

# Development of a Cell-free System to Study the Membrane Assembly of Photosynthetic Proteins of *Rhodobacter capsulatus*

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**Abstract.** A cell-free translation system from the facultatively photoheterotrophic bacterium *Rhodobacter capsulatus* is described. Synthesis of two proteins of the bacterium's photosynthetic apparatus (light-harvesting complex B870  $\alpha$  and  $\beta$ ) was performed by SP6 polymerase transcription of the subcloned genes, isolation of the mRNA and translation in vitro using a cell-free extract of *R. capsulatus* cells.

The integration of these proteins in vitro into added intracytoplasmic membrane vesicles (ICM) is demonstrated. Without addition of ICM ~70% of the synthesized B870 proteins were soluble. If, however, ICM were present during synthesis, the majority of the

soluble protein was found to associate with the membranes. The membrane-associated polypeptides could be solubilized only by detergent treatment but could not be extracted by treatment at alkaline pH ( $\text{Na}_2\text{CO}_3$ ), suggesting that the proteins had been firmly inserted into the lipid bilayer. Moreover, the B870  $\alpha$  and  $\beta$  proteins that integrated in vitro into ICM were also found to associate with pigment ligands and to assemble into a native reaction center/B870 complex. The native conformation of this complex isolated from ICM by Triton fractionation was demonstrated by microspectral analysis of the bound pigments.

**T**HE Gram-negative, facultatively photoheterotrophic bacterium *Rhodobacter capsulatus* is a good model organism for studying membrane differentiation because of its inducible apparatus for photosynthesis. The assembly of the photosynthetically active intracytoplasmic membrane (ICM)<sup>1</sup> system is induced by lowering either the oxygen tension or the light intensity (reviewed in references 9, 17, 40).

The photosynthetic complexes are composed of eight different integral membrane proteins, organized into two light-harvesting (LH) complexes, B870 (LH-I) and B800-850 (LH-II), and a reaction center (RC). Each LH-complex consists of two pigment-binding proteins in a 1:1 stoichiometry; B870  $\alpha$  and  $\beta$  ( $M_r = 12$  and  $7$  kD), and B800-850  $\alpha$  and  $\beta$  ( $M_r = 10$  and  $8$  kD). The B800-850 complex also contains the non-pigment-binding protein  $\gamma$  ( $M_r = 14$  kD). Each of these  $\alpha$  and  $\beta$  peptides has one  $\alpha$ -helical transmembrane segment of ~20 amino acids with the  $\text{NH}_2$  terminus facing the cytoplasm and the  $\text{COOH}$  terminus located in the periplasmic space (32-34). The RC consists of the two pigment-binding proteins L and M ( $M_r = 20.5$  and  $24$  kD), each possessing five membrane-spanning segments, and the non-pigment-binding protein H ( $M_r = 28$  kD), which is anchored within the membrane by a single hydrophobic stretch. The three RC proteins are found in the ICM in a 1:1:1 stoichiometry with their  $\text{NH}_2$  termini located in the cyto-

plasm (34). None of the eight polypeptides is synthesized with a cleavable,  $\text{NH}_2$ -terminal signal sequence (8).

The genes for the eight proteins of the photosynthetic complexes are organized into three operons: *puf* (B870  $\alpha$  and  $\beta$ , RC-L and M), *puh* (RC-H), and *puc* (B800-850). All of the structural genes have been sequenced (35, 38, 39).

Little is known about the assembly mechanism of the photosynthetic complexes. It was shown that (a) they appear to be inserted into distinct membrane areas (7, 15, 29); (b) the RC is not properly assembled if the B870 complex is missing (14); (c) pigments are required for the stability and proper assembly of the protein complexes (20).

The use of cell-free systems greatly facilitates the investigation of the molecular mechanism of protein integration into membranes. These systems allow the selective expression of individual proteins whose membrane transport can be reconstituted by the addition of exogenous membrane vesicles. Once such a system is established, the transport mechanism can be studied in more detail with the eventual aim of identifying molecular components of this process. Up to now, in prokaryotes, such an analysis using cell-free translation-transport systems has only been carried out for the plasma membrane of *Escherichia coli* (for a recent review see reference 30).

Here we report the development and characterization of a cell-free system from *R. capsulatus* for the study of the membrane integration of newly synthesized pigment-binding proteins. The two B870 proteins were synthesized in vitro by programming a membrane-free extract from *R. capsulatus* with

1. Abbreviations used in this paper: ICM, intracytoplasmic membrane vesicles;  $\text{Me}_2\text{SO}$ , dimethyl sulfoxide; TeaOAc, triethanolamine acetate.

specific mRNAs, obtained by transcription of the respective genes subcloned under the control of the heterologous SP6 promoter. These proteins are shown to integrate efficiently into exogenously added ICM prepared from *R. capsulatus* and to assemble into native pigment-containing protein complexes.

## Materials and Methods

### Construction and Large-Scale Preparation of Plasmid pSBC57

Plasmid pSBC57 was constructed by inserting the 4.8-kb Eco RI–Bam HI fragment of pBBC1 (19) containing the genes for B870  $\alpha$  and  $\beta$ , RC L and M and at least two additional open reading frames X and C2814 (3, 39) into the polylinker site of the SP6 promoter-containing vector pSP65 (Boehringer Mannheim GmbH, Mannheim, FRG). Plasmid DNA was purified by CsCl gradient centrifugation according to Maniatis et al. (23) and Godson and Vapnek (12).

