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1992

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Recommended Citation

Bollivar, David, "Association of Tetrapyrrole Intermediates in the Bacteriochlorophyll *a* Biosynthetic Pathway with the Major Outer-Membrane Porin Protein of *Rhodobacter capsulatus*" (1992). *Scholarship.* Paper 12.
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Biochemical Society
The Biochemical Journal, 1992, 282 (pt. 2); 471-476

Association of tetrapyrrole intermediates in the bacteriochlorophyll a biosynthetic pathway with the major outer-membrane porin protein of Rhodobacter capsulatus

D. W. Bollivar and C. E. Bauer

Rhodobacter capsulatus regulates synthesis of bacteriochlorophyll *a* in response to changes in oxygen partial pressure and light intensity. One early model proposed that this regulation involved a carrier polypeptide that functions to tether tetrapyrrole intermediates to the membrane. In the present study we isolated tetrapyrrole intermediates accumulated in three strains of *R. capsulatus* that contain mutations which block bacteriochlorophyll *a* biosynthesis at different steps of the magnesium branch of the pathway. Each of the tetrapyrrole intermediates was shown to be associated with the same 32 kDa polypeptide, as indicated by similar electrophoretic mobility and antigenic cross-reactivity with polyclonal antisera. The 32 kDa pigment-associated protein was further found to have an electrophoretic mobility, antigenic cross-reactivity and *N*-terminal sequence identical with those of the previously characterized major outer-membrane porin protein of *R. capsulatus*.

INTRODUCTION

In most species of photosynthetic bacteria, biosynthesis of bacteriochlorophyll *a* (Bchl *a*) is known to be tightly regulated by such environmental factors as oxygen partial pressure and light intensity. Early studies with *Rhodobacter* species such as *R. capsulatus* and *R. sphaeroides* demonstrated that the steady-state level of Bchl *a* increases 30–100-fold when growing cells are shifted from an aerobic to an anaerobic environment [1,2]. The level of Bchl *a* synthesized under anaerobic growth conditions can further vary up to 6-fold in response to changes in light intensity [1,2].

The mechanisms whereby the level of Bchl *a* biosynthesis is regulated by oxygen and light are still unsolved, but several results have provided an indication that regulation may occur via novel post-transcriptional events. The evidence for this conclusion is based in part on the abrupt cessation of Bchl *a* biosynthesis by the addition of oxygen or increased light intensity, [1–5], which occurs much more quickly than the rate at which expression of genes encoding enzymes involved in Bchl biosynthesis are affected by the same stimuli [6–8]. It has also been shown that regulation of Bchl *a* biosynthesis does not involve feedback inhibition of the synthesis of Bchl *a* or later intermediates in the pathway [9–15]. One early model, proposed by Lascelles [10,16], suggests that Bchl *a* biosynthesis may involve a membrane-bound carrier polypeptide associated in a non-covalent manner with intermediates in the pathway. This model is supported in part by observations that mutants blocked in Bchl *a* biosynthesis excrete tetrapyrrole intermediates into the culture supernatant fluid as a high-molecular-mass tetrapyrrole–protein–lipid–carbohydrate complex [14,17–20]. This model also accounts for the difficulty in observing enzymic activities *in vitro* using isolated tetrapyrrole intermediates as substrates. However, there is as yet no direct evidence for the involvement of the postulated carrier polypeptide in Bchl *a* biosynthesis.

More recently, a regulatory protein (PufQ) has been identified that controls the synthesis of Bchl *a* relative to the synthesis of light-harvesting and reaction-centre polypeptides [21]. PufQ has several features which led to the conclusion that it may function as the putative carrier polypeptide in Bchl *a* biosynthesis. These features include the existence of a hydrophobic domain containing similarities to known Bchl *a*-binding polypeptides as well as a similar size to a previously reported polypeptide thought to be associated with tetrapyrrole intermediates. The possibility that PufQ may function as a carrier polypeptide prompted our current investigation of the polypeptide component of the pigment–protein complex excreted by mutants of *R. capsulatus*. The results of our analysis demonstrate that excreted and membrane-bound Mg-tetrapyrrole intermediates obtained from several different steps of the pathway are each tightly associated with a similar 32 kDa polypeptide. We further show that the 32 kDa pigment-assoicated polypeptide has characteristics identical with those of the previously described major outer-membrane porin protein from *R. capsulatus*.

