Use of the Membrane-Impermeable Guanidinating Reagent 2-*S*-[^{14}C]Thiuroniumethanesulfonate To Demonstrate the Orientation of Light-Harvesting Proteins in *Rhodobacter sphaeroides*†

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ABSTRACT: The radiolabeled guanidinating reagent 2-*S*-[^{14}C]thiuroniumethanesulfonate reacts with the ϵ-amino groups of accessible lysyl residues of membrane proteins under relatively mild labeling conditions, yielding labeled homoarginyl residues. Model studies have shown that the resulting homoarginyl residues do act as new cleavage sites for trypsin, but only at a very slow rate of hydrolysis. The reagent has been shown to be impermeable to the intracytoplasmic membranes of *Rhodobacter sphaeroides*: when cytoplasmic-side-out chromatophores were treated with the reagent, it reacted with all four of the light-harvesting proteins, all of which have one or more lysyl residues on the N-terminal sides of their hydrophobic regions. However, when periplasmic-side-out vesicles, prepared by cytochrome *c* affinity chromatography, were treated with the guanidinating reagent, three of the light-harvesting proteins (B850α, B850β, and B870β) were not labeled. The only light-harvesting protein to be labeled (B870α) was the only one of the four to have a lysyl residue on the C-terminal side of its hydrophobic region. Guanidinated B870α polypeptides from both the cytoplasmic-side-out chromatophores and the periplasmic-side-out membrane vesicles were purified and digested with trypsin. The resulting peptide fragments were then separated by high-performance liquid chromatography and analyzed for radioactivity. The results have confirmed the asymmetric orientation of the light-harvesting proteins of *R. sphaeroides*, with their N-termini on the cytoplasmic side of the intracytoplasmic membrane. In the case of the B870α subunit, the protein has been shown to be transmembrane with its C-terminus on the periplasmic side of the membrane. The results have also demonstrated the effectiveness of the labeling reagent for future membrane–protein orientation studies.

Rhodobacter sphaeroides is known to contain two different LH[1] complexes, B870 and B800-850 (Aagaard & Sistrom, 1972), each composed of α- and β-subunits in a 1:1 ratio (Broglie et al., 1980; Cogdell et al., 1980; Bachmann et al., 1983). The bacteriochlorophyll:carotenoid:apoprotein ratio has been determined to be 1:1:1 for B870 (Broglie et al., 1980; Bachmann et al., 1983) and 6:3:4 for B800-850 (Kramer et al., 1984; Evans et at., 1988). However, the exact number of subunits per complex in vivo is under current investigation (Zuber et al., 1985; Vos et al., 1988). *Rhodobacter capsulatus* also has two LH complexes, but the B800-850 complex contains a γ-subunit, in addition to the α- and β-subunits, present in a 1:1:1 ratio (Shiozawa et al., 1982). Other members of the Rhodospirillaceae may have only one LH complex, composed of either α- and β-subunits (e.g., *Rhodospirillum rubrum*; Cogdell et al., 1982; Picorel et al., 1983) or α-, β-, and γ-subunits (e.g., *Rhodopseudomonas viridis*; Brunisholz et al., 1985). The primary structures of the α- and β-subunits for many of the Rhodospirillaceae have been determined by sequencing either the proteins themselves or their corresponding genes. [For reviews, see Drews (1985) and Zuber et al. (1985).] All are small molecular weight polypeptides of from 48 to 60 amino acids, and all have a single, centrally located hydrophobic domain. Their sequences thus suggest that they are intrinsic transmembrane proteins with their hydrophilic N-termini distributed either asymmetrically (on one side of the ICM only) or symmetrically (on either side of the ICM). On the basis of their sequences alone, it is also theoretically possible that the LH proteins are not transmembrane at all, but are only anchored in the membrane with both hydrophilic domains on the same side of the ICM.

Much of the early work to determine the orientation of the LH proteins in the Rhodospirillaceae was carried out before their sequences were known, and before adequate resolution of all of the LH proteins had been obtained during analysis by SDS–polyacrylamide gel electrophoresis. If an LH protein reacted with a reagent on only one side of the membrane, it was assumed to be asymmetrically distributed, but not necessarily transmembrane, and its orientation (with respect to its N- and C-termini) could not be ascertained. Also, if an LH protein reacted on both sides of the membrane, it could not be distinguished whether it was an asymmetrically oriented transmembrane protein or distributed symmetrically on both sides of the membrane.

In an earlier study from this laboratory, Francis and Richards (1980) used the lysine-specific reagents pyridoxal phosphate plus [^{3}H]KBH_4 to demonstrate that a band containing the B870α polypeptide was labeled from both sides of the ICM in *R. sphaeroides*, while a band containing the three

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[1] Abbreviations: LH, light harvesting; ICM, intracytoplasmic membranes; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; SDV, spheroplast-derived vesicles; CMA, chloroform/methanol (1:1 v/v) containing 0.1 M ammonium acetate; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; CF_α and CF_β, α- and β-subunits of the coupling factor ATPase; RC_H, RC_M, and RC_L, heavy, medium, and light subunits of the reaction center.

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other LH polypeptides was labeled from the cytoplasmic side of the membrane only. Bachmann et al. (1981) determined that the same two bands in *R. sphaeroides* were labeled from both sides of the membrane by a radioiodination procedure. Also, Cogdell et al. (1983) employed antibodies to demonstrate that the B800–850 LH complex of *R. sphaeroides* had antigenic sites on both sides of the membrane.

In studies carried out since the sequences of the LH proteins have become known, membranes of both orientations have been treated with proteolytic enzymes to determine the accessibility of the N- and C-termini of LH proteins. In *R. sphaeroides*, all four LH proteins have been demonstrated to be asymmetrically oriented (Takemoto et al., 1987; Tadros et al., 1988). Hydrophobic reagents have also been used to label LH proteins in *R. rubrum* (Meister et al., 1985; Gogel et al., 1986).