### Preparation of Protein Synthesis-competent Extracts from *R. capsulatus*

The preparation of extracts essentially followed previously described methods (26, 41). Cells from *R. capsulatus* strain 37b4 (8) were cultured photoheterotrophically in 1-liter screwcap bottles filled to the top with a malate-mineral-medium (8) to an optical density of  $A_{600} = 1.2$ – $1.5$  (all optical measurements were carried out in a spectrophotometer [model 7 UV/vis; Perkin-Elmer Cetus Corp., Cherry Hill, NJ]). Cells were washed and resuspended (1 g/ml) in S-30 buffer (10 mM triethanolamine acetate [TeaOAc] pH 8, 14 mM Mg(OAc)<sub>2</sub>, 60 mM KCl, and 1 mM DTT). PMSF dissolved in ethanol was added to 0.5 mM (26) and cells were lysed by two passages through a French pressure cell at 12,000 psi. After adding 1  $\mu$ l 1 M DTT/ml, the homogenate was centrifuged for 30 min at 31,000 g. The resulting supernatant (S-30) was then incubated in the dark for 1 h at 32°C with the following additions (per ml): 60  $\mu$ l 1 M TeaOAc, pH 8.0; 0.6  $\mu$ l 1 M DTT; 1.7  $\mu$ l 1 M Mg(OAc)<sub>2</sub>; 6  $\mu$ l 20 amino acids (1 mM each); 2  $\mu$ l 0.25 M ATP; 27  $\mu$ l 0.2 M phosphoenolpyruvate; and 5  $\mu$ g pyruvate kinase. Subsequently, the S-30 was dialyzed three times for 1 h each against 100 vol of S-30 buffer, containing KOAc instead of KCl. The dialyzed S-30 was used to prepare the membrane-free S-135 as described (26), except that centrifugation was carried out for 15 min.

### Cell-free SP6 Transcription

Before transcription, plasmid pSBC57 was linearized by the appropriate restriction enzymes. Transcription was performed as described (25) in 100- $\mu$ l reactions. Afterwards, DNA was digested with 40 U RNase-free DNase I (FPLC pure; Pharmacia Fine Chemicals, Uppsala, Sweden).

### Cell-free Translation

Translation of 200–300 ng mRNA was done in vitro in 25- $\mu$ l reactions according to Müller and Blobel (26), except that KOAc and Mg(OAc)<sub>2</sub> concentrations were lowered to 50 and 8 mM, respectively. 1- $\mu$ l membranes (see "Preparation of ICM") were added 5 min after starting protein synthesis. Translation was allowed to proceed for 45 min at 32°C, then stopped either by adding 5% TCA or by chilling on ice followed by fractionation as described below.

### Fractionation of Translation Mixtures

After translation was finished, samples chilled on ice were fractionated by centrifugation in the airfuge (Beckman Instruments, Fullerton, CA) over a two-step sucrose gradient consisting of 100  $\mu$ l 20% (wt/vol) and 50  $\mu$ l 50% sucrose in 40 mM TeaOAc pH 7.5. After a 10 min centrifugation at 30 psi in a rotor (model A100/18; Beckman Instruments), two fractions were removed as follows: the upper 50  $\mu$ l containing soluble material (SI), and the next 115  $\mu$ l containing membrane-bound (SII) material. The remaining 10  $\mu$ l were discarded. Fractions SI and SII were precipitated with 5% TCA. The pelleted material defined as fraction P was directly resuspended in sample buffer for SDS-PAGE. The recovery of labeled protein from the SI, SII,

and P-fractions was ~75–80% of total TCA-precipitable material before the gradient. Addition of carrier membranes did not change the distribution of the sample in the gradient, so addition was not made routinely.

### Preparation of ICM

Cells from *R. capsulatus* strain 37b4 were cultured photoheterotrophically as described for the preparation of cell extracts, harvested at an  $A_{600} = 1.0$  and washed once in membrane buffer (50 mM TeaOAc pH 7.5, 1 mM EDTA, 1 mM DTT). Cells (1 g/ml) were resuspended in membrane buffer containing 0.5 mM PMSF and passed twice through a French pressure cell at 18,000 psi. The homogenate was freed from unbroken cells and cell debris by a 10-min centrifugation at 11,000 g. The supernatant was layered onto a 5–40% (wt/vol) linear sucrose gradient poured on top of a 77% sucrose cushion. All sucrose solutions were prepared in membrane buffer containing 0.5 mM PMSF. Centrifugation was carried out in a swinging-bucket rotor (model TST 28.38/17; Kontron Analytical, Everett, MA) at 4°C for 4 h at 96,000 g. A broad, dark red band of chromatophores was recovered from the middle of the gradient, diluted threefold with membrane buffer, and collected by centrifugation for 2 h at 500,000 g. The pellet was resuspended in membrane buffer without EDTA containing 250 mM sucrose and the  $A_{260}$  was adjusted to 50. Membranes were stored in small aliquots at  $-70^\circ\text{C}$ .

Alternatively (experiments shown in Figs. 5 and 6), membranes were prepared from semiaerobically grown cells of *R. capsulatus* strains 37b4 and W1 (10). The preparation procedure differed from that described above as follows: to achieve low aeration, 1-liter Erlenmeyer flasks were filled with 800 ml malate-mineral medium containing a 50-ml inoculum and incubated at 30°C by rotation at 150 rpm; the membrane buffer used did not contain EDTA and DTT; the cell homogenate was centrifuged at 30,000 g for 20 min; 2.5 ml of the resulting supernatant were layered onto a discontinuous sucrose gradient composed of 5 ml each of 20, 30, 40, and 50% sucrose in membrane buffer; gradients were centrifuged in a rotor (model Ti60; Beckman Instruments) at 38,000 rpm for 16 h; ICM were collected from the 40/50% interphase by aspiration, pelleted in the Ti60 rotor at 60,000 rpm for 2 h, and resuspended as described above.

