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The membrane location of the B890-complex from *Rhodospirillum rubrum* and the effect of carotenoid on the conformation of its two apoproteins exposed at the cytoplasmic surface

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The membrane location of the two B890-apoproteins from *Rhodospirillum rubrum* has been investigated by comparing the effects of mild proteolysis with proteinase K, and immunoprecipitation, with antibodies prepared against the B890-complex and its individual apoproteins, upon membrane vesicles. By using chromatophores (inside-out vesicles) and spheroplasts (right-side vesicles) both membrane surfaces have been interrogated. The N-terminal regions of both aproproteins are located at the cytoplasmic membrane surface, while the C-termini are most probably located at the periplasmic surface. The conformation of the N-terminal regions of the apoproteins is changed by the presence of carotenoid. In the carotenoid-containing strain S1, for example, the α-apoprotein is insensitive to digestion by proteinase K, while in the absence of the carotenoid in the mutant G9$^+$ six amino acids are removed from the N-terminus of the α-aproprotein by proteinase K treatment.

Introduction

The antenna complex from *Rhodospirillum rubrum* (the B890-complex) is composed of two, closely interacting bacteriochlorophyll *a* molecules and one molecule of carotenoid (mainly spirilloxanthin) which are non-covalently bound to two low-molecular-weight, hydrophobic aproproteins (the B890-α-apoprotein, M_r 6079, and the B890-β-apoprotein, M_r 6101 [1–5]). In vivo, within the photosynthetic membrane, this light-harvesting complex most probably exists as an oligomer of this 'minimal unit' [1,2,6].

The two B890-apoproteins have been isolated, purified and their primary structures have been determined by sequential, automated Edman degradation [3–5]. The α-apoprotein contains 52 amino acids, while the β-apoprotein contains 54. The sequences of the two apoproteins isolated from both the wild-type strain, S1, and the well-known carotenoidless mutant, G9$^+$, are identical [4,5].

Based upon their primary structure the α- and β-apoproteins are only weakly homologous (approx. 6%); however, they do show one very striking similarity [5]. Hydropathy profiles of the two amino-acid sequences show quite clearly that the N- and C-terminal regions are polar, while a central region of 20–23 residues is strongly non-polar (for example, see Fig. 4 in Ref. 5). This has, of course, led to the suggestion (as illustrated in

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Fig. 1) that these two apoproteins should lie across the photosynthetic membrane [5,7]. If the central core, by analogy with the transmembrane segments of bacteriorhodopsin [8] and reaction centres from *Rhodopseudomonas viridis* [9], is assumed to be folded into an α-helix [5], then the apoproteins are only long enough to cross the membrane once. A recent analysis of the far ultraviolet CD-spectrum of the intact B890-complex has confirmed this predicted high α-helical content [10].

Once the primary structure of a membrane protein has been described, it is then possible to investigate its membrane location with a great deal of precision. Sites of surface-directed chemical modification or proteolysis can be determined with an exactness that is impossible without the sequence data. Brunisholz et al. [7], knowing the primary structures of the two B890-apoproteins from *R. rubrum*, investigated the effect of mild-proteolysis, with a range of proteases, on chromatophores (inside-out membrane vesicles) prepared from strain G9$^+$. Using the relatively non-specific protease, proteinase K, for example, they were able to show that this enzyme removed six amino acids from the N-terminus of the α-apoprotein and sixteen from the N-terminus of the β-apoprotein. In each case, by the end of the digestion, nearly all the B890-apoproteins were affected. No amino acid residues were removed from the C-termini of the apoproteins by any of the proteases tested. These results, therefore, very convincingly located the N-terminal regions of both apoproteins at the cytoplasmic surface of the photosynthetic membrane (that is the outer surface of the chromatophore membrane).