EXPERIMENTAL

Bacterial strains and growth conditions

R. capsulatus strains SB1003 (*rif*-10), BRP33 (*bchG33 crtf129 hsd-1 str-2*), CB1064 [*rif-10* Ωdel(*pufQ'-pufC2397*)], MB1007 (*bch1007*) and ZY5 (*bchL::Km*^{r} *rif-10*) have been described previously [21–25]. Cells were routinely grown heterotrophically or photosynthetically at 35 °C in PYS or RCV 2/3 PY medium as described previously [26]. Optimum production of tetrapyrrole intermediates was achieved by growth under dark low-aeration conditions in RCV$^+$, a synthetic malate minimal medium [27], supplemented with glucose, pyruvate and dimethyl sulphoxide [25,28].

Isolation of pigment–protein complexes

The excreted tetrapyrrole–protein complex was isolated from the culture supernatant fluid of cells grown to mid-stationary

Abbreviations used: Bchl *a*, bacteriochlorophyll *a*: LDAO, lauryldimethylamine oxide (dodecyldimethylamine oxide); ICM, intracytoplasmic membrane.

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phase in the dark under low-aeration conditions. An 800 ml culture of cells grown in RCV$^+$ was shaken at 90 rev./min until the cells were well into stationary phase (approx. 3 days incubation). Intact cells were removed by centrifugation at 16000 *g* ($r_{av.}$ 11.94 cm) for 10 min at 4 °C. Excreted tetrapyrrole–protein complex was then concentrated from the clarified culture supernatant by centrifuging at 70000 *g* ($r_{av.}$ 57.3 cm) for 2.0 h at 4 °C. The resulting pigmented pellet was resuspended to a final concentration of 6 mg/ml in 10 mM-Tris/HCl (pH 6.8)/1.0 mM-EDTA/2.0 mM-$MgCl_2$. A 30 % (w/v) solution of the detergent lauryldimethylamine oxide (LDAO; dodecyldimethylamine oxide; Calbiochem) was slowly added to the pigment–protein-complex suspension to give a final concentration of 0.25 % (w/v). A 0.5 ml sample of the solubilized pigment–protein complex was subsequently loaded on to a 12.75 ml step gradient composed of equal volumes of 15, 20 and 40 % sucrose (w/v) in the same buffer. After centrifugation at 100000 *g* ($r_{av.}$ 110.2 cm) for 13 h at 4 °C in an SW41 (Beckman) rotor, a green band was observed at the 20 %/40 %-sucrose interface. This band was isolated by fractionation and stored at −20 °C until further use.

For isolation of the membrane-bound pigment–protein complexes, cells were grown as described above, with the exception that the cells were harvested in early stationary phase (1.5 to 2 days growth). Cells were harvested by centrifugation at 10000 *g* ($r_{av.}$ 11.94 cm) for 10 min at 4 °C and subsequently washed in a volume equal to that of the growth medium of 50 mM-Tris/HCl (pH 7.5)/1.0 mM-EDTA/2.0 mM-$MgCl_2$. The cell pellet was then resuspended in the same buffer at a concentration of 1.0 g wet weight/3.0 ml of buffer and passed twice through a French-press cell (Aminco) at 103.5 MPa (15000 lbf/in^2). A few crystals of RNAase A and DNAase I were added to the resulting lysate, which was then incubated at 4 °C for 1.0 h with slight stirring. Intact cells and large debris were then removed by centrifugation at 16000 *g* ($r_{av.}$ 11.95 cm) for 15 min at 4 °C. The supernatant was then re-centrifuged at 150000 *g* ($r_{av.}$ 57.3 cm) for 2.0 h at 4 °C. The gelatinous pigmented layer of the pellet was resuspended in 0.1 M-sodium phosphate buffer, pH 7.5, to a final concentration of 12.0 mg of protein/ml, and subsequently diluted with an equal volume of the same buffer containing 0.5 % LDAO. Stirring of the solution at room temperature for 10 min was followed by centrifugation at 186000 *g* ($r_{av.}$ 57.3 cm) for 2 h at 4 °C. The pigmented pellet was then resuspended in 10 mM-Tris/HCl (pH 6.8)/1.0 mM-EDTA/2.0 mM-$MgCl_2$ to a concentration of 10 mg of protein/ml and then slowly diluted with an equal volume of the same buffer containing 0.6 % LDAO. A 0.5 ml volume of the suspension was then loaded on to a 12.75 ml sucrose step gradient and centrifuged as described above for the excreted pigment–protein complex. After centrifugation, the pigmented band present at the 20 %/40 %-sucrose interface was removed and either stored at −20 °C or concentrated by diluting with 10 mM-Tris/HCl (pH 6.8)/1.0 mM-EDTA/2.0 mM-$MgCl_2$ followed by centrifuging at 150000 *g* ($r_{av.}$ 57.3 cm) for 3 h at 4 °C. The resulting pellet was then resuspended in the same buffer and stored at −20 °C.