### SDS-PAGE

SDS-PAGE was performed with some modifications of the method of King and Laemmli (18). We used 1-mm-thick 15–20% acrylamide gradient gels. Gels were fluorographed by a modified method of Bonner and Laskey (4) and exposed to Fuji medical x-ray films (RX), usually overnight. Quantitation of the radioactivity in single polypeptide bands on SDS gels was carried out according to the method described by Walter et al. (36).

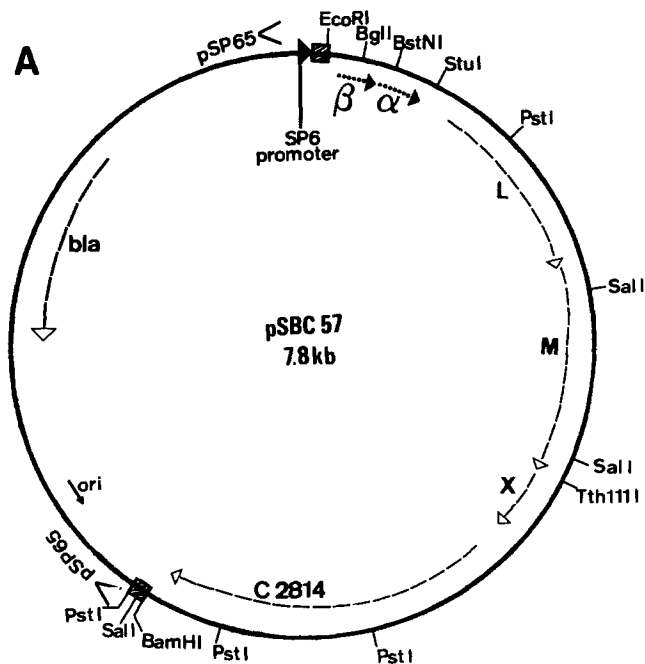
## Results

### Subcloning of the B870 Polypeptides under the Control of the SP6 Promoter

To selectively express individual pigment-binding proteins, specific mRNAs encoding only the proteins of interest were required. We therefore subcloned the *puf*-genes (B870  $\alpha$  and  $\beta$ , RC-L, and M) present on a 4.8-kb Eco RI–Bam HI fragment of pBBC1 into vector pSP65, creating a plasmid in which the *puf*-genes were now under the control of the heterologous SP6 promoter. The resulting plasmid pSBC57 is depicted in Fig. 1. After linearization with Stu I, plasmid pSBC57 was transcribed in vitro using SP6 RNA-polymerase (see Materials and Methods). Stu I has a unique restriction site behind the coding sequence for  $\alpha$  (Fig. 1).

### mRNA-dependent Synthesis and Identification of the B870 Polypeptides

To synthesize the B870  $\alpha$  and  $\beta$  polypeptides in vitro, we prepared an S-135 from a *R. capsulatus* homogenate completely free of endogenous mRNA (Fig. 2, lane 1). If this extract was programmed with full-length  $\beta\alpha$ -mRNA, two transla-



**Figure 1.** Construction of plasmid pSBC57. The construction of pSBC57 from pSP65 and an Eco RI-Bam HI fragment derived from pBBC1 is indicated. Shaded area, polylinker sequence of pSP65. The open reading frames X and C2814 have been described elsewhere (3, 5, 39).

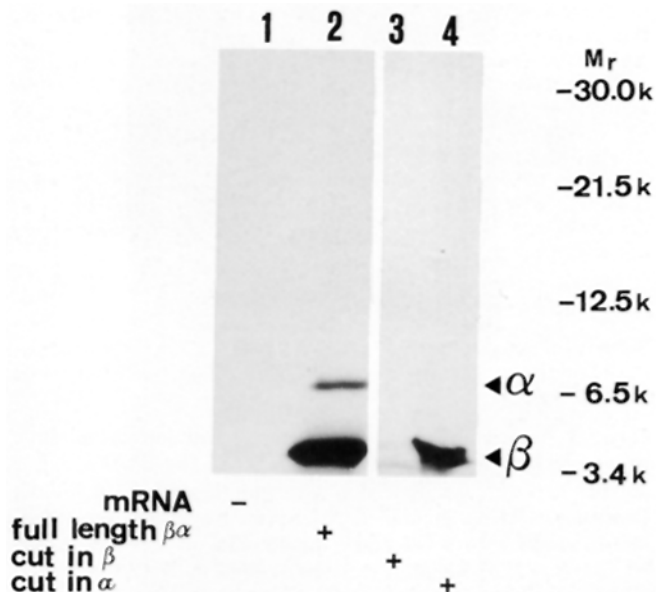
tion products of 6.7 and 4.7 kD were obtained (lane 2). These two proteins were recognized by antibodies raised against the B870 polypeptides (data not shown). That the two in vitro-synthesized proteins are in fact B870  $\alpha$  and  $\beta$  was demonstrated by their disappearance when transcripts were used which had been interrupted within the coding regions of  $\alpha$  or  $\beta$ . This is shown in Fig. 2, lanes 3 and 4. Linearizing pSBC57 DNA with Bgl I, which cuts within the coding region of  $\beta$  (Fig. 1) separates  $\alpha$  from the shared promoter. Thus, potentially only a truncated  $\beta$  peptide is left. This peptide however, is not detected (lane 3). In lane 4, mRNA was translated which was obtained by a linearization of pSBC57 with Bst NI, which cuts in the  $\alpha$ -gene and thus leaves  $\beta$  intact (Fig. 1). As expected, the 4.7-kD  $\beta$ -peptide is visible, whereas the  $\alpha$ -peptide is not. Clearly, the 4.7- and 6.7-kD proteins appeared only when the full-length coding regions for  $\alpha$  and  $\beta$  were contained in the transcripts. The failure to detect truncated forms of  $\alpha$  and  $\beta$  is probably due to rapid degradation as has been observed in other in vitro systems (24).

