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Crossed Immunoelectrophoretic Analysis of Chromatophore Membranes from *Rhodopseudomonas sphaeroides*

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Triton extracts of intracytoplasmic photosynthetic membranes (chromatophores) purified from *Rhodopseudomonas sphaeroides* were subjected to crossed immunoelectrophoresis with antiserum raised in rabbits to purified chromatophores. A total of 31 immunoprecipitates was visualized; 2 of the immunoprecipitates were identified as reduced nicotinamide adenine dinucleotide (EC 1.6.99.3) and L-lactate dehydrogenases by enzyme staining techniques. Reaction with a monospecific antiserum identified the photochemical reaction center. Photopigments were associated with a major precipitate in the pattern which was identified on the basis of immunological identity as light-harvesting bacteriochlorophyll *a*·protein complex. These results provide the basis for a detailed structural and functional analysis of the chromatophore membrane by crossed immunoelectrophoresis.

Growth of the facultative photoheterotrophic bacterium *Rhodopseudomonas sphaeroides* under low oxygen tension results in the development of intracytoplasmic (chromatophore) membranes in which the photosynthetic apparatus is localized. Formation of the chromatophore membrane is repressed under high aeration and derepressed upon reduction of oxygen tension. Fractionation and analysis of functional components of the *R. sphaeroides* chromatophore membrane has been restricted mainly to the photochemical reaction center (RC) (2, 13), the light-harvesting (LH) bacteriochlorophyll *a* (BCHL) complex (1, 5, 16), and the L-lactate dehydrogenase (LDH) (10). Additional chromatophore polypeptide components have been observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6, 15, 19), but the function of these components has remained largely unknown because this procedure results in the loss of any biological activity. Recently, crossed immunoelectrophoresis (CIE) has been applied to the study of bacterial membranes (7, 14, 17, 18). Since nondenaturing detergents are used for membrane disruption before CIE, both enzymatic and antigenic activities are usually retained, thus permitting a functional analysis of membrane components. In addition, a high order of resolution is achieved through CIE by virtue of the two-dimensional distribution of antigenic components. This communication describes a CIE analysis of chromatophores isolated from *R. sphaeroides*.

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Triton X-100 was selected as the detergent for the solubilization of chromatophores for CIE because a recent study demonstrated that it was superior to other detergents for the maximal recovery of *Micrococcus lysodeikticus* membrane components (4). Chromatophores were purified from phototrophically grown (15) *R. sphaeroides* NCIB8253 by differential and sucrose density gradient centrifugation (12). Chromatophore preparations for immunization were purified further by a second cycle of differential and rate-zone sedimentation. Previous reconstitution studies have shown that such chromatophore preparations are at least 95% free from protein of non-chromatophore origin after a single rate-zone sedimentation (11). The purified membranes were treated with Triton X-100 (detergent:protein ratio = 0.78) in 10 mM Tris-hydrochloride buffer (pH 7.5) for 1 h at 0°C in the dark. The insoluble residue was separated from the soluble extract by centrifugation at 50,000 rpm (165,000 × *g*) for 90 min. A fraction (28 to 48%) of the total protein, photopigments, and cytochromes was solubilized by detergent treatment of chromatophores. Comparison of the SDS-PAGE polypeptide patterns (not shown) of untreated chromatophores, soluble extracts, and insoluble residues after Triton treatment failed to indicate the absence of any

components in the solubilized preparation, although some differences in the extent of extraction were noted. The quantitative nature of CIE is not absolute, and measurements are made with reference to a standard control pattern. Therefore, although Triton treatment does not result in the complete solubilization of the chromatophore membrane, the use of such detergent extracts is adequate for CIE analysis because a representative sample of membrane components is obtained.

A schematic diagram of the CIE pattern obtained with the Triton extract of chromatophores analyzed by anti-chromatophore serum is shown in Fig. 1. Thirty-one immunoprecipitates are stained with Coomassie brilliant blue (Fig. 2a). The lack of immune reactions of partial identity between precipitates indicates that a random association of components has not occurred after solubilization. Because the most prominent immunoprecipitate, no. 25, is visible without staining due to the presence of photopigments (Fig. 2b), it seemed possible that this might represent the LH-BCHL complex. Acetone/methanol extraction of the excised precipitate and spectral analysis of this extract indicated the presence of both red and yellow carotenoid, as well as BCHL. There are two LH-BCHL·protein complexes in *R. sphaeroides* designated B850 and B875 on the basis of absorption maxima in the near infrared; the latter is the minor LH component in these chromatophore preparations, and it is extracted poorly with Triton. Co-electrophoresis experiments employing the crossed-line technique (8) were performed with the B850 complex (generous gift of C. N. Hunter) prepared by the procedure of Clayton and Clayton (1) by lauryl dimethyl amine oxide (LDAO) extraction. These immunoplates (not shown) indicate that immunoprecipitate no. 25 is the LH complex because an immune reaction of identity was observed between the anodic side of this immunoprecipitate and the purified LH complex. A spur was observed on the cathodic side of the precipitate in some experiments. There is evidence for some heterogeneity in this antigen in that a shoulder is frequently observed on the cathodic side (Fig.