We have recently developed a new membrane-impermeable radiolabeled guanidinating reagent, 2-*S*-[^{14}C]thiuroniumethanesulfonate, which reacts with accessible lysyl residues (Hundle & Richards, 1987), and have used it to study the labeling of the LH proteins in *R. sphaeroides*. The results have confirmed that all four polypeptides are asymmetrically oriented and are accessible to guanidination on their N-terminal region from the cytoplasmic side of the ICM. The results have also shown that the B870α protein has a transmembrane orientation with its C-terminal region accessible to gunidination from the periplasmic side of the ICM.

Materials and Methods

Materials. 2-*S*-Thiuroniumethanesulfonate was synthesized by the method of Hundle and Richards (1987) using either unlabeled thiourea or [^{14}C]thiourea purchased from Amersham Canada Ltd., Oakville, Ontario. In the latter case, the product was not crystallized before being diluted with unlabled guanidinating reagent; hence, its specific activity could not be accurately determined. The following materials were purchased from the sources indicated: L-arginyl-L-lysyl-L-aspartyl-L-valyl-L-tyrosine, horse heart cytochrome *c*, trypsin [treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone], CNBr-activated Sepharose 4B, and Sephadex LH-60 from Sigma Chemical Co., St. Louis, MO; Sephadex G-10 and protein molecular weight standards from Pharmacia Fine Chemicals, Lachine, Quebec; Whatmann DE-32 (DEAE-cellulose) and CM-52 (CM-cellulose) from Terochem Laboratories Ltd., Edmonton, Alberta; Brownlee Labs C_{18} Spheri-5 HPLC column from Chromatographic Specialties Inc., Brockville, Ontario; Polygram MN 300 cellulose TLC sheets from Brinkmann Instruments (Canada) Ltd., Rexdale, Ontario; Spectrapor 3 dialysis tubing from Canadawide Scientific Ltd., Ottawa, Ontario; En^{3}Hance from NEN Products, Du Pont Canada Inc., Lachine, Quebec; Scinti-Verse II from Fisher Scientific Co., Vancouver, BC; and Kodak XRP film from Kodak Canada Inc., Toronto, Ontario. All other chemicals were of reagent grade.

Preparation of Membrane Fractions. R. sphaeroides NCIB 8253 was grown phototropically as described by Francis and Richards (1980). The chromatophore fraction was prepared by French press disruption of 15 g (wet weight) of cells harvested after growth under normal light conditions (4500 lx) and suspended in 150 mL of 0.05 M potassium phosphate, pH 7.0, containing 10 mM EDTA, 5 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. The membranes were purified by the method of Francis and Richards (1980), resuspended (at a concentration of 4 mg of protein mL^{-1}) in 0.1 M potassium phosphate, pH 7.4, diluted 1:1 with glycerol, and kept forzen at −20 °C. The SDV fraction was prepared from 25 g (wet weight) of cells harvested after growth under high light intensities (15000 lx) and suspended in 500 mL of 0.12 M potassium phosphate, pH 8.0, containing 10 mM EDTA and 10 mM potassium ascorbate, by the lysozyme/osmotic shock method of Michels and Konigs (1978). Following removal of unbroken cells and spheroplasts by centrifugation at 15000*g* for 20 min at 4 °C, the SDV fraction was sedimented by centrifugation at 50000*g* for 30 min at 4 °C. The pellet was resuspended in 0.05 M potassium phosphate, pH 7.4, and purified by centrifugation for 16 h at 4 °C in 30–55% (w/w) continuous sucrose density gradients by the method of Takemoto and Bachmann (1979), using an SW27 rotor at 20000 rpm. The major pigmented band was collected, dialyzed at 4 °C against 0.1 M potassium phosphate buffer, pH 7.4, and sedimented by centrifugation at 50000*g* for 30 min at 4 °C. The pellet was resuspended in 0.05 M potassium phosphate buffer, pH 7.4, diluted 1:1 with glycerol, and kept frozen at −20 °C.

Purification of the SDV Fraction by Cytochrome c Affinity Chromatography. Horse heart cytochrome *c* was covalently linked to CNBr-activated Sepharose 4B by the method of Lotscher et al. (1979). The gel was packed into a 0.9 × 17 cm column and equilibrated with 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA. The SDV fraction (containing 8–10 mg of protein) was suspended in 0.15 M NaCl to remove any endogenous cytochrome c_2, sedimented by centrifugation at 50000*g* for 30 min at 4 °C, and resuspended in 1.2 mL of the equilibration buffer described above containing 0.15 M KCl. After addition of the sample to the affinity column, it was developed with 25 mL of the equilibration buffer containing 0.15 M KCl, followed by a continuous KCl gradient formed between 50 mL each of the equilibration buffer and the equilibration buffer containing 1.5 M KCl. The eluate was monitored at 280 nm with an LKB Uvicord II flow monitor. After determination of the location of the desired band (cf. Results and Discussion), the KCl gradient was routinely replaced by a fixed concentration (0.75 M) of KCl in the equilibration buffer. The eluted SDV fraction was dialyzed at 4 °C against 0.1 M potassium phosphate, pH 7.4, diluted 1:1 with glycerol, and kept frozen at −20 °C. The column could be washed with the equilibration buffer containing 0.1% (v/v) Triton X-100, followed by the equilibration buffer containing no additives, and reused with no noticeable loss of activity.