### Integration into ICM of B870 Polypeptides Synthesized In Vitro

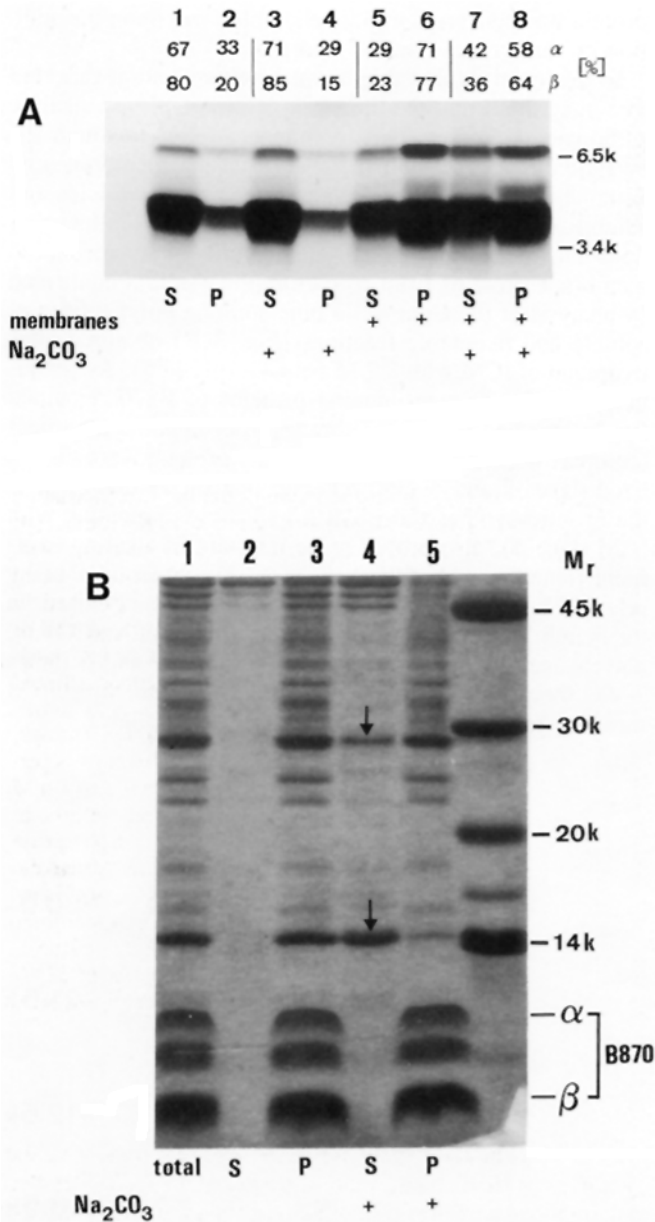
To study the integration of the B870  $\alpha$  and  $\beta$ -polypeptides into membrane vesicles of *R. capsulatus* the membrane-free S-135 was supplemented with ICM prepared from phototrophically grown *R. capsulatus* cells (see Materials and Methods). As shown in Fig. 3, both proteins became associated with the exogenously added ICM. In this experiment, translation products were separated into soluble (S) and pelletable (P) material. In the absence of ICM, most of  $\alpha$  and  $\beta$  remained soluble (lanes 1 and 2), whereas the majority of each

protein was converted into a pelletable form upon the addition of membranes (lanes 5 and 6).

To demonstrate that this membrane association reflected true integration into the lipid bilayer, we employed alkaline carbonate extraction. This treatment, applied to rough ER by Fujiki et al. (11) and to *E. coli* plasma membranes by Watanabe et al. (37), breaks open membrane vesicles and solubilizes loosely attached proteins without influencing lipid-integrated material. The applicability of this method to membrane proteins from *R. capsulatus* was first confirmed by analysis of the Coomassie blue-staining polypeptides of soluble and pelletable fractions (Fig. 3 B) obtained after treatment of ICM with 0.2 M  $\text{Na}_2\text{CO}_3$  (pH 11.5). As we anticipated, the pigment-binding proteins of B870 remained firmly attached to the membranes after alkaline treatment (compare lanes 3 and 5), whereas other proteins were solubilized (lanes 2 and 4, arrows). Fig. 3 A shows that most of the in vitro synthesized B870  $\alpha$  and  $\beta$  cosedimenting with ICM (lane 6), also proved to be resistant to alkaline treatment (lane 8), with only 13% of both polypeptides being released by  $\text{Na}_2\text{CO}_3$  (compare lanes 5 and 7). Because in the membrane-free control the mere addition of  $\text{Na}_2\text{CO}_3$  did not change the sedimentation behaviour of  $\alpha$  and  $\beta$  (lanes 1-4), their alkaline-resistant cosedimentation with ICM must be due to an integration into the lipid bilayer.



**Figure 2.** Cell-free synthesis of the B870  $\alpha$  and  $\beta$  proteins. Shown are [ $^{35}\text{S}$ ]methionine-labeled proteins synthesized in an S-135 from mRNAs of various lengths. Proteins were separated by SDS-PAGE and visualized by fluorography. The mRNAs used for this experiment were prepared by SP6 polymerase transcription of pSBC57 linearized with different restriction enzymes: Stu I (lane 2) cuts after the gene for  $\alpha$  giving rise to a full-length mRNA in which the SP6 promoter is followed by the coding sequence of  $\beta$  and then  $\alpha$ . Bgl I (lane 3) cuts in the gene for  $\beta$ , leaving only a truncated  $\beta$ -gene and no  $\alpha$ . Bst NI (lane 4) cuts in the gene for  $\alpha$  without affecting  $\beta$ . Lane 1 is the mRNA-free control. Marker proteins are: [ $^{14}\text{C}$ ]methylated insulin B-chain (3.4 kD), aprotinin (6.5 kD), cytochrome c (12.5 kD), soybean trypsin inhibitor (21.5 kD), and carbonic anhydrase (30 kD).



