800–850 was revealed by subtraction of the absorption spectrum of this fraction from that of the upper pigmented band. In thin sections of the revertant cells, both tubular and vesicular internal membranes were observed. The presence of vesicular ICM in mutant NF57, which produces B800–850 but lacks the B875 and reaction center proteins, indicates that B800–850 is both necessary and sufficient for the formation of this structure and that the membrane maturation process can be completed in the absence of B875–reaction center core particles. Overall, these results are consistent with the pos-

sibility that in the absence of B800–850, morphogenesis of internal membranes is incomplete and is arrested at a tubular stage. Development of ICM to the stage which gives rise to discrete chromatophore vesicles upon cell disruption appears to require the B800–850 protein. Either this protein has specific properties necessary for these morphological changes or the changes ultimately result from an increased protein:lipid ratio.

In other ultrastructural studies of *R. sphaeroides*, tubular internal membranes were observed during early stages of induction of ICM formation under anaerobic conditions in the light (Chory et al., 1984). In contrast, both large lamellar and vesicular ICM were demonstrated in the R26 carotenoidless mutant (Lommen & Takemoto, 1978), while Sprague and Varga (1986) have noted that in a mutant which lacked both B800–850 and carotenoids, tubular as well as vesicular internal membranes were seen. The R26 mutant has been shown to have multiple defects (Davidson & Cogdell, 1981; Theiler et al., 1984, 1985), and a role for carotenoids in the development of the ICM remains to be established. Since genetic, spectral, and biochemical evidence suggests that the M21 mutant is defective only in the *puc* genes, it appears that in this strain it is solely the absence of the B800–850 complex from the membrane which causes accumulation of tubular structures.

When membranes of mutant M21 were subjected to lithium dodecyl sulfate electrophoresis, much of the B875 was present in complexes of higher oligomerization states. This is not surprising in view of recent singlet–singlet annihilation measurements; this procedure provides an estimate of domain sizes, i.e., the number of functionally interconnected antenna BChl molecules (Vos et al., 1986). Domains of ~330 B875 BChl's were estimated in membranes of strain M21, which approximates to 3 B875–reaction center core structures, containing about 100 B875 BChl's in close association (Vos et al., 1987). The B875 multimers seen in annihilation studies of M21 membranes are not observed in the wild type, since the B875–reaction center core structures that are closely associated in the mutant are thought to be separated by B800–850 units in wild-type membranes (Vos et al., 1986).

The oligomeric complexes in lithium dodecyl sulfate gels of M21 membranes banded in the position of the complexes of intermediate migration in the wild type which contain B800–850 and B875 in various associations. The possibility that intermediate bands in the wild type arise from associations that exist within the membrane was suggested by their absence after reelectrophoresis of mixtures of B800–850 and B875 (Hunter et al., 1982) as well as evidence that in several of these bands excitation energy was transferred from B800–850 to B875 (Hunter et al., 1981b). Therefore, the different pigment–protein aggregates contained within the detergent micelles and separated by the electrophoretic procedure may represent various portions of the assembled complexes within the membrane.

Radiolabeling studies by Hunter et al. (1982) and Reilly et al. (1986) have demonstrated a preponderance of reaction center, B875, and intermediate complexes during early stages of assembly of photosynthetic units, followed by subsequent appearance of the major B800–850 oligomers. Since a family of oligomeric B875 complexes was observed in mutant M21, it is possible that during formation of photosynthetic units, B800–850 associates initially with higher B875 oligomers at the peripheries of previously assembled core particles. The lower B875 bands, as well as isolated reaction center particles, may arise from inner core regions. A requirement for preassembled B875 oligomers for formation of intermediate complexes is also supported by results with mutant NF57 in which intermediate bands were absent and virtually all B800–850 was present in higher oligomers. These higher B800–850 oligomers may arise from the "lakes" of this complex at peripheries of the photosynthetic units which in NF57 consist of domains of ~365 B850 BChl's (Vos et al., 1987). In the wild type, excitation energy from the large B800–850 assemblies may be transferred to the B875 core structures through sites of B800–850/B875 association. The data of Vos et al. (1986) were explained by two to three such contact sites per B800–850 complex. The existence of oligomeric B800–850 complexes, as well as B875-associated B800–850, appears to be necessary for energy transfer from more peripheral B800–850 to B875–reaction center units as well as between such units. Both in developing photosynthetic membranes which lacked a full complement of B800–850 (Hunter et al., 1985) and in phospholipid-enriched membranes in which B800–850 multimers were dissociated from the core particles (Westerhuis et al., 1986), excitations were confined to the individual core units. In addition, an obligatory role for B875 in exciation energy transfer from B800–850 to the reaction center has been suggested from studies in a *R. sphaeroides* mutant strain that lacked B875 (Meinhardt et al., 1985). In this strain, only limited energy transfer between B800–850 and the reaction center was demonstrated.