Separation of Labeled LH Proteins from Guanidinated Membrane Fractions. Chromatophore and SDV fractions were purified and sedimented from their storage buffers as described above. Each was resuspended to a final concentration of 20 mg mL^{-1} in 10 mL of 0.1 M potassium phosphate, pH 9.0, containing 0.2 M 2-*S*-[^{14}C]thiuroniumethanesulfonate, and incubated for 2 h at 30 °C. (The membrane labeling was done in two separate experiments; hence, the specific activities of the guanidinating reagents employed in each case, although unknown, were undoubtedly different.) Both fractions were then resedimented and washed repeatedly with 0.1 M potassium phosphate, pH 7.4, until no radioactivity was detected in the supernatant. The sedimented membranes were then lyophilized and stored at −20 °C. Labeled LH proteins were isolated from the lyophilized labeled chromatophore and SDV fractions by extracting repeatedly at 4 °C in dim green light with a total of 32 mL of chloroform/methanol (1:1 v/v) containing 0.1 M ammonium acetate (CMA). The four LH proteins in the extract were then purified by the method of Theiler et al. (1984a). All four of the purified LH protein fractions were dialyzed against distilled water, using Spec-

trapor 3 dialysis tubing with a molecular weight cutoff value of 3500. The LH polypeptides were recovered after they had precipitated at the chloroform/water interface (which developed inside the tubes), lyophilized, and stored at −20 °C.

Action of Trypsin on a Guanidinated Pentapeptide Containing a Residue of Homoarginine. A pentapeptide, L-arginyl-L-lysyl-L-aspartyl-L-valyl-L-tyrosine (3 μmol; 2 mg), was guanidinated by incubation with unlabeled 2-*S*-thiuronium-ethanesulfonate (1 mmol; 184 mg) in 5 mL of 0.1 M potassium phosphate, pH 9.5, for 2 h at 37 °C. The sample was applied to a Sephadex G-10 column (1.0 × 28 cm) and developed with distilled water at a flow rate of 8 mL h^{-1}. The guanidinated pentapeptide was found to be contained in a fraction eluted just prior to the large excess of guanidinating reagent. The first fraction was lyophilized and resuspended in 1.0 mL of 0.05 M ammonium bicarbonate, pH 8.0, to which was added 0.5 mL of the same buffer containing 25 μg of trypsin, and the mixture was incubated for 30 min at 37 °C. Half of the sample was removed for analysis, and the remainder was allowed to react for a total of 3 h. A similar trypsin hydrolysis (but for 30 min only) was also carried out with the unmodified pentapeptide (1.5 μmol; 1 mg). The reactions were terminated by boiling for 90 s. The hydrolysis products were analyzed by TLC on 0.1-mm cellulose sheets developed with *n*-butanol/water/pyridine/acetic acid (50:40:33:1 v/v). Samples were visualized in ultraviolet light after using the fluorescamine spray reagent of Stephens (1978).

Separation of Tryptic Fragments of the Purified Guanidinated B870α LH Protein by HPLC. The lyophilized B870α polypeptides purified from the radiolabeled chromatophore and SDV fractions (200 μg each) were dispersed in 0.2 M potassium phosphate, pH 8.1, by the method of Cohen and Kaplan (1981) using a Bronwill Biosonic III ultrasonicator equipped with a microprobe at a setting of 30. The suspensions were incubated with 5 μg of trypsin for 6 h at 37 °C. The reactions were terminated by the addition of 25 μL of formic acid, and the samples were lyophilized and redissolved in 0.1 mL of formic acid. The products were separated by HPLC on a C_{18} Spheri-5 column (0.4 × 30 cm) at ambient temperature using a Waters Model 481 detector set at 280 nm connected to a Waters Model 840 data system. Elution was accomplished by a modification of the method of Miller et al. (1987), using a linear gradient formed between 10% (v/v) aqueous formic acid and tetrahydrofuran at a flow rate of 0.6 mL min^{-1} and a column pressure varying between 8.3 and 11.0 MPa (1200–1600 psi) maintained by two Waters Model 510 solvent pumps. Bands absorbing at 280 nm were collected by hand and counted directly in the scintillation counter.

Other Methods. SDS–polyacrylamide gel electrophoresis was accomplished in 1.5-mm mini-slab gels (10.0 × 8.4 cm) prepared from 12–18% (w/v) acrylamide solutions and were run for 4–5 h at 75 V with the buffer system of Laemmli (1970). Samples were heated at 100 °C for 90 s in the sample buffer of Schumacher and Drews (1978) and centrifuged for 10 min at 13 000 rpm in a Johns Scientific microcentrifuge prior to electrophoresis. Gels stained with Coomassie blue R-250 were destained, photographed, and then further destained to minimize quenching during fluorography. The gels were impregnated with En^{3}Hance, dried with a Bio-Rad gel drying apparatus, and exposed to Kodak XRP film for 8 weeks at −70 °C. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Radioactivity was determined in 10 mL of Scinti-Verse II on an LKB-Wallac Model 1217 RackBeta liquid scintillation counter.

Results and Discussion

In order to demonstrate the asymmetric orientation of an intrinsic bacterial membrane protein by a labeling procedure, it is necessary to prepare membranes of both the cytoplasmic-side-out and periplasmic-side-out orientations. Members of the Rhodospirillaceae are ideal organisms for this purpose, since membrane fractions of each orientation can be easily prepared from them. Chromatophore vesicles, derived from the ICM of these bacteria during cellular disruption by ultrasonication or pressure release in the French pressure cell, are oriented predominantly cytoplasmic side out (Michels & Konigs, 1978; Lommen & Takemoto, 1978). The SDV fraction consists of a mixed population of cytoplasmic- and periplasmic-side-out vesicles; however, the proportion of the latter can be greatly increased in *R. sphaeroides* by growing the cells in very high light intensities, thereby reducing the total amount of ICM (Takemoto & Bachmann, 1979). The remaining ICM consist of closely appended single invaginations of the cytoplasmic membrane which lack extensive internal elaborations of the membrane. Hence, during the osmotic shock procedure, a greater proportion are inverted to form vesicles (also containing portions of the cytoplasmic membrane) with a periplasmic-side-out orientation. A near-infrared spectrum of the SDV fraction revealed that the predominant light-harvesting form of bacteriochlorophyll was the B870 complex, with only a shoulder at 850 nm apparent (data not shown). This was anticipated because the high light intensity used to grow the cells is known to repress the B800–850 complex (Aagaard & Sistrom, 1972). In contrast, a near-infrared spectrum of the chromatophore fraction revealed that it contained predominantly the B800–850 complex, with only a shoulder at 870 nm apparent (data not shown).