**Figure 3.** Association of B870 polypeptides with intracytoplasmic membrane vesicles is Na<sub>2</sub>CO<sub>3</sub> resistant. (A) The B870  $\alpha$  and  $\beta$  polypeptides were synthesized in vitro with (lanes 5–8) or without (lanes 1–4) added membranes. After protein synthesis was terminated, samples were incubated for 15 min on ice with 0.2 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.5). Controls received an equal amount of buffer (40 mM TeAOAc, pH 7.5). Subsequently, samples were centrifuged in the airfuge at 30 psi for 10 min. Supernatants (S) were removed and those of Na<sub>2</sub>CO<sub>3</sub>-containing reactions were neutralized by adding acetic acid before precipitation with TCA and preparation for SDS-PAGE. The pellets (P) were directly dissolved in SDS-PAGE sample buffer. The radioactivity in each  $\alpha$  and  $\beta$ -band was determined and is indicated as a percentage at which 100% was defined as the total counts recovered from the two subfractions for either  $\alpha$  or  $\beta$ . (B) 5- $\mu$ l aliquots of ICM were incubated as described for A. Protein bands were stained with Coomassie brilliant blue. Lane 1 shows the total protein content of a non-centrifuged ICM sample; lanes 2 and 3 represent the supernatant (S) and pellet (P), respectively, resulting after centrifugation of a mock-treated sample; lanes 4 and 5 represent the S and P fractions, respectively, from Na<sub>2</sub>CO<sub>3</sub>-treated ICM.

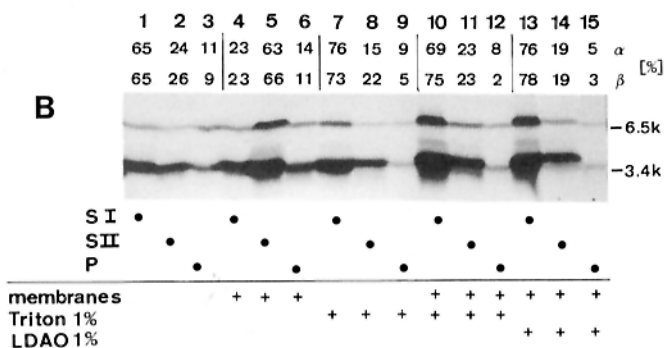
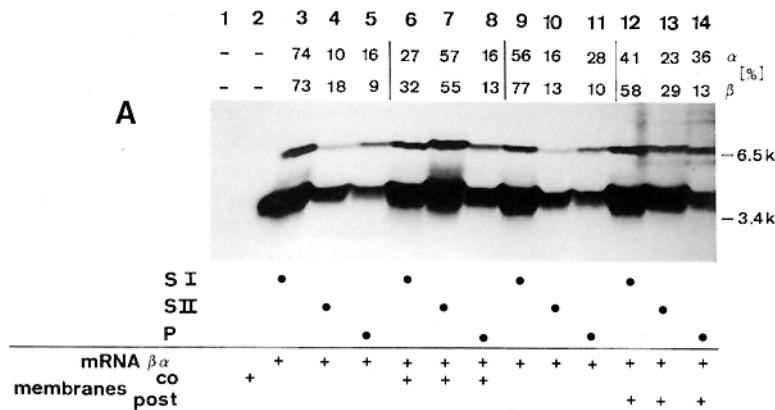
Even in the absence of exogenously added ICM some of the in vitro synthesized B870 proteins were found in the pellet fraction (Fig. 3 A, lane 2) presumably due to aggregation. To discriminate therefore between true membrane integration and mere aggregation, the previous separation into soluble and pelletable translation products was extended (Fig. 4) by fractionating newly synthesized proteins into soluble (SI), membrane-bound (SII), and aggregated (P) forms using a two-step sucrose gradient (Materials and Methods). The reliability of the fractionation protocol has recently been verified using an established *E. coli* cell-free translation-translocation system (1). Moreover, the *R. capsulatus* ICM could be easily detected in the SII fraction by their intense pigmentation.

Fig. 4 A shows the  $\beta\alpha$ -mRNA translation products fractionated in the above mentioned manner and separated by SDS-PAGE. When translated in the absence of membranes the bulk of newly synthesized  $\alpha$  and  $\beta$  was soluble (74 and 73%, respectively; lanes 3–5). The comparably small amount of protein in fraction SII from membrane-free samples (lane 4: 10% of  $\alpha$ ; 18% of  $\beta$ ) is probably due to an incomplete separation of fractions SI and SII and not to residual membranes in the S-135 (see below). Upon cotranslational addition of ICM, however, almost 60% of  $\alpha$  and  $\beta$  was now recovered from the membrane fraction SII (lane 7). The amount of pelleted  $\alpha$  and  $\beta$  was unaffected by the addition of ICM (lanes 5 and 8). These results suggest that up to two-thirds of the soluble species can associate with ICM in our cell-free system.

Fig. 4 A, lanes 9–14 show an analogous experiment in which membrane-free protein synthesis was inhibited after 45 min by puromycin; membranes were added posttranslationally and the sample was incubated for another 30 min (lanes 12–14). A control (lanes 9–11) was treated identically except that ICM were omitted. Only small amounts of  $\alpha$  and  $\beta$  shifted from SI to SII upon the posttranslational addition of ICM (compare lanes 10 and 13), indicating that the posttranslational membrane association is much less efficient than the cotranslational one. The most important aspect of this experiment, however, is that the observed membrane association of de novo-synthesized B870  $\alpha$  and  $\beta$  was not dependent on the mere presence of ICM, but in its requiring the addition of ICM early during protein synthesis reflected a specific process occurring only during a distinct time window.