N-Terminal-sequence analysis

The protein component of the pigment–protein complex was further purified for *N*-terminal-sequence analysis. For this analysis the pigment–protein complex was obtained from the membrane fraction of MB1007 as described above and then further subjected to size-exclusion chromatography on a Superose 12 (Pharmacia) f.p.l.c. column that had been equilibrated with 0.05 % Triton X-100. The major peak, which contained electrophoretically pure 32 kDa protein, was subsequently sequenced by automated Edman degradation on a model 470A gas-phase protein sequencer (Applied Biosystems) [29]. The results were analysed by using an on-line model 120A PTH (phenylthiohydantoin) Analyzer (Applied Biosystems).

Isolation of membrane fractions and the major outer-membrane porin protein

Purified intracytoplasmic membrane (ICM) preparations were obtained from photosynthetically grown cells by using sucrose-density-gradient centrifugation as detailed previously [30]. Outer-membrane preparations were obtained from aerobically grown cells as described by Flamman & Weckesser [31]. Sucrose gradients were fractionated using a gradient fractionator (ISCO) with the absorbance at 280 nm and 260 nm recorded and used to localize membrane fractions as described by Lane & Hurlbert [30]. Peak fractions were subsequently assayed for succinate dehydrogenase activity [32] and level of 2-oxo-3-deoxyoctonate [33], to localize inner- and outer-membrane fractions respectively. The fractions were subsequently concentrated by centrifugation at 186000 *g* ($r_{av.}$ 57.3 cm) at 4 °C for 3 h in 5.0 mM-Tris/HCl (pH 8.1)/1.0 mM-dithiothreitol followed by resuspending the pellet in 10 mM-Tris/HCl, pH 6.8, to a final concentration of 10 mg of protein/ml, which was stored at −20 °C.

The major outer-membrane porin protein was isolated as described by Flamman & Weckesser [31], with porin activity being demonstrated for the isolated porin protein using a liposome-swelling assay [34,35].

Gel electrophoresis and Western-blot analysis

SDS/PAGE was performed using a modified Laemmli [36] procedure. For Western-blot analysis, antibodies were raised against a purified 32 kDa protein preparation obtained from whole MB1007 cells. Approx. 200 μg of protein were excised from preparative SDS/PAGE gels, emulsified with Freund's complete adjuvant (Difco) and injected subcutaneously into virgin female New Zealand White rabbits. Booster injections containing 200 mg of similarly prepared protein were emulsified with Freund's incomplete adjuvant (Difco) and injected every third week for an additional 9-week period. The rabbits were anaesthetized with Innovar at a dose of 0.125 ml/kg body weight before bleeding. Serum was prepared as described in [37]. Transfer of protein from SDS/PAGE gels on to nitrocellulose (Schleicher and Schuell) was accomplished using an electrophoretic blotting apparatus (TransBlot Cell; Bio-Rad) using buffers described in [37a]. Probing for antigen was performed using the Immun-Blot GAR-AP Kit (no. 170–6460; Bio-Rad) as described by the manufacturer using antisera or preimmune sera that were diluted 1:40000 before use.

