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Assessment of *Rhodopseudomonas sphaeroides* Chromatophore Membrane Asymmetry Through Bilateral Antiserum Adsorption Studies

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The asymmetric structure of the *Rhodopseudomonas sphaeroides* chromatophore membrane was examined in detail by crossed immunoelectrophoresis techniques. Because these methods are quantitative and allow increased resolution and sensitivity, it was possible to analyze simultaneously the relative transmembrane distribution of a number of previously identified antigenic components. This was demonstrated by analysis of immunoglobulin samples that were adsorbed by preincubation with either isolated chromatophores or osmotically protected spheroplasts. The photochemical reaction center, the light-harvesting bacteriochlorophyll *a*-protein complex, the L-lactate dehydrogenase, and reduced nicotinamide adenine dinucleotide dehydrogenase (EC 1.6.99.3) were found to be exposed on the chromatophore surface (cytoplasmic aspect of the membrane within the cell). Other antigenic components were found to be exposed on the surface of spheroplasts (periplasmic aspect of the in vivo chromatophore membrane). Antigens with determinants expressed on both sides of the chromatophore membrane were also identified. Charge shift crossed immunoelectrophoresis confirmed the suggested amphiphilic character of the pigment-protein complexes and identified several additional amphiphilic membrane components.

Growth of the facultative photosynthetic bacterium *Rhodopseudomonas sphaeroides* under reduced oxygen tension results in the formation of a differentiated intracytoplasmic membrane designated the chromatophore membrane (21). The photosynthetic apparatus is localized within this structure, which upon disruption gives rise to an essentially uniform population (17) of sealed vesicles (chromatophores) that can be readily purified (21). Ultrastructural studies in this (1) and in the related organism *Rhodospirillum rubrum* (2, 15) have suggested that the chromatophore membrane is continuous with the peripheral cytoplasmic membrane. This was supported by the isolation from osmotically lysed spheroplasts of a membrane fraction containing both chromatophore and cell envelope components (5). Furthermore, photooxidation studies on the localization of cytochrome c_2 (29) have indicated that the soluble contents of the chromatophore membrane of *R. sphaeroides* are continuous with the periplasm.

It would be expected from such membrane continuity that isolated chromatophores are oriented inside-out with respect to the plasma membrane. This has been demonstrated in studies of proton movement (32), diffusion potential-induced carotenoid band shifts (19), and light-dependent transport activities (20). Freeze-fracture electron microscopy (17) has confirmed that the isolated chromatophores, which have the same orientation as the intracytoplasmic chromatophore membrane in situ, have the reverse orientation of the plasma membrane in the intact cell. It is possible to isolate spheroplast-derived vesicles from *R. sphaeroides* that are mostly oriented in the same manner as the plasma membrane (17, 20) and opposite to that of isolated chromatophores.

We have recently applied the technique of crossed immunoelectrophoresis (CIE) to the study of the chromatophore membrane of *R. sphaeroides* (4). Through use of this technique, 31 distinct immunoprecipitates were resolved from a Triton X-100 extract of chromatophores. Since nondenaturing conditions were used, the retention of biological activity by solubilized membrane components permitted the recognition of NADH dehydrogenase (EC 1.6.99.3) and L-lactate dehydrogenase (LDH) in the two-dimensional immunoprecipitate pattern. Moreover, the immunoprecipitates formed by the photochemical reaction center (RC) and the light-harvesting bacteriochlorophyll *a* (BCHL)-

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protein complex (LH) were also identified. The quantitative nature of CIE permits comparison of antiserum samples adsorbed with intact membranes. The present study was undertaken to assess the sidedness of the chromatophore membrane by CIE and investigate the localization of the components that we have resolved previously (4) by this technique.

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MATERIALS AND METHODS

Cultivation of organism. *R. sphaeroides* NCIB 8253 was grown phototrophically at 170 foot-candles (1,830 lx) in a chemically defined medium supplemented with 0.1% yeast extract (3). A low light intensity was selected to obtain cells which produce a high yield of chromatophores (21). Additionally, spheroplasts prepared from such cells are more stable to osmotic lysis than those prepared from cells grown under high illumination (14, 20). Cultures were harvested in mid to late exponential growth.

Preparation of spheroplasts. Harvested cells were washed twice in 1.0 mM Tris-hydrochloride (pH 7.5) and resuspended to 6.5 OD_{680} (optical density units at 680 nm) in 20% (wt/wt) sucrose prepared in the same buffer (Tris-sucrose). This suspension was stirred at room temperature for 10 min followed by 1/10-volume additions of lysozyme (EC 3.2.1.17; Worthington Biochemicals Corp., Freehold, N.J.; 6.4 mg/ml in Tris-sucrose) and EDTA (20 mg/ml in Tris-sucrose). The suspension was stirred at room temperature for 20 min after each addition. The osmotically sensitive spheroplasts were collected by centrifugation and resuspended in Tris-sucrose to 30 OD_{680}.