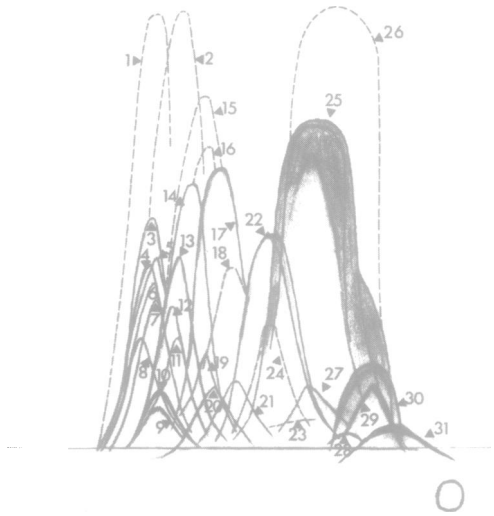

FIG. 1. *Schematic drawing of the CIE pattern obtained with Triton extracts of chromatophores analyzed with anti-chromatophore serum and stained with Coomassie brilliant blue (Fig. 2a). Sample was applied to well at lower right, current was applied for 75 min, and the components of the antigen were separated across the bottom of the slide on the basis of electrophoretic mobility (anode at left). Resolved antigens were then electrophoresed into top gel containing anti-chromatophore antibodies (anode at top) resulting in immunoprecipitation. Antiserum was raised in rabbits to purified chromatophores. Antigen was administered, and serum was obtained, purified, and stored as described by Collins and Salton (4). Conditions for electrophoresis and for treatment of immunoplates have been described in detail (4).*

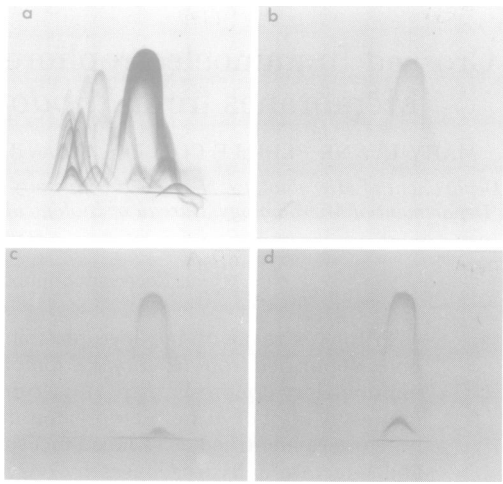

FIG. 2. *CIE pattern obtained with 1% Triton extracts of chromatophores (30 μg of protein) analyzed with anti-chromatophore serum (60 μl/ml). (a) Stained with Coomassie brilliant blue; (b) unstained; (c) stained for NADH dehydrogenase activity; (d) stained for LDH activity. Wet immunoplates were incubated in enzyme staining reagents for ca. 30 min. LDH was recognized by staining with the following incubation mixture: tetranitro blue tetrazolium, 3 mg; 1.0 M L-lactate (pH 7.0), 1 ml; phenazine methosulfate, 0.25 mg; 50 mM Tris-hydrochloride (pH 7.5), 9 ml. NADH dehydrogenase was stained by reaction with the following mixture: NADH, 7 mg; tetranitro blue tetrazolium, 6 mg; 50 mM Tris-hydrochloride (pH 7.5), 20 ml. Note that immunoprecipitate no. 25 is visible in (c) and (d) by virtue of the intrinsic photopigments. This precipitate was not stained by the zymogram reagents.*

3). This may represent dissociation products which lack some components, reflected in a reduction of electrophoretic mobility and the loss of antigenic determinants. Immunoprecipitate no. 25 is formed by more than one boundary (Fig. 1); such a pattern has been observed in CIE of other bacterial antigens (M. L. P. Collins and M. R. J. Salton, unpublished data).

NADH dehydrogenase, immunoprecipitate no. 28, may be identified on the basis of specific enzyme staining for this activity (Fig. 2c). The LDH precipitate does not stain with Coomassie brilliant blue, but is visible by zymogram staining, demonstrating amplification by this technique (Fig. 2d). Attempts to demonstrate succinate dehydrogenase (EC 1.3.99.1) and ATPase (EC 3.6.1.3) by zymogram staining techniques (14) were not successful. The failure to observe ATPase activity is probably attributable to the release of this enzyme from the membrane by treatment with low ionic strength buffer during the isolation procedure. During prolonged incubation, one precipitate was capable of reducing tetrazolium in the presence of succinate, as well as several other electron donors. Thus, no definitive identification of succinate dehydrogenase has as yet proved possible.