Analytical methods

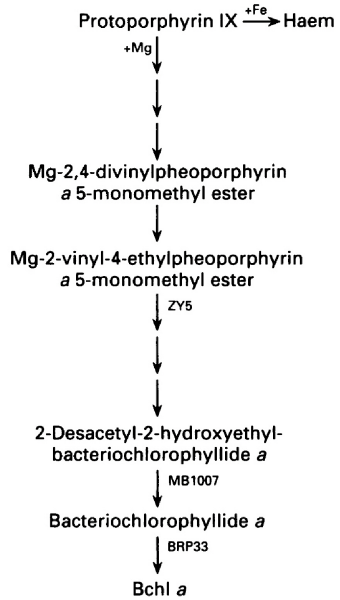
Protein concentration was determined using a Bradford assay [38] (Bio-Rad) or with a modified Lowry assay [39]. Spectral scans and absorbances were recorded using a Beckman DU-50 spectrophotometer. The ratio of protochlorophyllide to protein was determined from the pigment–protein complex isolated from the membrane fraction of ZY5. A 100 μl volume of isolated pigment–protein complex was first dried in darkness in a rotary vacuum desiccator (Speed-Vac; Savant). The pigment was then solubilized by resuspension in 5.0 ml of cold (−20 °C) acetone/methanol (7:2, by vol), followed by 5.0 ml of diethyl ether. The solubilized pigments were clarified by centrifugation at 10000 *g* ($r_{av.}$ 11.94 cm) at 4 °C for 10 min. The supernatant fluid was dried under a stream of nitrogen, and the dried tetrapyrrole was resuspended in diethyl ether. Molarity was calculated by using a previously determined absorption coefficient for protochlorophyllide [40].

RESULTS

Isolation of excreted and membrane-bound tetrapyrrole–protein complexes

Mutants of *R. capsulatus* that disrupt the Bchl *a* biosynthetic pathway produce large amounts of tetrapyrrole intermediates preceding the position in the pathway that is blocked. For example, as a consequence of a mutation in the *bchL* gene (Scheme 1), strain ZY5 accumulates Mg-2,4-divinylpheoporphyrin a_5 monomethyl ester and Mg-2-vinyl-4-ethylpheoporphyrin a_5 monomethyl ester (mono- and di-vinyl versions of protochlorophyllide respectively) [25]. As shown in Fig. 1, the accumulated protochlorophyllide, which can be observed by its characteristic absorbance maximum at 640 nm, is excreted into the culture medium of stationary-phase cells (Fig. 1*a*; broken line). In accordance with previous studies, we found that the excreted tetrapyrrole is part of a high-molecular-mass pigment–protein complex that can be readily concentrated from the culture supernatant fluid by high-speed centrifugation (see the Experimental section). Also in agreement with previous studies [14–17] are the results of SDS/PAGE analysis of the concentrated pigment–protein complex, which show the presence of numerous polypeptides (Fig. 2, lane 1). The results of the PAGE analysis of the excreted complex should be interpreted with caution, however, since high-speed centrifugation will pellet polypeptides other than those specifically associated with the pigment (e.g. bacteriophage-like particles that are continually excreted from *R. capsulatus*). The excreted pigment–protein complex was therefore further purified by treatment with the non-ionic detergent LDAO followed by centrifugation through a sucrose-density step gradient. Under such conditions a pigmented band forms at the 20%/40%-sucrose interface that has a similar absorbance profile (Fig. 1*b*; broken line) to that of the crude complex excreted into the culture media. Electrophoresis of the isolated pigment–protein complex shows the presence of a single prominent polypeptide with an approximate size of 32 kDa as indicated by electrophoretic mobility (Fig. 2, lane 2).

We also undertook to determine whether or not the same 32 kDa polypeptide is associated with the tetrapyrrole before being excreted into the culture medium. For this analysis, ZY5 cells were harvested at early stationary phase, and the pigment–protein complex was extracted from whole-cell lysate by differential solubilization of membrane fractions with LDAO, followed by sucrose-density-gradient centrifugation. As shown in Fig. 1*b* (continuous line), the absorbance spectrum of the pigment–protein complex purified from the membrane fraction is



Scheme 1. Pathway of Bchl *a* synthesis in *R. capsulatus*

The Bchl *a* biosynthetic pathway, as well as that for various intermediates accumulated by mutations in ZY5, MB1007 and BRP33, have been described previously [22,24,25,28].