In order to purify periplasmic-side-out vesicles from those of the opposite orientation, we have taken advantage of the binding site for cytochrome c_2 on the periplasmic side of the membrane. Brudvig et al. (1983) had previously observed that horse heart cytochrome *c* could bind to reaction centers of both *R. sphaeroides* and *R. capsulatus* and had used cytochrome *c* linked Sepharose 4B affinity chromatography to purify reaction centers from the R-26 mutant of *R. sphaeroides*. Similar procedures have been used by other workers to separate mitoplasts from inverted submitochondrial vesicles (Gautheron et al., 1979; Lotscher et al., 1979).

Horse heart cytochrome *c* linked Sepharose 4B affinity chromatography was, therefore, used to purify vesicles with the periplasmic-side-out orientation from the SDV fraction, which was prepared as described under Materials and Methods (Figure 1). Fraction I was eluted with the equilibration buffer containing 0.15 M KCl (low ionic strength). It likely contained vesicles oriented cytoplasmic side out and therefore lacking binding sites for cytochrome *c*. The equilibration buffer was then changed to include a gradient of increasing KCl concentration; fraction II was eluted between 0.3 and 0.6 M KCl. Increased ionic strength is known (Brudvig et al., 1983; Bosshard et al., 1987) to disrupt the interaction between cytochrome *c* and its binding site. Hence, this fraction must have contained vesicles oriented predominantly periplasmic side out.

The periplasmic-side-out SDV fraction (fraction II of Figure 1) and a cytoplasmic-side-out chromatophore fraction (derived from cells disrupted in a French pressure cell as described under Materials and Methods) were subsequently labeled with the ^{14}C-guanidinating reagent. Analysis by SDS–polyacrylamide gel electrophoresis (Figure 2) revealed that the two fractions had much different protein contents. While the chromatophore fraction contained predominantly proteins

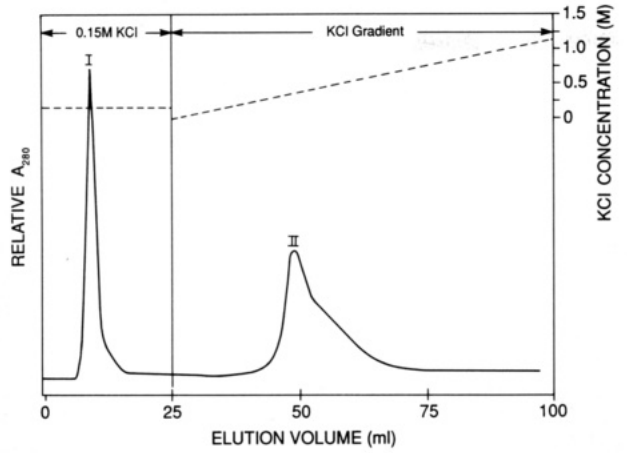

FIGURE 1: Cytochrome *c*–Sepharose 4B affinity chromatography of spheroplast-derived vesicles. The dashed line indicates the approximate KCl concentration of the eluate. Peaks I and II represent cytoplasmic-side-out and periplasmic-side-out vesicles, respectively.

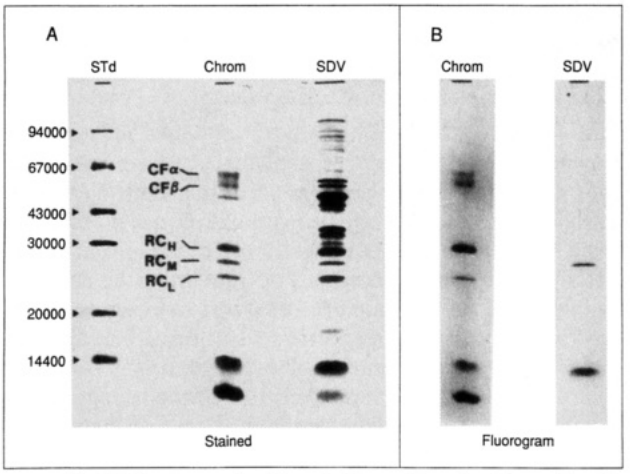

FIGURE 2: SDS–polyacrylamide gel electrophoresis of cytoplasmic-side-out chromatophores (Chrom) and periplasmic-side-out SDV previously labeled with 2-*S*-[^{14}C]thiuroniumethanesulfonate. (A) Gel stained with Coomassie blue; (B) fluorogram. Protein M_r standards (Std) employed were phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), and α-lactalbumin (14 400).

which function in photosynthesis (RC, LH, and coupling factor polypeptides), the SDV fraction contained numerous other protein bands (in addition to the photosynthetic proteins), due to the presence of cytoplasmic membrane proteins in the vesicles. The pattern of labeling by the guanidinating agent was also completely different. The major proteins labeled in the chromatophore fraction included polypeptides with M_r values similar to the α- and β-subunits of the coupling factor (M_r 55 000 and 51 000), the RC_H (M_r 28 000), the RC_L (M_r 21 000), and two bands containing LH polypeptides (M_r 12 000 and 10 000). In the case of the SDV fraction, polypeptides with M_r values similar to the B870α (M_r 12 000) and RC_M (M_r 24 000) subunits were observed to be heavily labeled. Since no activity was found in the RC_L band (which also contains two periplasmic lysine residues, one of which is presumably more accessible than the single periplasmic lysine residue of the RC_M subunit), it is likely that some other labeled polypeptide comigrated in the same region of the gel as the RC_M polypeptide. No radioactivity was detected in the α- and β-subunits of the coupling factor or the RC_H polypeptide of the SDV fraction after 8 weeks of fluorography, however. This result confirmed that the guanidinating reagent had not penetrated the membrane during the time that it was in contact with the membranes. It also demonstrated the opposite orientation of the two membrane preparations, thereby confirming the effectiveness of the cytochrome *c* affinity chromatography. While it would have been preferable to have labeled both sides of the *same* membrane preparation, the polypeptide for which an asymmetric orientation was demonstrated (the B870α polypeptide; cf. below) is a major component of both of the membrane preparations used.