Preparation of chromatophores. Washed cells were disrupted in a French pressure cell, and the resulting extracts were subjected to differential and rate-zone sedimentation procedures described previously (22). A linear 5 to 35% (wt/wt) sucrose gradient prepared over a cushion of 60% (wt/wt) sucrose was used for rate-zone sedimentation. The purified chromatophores were resuspended in 10 mM Tris-hydrochloride (pH 7. 5).

Preparation of antiserum. Chromatophores subjected to two cycles of rate-zone sedimentation were administered to rabbits by subcutaneous injection (1.0 mg of protein) in 50% Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.). Booster injections were administered according to a schedule described previously (6). Sera obtained from weekly bleedings were pooled. Antisera were purified and concentrated (5- to 10-fold) by $(NH_4)_2SO_4$ fractionation and dialysis (12). Purified immunoglobulins were stored in 100 mM NaCl-15 mM NaN_3 at 4°C.

Preparation of soluble membrane extracts. Chromatophores (12.8 mg of protein per ml) were treated with 1% Triton X-100 (detergent/protein = 0.78) in 10 mM Tris-hydrochloride (pH 7.5) with or without 1 mM phenylmethylsulfonyl fluoride (PMSF) at 0°C for 1 h in the dark. The soluble extract was separated from the insoluble residue by centrifugation at 50,000 rpm (165,000 × *g*) for 90 min in a Beckman type 50 Ti rotor. Soluble extracts were divided into equal portions and stored at −70°C until required for use. The addition of PMSF to the solubilization buffer did not affect the immunoelectrophoretic pattern.

Adsorption of antichromatophore immunoglobulins. Portions (0.20 ml) of antichromatophore immunoglobulins were incubated for 1 h at room temperature with freshly prepared chromatophores (0 to 4.6 mg of chromatophore protein) in a total volume of 0.32 ml in 10 mM Tris-hydrochloride (pH 7.5). Visible agglutination of chromatophores occurred immediately upon mixing with antibody, and the resulting aggregates were collected by centrifugation for 10 min at 2,500 rpm. The adsorbed immunoglobulins in the supernatant solution were recovered and stored at −20°C until analyzed by CIE. Chromatophores disrupted with Triton X-100 were also used for antibody adsorption. Immunoglobulin samples were adsorbed with spheroplasts (0 to 5.7 OD_{680}) under the same conditions except that the spheroplasts were stabilized by 10% (wt/wt) sucrose. It was not technically possible to directly relate the amount of chromatophore membrane available for antibody binding in spheroplast and chromatophore preparations. Conditions were chosen that resulted in progressive adsorption of antibody from samples until too little remained to immunoprecipitate the affected antigens.

To assess the antibody composition of the adsorbed immunoglobulin samples, molten agarose was added to the thawed samples (final volume, 3 ml) and applied to the top portion of plates for CIE. These plates were electrophoresed, processed, and stained as described below. Calculation of the area subtended by the individual immunoprecipitate peaks has been described by others (24, 25). The slopes of the progressive adsorption curves were determined by the method of least squares. Results presented here are from antibody samples adsorbed in triplicate. These findings were further confirmed by independent experiments performed with antibodies obtained from other rabbits. However, some differences in the CIE pattern obtained with sera from other rabbits prevented corroboration of results for every immunoprecipitate.

Immunoelectrophoretic procedures. The procedures used for CIE have been detailed in reports from this laboratory and others (6, 33, 37). In these experiments, Tris-barbital (4.48 g of Tris, 8.86 g of diethylbarbituric acid per liter, pH 8.6) buffered the agarose gel, which was cast on glass slides (2 by 2 inches [ca. 5 by 5 cm]). Unless otherwise indicated, electrophoresis was conducted for 75 min at 150 V in the first dimension and at 55 V for 12 to 16 h in the second dimension in chambers maintained at 10°C. Slides were repeatedly washed and pressed in 100 mM NaCl and dried before staining with 0.25% Coomassie brilliant blue (R-250) in methanol-water-glacial acetic acid (227:227:46).

Charge shift CIE was performed essentially as described by others (26). Procedures for enzyme staining of immunoplates have been described (4, 6, 34). No activity for malate dehydrogenase (EC 1.1.1.37), glycerol 3-phosphate dehydrogenase (EC 1.1.99.5), glutamate dehydrogenase (EC 1.4.1.2), or succinate dehydrogenase (EC 1.3.99.1) could be detected by enzyme

staining procedures even when PMSF was included in the buffer for membrane solubilization. In addition, no LDH could be demonstrated by enzyme staining of immunoplates prepared with antibodies obtained from some rabbits. This observation suggests that some sera may contain antibodies which are more effective in the inhibition of enzyme activity of certain antigens. Alternatively, these sera may lack antibodies specific for this enzyme antigen. This variability among antisera may account for the discrepancy between the findings of the present study and another (8) in which succinate and malate dehydrogenases were demonstrated by the application of enzyme-staining techniques to CIE plates prepared with extracts of chromatophore preparations. Moreover, the membrane extracts used in the latter study contained cell envelope material indicated by the presence of an outer membrane antigen (8); it is possible that the cytoplasmic membrane also was the source of some of the antigens observed, including those exhibiting enzymatic activity.