It was consistently observed that the amount of  $\alpha$  and  $\beta$  recovered from the three subfractions increased in the presence of ICM (compare lanes 3–5 with 6–8 and 9–11 with 12–14). It remains unexplained exactly why this is. In any case, it was clearly not due to endogenous  $\alpha$  and  $\beta$  mRNA associated with ICM as shown by the control (lane 2) and the posttranslational assay (lanes 12–14), in which puromycin prevented any protein synthesis after the addition of ICM.

Additional evidence for B870  $\alpha$  and  $\beta$  being truly integrated into ICM is provided by the experiment shown in Fig. 4 B, which was based on the fact that lipid-integrated proteins require the addition of a detergent for solubilization. In our fractionation assay solubilization would be expected to result in a shift from SII to SI. The translation reactions were therefore treated with either 1% Triton X-100 or 1% lauryldimethylamine oxide (LDAO) before fractionation. Lanes



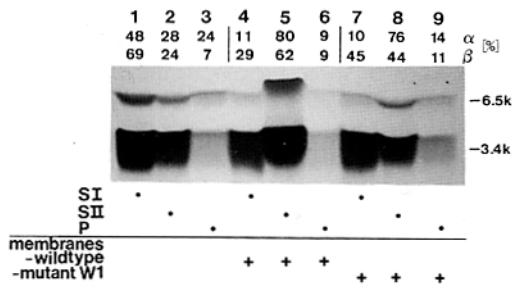
**Figure 4.** Subfractionation of the in vitro translation products reveals specific membrane integration. (A) B870  $\beta\alpha$ -mRNA directed translation reactions were fractionated into soluble (SI), membrane-associated (SII), and pelletable material (P) as described in Materials and Methods. Membranes were omitted or added either co- or post-translationally as indicated. Lanes 1 and 2 show controls without added mRNA. Membranes do not harbor endogenous, protein-synthesizing activity (lane 2). In posttranslational assays, protein synthesis in the absence of membranes was performed for 45 min, stopped by the addition of 1 mM puromycin, and incubation was continued for 30 min in the presence of membranes, ATP, creatine phosphate and creatine phosphokinase, which were readded to the same concentrations as during the first 45 min of incubation. (B) Before subfractionation, translations were incubated with 1% Triton X-100 (TX-100) or lauryldimethylamine oxide (LDAO) for 10 min at 32°C. Controls that received H<sub>2</sub>O instead of detergent were treated identically.

1-3 depict membrane-free and lanes 4-6 depict membrane-containing controls that did not receive detergent. After detergent treatment with either Triton or LDAO (lanes 10-15) the bulk of the  $\alpha$  and  $\beta$  proteins was in fact shifted from the membrane fraction SII (lane 5) to the soluble fraction SI (lanes 10 and 13). The aggregated material was also solubilized to some extent by the detergents (compare lane 6 with lanes 12 and 15) which might indicate that some of  $\alpha$  and  $\beta$  in the pellet is due to cross-contamination with membranes. The amount of  $\alpha$  and  $\beta$  recovered from the SII of the membrane-free sample (lane 2) was only slightly lowered by detergent (lane 8). This fact is an additional strong indication for the S-135 being almost completely membrane free.

#### Assembly of De Novo Synthesized B870 $\alpha$ and $\beta$ into Membrane-bound, Pigment-containing Complexes

Finally we wanted to examine whether in vitro synthesized B870  $\alpha$  and  $\beta$  polypeptides would assemble into pigment-containing complexes when integrated into the lipid bilayer of ICM. To this end we prepared membranes of the pigment-deficient mutant strain W1 (10). W1 cells cannot grow phototrophically, yet, like wild-type cells, they induce ICM when cultured semiaerobically in the dark (10, 21). The results

which were obtained when W1-ICM were present during the synthesis of B870  $\alpha$  and  $\beta$  are illustrated in Fig. 5. The two polypeptides partitioned into the fraction of the mutant ICM to different degrees (lane 8) when compared with wild-type membranes that had also been prepared from semiaerobically grown cells (lane 5). Whereas as much  $\alpha$  was found in the mutant as in the wild-type membranes, association of  $\beta$  with W1-ICM was considerably reduced. The reason for the different behavior of both proteins towards W1-ICM is not clear. Most strikingly however, the discrete decrease in the electrophoretic mobility of  $\alpha$  and  $\beta$  that was consistently observed for the ICM-associated species (lane 5; see also Fig. 4, lane 7) was completely absent if W1-ICM were used (Fig. 5, lanes 5 and 8). This increase in the apparent molecular weight upon membrane association of in vitro synthesized B870  $\alpha$  and  $\beta$  has also been observed by others (16) and has been explained by a tight, detergent-resistant association with pigments. In agreement with this B870  $\alpha$  and  $\beta$  retained their higher apparent molecular weights after solubilization with Triton and LDAO (Fig. 4 B, lanes 10 and 13). Moreover, as shown in Fig. 5, ICM of a pigment-deficient mutant strain did not give rise to a change in the electrophoretic mobility of B870  $\alpha$  and  $\beta$ . This finding therefore strongly suggests that