Table I: Specific Radioactivities of Light-Harvesting Proteins Purified from Chromatophores and Spheroplast-Derived Vesicles Treated with 2-*S*-[^{14}C]Thiuroniumethanesulfonate

LH protein	specific activity [dpm (mg of protein)$^{-1}$] of LH proteins derived from: chromatophores	SDV
B870α	25 700	24 300
B850α	23 900	nd[a]
B870β	24 600	nd
B850β	18 900	nd

[a] nd means not detected.

The LH proteins of the ICM of *R. sphaeroides* are ideal models to test the effectiveness of the recently developed, membrane-impermeable radiolabeled guanidinating agent, 2-*S*-[^{14}C]thiuroniumethanesulfonate. The primary sequences of all four LH proteins of the parent strain are known (Theiler et al., 1984b, 1985), and methods for the isolation and separation of the hydrophobic LH proteins have recently been established (Theiler et al., 1984a). Examination of their sequences reveals that all four have between 1 and 2 lysyl residues on the N-terminal side, whereas only the B870α polypeptide has a lysyl residue on the C-terminal side of the hydrophobic membrane sector. Since the guanidinating reagent is known to react with accessible lysyl residues (Hundle & Richards, 1987), three of the four LH proteins should be labeled in one membrane preparations *only*, if they are asymmetrically oriented in the membrane, while the B870α polypeptide should be labeled in membranes of both orientations.

The LH proteins were extracted from both the radiolabeled chromatophore and SDV fractions, and the four LH polypeptides from each fraction were separated and purified as described under Materials and Methods. The specific activities of the purified LH proteins are given in Table I. In the case of the guanidinated chromatophores, all four of the LH proteins were found to be radiolabeled. Their specific activities were found to be similar, indicating that a similar number of residues had been labeled in each case (most likely two lysine residues of the B870α subunit, which has a blocked N-terminus, and one lysine plus the N-terminal residues of the other three subunits). Only the B870α polypeptide was radiolabeled in the guanidinated SDV fraction. Its specific activity, while similar to those of the chromatophore-labeled subunits, cannot be compared with the latter because they were obtained with a different sample of labeled guanidinating reagent (with a different initial specific activity) from that used for labeling the SDV fraction.

The purified LH proteins were also run on SDS–polyacrylamide gel electrophoresis, and fluorography was carried out by placing the dried gels in contact with X-ray film for 8 weeks. The results are shown in Figures 3 and 4 for the chromatophore- and SDV-derived LH proteins, respectively. In both cases, the Coomassie blue stained gels revealed that none of the LH proteins was contaminated with a significant amount of any other protein. The fluorograms confirmed the results of Table I and demonstrated that the radioactivity was

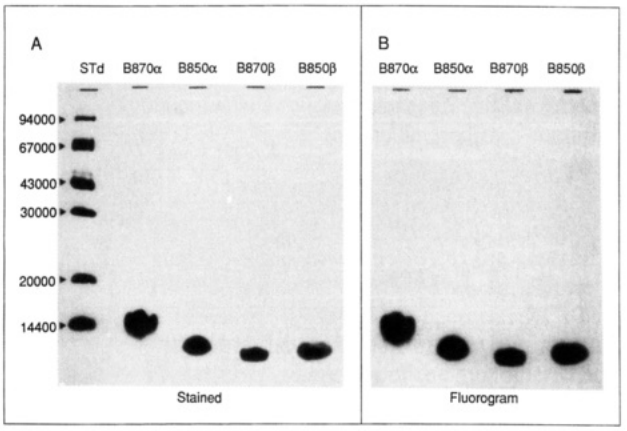

FIGURE 3: SDS–polyacrylamide gel electrophoresis of LH proteins purified from cytoplasmic-side-out chromatophores previously labeled with 2-*S*-[^{14}C]thiuroniumethanesulfonate. (A) Gel stained with Coomassie blue; (B) fluorogram. Protein M_r standards (Std) as in Figure 2.

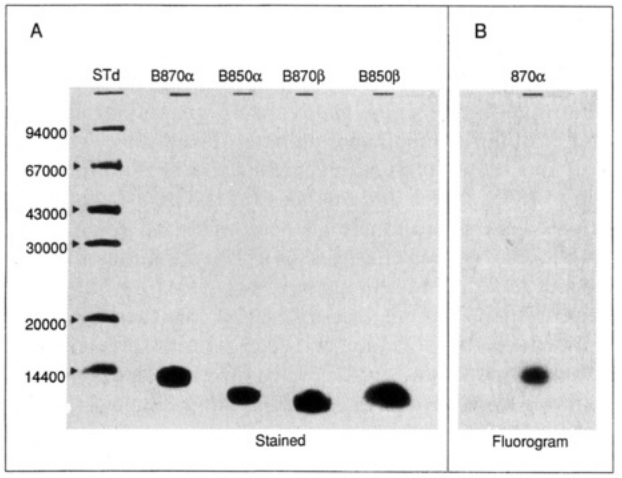

FIGURE 4: SDS–polyacrylamide gel electrophoresis of LH proteins purified from periplasmic-side-out SDV previously labeled with 2-*S*-[^{14}C]thiuroniumethanesulfonate. (A) Gel stained with Coomassie blue; (B) fluorogram. Protein M_r standards (Std) as in Figure 2.

indeed contained in and limited to the LH proteins.