Analytical procedures. Protein was determined by the method of Lowry et al. (18), with 0.1% sodium dodecyl sulfate included to solubilize particulate protein and prevent interference by Triton X-100. Bovine serum albumin (Miles Laboratories, Inc., Kankakee, Ill.) was used as the standard.

Materials. Agarose of the type HGT was obtained from Marine Colloids, Rockland, Maine. Triton X-100 was obtained from Research Products International, Elk Grove Village, Ill. Cetyltrimethylammonium bromide (CTAB) and PMSF were obtained from Sigma Chemical Co., St. Louis, Mo., and deoxycholate (DOC) was obtained from Schwarz/Mann, Orangeburg, N.Y. All other chemicals were from standard commercial sources.

RESULTS

Antiserum adsorption studies. Preincubation of antibody preparations with either isolated chromatophores or osmotically protected spheroplasts resulted in a reduction in the concentration of some populations of antibodies. This alteration in the composition of antiserum samples was reflected in the immunoprecipitate patterns generated when these samples were analyzed in CIE. The enzyme-stained immunoplates obtained with antibody samples adsorbed with increasing amounts of chromatophores (Fig. 1) indicated that antibodies specific for both NADH dehydrogenase and LDH were bound to chromatophores during the preincubation step and consequently removed from the antibody sample. The areas subtended by the

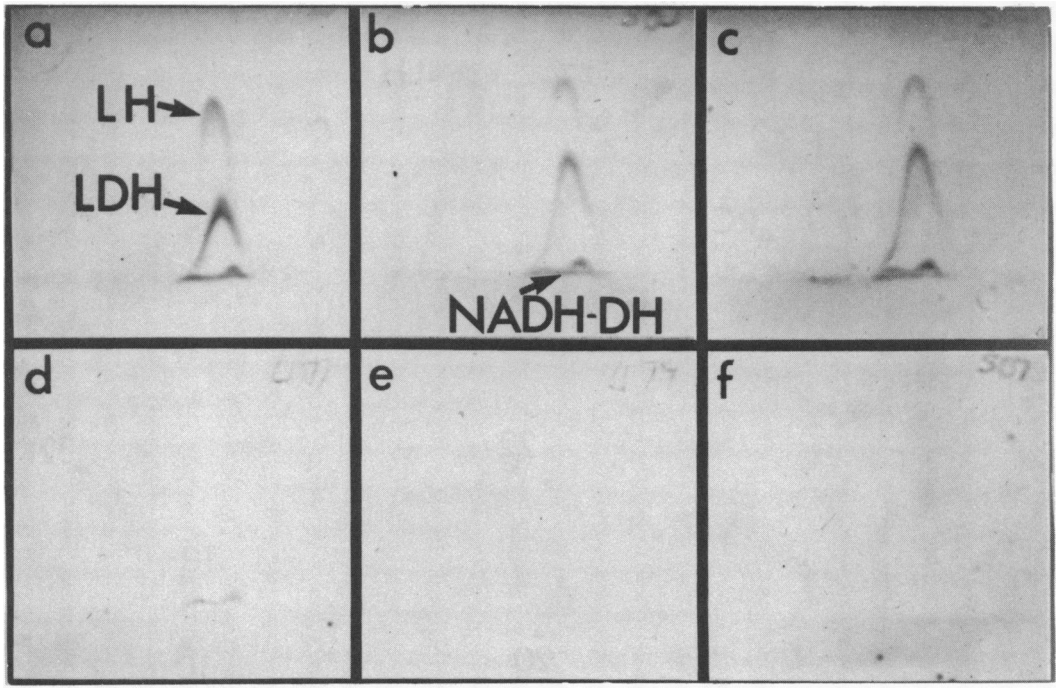

FIG. 1. *CIE of Triton extract of chromatophores precipitated with antibody samples that were adsorbed with chromatophores. Immunoplates were stained sequentially for LDH and NADH dehydrogenase (NADH-DH). The BCHL LH is visible by virtue of intrinsic photopigments. Antibody preparations were adsorbed with the following levels of chromatophore protein: (a) 0 mg; (b) 0.58 mg; (c) 0.77 mg; (d) 1.55 mg; (e) 3.10 mg; (f) 4.64 mg. In all cases, 15 μg of extracted chromatophore protein was applied to the origin at the bottom right. Anode at left (first dimension) and top (second dimension).*

immunoprecipitates formed by these enzyme molecules and corresponding specific antibodies increased progressively (Fig. 1). Since this area is proportional to the ratio of antigen to antibody present, this increase reflected a gradual reduction in antibody concentration because the antigen loading was constant. Incubation with sufficient quantities of chromatophore membrane reduced the amount of specific antibody to a level too low to precipitate the corresponding antigen. The LDH immunoprecipitate was not formed on immunoplates in Fig. 1d–f because too little specific antibody was present.