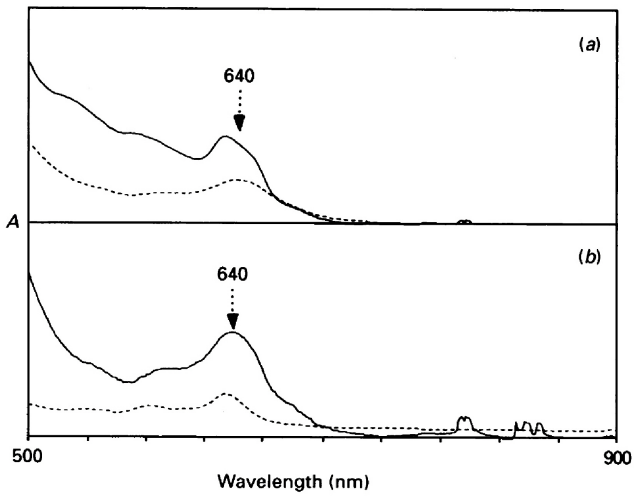


Fig. 1. Spectral analysis of protochlorophyllide accumulated by ZY5

(*a*) The continuous line is an '*in vivo*' spectrum of whole cells [48]. The broken line is the spectrum of the excreted pigment–protein complex after concentration by ultracentrifugation. (*b*) The broken line is the spectrum of the pigment–protein complex purified from the culture supernatant of ZY5. The continuous line is the spectrum of the pigment–protein complex purified from the membrane fraction of ZY5.

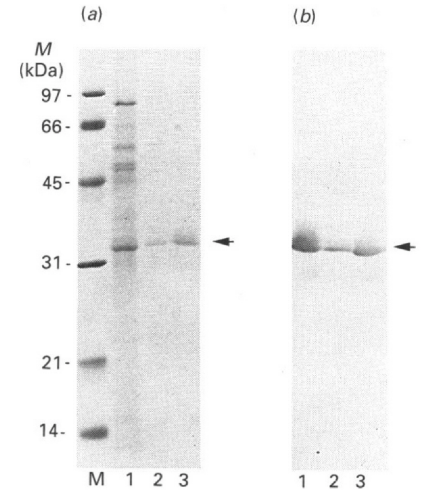


Fig. 2. SDS/PAGE and Western-blot analysis of excreted and purified pigment–protein complexes

(*a*) Coomassie Blue-stained SDS/PAGE polypeptide profile. Lane 1 is 25 μg of the excreted pigment–protein complex concentrated from the culture supernatant of ZY5 by ultracentrifugation. Lane 2 is 5 μg of the excreted pigment–protein complex after isolation by sucrose-density centrifugation. Lane 3 is 5 μg of the pigment–protein complex isolated from the membrane fraction of ZY5. M denotes molecular-mass (*M*) markers. (*b*) Western-blot analysis of the polypeptides associated with the tetrapyrrole. Lanes 1, 2 and 3 are as indicated in (*a*).

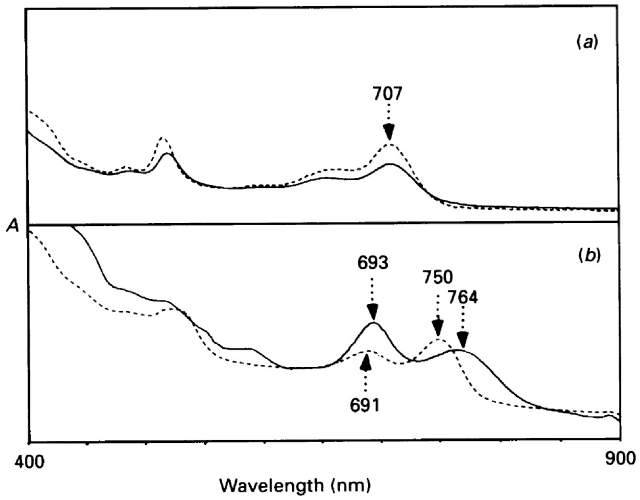


Fig. 3. Spectral analysis of tetrapyrroles accumulated in MB1007 and BRP33

(*a*) The solid line is an '*in vivo*' spectrum of strain MB1007, which accumulates a characteristic Mg-tetrapyrrole intermediate with an absorbance maximum at 707 nm. The broken line is the spectrum of the pigment–protein complex purified from the membrane fraction of MB1007. (*b*) The continuous line is an '*in vivo*' spectrum of BRP33 which accumulates tetrapyrrole intermediates which absorb at 693 and 764 nm. The broken line is the spectrum of the pigment–protein complex purified from the membrane fraction of BRP33.