In order to demonstrate an asymmetric, transmembrane orientation for the B870α protein, however, it must be digested by trypsin and the labeling pattern of the trypsin fragments examined. There was some disagreement in the literature, however, as to whether trypsin would recognize the newly created homoarginyl residues in the B870α protein as sites for hydrolysis. Weil and Tilka (1957), using α-lactalbumin, and Shields et al. (1959), using mercuripapain, observed that homoarginyl residues were not substrates for trypsin hydrolysis, even after 24 h. Kennelly et al. (1981), however, observed the trypsin-catalyzed cleavage of guanidinated cytochrome *c* (coupled to an innert gel matrix) on the carboxyl side of homoarginyl residues. Guanidinated cytochrome *c* which was not coupled to the gel was also cleaved by trypsin at homoarginyl residues when the reaction was carried out for 12 h.

We chose to study the homoarginine specificity of trypsin by the use of a model pentapeptide containing a lysyl residue, Arg-Lys-Asp-Val-Tyr. The unmodified pentapeptide was treated with trypsin for 30 min, while a guanidinated pentapeptide preparation was treated with trypsin for both 30 min and 3 h. The hydrolysates were analyzed by TLC on cellulose sheets. In addition to the uncleaved pentapeptide (R_f 0.30), the hydrolysate of the unmodified sample exhibited spots at R_f 0.11 and 0.50. On the basis of the known specificity of trypsin, these should be due to the dipeptide Arg-Lys and the

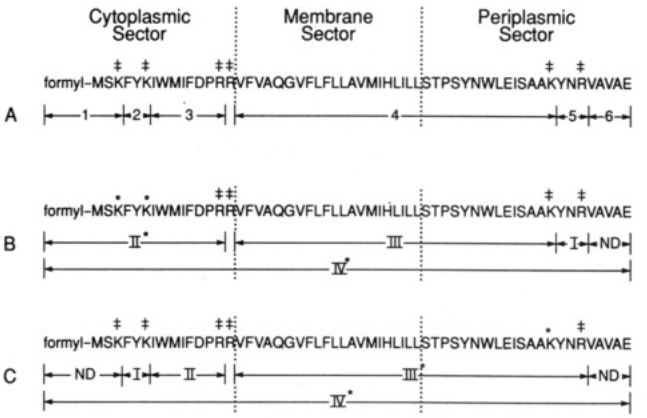

FIGURE 5: Primary structure of B870α showing the approximate location of the cytoplasmic, membrane, and periplasmic sectors. (A) Unmodified B870α; (B) B870α guanidinated from the cytoplasmic side; (C) B870α guanidinated from the periplasmic side. Lysyl and arginyl residues which are potential sites of trypsin hydrolysis are marked with a double dagger; lysyl residues potentially modifiable to homoarginyl residues are marked with an asterisk. The trypsin fragments which would be produced under the assumption that homoarginyl residues are *not* hydrolyzed are indicated under the sequences; those which should be radiolabeled are marked with an asterisk. ND means that the fragment would not be detected at 280 nm due to the lack of tyrosyl or tryptophanyl residues.

tripeptide Asp-Val-Tyr, respectively, although we had no known samples of these peptides for comparison. Trypsin did not hydrolyze the N-terminal L-arginyl residue since neither free L-arginine (R_f 0.21) nor L-lysine (R_f 0.16) was present. After 30 min of its exposure to trypsin, only the uncleaved guanidinated polypeptide (R_f 0.36) was detected in the hydrolysate of this compound. However, after 3 h of exposure, three additional spots were visible at R_f values of 0.22, 0.44, and 0.51. The latter (most likely due to the tripeptide Asp-Val-Tyr) was visible only as a minor spot, indicating that trypsin had only very slowly hydrolyzed the guanidinated pentapeptide at the L-homoarginyl residue. A more intense spot appeared at R_f 0.44, which may have been due to the *tetrapeptide* L-homoarginyl-Asp-Val-Tyr. It is known that the guanidinating reagent also reacts with the N-terminal amino group of peptides, since *N*-amidinoglycine (guanidinoacetate) was isolated after guanidination of glycylglycine followed by acid hydrolysis (Hundle, 1988). Hence, the resulting N-terminal N^{α}-amidino-L-arginyl residue may have served as a trypsin hydrolysis site (to a greater extent than the L-homoarginyl residue), resulting in the formation of the tetrapeptide. The third spot (at R_f 0.22) corresponded to a known sample of L-homoarginine, which could have arisen from hydrolysis of the dipeptide N^{α}-amidino-L-arginyl-L-homoarginine. However, the hydrolysis product expected in greater amounts (N^{α}-amidino-L-arginine) may have had the same R_f value as L-homoarginine and, therefore, may have gone undetected. We were not, however, able to confirm any of the above hypotheses for lack of authentic samples for comparison.

Examination of the sequence of the B870α polypeptide (Figure 5) reveals that it contains three lysyl and three arginyl residues: two of each on the N-terminal side and one of each on the C-terminal side of the hydrophobic region. Hydrolysis of the unmodified polypeptide by trypsin should yield a maximum of six oligopeptides (plus, perhaps, a single arginine from the Arg-Arg in the sequence) as indicated in Figure 5A. If the N- and C-terminal ends were oriented toward the cytoplasmic and periplasmic sides of the membrane, respectively, guanidination of the B870α polypeptide in chromatophores would convert the two lysyl residues on the N-terminal end to labeled homoarginyl residues (cf. Figure 5B), while guan-

Table II: Radioactivities of HPLC-Purified Trypsin Fragments of B870α Isolated from Chromatophores and Spheroplast-Derived Vesicles Treated with 2-*S*-[^{14}C]Thiuroniumethanesulfonate

peak no.[a]	radioactivity (dpm) of trypsin fragments of B870α derived from: chromatophores	SDV
I	31	31
II	1361	28
III	29	1239
IV	477	320

[a] Refer to Figure 6.

idination in the SDV fraction would similarly convert the lysyl residue on the C-terminal end (cf. Figure 5C). Note that the N-terminal methionine amino group is formylated and would, therefore, not be guanidinated in chromatophores.