These data suggest that these enzyme molecules exist on the cytoplasmic face of the chromatophore membrane within the cell because, after isolation of the chromatophore vesicles, the antigenic determinants were available to form complexes with specific antibody. Evidence that these antigenic determinants of the dehydrogenases are not expressed on the interior surface of the isolated chromatophore (i.e., periplasmic aspect of chromatophore membrane in the intact cell) was provided by the failure of antibody specific for these enzymes to be adsorbed by spheroplasts. Immunoplates (not shown) prepared with antibody samples adsorbed with spheroplasts showed that the level of antibody directed against these antigens was virtually unchanged by exposure to spheroplasts. Spheroplasts, rather than spheroplast-derived vesicles, were chosen for these adsorption studies because these vesicles have been shown (8, 17, 25) to exhibit at least slight perturbations in membrane structure.

When immunoplates were stained for protein, the adsorption of antibodies directed against other antigens could be assessed. Examination of these immunoplates revealed that antibodies specific for additional antigens were efficiently adsorbed with chromatophores (Fig. 2). Included in this group are the LH (see also Fig. 1) and RC immunoprecipitates. Antibody forming some immunoprecipitates was adsorbed by spheroplasts (Fig. 3). Prominent among this group were the immunoprecipitates formed by the fast-moving antigens, including immunoprecipitate 14. (For a complete nomenclature of the

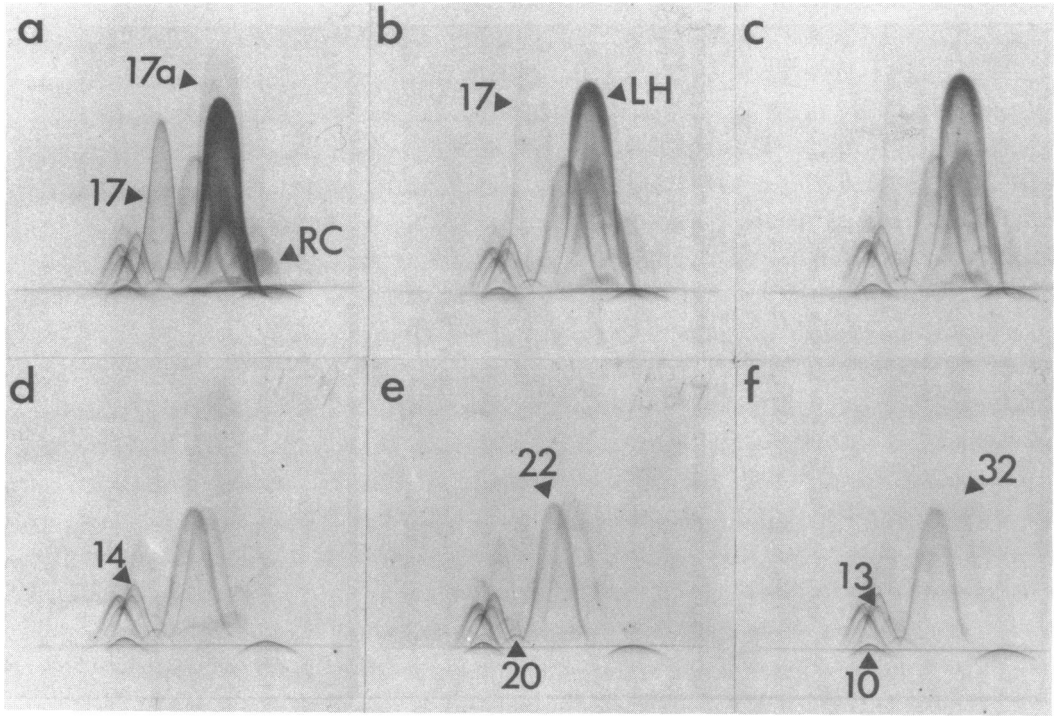

FIG. 2. *Immunoprecipitate pattern of CIE plates (Fig. 1) after staining for protein with Coomassie brilliant blue. Numerical designations of immunoprecipitates are from reference 4. Number 32 was not observed previously (4) because it was masked by no. 25, LH. It is observed in these immunoplates when the relative levels of antibodies against antigens 25 and 32 are altered by serum adsorption. In some unadsorbed serum pools, the relative levels of antibodies against these antigens are such that distinct immunoprecipitates 25 and 32 are resolved. Immunoplates not shown.*