The RC was identified by reaction with antiserum raised to purified RCs. This monospecific antiserum was incorporated in the gel intermediate between the sample and reference gel (Fig. 3a). Comparison of this immunoplate with the control (Fig. 3b), which was run without monospecific serum, indicates that immunoprecipitate no. 30 is formed by the RC in combination with its corresponding specific antibody. The precipitation of no. 31 in the intermediate gel is not due to reaction with the anti-RC antibodies, but is caused by electroendosmosis. This process causes flow of buffer toward the cathode. This backward movement carries anti-chromatophore antibodies from the reference gel, causing this immunoprecipitation in the intermediate gel. The effect is more pronounced with antigens of low electrophoretic mobility. Comparison of the control plate (Fig. 3b) with the plate in which monospecific serum was included (Fig. 3a) reveals that only precipitate no. 30 reacted with anti-RC antibodies.

By providing a means for both high resolution and unambiguous identification, CIE permits a critical comparison of the composition of different preparations. The zwitterionic detergent LDAO has been used extensively in studies performed on *R. sphaeroides* (1, 2, 10, 13). The similarity of the CIE patterns of extracts prepared with Triton (Fig. 2a) or LDAO (Fig. 4a) suggests that there is little difference between these extracts. This is consistent with the findings of Collins and Salton (4). Their study of the solubilization of *M. lysodeikticus* membranes by a variety of nondenaturing detergents revealed that the action of these agents is nonselective and that extracts prepared with different detergents are qualitatively similar.

Extracts of chromatophores from the carotenoidless R-26 mutant produced a CIE profile (Fig. 4b) which is similar to that of wild-type chromatophores. RCs were identified on the basis of reaction with monospecific serum. The immunoprecipitate designated LH showed im-

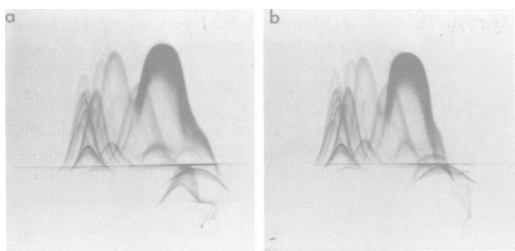

FIG. 3. *Identification of RC by CIE with intermediate gel containing anti-RC antibodies. (a) Anti-RC plate. Lower gel contained no antibody; top reference gel contained anti-chromatophore antibodies; intermediate gel contained anti-RC antibodies. Slides were stained with Coomassie brilliant blue. Anti-RC was a gift of M. Y. Okamura, University of California, San Diego; (b) Control plate. Lower gel contained no antibody; top reference gel contained anti-chromatophore antibodies.*

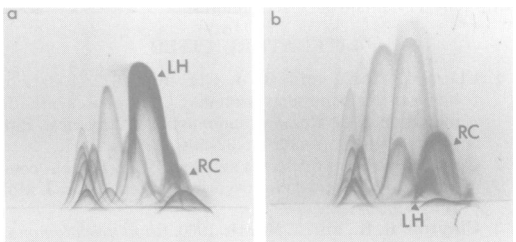

FIG. 4. *CIE analysis of (a) wild-type chromatophore extract prepared with LDAO (gift of Onyx Chemical Co., Jersey City, N.J.) under conditions used for Triton extraction (detergent-protein ratio = 0.78). (b) Triton extract of chromatophores prepared from carotenoidless R. sphaeroides R-26 (3). The mutant was grown photosynthetically under an atmosphere of 95% N_2–5% CO_2 after a period of dark adaptation to prevent photooxidative killing. When standard isolation procedures (12) were applied to extracts from this mutant strain, only a portion of the pigmented material banded in the position of chromatophores obtained from wild-type cells; the remainder banded with the cell envelope. These sedimentation properties are consistent with the ultrastructural appearance of cells of this mutant (9). Reaction centers designated RC; Light-harvesting BCHL·complex designated LH.*

munological identity or partial identity with the LH complex purified from the wild type. Comparison of this immunoplate (Fig. 4b) with that run with extracts of wild-type chromatophores (Fig. 2a) at the same level of antigen loading revealed that the R-26 chromatophore extract is enriched in RC (no. 30) and contains reduced levels of the LH-reactive antigen in comparison to extracts prepared from the wild type. The latter finding is consistent with observations made by SDS-PAGE (19). This similarity in the CIE profiles of extracts of chromatophores from the R-26 strain and those of the wild type suggest that this mutation is expressed by limited differences in overall membrane antigenic structure.

There are many immunoprecipitates in the chromatophore CIE pattern which are awaiting identification. It is possible that these will include enzymes involved in energy conservation, phospholipid synthesis, and transport. The results in the present communication provide the basis for further studies of chromatophore structure and function by CIE.

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