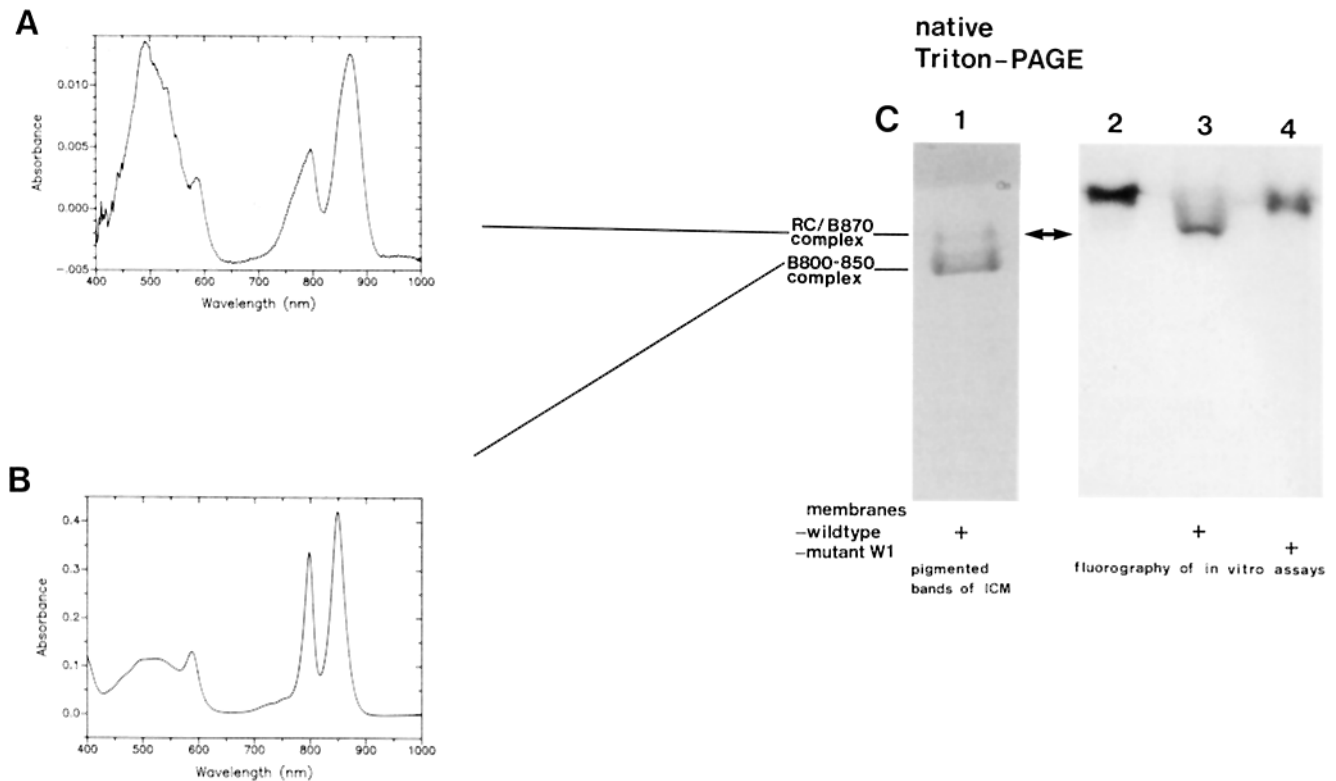
**Figure 5.** B870 polypeptides which integrated *in vitro* into ICM associate with membrane-derived pigments. B870  $\alpha$  and  $\beta$  were synthesized *in vitro* and the translation products were analyzed as described in the legends to previous figures. Synthesis was performed either in the absence of ICM (lanes 1-3), in the presence of wild-type ICM (lanes 4-6) or in the presence of ICM from the pigment-deficient mutant strain W1 (lanes 7-9). Membranes had been prepared from semiaerobically grown cells (Materials and Methods).

*in vitro* synthesized B870  $\alpha$  and  $\beta$  do in fact associate with membrane-derived pigments when integrated into wild-type ICM.

A direct demonstration of the assembly of *in vitro* synthesized B870  $\alpha$  and  $\beta$  polypeptides into functional, supramo-

lecular complexes is presented in Fig. 6. In these experiments pigment-containing protein-complexes were isolated by mild detergent fractionation using Triton X-100 (28). When an aliquot of ICM (4  $\mu$ l) prepared from semiaerobically grown cells was solubilized by Triton X-100 and fractionated by Triton-PAGE at 4°C in the dark, two pigmented bands were resolved (Fig. 6 C, lane 1). The absorption spectrum of each band, obtained by scanning the respective part of the gel with a microspectrophotometer, revealed the absorbance pattern characteristic of RC/B870 and B800-850 complexes, respectively. Fig. 6 A shows the absorbance peak at 870 nm of B870 and a shoulder in the 780 nm range typical for the reaction center (22). B depicts the sharp absorbance peaks of B800-850.

When B870 proteins synthesized *in vitro* in the presence of ICM were analyzed by Triton-PAGE and fluorography, the major part of the radioactively labeled proteins was recovered from the B870-containing band (Fig. 6 C, lanes 1 and 3, *double arrow*). As expected no radioactivity was found in the B800-850 complex, because no B800-850 proteins had been synthesized. If the synthesis proceeded in the absence of ICM however, the radioactive proteins migrated with different electrophoretic mobilities (lane 2), i.e., to a place on the Triton-gel, which does not contain pigment-protein



**Figure 6.** *In vitro* synthesized B870  $\alpha$  and  $\beta$  polypeptides assemble into a pigment-containing RC/B870 complex of ICM. (A and B) Absorption spectra of the two pigmented bands shown in C, lane 1. A gel strip as that shown in C, lane 1, was vertically scanned by monitoring the absorption spectra. Those of the two major pigmented bands are shown. Gel scanning was performed as described (22). Note the considerably higher intensity of the signals in B versus A which fits well with the different degree of pigmentation of the two bands separated by Triton-PAGE. This in turn, is a reflection of the significantly larger occurrence of B800-850 in ICM compared with RC/B870 under semiaerobic growth conditions (31). (C) Solubilization of pigment-containing protein complexes from ICM and resolution by Triton-PAGE according to methods published in reference 28. Lane 1 shows the two pigmented bands thus obtained from 4  $\mu$ l ICM. Lanes 3 and 4 are fluorographs of the B870 polypeptides synthesized *in vitro* in the presence of 1  $\mu$ l ICM from either wild-type strain 37b4 (lane 3) or the pigment-deficient mutant W1 (lane 4). Lane 2 is the ICM-free control. Samples were resolved by Triton-PAGE under identical conditions as described for lane 1.