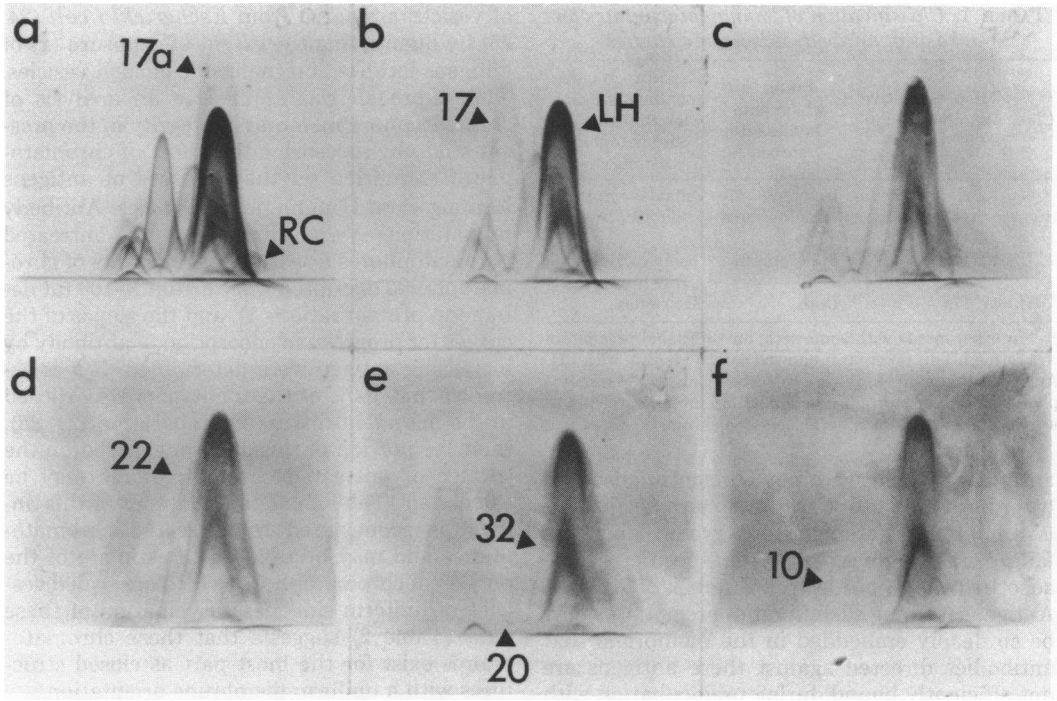

FIG. 3. *CIE of Triton extract of chromatophores (15 μg of protein) precipitated with antibody samples that were adsorbed with spheroplasts. Immunoplates were sequentially stained for dehydrogenase activities and protein. Antibody preparations were adsorbed with the following amounts of spheroplasts expressed in OD_{680} units: (a) 0; (b) 0.57; (c) 1.14; (d) 1.70; (e) 2.84; (f) 5.68.*

immunoprecipitates in the CIE pattern, see reference 4.) These highly mobile antigens were precipitated by antibody samples that were maximally adsorbed with chromatophores (Fig. 2f). In the series of plates (Fig. 3) prepared with antibody samples adsorbed with spheroplasts, these immunoprecipitates gradually increased in area (Fig. 3a–c) and disappeared (Fig. 3a–f) from the pattern. This observation suggests that the localization of the corresponding antigenic determinants is on the periplasmic face (i. e., the inside of the isolated chromatophore) which is accessible in spheroplasts for antibody binding. These data indicate that many of these antigens have an asymmetric distribution of determinants.

Provided that suitable determinants are available for immunoadsorption, the formation of an immunoprecipitate by an antigen which is exposed on both sides of the membrane would be affected by preincubation of antibody samples with either chromatophores or spheroplasts. Indeed, this was found to be the case for antigen 17, which was absent from immunoplates (Fig. 2d–f, Fig. 3c–f) prepared with immunoglobulins preincubated with either chromatophores or spheroplasts. The RC immunoprecipitate likewise disappeared from the pattern.

On the basis of adsorption studies with this antiserum, three classes of immunoprecipitates may be recognized (Table 1). Antibodies forming those of class I (the LDH was typical of this group) were adsorbed by chromatophores, suggesting that determinants of antigens forming immunoprecipitates of this class are expressed on the cytoplasmic face of the chromatophore membrane as it exists within the cell. Antibodies forming class II immunoprecipitates were adsorbed by spheroplasts, suggesting that the corresponding antigenic determinants are present on the periplasmic face of the chromatophore membrane. Moreover, the adsorption of antibodies forming this class of immunoprecipitates by detergent-disrupted chromatophores, but not by untreated chromatophores, is also consistent (24, 25) with the localization of these antigens on the inside of isolated chromatophores. Antibodies forming some immunoprecipitates (class III) were efficiently adsorbed by preincubation with either chromatophores or spheroplasts. This observation suggests that such antigens (e.g., no. 17) may be transmembrane proteins which span the bilayer. Some immunoprecipitates (e.g., no. 10, 20, and 32) were formed by

TABLE 1. *Classification of immunoprecipitates on the basis of antibody adsorption studies*

Class	Representative immunoprecipitates	Prepn to which antibodies adsorbed	Exposure of corresponding antigenic determinants on chromatophore membrane
I	LDH, NADH dehydrogenase, LH[a]	Isolated chromatophores	Outside (cytoplasmic face in vivo)
II	13, 14, 22	Spheroplasts	Inside (periplasmic face in vivo)
III	17, 17a	Both	Both sides