The purified, guanidinated B870α polypeptides from both the chromatophore and SDV fractions were treated with trypsin for 6 h, and the hydrolysates were separated by HPLC on a C_{18} Spheri-5 column as described under Materials and Methods. The absorbance of the eluate was monitored at 280 nm; hence, only peptides containing tryptophan and/or tyrosine could be detected. Assuming that trypsin did *not* hydrolyze the guanidinated peptides at homoarginyl residues to a significant extent, the guanidinated B870α polypeptide from the chromatophore fraction should yield a total of four fragments; only three contain tryptophan and/or tyrosine and would, therefore, be detectable, and only one of these should be labeled (cf. Figure 5B). Similarly, the guanidinated polypeptide from the SDV fraction should yield a total of five fragments; again, only three would be detectable, and only one of these (which would be different from the one labeled in chromatophores) should be labeled (cf. Figure 5C).

The results of the HPLC separations of the trypsin-treated, guanidinated B870α polypeptides are shown in Figure 6, and the radioactivities contained in the tryptic fragments recovered from the HPLC column are given in Table II. These results are consistent with, and best interpreted by, the assumption that trypsin has not significantly hydrolyzed the newly created homoarginyl residues. Hydrolysates from both the chromatophores (Figure 6B) and SDV fraction (Figure 6C) contained the unhydrolyzed B870α polypeptide (the major band in Figure 6A, and labeled as band IV in both Figure 6B,C). As predicted above, only three additional bands were detected in each sample (representing a different set of three tryptic fragments, each fragment, therefore, having a different retention time); only one (in addition to the undigested B870α polypeptide) was radiolabeled in each case (Table II). A likely assignment of these peaks is indicated in Figure 5. Band II in the chromatophore-labeled sample was found to be radiolabeled and is thus assigned as the N-terminal fragment (Figure 5B). Band III in each sample is assigned as the large fragment containing the hydrophobic region, because it was this band that was radiolabeled in the SDV-labeled sample (Table II). Finally, band I in both samples is assigned as a tripeptide. These would be different in each case, but since they have similar structures, they should, therefore, have similar properties. This would leave band II in the SDV-labeled sample to be assigned as the remaining oligopeptide indicated in Figure 5C. As amino acid sequences of the recovered peptides were not obtained, the above assignments must be considered to be tentative. However, just the fact that different peptides were produced by trypsin from the two preparations and, in addition, that the pattern of labeling of the peptides was different has confirmed the asymmetric,

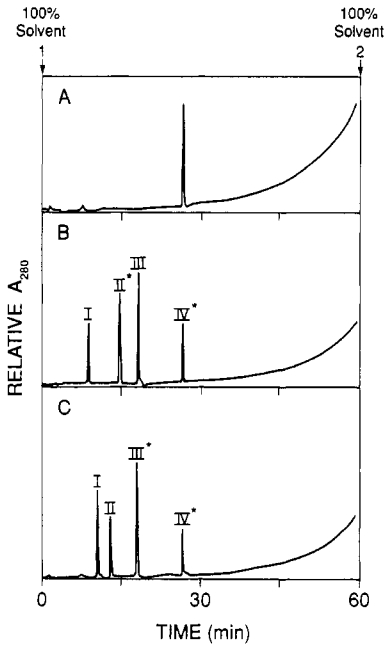

FIGURE 6: HPLC separation of (A) unmodified B870α, (B) trypsin digest of B870α isolated from guanidinated cytoplasmic-side-out chromatophores, and (C) trypsin digest of B870α isolated from guanidinated periplasmic-side-out SDV. The separation was accomplished on a C_{18} Spheri-5 column in a linear gradient formed between 10% aqueous formic acid (solvent 1) and tetrahydrofuran (solvent 2). Bands found to be radiolabeled (cf. Table II) are marked with an asterisk.

transmembrane orientation of the B870α polypeptide.

Membranes of both orientations have also been treated with proteolytic enzymes to determine the accessibility of N- and C-termini of LH proteins from several different members of the Rhodospirillaceae. The N-termini of the following LH proteins were found to be accessible to proteolysis from the cytoplasmic side of the ICM: both the B870α and B870β subunits in the carotenoid$^-$ mutant (G-9$^+$) of *R. rubrum* (Brunisholz et al., 1984); both the B870α and B870β subunits in the B800–850$^-$ mutant (Ala$^+$) of *R. capsulatus* (Tadros et al. 1986a); and both the B850α and B850β subunits in the B870$^-$ (and RC$^-$) mutant (Y5) of *R. capsulatus* (Tadros et al., 1986b). In the case of *R. rubrum*, when the parent (S1) strain was investigated, the B870α subunit was found to be protected in some way from proteolysis (Brunisholz et al., 1986), while in the parent (37b4) strain of *R. capsulatus*, both the B870α and B850α subunits were also found to be protected from proteolysis (Tadros et al., 1987). In the latter case, the C-termini of the two α-subunits were found to be accessible to proteolysis from the periplasmic side of the ICM, while the C-termini of the two β-subunits were protected (Tadros et al., 1987). A similar situation has been demonstrated with both LH complexes in the parent strain of *R. sphaeroides*: the N-termini of both the B870β and B850β subunits have been shown to be accessible to proteolysis from the cytoplasmic side of the ICM, while the N-termini of the B870α and B850α subunits are protected (Takemoto et al., 1987; Tadros et al., 1988). The situation for the C-termini is exactly the reverse: the B870α and B850α subunits are accessible to proteolysis from the periplasmic side of the ICM, while the C-termini of the B870β and B850β subunits are protected (Takemoto et al., 1987; Tadros et al., 1988).