This overall supramolecular organization of bacterial photosynthetic units is also supported by freeze–fracture studies in *Rhodopseudomonas palustris* which suggested that subunits of discrete size were added to core particles in membranes of cells grown at different light intensities (Varga & Staehelin, 1983). A similar trend in diameters of intramembrane particles was reported in *R. sphaeroides* (Yen et al., 1984). Further support is provided from chemical cross-linking studies in *R. capsulatus* using reversibly cleavable reagents as well as mild detergent fractionation procedures (Peters et al., 1983; Drews, 1985); apparent oligomeric forms of B800–850 and associations between B875 and the H subunit of the reaction center were observed. Associations observed in oligomeric pigment–protein complexes isolated from detergent-solubilized *Rp. palustris* membranes have also been interpreted to reflect an overall organization similar to that proposed above (Varga & Staehelin, 1985).

Here, oligomerization states of the various pigment–protein complexes were examined from their behavior in lithium dodecyl sulfate–polyacrylamide gel electrophoresis.[3] A molecular weight could be estimated only with the B875 complex of highest mobility, since this was the sole band which behaved ideally in Ferguson plots. Although these data were consistent with an $(\alpha\beta)_3$ structure, the observation of an oligomeric series of B875 complexes in the M21 mutant suggests that single α/β heterodimers can be added to $(\alpha\beta)_3$ units to form the higher aggregation states. Singlet–singlet annihilation data indicated that after lithium dodecyl sulfate electrophoresis, the major B875 band, isolated from the wild type, contained 8 connected BChl's while B800–850 bands contained >25 connected B850 BChl's (van Grondelle et al., 1983); this is in general accord with electrophoretic estimates of oligomerization states.

[3] A preliminary computer-assisted sedimentation equilibrium analysis in an analytical ultracentrifuge was consistent with a molecular weight near 130000 for the major B800–850 band derived from lithium dodecyl sulfate gels, while with B875 preparations large aggregates were observed during sedimentation ($M_r \geq 1\,000\,000$) (A. T. Greway, T. F. Kumosinski, and R. A. Niederman, unpublished results).

Although lithium dodecyl sulfate–polyacrylamide gel electrophoresis provided the earliest technique for isolation of essentially homogeneous B875 preparations (Broglie et al., 1980), more conventional preparative procedures have subsequently been reported for *R. sphaeroides* (Bachmann et al., 1983; Seban et al., 1985). Nevertheless, this electrophoretic procedure still serves as a useful analytical method for characterization of mutant strains with defects in pigment–protein complexes and for studies of their supramolecular organization, stability, and assembly in photosynthetic membranes.