[a] In experiments with some sera, an additional precipitate was formed. This precipitate showed partial identity with LH. Antibodies forming LH were adsorbed only by chromatophores; antibodies forming the additional immunoprecipitate were adsorbed to both spheroplasts and chromatophores.

antibodies which were not efficiently adsorbed by either chromatophores or spheroplasts. This is probably a heterogeneous group, since the failure of antibodies of a certain specificity to be adsorbed may be due to a number of reasons. Antigens forming such immunoprecipitates may be so deeply embedded in the membrane that antibodies directed against these antigens are not efficiently bound during preincubation with either chromatophores or spheroplasts. Alternatively, this may reflect a low-affinity association between these antigens and corresponding antibody or the effect of steric factors preventing antibody binding. Also, the observed failure of these antibodies to bind to membranes may be due to the presence of only low levels of the corresponding antigen in the adsorbing membrane preparation. The latter possibility appeared to be the case for immunoprecipitate 10, which was the only immunoprecipitate not lost from the CIE pattern obtained with antibody samples maximally adsorbed with detergent-disrupted chromatophores (not shown).

The reciprocal of the area subtended by an immunoprecipitate is linearly related to the amount of antigen used in the adsorption step. This relationship is defined by: $1/A_v = 1/A_0 - Kxv$ (24), where A_v and A_0 are the areas delineated by an immunoprecipitate adsorbed with v and 0 volume of an adsorbing antigen suspension, x is the degree to which the determinants are exposed in the adsorbing antigen, and K is a constant for a given set of antigenic determinants under established experimental conditions. Included in K are the concentration of the antigenic determinants in the adsorbing material and an agglutination constant for the binding of antibody by these determinants. The slope of the progressive adsorption curve for an antigen is $-Kx$.

This relationship has recently been used to evaluate the sidedness and degree of uniformity of vesicles prepared from *Escherichia coli* (24, 25) by quantifying the extent of exposure (x) of antigens localized on the inside of such vesicles. This approach was applied to an analysis of chromatophore membrane integrity in the present study by measuring the effect of chromatophore disruption on the exposure of antigens forming class II immunoprecipitates. Antibody preparations were adsorbed with untreated chromatophores or with equal amounts of chromatophores disrupted with Triton X-100 (at detergent/protein ratio = 1), and the slopes of the curves for progressive adsorption of antibody by increasing antigen were determined. If it is assumed that 100% of these antigens are exposed in the detergent-disrupted preparation (24, 25), then the portion of this antigen exposed on the surface of untreated chromatophores may be calculated (Table 2). The remainder of this antigen is sequestered in untreated chromatophores and may be used as an estimate of the portion of chromatophores which are sealed vesicles of uniform sidedness. Evaluation of these data (Table 2) suggests that these chromatophores exist for the most part as closed structures with a uniform membrane orientation.

In this series of experiments, the class I immunoprecipitate NADH dehydrogenase exhibited the adsorption behavior predicted (24, 25) of an immunoprecipitate formed by an antigen which is situated on the outer surface of the membrane; the rate of adsorption of specific antibody was not affected by Triton disruption of the membrane. This was not the case for the LH; antibodies forming this immunoprecipitate were more effectively removed by disrupted than untreated chromatophores, despite the surface location of the corresponding antigen (Table 1). This increased exposure of LH antigen in Triton-disrupted preparations is explicable on the basis of the probable polymeric nature of this antigen (31). Detergent treatment may relieve steric constraints that prevent binding of antibody by the repeating antigenic determinants present.

TABLE 2. *Evaluation of chromatophore membrane sidedness by measurement of effect of chromatophore disruption on exposure of antigens*

Immunoprecipitate	x(disrupted chromatophores)/x(untreated chromatophores)[a]	% of antigen exposed in untreated chromatophores	Estimation of uniformity of chromatophore sidedness (%)
7	8.6–9.5	10–12	88–90
14	6.6–8.2	12–15	85–88

[a] Correlation coefficient for curves of progressive adsorption was between −0.95 and −0.99. Values reported are ranges from two adsorption series.

Charge shift experiments. To further elucidate the organization of the chromatophore membrane, charge shift CIE (26) was used to assess the amphiphilic character of these antigenic components. Amphiphilic proteins bind ionic detergents and consequently exhibit altered mobility when electrophoresed in the presence of such charged surfactants (13). Among those antigens that displayed retarded or enhanced migration toward the anode in the presence of cationic (CTAB) or anionic (DOC) detergents, respectively, were those forming no. 17a, 32, LH, and RC (Fig. 4). It is interesting to note that the previously described (4) cathodic shoulder of LH exhibited a greater charge shift than did the LH, suggesting that the LH antigen is heterogeneous in its amphiphilicity as well as its electrophoretic mobility. The migration of the antigen forming immunoprecipitate 17 from the origin was retarded in the presence of CTAB, but in the presence of DOC it fused with no. 17a, which was shifted substantially toward the anode. The altered mobility of no. 17 in the presence of CTAB may therefore reflect electrostatic binding rather than hydrophobic interaction.