Our results have thus confirmed the work of Takemoto et al. (1987) and Tadros et al. (1988) that the LH proteins of *R. sphaeroides* are asymmetrically oriented within the ICM with their N-terminal residues on the cytoplasmic side. The

results with the B870α polypeptide, however, are the first in which a LH polypeptide in the parent strain of *R. sphaeroides* has been shown to react with a labeling reagent at amino acid residues on different sides of the hydrophobic membrane sector when added to ICM preparations of two different orientations, thus demonstrating the transmembrane orientation of this protein. They have also demonstrated the effectiveness of the radiolabeled guanidinating reagent for future membrane–protein orientation studies. This reagent has been previously shown to have several distinct advantages, requiring lower temperatures and shorter reaction times than other methods (Hundle & Richards, 1987); and since the reagent is a small molecular weight, water-soluble zwitterion, it is much more likely that reactive residues in hydrophilic regions of membrane proteins will be accessible to it than during methods requiring macromolecules such as proteolytic enzymes. The results of Takemoto et al. (1987) and Tadros et al. (1988) provide such an example, where only the β-subunits (but not the α-subunits) of both LH complexes in *R. sphaeroides* were accessible to protease K treatment from the cytoplasmic side of the membrane. Results with *R. capsulatus* have indicated that it may have been the presence of both the B870 and B800–850 complexes which somehow hindered access of the protease to both α-subunits (Tadros et al., 1987). However, even in the presence of both LH complexes, at least one lysine residue in all four subunits of *R. sphaeroides* was accessible to the guanidinating reagent used in the present study.

Radioiodination has also been used to label LH polypeptides in *R. capsulatus* (Webster et al., 1980; Peters & Drews, 1983), *R. sphaeroides* (Bachmann et al., 1981), and *R. viridis* (Jay & Lambillotte, 1985). This method is known to label accessible tyrosine (and, to a lesser extent, histidine and phenylalanine) residues (Koshland et al., 1963). Not all residues capable of iodination were found to have reacted in every case, however. For example, in *R. capsulatus*, a band containing B850α, known to contain a tyrosine residue on the periplasmic side of the hydrophobic membrane sector (Tadros et al., 1983), was observed to be either unlabeled (Webster et al., 1980) or only weakly labeled (Peters & Drews, 1983) from the periplasmic side of the membrane. Hence, this tyrosine residue must have been inaccessible to the reagents employed. Our results have indicated that all three lysine residues in the B870α subunit were accessible to the guanidinating reagent. Furthermore, at least one lysine residue was accessible from the cytoplasmic side of the membrane in the three other LH subunits, although in the case of the B850α and B850β subunits, a second lysine residue much closer to the membrane sector may not have been accessible.

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Light-Dependent Degradation of the D1 Protein in Photosystem II Is Accelerated after Inhibition of the Water Splitting Reaction†

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ABSTRACT: Strong illumination of oxygen-evolving organisms inhibits the electron transport through photosystem II (photoinhibition). In addition the illumination leads to a rapid turnover of the D1 protein in the reaction center of photosystem II. In this study the light-dependent degradation of the D1 reaction center protein and the light-dependent inhibition of electron-transport reactions have been studied in thylakoid membranes in which the oxygen evolution has been reversibly inhibited by Cl^- depletion. The results show that Cl^--depleted thylakoid membranes are very vulnerable to damage induced by illumination. Both the D1 protein and the inhibition of the oxygen evolution are 15–20 times more sensitive to illumination than in control thylakoid membranes. The presence, during the illumination, of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) prevented both the light-dependent degradation of the D1 protein and the inhibition of the electron transport. The protection exerted by DCMU is seen only in Cl^--depleted thylakoid membranes. These observations lead to the proposal that continuous illumination of Cl^--depleted thylakoid membranes generates anomalously long-lived, highly oxidizing radicals on the oxidizing side of photosystem II, which are responsible for the light-induced protein damage and inhibition. The presence of DCMU during the illumination prevents the formation of these radicals, which explains the protective effects of the herbicide. It is also observed that in Cl^--depleted thylakoid membranes, oxygen evolution (measured after the readdition of Cl^-) is inhibited before electron transfer from diphenylcarbazide to dichlorophenolindophenol. The latter activity is dependent on functional electron transfer in photosystem II between the electron donor tyrosine Z and the first quinone acceptor, Q_A. The kinetics for the inhibition of the electron transfer from diphenylcarbazide to dichlorophenolindophenol were approximately similar to the kinetics for the degradation of the D1 protein. Together these results indicate that the light-dependent degradation of the D1 protein is triggered by the accumulation of P_{680}^+ and/or tyrosine Z^+, both of which are highly oxidizing. It is proposed that similar reactions also trigger the degradation of the D1 protein in vivo and possible mechanisms for this are discussed.

Photosystem II (PSII)[1] is a large, multisubunit enzyme (Andersson & Åkerlund, 1987) that catalyzes the light-driven reduction of plastoquinone with electrons derived from water (Andréasson & Vänngård, 1988; Rutherford, 1989). The reaction center in PSII is composed of two hydrophobic proteins, D1 and D2, which are homologous of each other and of the L and M subunits in the photosynthetic reaction center from purple bacteria. The heterodimer of the L and M sub-

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[1] Abbreviations: DPC, 2,2′-diphenylcarbonic dihydrazide; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P_{680}, primary electron donor chlorophyll(s) of PSII; Pheo, pheophytin that functions as the intermediate electron acceptor to P_{680}; PSI, photosystem I; PSII, photosystem II; Q_A, first quinone acceptor in PSII; Q_B, second quinone acceptor in PSII; S_0–S_4, charge storage states of the oxygen-evolving complex; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Tyr_Z, tyrosine 161 on the D1 protein—electron carrier between P_{680} and the Mn cluster; Tyr_D, tyrosine 160 on the D2 protein—accessory electron donor in PSII.

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