complexes (compare lanes 1 and 2). As an additional demonstration for the specificity of the comigration of the in vitro membrane-integrated B870  $\alpha$  and  $\beta$  proteins with the native RC/B870 complex, a sample containing W1 mutant ICM is shown in lane 4. In this case, the radioactively labeled proteins again did not comigrate with the RC/B870 complex (lanes 3 and 4), although they had been shown to associate with the mutant ICM (Fig. 5). Obviously the in vitro synthesized B870  $\alpha$  and  $\beta$  proteins did not assemble into the supramolecular RC/B870 structure due to a lack of pigment ligands in the mutant ICM. Consequently, the results illustrated in Fig. 6, indicate clearly that in the presence of wild type ICM a significant part of de novo-synthesized B870  $\alpha$  and  $\beta$  not only integrates into the lipid bilayer but further assembles into a pigment-containing RC/B870 complex whose native structure is revealed by its authentic absorbance profile.

## Discussion

We report here the development of a cell-free protein-synthesizing system from *R. capsulatus*. The two pigment-binding proteins  $\alpha$  and  $\beta$  of the B870 light-harvesting complex were expressed from isolated  $\beta\alpha$ -mRNA using a high-speed membrane-free supernatant (S-135) of a *R. capsulatus* cell extract. Although our results clearly show that the two translation products of  $\beta\alpha$ -mRNA are indeed the B870 polypeptides  $\alpha$  and  $\beta$ , their apparent molecular masses of 6.7 and 4.7 kD differ somewhat from those previously reported (27). This is most likely due to different electrophoretic conditions as varying molecular masses were also described by others for light-harvesting complex proteins of *R. sphaeroides* (2, 16).

Up to now no attempt had been made to show membrane integration in vitro of photosynthetic proteins in bacteria. Chory and Kaplan described a translation system based on an S-30 from *R. sphaeroides* (6). Only Hoger et al. (13) reported membrane-association of pigment-binding proteins of *Rhodobacter sphaeroides* in vitro; however, their cell-free system was not clearly shown to be membrane dependent. We report here that the in vitro-synthesized proteins were found to integrate into exogenously added ICM. The availability of such a membrane-dependent system is a prerequisite for the study of membrane integration of pigment-binding proteins.

Because pigment-binding proteins are synthesized without a cleavable signal sequence (8), proteolytic processing could not be used as a marker for membrane integration, unlike with the majority of exported *E. coli* proteins studied so far in vitro (30). Integration of the de novo-synthesized proteins into the lipid bilayer of the membranes however, could be demonstrated by: (a) the fact that membrane association of newly synthesized B870  $\alpha$  and  $\beta$  could not be reversed by  $\text{Na}_2\text{CO}_3$  in contrast to other ICM proteins; (b) the detergent-solubility of the membrane-integrated proteins; and (c) the finding that membrane integration of the pigment-binding polypeptides was not dependent on the mere presence of ICM, but specifically required the addition of membranes cotranslationally to proceed efficiently. A conceivable reason for the low degree of posttranslational ICM association is the acquisition of a stable tertiary structure of the B870 polypeptides when synthesized to completion in the absence of membranes. Such a stable conformation would be incompatible with membrane integration unless chaperonins or un-

foldases were available as found for the membrane transport of many other proteins (30).

The use of pigment-deficient membrane vesicles revealed that newly synthesized B870  $\alpha$  and  $\beta$  proteins did not only integrate into the lipid bilayer of ICM but also became associated with pigment ligands within the plane of the membrane. This conclusion is drawn from a characteristic decrease in the electrophoretic mobilities of B870  $\alpha$  and  $\beta$  on SDS gels after association only with wild type ICM but not with pigment-deficient mutant membranes. Furthermore, the binding of in vitro integrated B870 polypeptides to membrane pigments could directly be demonstrated. Isolation of native pigment-protein complexes disclosed that a significant amount of de novo-synthesized B870  $\alpha$  and  $\beta$  protein had assembled into a supramolecular structure characteristic of a RC/B870 complex. Thus in vitro-integrated B870 proteins must have associated with membrane-located pigment ligands. These in vitro-assembled light-harvesting complexes then interacted further with reaction center complexes to form functional units as evidenced by the authentic absorption spectrum of an intact RC/B870 unit.

In summary, we have shown that synthesis and membrane integration of two proteins of the photosynthetic apparatus of *R. capsulatus* can be reproduced in a homologous cell-free system. Approximately 50% of the B870 polypeptides are thus integrated into cotranslationally added ICM. Moreover, the de novo-integrated B870 polypeptides were found to associate with preexisting pigments and RC-complexes to form native photosynthetic RC/B870 units. This cell-free system now offers the opportunity to study integration and assembly of pigment-binding proteins in mutant membranes lacking individual pigment-binding proteins. Furthermore, it should be suitable to address the question of the specificity of the protein insertion into distinct membrane subfractions as suggested by others (7, 29). These studies should shed some light on the complex regulation of synthesis, integration and assembly of the photosynthetic apparatus from *R. capsulatus*. In addition, the system should prove useful in general for the investigation of protein integration into bacterial membranes.

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