DISCUSSION

The asymmetric distribution of components in membrane bilayers has been the subject of much interest in recent investigations (24, 25, 27, 28, 30). The chromatophore membrane provides an excellent experimental system in which to study membrane sidedness because it is possible to examine both faces of the membrane by using essentially native structures. In the present study, the unique structural arrangement of the intracytoplasmic membranes of *R. sphaeroides* was exploited in bilateral antiserum adsorption studies. Such experiments using isolated chromatophores and osmotically protected spheroplasts confirmed that the chromatophore membrane is asymmetric. Furthermore, the localization of several components has been indicated (Table 1) by the adsorption of corresponding antibody by either side of the chromatophore membrane. In interpreting these data, it must be considered that there may be aspects of a membrane component which do not elicit an antibody response in the animal. To minimize the possibility of overlooking the exposure of an antigen on the membrane surface due to the failure of the antiserum to recognize such a nonimmunogenic moiety, these adsorption studies were also conducted with three additional antiserum pools from other rabbits. The results essentially confirmed those presented in Table 1. Nonetheless, these surface exposures must be considered minima since it is possible that some antigens forming class I or class II immunoprecipitates may be exposed on both sides of the membrane, but that only the aspect exposed on one face may be detected by these immunochemical procedures.

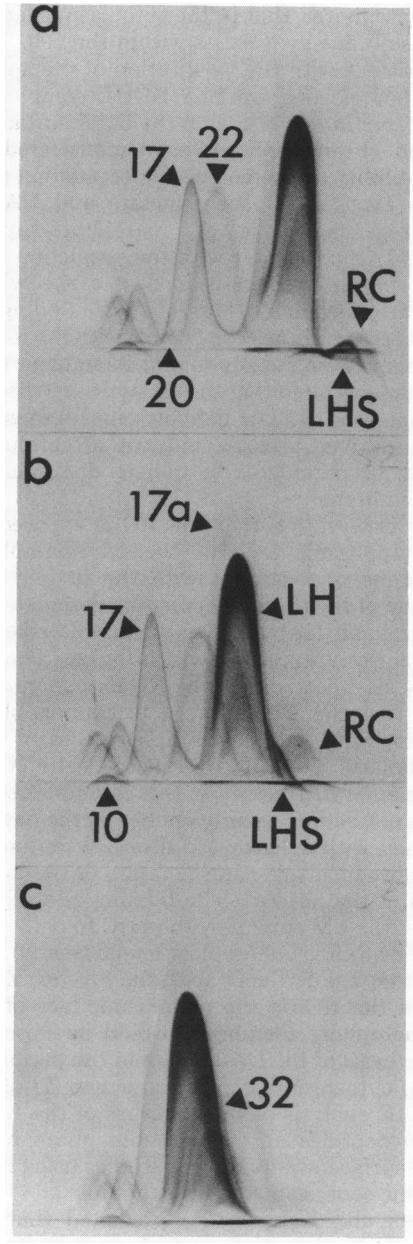

FIG. 4. *Analysis of amphiphilicity of chromatophore membrane antigens by charge shift CIE. Electrophoresis for 90 min in first dimension was performed in the presence of: (a) 0.05% CTAB, 1% Triton X-100; (b) 1% Triton X-100; and (c) 0.25% DOC, 1% Triton X-100. LHS, Shoulder of LH.*

By these CIE techniques, the LH antigen was shown to be exposed on the outer surface of

chromatophores, that is, the cytoplasmic face of the membrane as it exists within the cell. This is consistent with the localization of the apparently homologous antenna BCHL complex in *Rhodospirillum rubrum* on the outer surface of isolated chromatophores as demonstrated by susceptibility to chymotrypsin degradation (23).

The exposure of the L-lactate and NADH dehydrogenases at the chromatophore surface (Table 1) is consistent with the prediction that these enzymes are positioned at a site where substrate is available. Moreover, the findings of the present study are compatible with the recent investigation of Takemoto and Bachmann (36), who demonstrated that the catalytic activities of succinate and NADH dehydrogenases in spheroplast-derived vesicles, but not in chromatophores, are stimulated by toluene disruption of the membrane.

The observation that the RC is exposed on the cytoplasmic face of the chromatophore membrane is consistent with the finding (11) that one of the three RC subunits designated as H is available for hydrolysis when isolated chromatophores are treated with pronase. The apparent disappearance of the RC immunoprecipitate from the CIE pattern of immunoplates prepared with antibody samples adsorbed with spheroplasts (Fig. 3) suggests that the RC is transmembrane; however, this immunoprecipitate is not defined clearly enough in the pattern obtained with spheroplast-adsorbed antibodies to allow conclusive determination of the localization of the antigen. The binding of antibody against the LM subunits of the RC to both sides of the disrupted spheroplast membrane (9) suggests that the RC does span the bilayer. Exposure of the RC on the periplasmic face of the chromatophore membrane would be expected on the basis of the localization in the periplasm (29) of cytochrome c_2, the reductant (7) of the RC. CIE analysis of the exposure of the L and M polypeptides on the chromatophore membrane surface awaits further studies using monospecific sera against these subunits.