However, working with *R. rubrum* it is also possible to make right-side-out vesicles (spheroplasts [11]) and so to look for the presence of the

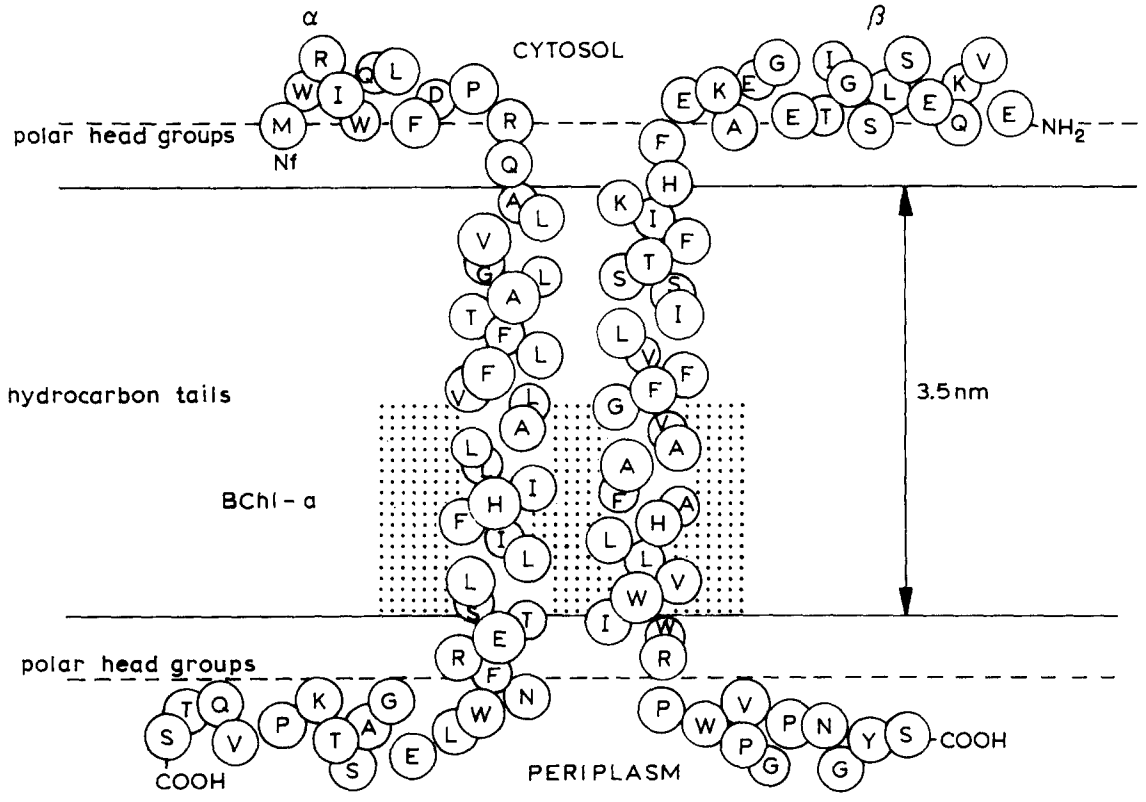

Fig. 1. The proposed model of how the two antenna apoproteins of the B890-complex from *R. rubrum* are organised with respect to the photosynthetic membrane. This model is based upon the data presented in Refs. 5 and 7. The amino acids are represented by the one letter code.

antenna apoproteins at the opposite, periplasmic-facing membrane surface. In this report we describe the results of a set of experiments designed to test whether the B890-apoproteins from *R. rubrum* can also be detected at the periplasmic surface, and so determine whether they actually do span the photosynthetic membrane.

We have used both mild-proteolysis with proteinase K and immunoprecipitation to look for surface-located antenna apoproteins in both spheroplasts and chromatophores. Also, by comparing the results obtained with wild-type membranes with those obtained from the mutant, $G9^+$, we discovered an interesting effect of the carotenoid on the conformation of the antenna apoproteins.

Materials and Methods

Growth of cells and preparation of the membrane vesicles

Cells of *R. rubrum* strain S1 and the carotenoidless-mutant $G9^+$ were grown anaerobically in the light at 30°C, with succinate as the main carbon source [12]. The cells were harvested by centrifugation, washed in 20 mM Tris-HCl (pH 8.0) and disrupted in the presence of a little DNAase, by passage through a French-pressure cell at approx. 154 MPa. Chromatophores were then isolated from the broken cells by differential centrifugation [13] and resuspended in a minimal volume of 20 mM Tris-HCl (pH 8.0). For the proteolysis or the immunoprecipitation experiments the chromatophores were kept on ice and used on the day of their preparation.