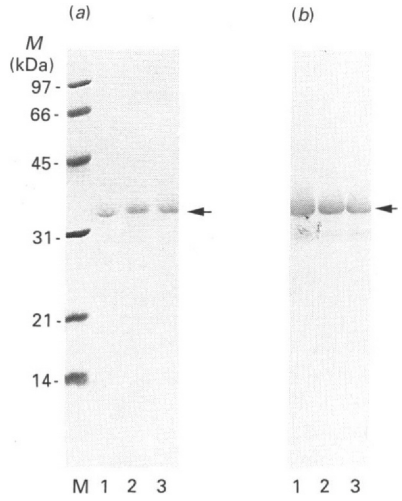


Fig. 4. SDS/PAGE and Western-blot analysis of pigment–protein complexes from ZY5, MB1007 and BRP33

(*a*) Lanes 1, 2 and 3 are the Coomassie Blue-stained SDS/PAGE polypeptide profiles of 5 μg of pigment–protein complexes isolated from membrane fractions of ZY5, MB1007 and BRP33 respectively. M denotes molecular-mass (*M*) markers. (*b*) Western-blot analysis of the protein samples separated by SDS/PAGE as described in (*a*).

similar to the pigment–protein complex purified from the culture supernatant (Fig. 1*b*, broken line). SDS/PAGE (Fig. 2*a*) and Western-blot (Fig. 2*b*) analyses of the membrane-bound pigment–protein complex also show the presence of a single prominent polypeptide exhibiting the same electrophoretic mobility and immunological cross-reactivity as is observed for the 32 kDa polypeptide isolated from the excreted complex.

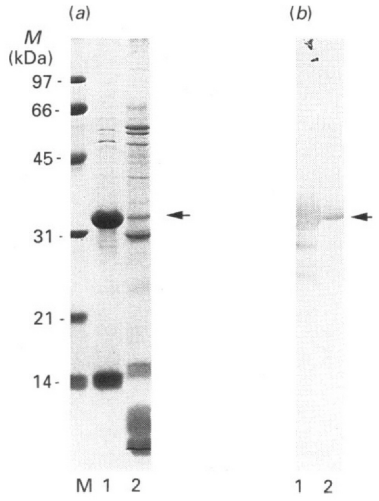


Fig. 5. SDS/PAGE and Western-blot analysis of membrane fractions isolated from SB1003

(*a*) Lanes 1 and 2 are the Coomassie Blue-stained SDS/PAGE profiles of 25 μg isolated outer-membrane and isolated ICM fractions respectively. M denotes molecular-mass (*M*) markers. (*b*) Western-blot analysis of the protein samples separated by SDS/PAGE as described in (*a*).

Isolation of pigment–protein complexes at additional steps of the pathway

An analysis was undertaken to determine whether a similar protein is associated with tetrapyrrole intermediates accumulated by mutants blocked at later stages of the Bchl *a* biosynthetic pathway. For this analysis, tetrapyrrole–protein complexes were isolated from the membrane fraction of MB1007, which accumulates the intermediate 2-devinyl-2-hydroxyethylchlorophyllide *a* and with strain BRP33 which accumulates the penultimate tetrapyrrole in the pathway, bacteriochlorophyllide *a* (Scheme 1; [22]). Absorbance spectrophotometry demonstrates that the pigment–protein complex isolated from MB1007 exhibits a major absorbance peak at 707 nm (Fig. 3*a*; broken line) which is the same spectrum observed from intact cells (Fig. 3*a*; solid line). Absorbance spectrophotometry of the pigment–protein complex isolated from BRP33 shows major absorbance peaks at 691 and 750 nm (Fig. 3*b*; broken line) which are slightly blue-shifted from the 693 and 764 nm peaks observed *in vivo* (Fig. 3*b*; solid line). SDS/PAGE polypeptide profiles of the pigment–protein complexes isolated from MB1007 and BRP33 also demonstrate the presence of a single polypeptide which exhibits an electrophoretic mobility and antigenic cross-reactivity that is identical with that of the 32 kDa polypeptide observed in ZY5 (Figs. 4*a* and 4*b*). The results of this analysis suggest that tetrapyrrole intermediates accumulated in strains containing mutations at early or later steps in the Mg branch of the Bchl *a* biosynthetic pathway are each associated with a similar 32 kDa polypeptide.