Owen and Kaback (25) predicted that the progressive adsorption curve for antibodies directed against a transmembrane antigen would be biphasic and that a plateau would be reached. The results observed here for immunoprecipitates 17 and 17a are not consistent with this hypothesis and instead show progressive adsorption at a constant rate until insufficient antibody remains to form the immunoprecipitates (Figs. 2 and 3). The precipitation of an antigen in immunoelectrophoresis requires a minimum concentration of specific antibody. If the population of antibody is sufficiently reduced by the removal of a subpopulation with specificity for certain antigenic determinants, an immunoprecipitate will not be formed, and a soluble immune complex with an insufficient ratio of antibody to antigen will be electrophoresed off the immunoplate. Also, the adsorption behavior of class III immunoprecipitates is consistent with the possibility that there are only two antigenic determinants per molecule which are distributed on opposite sides of the membrane bilayer. If antibody to only one determinant remained after antiserum adsorption, immunoprecipitation, which requires antibodies to at least two determinants, would not occur.

The adsorption behavior of immunoprecipitates 17 and 17a is consistent with the presence of the corresponding antigenic determinants on both sides of the chromatophore membrane bilayer (Table 1). The possibility that the presence of these determinants on both sides of the bilayer is due to dislocation of the antigen during cell disruption, rather than a true transmembrane localization in the native structure, cannot be excluded.

In these studies, spheroplasts were used to approach the periplasmic aspect of the chromatophore membrane. The possibility that the adsorption of antichromatophore antibody by spheroplasts is due to the binding of these antibodies to shared or cross-reacting antigens in the cytoplasmic membrane, rather than antigens expressed on the periplasmic aspect of the chromatophore membrane, cannot be entirely ruled out. The presence of such antigens has been suggested in previous experiments (M. L. P. Collins, unpublished data) in which the particulate fraction from aerobically grown cells was analyzed in CIE and found to contain antigens that were precipitated by antichromatophore antibodies. However, the results of adsorption experiments with disrupted chromatophores, in the present study, are consistent with the localization of antigens forming class II immunoprecipitates on the inner face of the chromatophore membrane. Moreover, the finding that the BCHL-associated components specific to chromatophores are not among the shared or cross-reacting antigens (Collins, unpublished data), together with the adsorption of anti-RC antibody by spheroplasts in the present study, confirms that the periplasmic aspect of the chromatophore membrane is indeed exposed in spheroplasts.

Quantitative evaluation of the degree of continuity and uniformity in sidedness of our chromatophore preparations can be made on the basis of the relative degree of exposure of antigens forming class II immunoprecipitates in dis-

rupted and untreated chromatophores. These studies (Table 2) are in agreement with the result of a previous study (16) in which the photooxidation of exogenously added ferrocytochrome *c* was examined. From the marked increase in the steady-state levels of this reaction after disruption of the membranes with sodium cholate, it is suggested that nearly 80% of the chromatophores in our typical preparations have the expected orientation. This is in close agreement with results of freeze-fracture electron microscopy (17) in which 79% of such purified chromatophores were found to be oriented in the same manner as the intracellular vesicle membranes in whole cells. Furthermore, in a recent CIE analysis (8) in which immunoadsorption with an *R. sphaeroides* chromatophore preparation was compared with that obtained with preparations solubilized with Triton, it was found that about 90% of the chromatophores had such an "inside-out" orientation.

Several antigenic components were observed here to bind detergents in charge shift CIE. The amphiphilic nature of the LH and RC components, shown by their bidirectional shifts, is consistent with their amino acid compositions (10, 35) and, in the case of LH antigen, with its solubility in apolar solvents (S. J. Tonn and P. L. Loach, Abstr. Int. Congr. Biochem., p. 165, 1979). The charge shift manifested by no. 17a is consistent with the amphiphilic character expected of a protein that penetrates the hydrophobic lipid bilayer and spans the membrane (Table 1). Immunoprecipitates 10 and 20 were not shifted in this experiment, suggesting that the corresponding antigens are not amphiphilic. Therefore, it is likely that the reason that antibodies against these components were not efficiently adsorbed by preincubation with either chromatophores or spheroplasts is related to the level of antigen present in the adsorbing membrane, affinity of antibody binding, or steric hindrance. The failure of no. 22 to exhibit a charge shift suggests that this component is also hydrophilic. This is consistent with the observation that this antigen is a peripheral membrane protein, since it is found in the cytoplasm as well as in chromatophores (Collins, unpublished data).

This study has permitted a simultaneous assessment of the transmembrane localization of a large variety of the antigenic components within the chromatophore membrane, including several functional entities such as specific dehydrogenases and the BCHL-protein complexes involved in harvesting light energy and primary photochemistry.

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LITERATURE CITED

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