Spheroplasts were prepared by a slight modification of the method of Markwell and Lascelles [14]. Cells were grown in 500 cm^3 flat-sided bottles for approx. 15 h, in relatively high-light (more than 6 $mW \cdot cm^{-2}$), harvested by centrifugation and resuspended in the lysozyme digestion buffer at an absorbance$_{680}$ of 40 cm^{-1}. The lysozyme digestion buffer contained 25% w/v sucrose (as an osmotic buffer), 100 mM Tris-HCl (pH 6.0) (in our hands this is the optimal pH for good yields of spheroplasts, and even though Tris is only a week buffer at this pH it gives much better yields of spheroplasts than does phosphate – Roberts, L. and Cogdell, R.J. unpublished observations) and 6 mM EDTA. The cells were incubated at 37°C for 1 h, in a shaking water bath, in the presence of 2 mg/ml lysozyme (Sigma egg-white lysozyme grade 1, EC 3.2.1.17). After the hour's incubation the reaction was stopped by the addition of 80 mM $MgCl_2$. The mixture was then subjected to a differential centrifugation and two pellets were obtained. A low-speed pellet was produced by centrifuging at $5700 \times g$ for 10 min and a high-speed pellet by centrifuging at $10500 \times g$ for 10 min. These two pellets were resuspended in a minimal volume of 25% sucrose/100 mM Tris HCl (pH 6.0) and examined under the light microscope. By comparing the two pellets with a sample of untreated whole cells the yield of spheroplasts was determined. Usually the low-speed pellet provided the higher yield of spheroplasts (yields of greater than 85% were treated as acceptable). This pellet was then washed twice by centrifugation (to remove excess lysozyme) and resuspended in 25% sucrose, 100 mM Tris-HCl (pH 8.0). On several occasions we checked our observations obtained under the light microscope by examining spheroplasts and whole cells in the electron microscope. The whole cells were clotted in fibrin (Diagnostic Reagents Ltd., Thame, Oxfordshire, U.K.) for ease of handling during the fixation procedure. The 'clot' was cut into small pieces (approx. 1 mm^3) and fixed in 3% v/v glutaraldehyde, 200 mM sodium cacodylate (pH 7.5) for 12–18 h. It was then rinsed in 200 mM Na cacodylate (pH 7.5) (three changes of 10–15 min each) and post-fixed in 1% w/v osmium tetroxide, 200 mM Na cacodylate (pH 7.5) for 1–3 h. The samples were then briefly rinsed in distilled water, block stained in 2% w/v aqueous Uranyl acetate, dehydrated in an acetone series and embedded in SPURR resin (for approx. 12 h at 60°C). Thin sections were cut (approx. 60 nm thick) with an LKBIII ultratome. These were picked up on naked 300 mesh copper grids, stained with saturated ethanolic Uranyl acetate for 10 min [15] and lead citrate for 2 min [16], and then examined in a Philips EM301 electron microscope. The spheroplasts were prepared for the electron microscope in a similar way, except that the fibrin clotting procedure was not required and 3% w/v sucrose was added to the glutaraldehyde fixative.

We were able to confirm by this electron mi-

croscopy that we had indeed made spheroplasts in high yield. The spheroplasts typically have sections of intact cell wall separated by digested portions. Since it is important for our topological studies not only to produce spheroplasts in high yield, but to ensure that they remain intact, we also routinely checked for their degree of intactness. The degree of intactness was estimated by monitoring the release of the soluble cytoplasmic enzyme malate dehydrogenase [17]. The activity of this enzyme was assayed spectrophotometrically as described by Markwell and Lascelles [14]. A solution of whole cells was divided into two equal volumes. One half was completely disrupted by passage through the French press. These broken cell membranes were then pelleted by centrifugation at $220\,000 \times g$ for 1 h. The supernatant was saved and the pellet washed two more times by resuspending in 20 mM Tris-HCl (pH 8.0) and centrifuging. The supernatants from both washes were also saved. The total activity of malate dehydrogenase in these supernatants was then determined. This value was then compared with the amount of malate dehydrogenase activity that was released when the other half of the sample of cells was converted into spheroplasts by lysozyme digestion. We also checked for the latency of malate dehydrogenase activity in the spheroplasts by assaying before and after osmotic lysis. The intactness of the spheroplasts was routinely estimated to be greater than 70–80% by these two methods.

Preparation of the antibodies to the B890-complex and the individual antenna apoproteins

The B890-complex from strain S1 was prepared from reaction centre depleted membranes as previously described [1]. Its purity was checked by SDS-polyacrylamide gel electrophoresis [18] and only samples which showed no other protein bands, except the two antenna apoproteins, were used to raise the antibodies. The individual antenna apoproteins were isolated and purified from freeze-dried chromatophores as described by Brunisholz et al. [5], by organic solvent extraction and chromatography on Sephadex LH-60.