The 32 kDa pigment-associated polypeptide is the major outer membrane porin protein

The 32 kDa polypeptide was localized to a particular cellular compartment by performing Coomassie Blue staining and Western-blot analysis of SDS/PAGE-separated polypeptides from isolated ICM and outer-membrane fractions. As shown in Fig. 5(*b*), there is strong binding of the 32 kDa antisera to a polypeptide in the outer-membrane fraction of the cell, and very weak binding of the antisera to PAGE-separated polypeptides from the ICM fraction. Coomassie Blue staining of the gel (Fig. 5*a*) further demonstrates that the antiserum is recognizing the

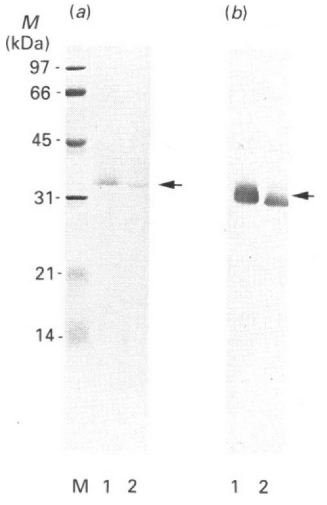


Fig. 6. SDS/PAGE and Western analysis of isolated porin and isolated pigment–protein complexes

(*a*) Lanes 1 and 2 are the SDS/PAGE polypeptide profiles of 5 μg of the porin complex isolated from wild-type cells and 5 μg of the pigment–protein complex isolated from ZY5 respectively, which were boiled for 3 min before electrophoresis. (*b*) Western-blot analysis of a gel similar to that described in (*a*).

major protein component of the *R. capsulatus* outer membrane which has the same electrophoretic mobility as the 32 kDa pigment-associated polypeptide.

The major outer-membrane protein from *R. capsulatus* has previously been isolated and characterized by Flamann & Weckesser [31,41–44]. Their analysis demonstrated that it has an approximate molecular mass of 33 kDa and is both structurally and functionally similar to porins characterized in several other Gram-negative species. A comparative analysis was therefore undertaken with the porin complex isolated from wild-type *R. capsulatus* and the 32 kDa pigment-associated polypeptide isolated from strain ZY5. As shown in Fig. 6(*a*) and 6(*b*) (lanes 1 and 2), the isolated porin and 32 kDa polypeptides exhibit identical electrophoretic mobilities and similar extents of antibody cross-reactivity as measured by Western-Blot analysis. In addition, the *N*-terminal sequence of the 32 kDa pigment-binding polypeptide obtained from MB1007 (Glu-Val-Lys-Leu-Ser-Gly-Asp-Ala-Arg-Met-Gly) exactly matches the previously published *N*-terminal sequence for the *R. capsulatus* porin [45]. From these results we conclude that the pigment-binding polypeptide is the major outer-membrane porin protein from *R. capsulatus*.