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Binding of Hydroxylamine to the Water-Oxidizing Complex and the Ferroquinone Electron Acceptor of Spinach Photosystem II†

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ABSTRACT: The reaction between spinach photosystem II (PSII) membranes and hydroxylamine has been investigated by equilibrium titrations and flash-induced reactions with electron paramagnetic resonance (EPR) spectroscopy to monitor the odd-electron species, O_2 evolution rate, and manganese binding. Two high-affinity sites for NH_2OH reaction have been characterized. Binding to the first site occurs within the water-oxidizing complex (WOC) and produces the well-known two flash shift in O_2 evolution. The usual two-electron shift in O_2 yield is accompanied by a parallel two-electron shift in the yield of the S_2 multiline EPR signal. This reaction occurs in two steps—an initial reversible reduction of manganese by two electrons at low concentrations ($\leq$5 NH_2OH/PSII) followed by, at higher concentrations, further reduction that is irreversible due to the release of 3 out of 4 Mn/PSII. The titration curve suggests that 2–3 Mn/PSII are released cooperatively, presumably from a common site. Binding to the second high-affinity site ($\leq$6 NH_2OH/PSII) produces a structural change in the ferrosemiquinone electron acceptor that is characterized by the conversion of the normal form of its EPR signal from $g = 1.9$ to a new form having $g = 2.1$. This structural change is blocked by herbicides, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea, which block access to the Q_B acceptor site. The two flash delay in turnover seen at room temperature is lost at low temperatures (150–500 K) due to a block in multiple turnovers caused by NH_2OH. The site for the low-temperature blockage is undetermined but correlates with the structural change at the ferroquinone site. This suggests that the reoxidation of Q_A^- by Q_B following turnover is blocked, resulting instead in recombination upon warming. The reversible loss of both of the S_2-state EPR signals, the multiline and the $g = 4.1$ signals, caused by NH_2OH, titrated with identical curves, suggesting a common chemical reactivity and hence origin for these signals. The reaction between the S_2 state and NH_2OH occurs in less than 10 s and is considerably faster than binding to the (dark) S_1 state. The reversible binding of NH_2OH produces no stable paramagnetic products in the dark. The release of Mn by NH_2OH is followed by reduction of the oxidized donor D^+ responsible for EPR signal II_{slow} and signal II_{dark}, confirming earlier work establishing the accessibility of this donor to the aqueous phase through the Mn binding site [Ghanotakis, D. F., & Babcock, G. T. (1983) *FEBS Lett. 153*, 231–234].

Insight into the mechanism of O_2 evolution from the water-oxidizing complex (WOC) of photosynthetic membranes has come from studies of inhibitors which are substrate analogues such as NH_2OH (Bennoun & Joliot, 1969; Bouges, 1971; Cheniae & Martin, 1971).

There is considerable circumstantial evidence suggesting that NH_2OH binds to manganese within the active site of the complex [reviewed in Radmer and Cheniae (1977)]. Unlike substituted amines which inhibit O_2 evolution reversibly at high (millimolar) concentrations in direct proportion to their basicity (Ghanotakis et al., 1983), NH_2OH at comparable concentrations releases between two and four Mn(II) ions per photosystem II (PSII) (Cheniae & Martin, 1971; Yocum et al., 1981) and extrinsic proteins (Tamura & Cheniae, 1985), while being consumed at least partially to form N_2 (Radmer, 1983). In the mechanism proposed by Radmer (1983) a two-step process involving initial reduction of S_1 to S_0 occurs, followed by displacement of water bound to Mn by another molecule of NH_2OH. This suggests that reduction to the more labile Mn(II) oxidation state precedes the release of manganese. N- and O-substituted derivatives of NH_2OH were found to be less reactive, in a manner which correlated with their size rather than their redox potential (Radmer & Ollinger, 1983, 1984). The evidence which has accumulated to date has been unable to determine if the reduction of Mn occurs by an outer-sphere electron-transfer mechanism or by direct binding to Mn.

Preincubation with NH_2OH at low (micromolar) concentrations in the dark (S_1 state) results in the retardation of the flash-induced yield of O_2 by two electrons (Bouges, 1971). Similar results were found with H_2O_2 (Velthuys & Kok, 1978). Other phenomena coupled to electron transport such as PSII fluorescence emission (Theg et al., 1984; Itoh et al., 1984), proton release during short flashes (Foster & Junge, 1986a), and UV absorption transients attributed to Mn oxidation (Witt

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