Rabbit antibodies were raised against the B890-complex and the individual antenna apoproteins as described by De Marcucci et al. [19]. All the immunoprecipitation experiments were carried out with the antiserum, without any further purification. Control serum was obtained from non-injected rabbits. The titre of the individual antisera and their specificity was checked by immunoblotting on nitrocellulose filters. Purified antenna apoproteins were resolved on 16% w/v acrylamide SDS-polyacrylamide gels and transferred electrophoretically on to nitrocellulose paper, essentially as described by Towbin et al. [20]. The transfer buffer was supplemented with 0.02% w/v SDS. The nitrocellulose paper was then incubated with the antisera (diluted 1 : 50) using the modification suggested by Batteiger et al. [21]. The specific antigen-antibody conjugates were then detected, after incubation with ^{125}I-labelled protein A (60 000–80 000 d.p.m. cm^{-3}), by autoradiography as previously described [19].

Treatment of the membranes with proteinase K

The protein concentration of the chromatophores or the spheroplasts to be digested was determined by the Tannin method [22]. In the case of the chromatophores the digestion was carried out in 10 mM Tris-HCl (pH 8.0), while with the spheroplasts 25% w/v of sucrose was added to the buffer as an osmotic support. The membranes were preincubated at 37°C for 5 min in a shaking water bath and then proteinase K (Boehringer of Sigma type X) from *Tritirachium album*, EC 3.4.21.14, was added in the ratio of 1 mg of enzyme to 20 mg of chromatophore or spheroplast protein. Routinely between 500 and 1000 mg of chromatophore or spheroplast protein was digested at a time. Samples were withdrawn at zero time, after 10 min and after 1 h of digestion. The reaction was stopped by chilling the samples on ice and by adding the protease inhibitor PMSF (phenylmethylsulphonylfluoride) to a final concentration of 2.5 mM. The membranes were then pelleted by centrigution at $220\,000 \times g$ for 1 h, resuspended in water and freeze-dried. The antenna apoproteins were extracted and purified from the freeze-dried membranes as described by Brunisholz et al. [5]. In all cases the in vivo bacteriochlorophyll *a* absorption bands in the NIR were unaffected by the digestion with proteinase K.

The effect of this mild proteolysis on the antenna apoproteins was determined by sequence

analysis [7]. The proteinase-K-digested apoproteins were subjected to manual Edman degradation (N-terminal amino-acid sequence determination), carboxypeptidase degradation and hydrazinolysis [23]) (C-terminal amino-acid sequence determination) and total amino-acid analysis. The details of each of these methods have been presented elsewhere [7].

Immunoprecipitation

Chromatophores and spheroplasts were challenged with each of the various antibodies to see whether they were agglutinated. Membranes were made up in 1 cm^3 of a mixture of buffer (for chromatophores the buffer was 150 mM sodium chloride/20 mM sodium phosphate (pH 7.4), for spheroplasts 40% w/v sucrose was added to this buffer as an osmotic support) and antiserum to give an absorbance, at the NIR bacteriochlorophyll *a* absorption band of 0.5 cm^{-1} (the peak wavelength was 880 nm for strain S1 and 872 nm for $G9^+$). In this way a set of test tubes were set up each containing a final volume of 1 cm^3, but with increasing amounts of antiserum. A similar set of control tubes were made up using equal volumes of control serum.

The tubes were incubated at 37°C for 30 min and then for 4 h at 4°C. Any agglutinated material was removed by a low-speed centrifugation of $1000 \times g$ for 5 min and the concentration of the membranes remaining in solution was assayed spectrophotometrically by measuring the absorbance at 880 nm or 872 nm depending upon the strain being tested.

Results and Discussion

Digestion with proteinase K

Chromatophores from both strains S1 and $G9^+$ were incubated with proteinase K. Aliquots were removed at zero time and after 10 min and 60 min of digestion, and the effect of the proteolysis on the primary structure of the antenna apoproteins was determined.