DISCUSSION

Several early studies by Lascelles and co-workers demonstrated that *R. sphaeroides* mutants in which Bchl *a* biosynthesis is blocked accumulate large amounts of tetrapyrrole intermediates in the membrane fraction as well as in the culture supernatant fluid [10,11]. The amount of pigment excreted into the culture fluid was further shown to be increased by the addition of detergent to the culture media. These results, as well as evidence that pigment biosynthesis may be occurring in membrane fractions of the cell [9], led to the proposal that Bchl *a* biosynthesis may involve a carrier polypeptide that functions to tether tetrapyrrole intermediates to the membrane [10,16]. Subsequent analysis of tetrapyrrole intermediates excreted from *R. sphaeroides*, *R. rubrum* and *R. capsulatus* cultures demonstrated that the pigment was part of a high-molecular-mass pigment–protein–carbohydrate–lipid complex [14,17–20]. Previous analyses of the protein component of excreted pigment–protein complexes have, however, given varying results. Oelze & Drews [18] isolated a 2-devinyl-2-hydroxyethylchlorophyllide *a*–protein complex excreted from *R. rubrum* and concluded that there were three polypeptides associated with this complex with molecular masses of approx. 16, 32 and 65 kDa. Utilizing several different procedures, Richards *et al.* [19] reported three different molecular-mass determinations of 9, 36 and 56 kDa for the major polypeptide components of the excreted pigment–protein complex of *R. sphaeroides*. Since 36 and 54 are multiples of 9, they proposed the putative carrier polypeptide had a unit size of 9000. This conclusion, however, should be viewed with caution, since the 9 kDa polypeptide was obtained from an unpurified pigment–protein complex. A pigment–protein complex excreted from *R. capsulatus* was previously characterized by Drews [17] who reported that three polypeptides of 32.4, 42 and 45.5 kDa were associated with the excreted pigment. The latter study is supported, in part, by our study, which shows the association of tetrapyrrole intermediates with a 32 kDa polypeptide.

As noted above, the current investigation was undertaken in an attempt to provide evidence for involvement of a specific 'carrier polypeptide' in Bchl *a* biosynthesis, as proposed by Lascelles [10,16]. However, our observation that the 32 kDa pigment-binding polypeptides appears to be the major outer-membrane porin protein of *R. capsulatus* does not support the carrier-polypeptide model. It suggests, rather, that tetrapyrrole intermediates may be associated in a non-specific fashion with the major outer-membrane porin complex. We note that the 'association' of pigment with the porin involves a substantial amount of pigment. Using a previously reported absorption coefficient for protochlorophyllide [40], we calculate that there are approx. 1.5–1 tetrapyrrole molecules bound per 32 kDa polypeptide in the pigment–protein complex isolated from ZY5. The pigment interaction with the 32 kDa polypeptide also appears to be rather tight, as evidenced by the observation that the pigment–protein complex remains intact even when subjected to size-exclusion chromatography in the presence of non-ionic detergents such as LDAO and Triton X-100 (D. W. Bollivar & C. E. Bauer, unpublished work).

It is unclear why the tetrapyrrole intermediates become associated with the porin nor how this complex is excreted into the culture medium. The *R. capsulatus* porin is well characterized, with its crystal structure resolved to 0.6 nm [41–46]. The porin was found to have several channels in the size range 0.5–1.0 nm in diameter with an exclusion limit of 500 Da. Since the sizes of tetrapyrrole intermediates are larger than the reported porin exclusion limit, it is possible that intermediates are simply being excreted by the cell and are becoming non-specifically lodged within the porin complex. A more extensive analysis will have to be undertaken to determine how the pigment–porin complex is excreted into the culture supernatant fluid. Presumably, excretion of the pigment–porin complex is either an attempt by a viable cell to expel the porin complex once its has become disrupted by the tetrapyrrole or is simply the result of cell lysis that occurs in aged cultures. Interestingly, Biel [47] has recently reported that wild-type *R. capsulatus* cultures excrete coproporphyrin bound to a polypeptide that has the same electrophoretic mobility and *N*-terminal sequence (A. Biel, personal communication) as reported in this study, thereby suggesting that association of the porin with tetrapyrroles occurs early in the pathway and may be a general feature of *R. capsulatus* physiology.

Although we believe that the results of this study cast doubt on the existence of a carrier polypeptide, we cannot formally rule out its presence. It is possible that a carrier protein could simply be releasing the tetrapyrrole into the periplasm and that the 'free' tetrapyrrole subsequently binds to the porin. Another

possibility is that a pigment–carrier protein complex could itself by interacting with the porin and that the carrier polypeptide is stripped off during subsequent purification steps. A more direct approach to test for the presence or absence of such a protein will therefore have to be undertaken to rule out these possibilities.

We thank Dr. Howard Gest and members of the Photosynthetic Bacteria Group for critical reading of this manuscript and for helpful discussions. This work was supported by a grant from the National Institutes of Health (grant GM40941).

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Received 3 June 1991/27 August 1991; accepted 6 September 1991