The products of the digestion were extracted into organic solvent and separated by passage down a column of Sephadex LH-60. The various bands eluted from this column were collected and analysed (as described in the Materials and Methods) for their N- and C-terminal sequences and their amino acid composition. The data are presented in Table I. By reference to the amino-acid sequence of the intact antenna apoproteins it was determined that with $G9^+$ chromatophores proteinase K digestion removes six amino acids from the N-terminus of the α-apoprotein and sixteen amino acids from the N-terminus of the β-apoprotein. The digestion of the β-apoprotein is complete within 10 min of incubation with pro-

TABLE I

THE MAJOR DIGESTION PRODUCTS PRODUCED BY PROTEINASE K TREATMENT OF THE CHROMATOPHORES FROM *R. RUBRUM* STRAINS S1 AND $G9^+$

The fractions from the LH-60 column were analysed as described in Materials and Methods for their N- and C-terminal sequences, and their amino-acid composition. The major fractions were identified by reference to their primary structures as described by Brunisholz et al. [7].

Strain	Time of digestion (min)	Main products identified after Sephadex LH-60 chromatography
$G9^+$	10	Uncleaved reaction centre L and M polypeptides. α-apoprotein, mainly uncleaved but with some chains cleaves between Gln-6 and Leu-7. β-apoprotein cleaved between Lys-16 and Glu-17.
	60	Uncleaved reaction centre L and M polypeptides. Some L polypeptides lacking the first 16 N-terminal amino acids. A C-terminal fragment of the M polypeptide of approx. 65 amino acids. α-apoprotein cleaved between residues Gln-6 and Leu-7. β-apoprotein cleaved between residues Lys-16 and Glu-17.
S1	10	Uncleaved reaction centre L and M polypeptides. α-apoprotein uncleaved. β-apoprotein cleaved between residues Glu-5 and Ser-6.
	60	Uncleaved reaction centre L and M polypeptides. Reaction centre L polypeptide lacking 16 N-terminal amino acids. α-apoprotein uncleaved. β-apoprotein cleaved between residues Glu-5 and Ser-6, and in low yield cleaved between residues Lys-16 and Glu-17.

teinase K, while after 10 min the α-apoprotein is only partially digested. The C-termini of both apoproteins were unchanged by the proteinase K treatment (data not shown). These results are in complete agreement with those previously reported [7] and confirm the location of N-terminal regions of both antenna apoproteins at the cytoplasmic surface of the photosynthetic membrane.

However, as can be seen in Table I, rather different results are obtained with wild-type chromatophores. In this case the α-apoprotein is almost completely unaffected by the proteinase K and the time-course of the digestion of the β-apoprotein is changed. After 10 min the β-apoprotein has lost 5 amino acids from the N-terminus and only after 1 hour's digestion do some β-apoprotein molecules lose 16 N-terminal amino acids. Again the C-termini of both apoproteins were unaffected (data not shown).

The only difference in the antenna complex from strain S1, as compared to the complex from $G9^+$, is the presence of the carotenoid, spirilloxanthin [1,2,4,5]. The antenna apoproteins from both strains S1 and $G9^+$ have identical primary structures. It is clear therefore that the presence of the carotenoid has altered the structure of the N-terminal regions of the apoproteins, so that the β-apoprotein is less susceptible to proteolysis and so that the α-apoprotein is now highly protected.

Treatment of spheroplasts from either strain S1 or $G9^+$ with proteinase K did not cause any changes in the sequence of the two antenna apoproteins at either their respective N- or C-termini (data not shown). This negative result is, however, ambiguous. It could mean that the antenna apoproteins are not present at the periplasmic membrane surface or, on the other hand, it could mean that they are present, but resistant to proteolytic attack (rather in the way the N-terminus of the α-apoprotein is resistant to proteinase K in chromatophores from strain S1).

Investigation of the surface location of the B890-apoproteins with antibodies prepared to either the intact B890-complex from strain S1 or to the individual apoproteins

It is very often an advantage in studies on the membrane topology of a given membrane protein to use more than one method of locating that protein, in order to overcome the problem of the ambiguous nature of negative results. In the present study we have chosen to use immunoprecipitation as an alternative method, however quite clearly the approach lacks the detailed resolution that can be achieved by the combination of proteolysis and subsequent amino acid sequencing. The reaction with monospecific, polyclonal antibodies can only determine whether a given protein is surface located, it cannot, directly, show which domain in the protein is responsible for the interaction.

The immunogenic behavior of our antisera were initially characterised by immune-blotting. The antenna apoproteins were challenged with all three antisera (anti-B890, anti-B890-α and anti-B890-β) (Fig. 2). The antiserum raised to the B890-complex 'lit up' both antenna apoproteins, while the anti-

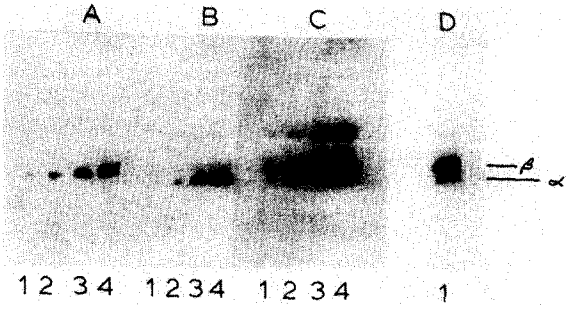

Fig. 2. Immunological detection of the antenna apoproteins with the three different antisera. Varying amounts of the antenna apoproteins were separated by SDS-polyacrylamide electrophoresis on a 16% (w/v) polyacrylamide slab gel. The gel was divided into four parts. Part D was stained with Coomassie blue. Parts A, B and C were transferred onto nitrocellulose paper for incubation with the antisera. Bound antibodies were detected by autoradiography after incubation with ^{125}I-labelled protein A as described in the Methods. A-incubated with anti-B890-β, B-incubated with anti-B890-α and C-incubated with anti-B890. In A–C lanes 1–4 contain 0.6, 1.2, 3.0 and 6.0 μg of antenna apoproteins, respectively, and in D lane 1 contains 6.0 μg of antenna apoproteins. In D the faintly staining band above the β-apoprotein band is an aggregate of the antenna apoproteins. In C, this band is seen reacting with anti-B890. In D it can also be seen that the β-apoprotein appears to be present in a much higher amount than the α-apoprotein, this is an artefact of the staining, since the β-apoprotein reacts more strongly with the Coomassie blue. It should also be pointed out that there is a small amount of cross-reaction of the anti-B890-α antiserum with the B890-β-apoprotein. This was probably due to a small (2–5%) level of cross-contamination of the B890-α-apoprotein used to raise the antibodies with B890-β-apoprotein.

B890-α and anti-B890-β sera selectively 'lit up' their respective antigens. In the case of the apoprotein-specific antisera the titres were very similar.

The anti-B890 serum was tested for its ability to agglutinate chromatophores prepared from both strains S1 and G9$^+$. In both cases (Fig. 3) the anti-B890 completely immunoprecipitated the chromatophores. Control serum caused no agglutination in either case. Note, however, the difference in titre between the two sets of chromatophores. Chromatophores from strain S1 were always preferentially agglutinated by the anti-B890. This was a very reproducible feature of this agglutination reaction and its possible significance will be discussed further below. This strong agglutination reaction with the anti-B890 serum confirms the presence of the B890-complex at the cytoplasmic face of the photosynthetic membrane.

The anti-B890 serum was also tested for its ability to agglutinate spheroplasts prepared from both S1 and G9$^+$. In both cases the anti-B890 serum caused a strong agglutination reaction; however, unlike with chromatophores the extent of the reaction was never more than 80%. This probably reflected the presence of some whole cells in with the spheroplasts. Whole cells were not agglutinated by the anti-B890. In Fig. 3, where the antiserum was tested against chromatophores, the relative titre for S1 and G9$^+$ membranes could be accurately compared. This was not possible with the spheroplasts. Spheroplasts, especially these from G9$^+$, tend to aggregate spontaneously during the rather long incubations required for the agglutination test and they are rather light-scattering, and so it is not possible to compare accurately equal amounts of spheroplasts from S1 and G9$^+$ as it is for the chromatophores. For this reason data is only shown for strain S1 (Fig. 4).

It is clear from Fig. 4 that the B890-complex is also present at the periplasmic membrane surface. Since the N-termini of the antenna apoproteins are both at the cytoplasmic surface, and since the size of apoproteins probably means that they are only able to cross the photosynthetic membrane once, this suggests that their C-terminal regions must be located at the periplasmic surface. The spheroplasts were consistently agglutinated by the antisera raised to the individual apoproteins. This locates both apoproteins at the periplasmic surface. However, since these antisera has a lower titre than the anti-B890 antiserum and because of the tendency of the spheroplasts to aggregate spontaneously we were not able to show any reproducible difference in the titre of these antisera in agglutinating the spheroplasts.

In contrast, with chromatophores there was a marked difference in the effectiveness of anti-B890-α and anti-B890-β in producing agglutina-

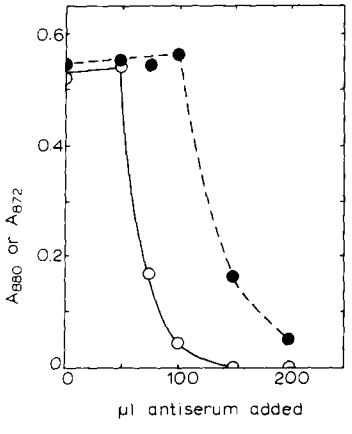

Fig. 3. Determination of the titre for the immunoprecipitation of chromatophores from *R. rubrum* S1 and G9$^+$ with anti-B890. ●------●, G9$^+$ chromatophores (A_{872}); ○——○, S1 chromatophores (A_{880}). As described in Materials and Methods equal concentrations of chromatophores were challenged with anti-B890 serum and their relative titre determined.

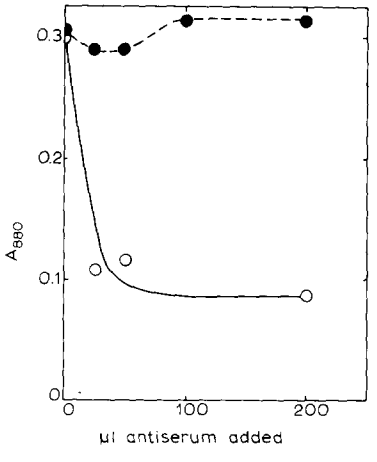

Fig. 4. Agglutination of spheroplasts prepared from *R. rubrum* S1 by anti-B890. ○——○, agglutination with anti-B890; ●------●, control serum. The experimental conditions are described in Materials and Methods.

tion. The titre of these two antisera as determined by immunoblotting was very similar; however, with the chromatophores only anti-B890-β caused agglutination. This is illustrated in Fig. 5 for chromatophores from strain S1.

Treatment of chromatophores with proteinase K showed that the presence of the carotenoid in strain S1 protected the α-apoprotein against proteolysis. This difference between the conformation of the N-termini of the apoproteins in the presence of carotenoid was confirmed by antibody studies. The titre with the anti-B890 serum for agglutination of the chromatophores was markedly different for the strain S1 and the $G9^+$ mutant. In the proteolysis experiments the β-apoprotein was the most sensitive to proteinase K. It is interesting therefore that only the anti-B890-β serum was able to agglutinate the chromatophores. A simple explanation that rationalises all this data would be the suggestion that the N-terminal region of the β-apoprotein is folded over the N-terminal region of the α-apoprotein. This would explain the greater sensitivity of the β-apoprotein to proteolytic attack and the inability of the anti-B890-α serum to agglutinate chromatophores. The presence of the carotenoid then modulates this situation causing a change in conformation such that the α-apoprotein is even more resistant to proteolysis. It will be interesting to see whether further work locates the carotenoid binding site near to the N-terminal regions of the apoproteins.

Brunisholz et al. [7] showed that proteinase K digestion of $G9^+$ chromatophores removed 16 amino acids from the N-terminus of the L subunit of the reaction centre. During our present study we were also able to show that proteinase K removed the same 16 amino acids from the N-terminus of the L subunit of the reaction centre of S1 chromatophores (data not shown).

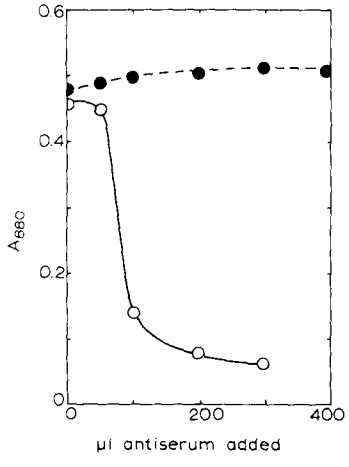

Fig. 5. Determination of the relative effectiveness of anti-B890-α and anti-B890-β anti-sera in agglutinating chromatophores from *R. rubrum* S1. ●------●, anti-B890-α; ○——○, anti-B890-β. As described in Materials and Methods equal concentrations of chromatophores were challenged with anti-B890-α or anti-B890-β and the effectiveness of each anti-serum in producing agglutination was determined by measuring the amount of membranes remaining in solution after centrifugation at $1000 \times g$ for 5 min.

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