Immunological Probes for Bacteriorhodopsin

IDENTIFICATION OF THREE DISTINCT ANTIGENIC SITES ON THE CYTOPLASMIC SURFACE*

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We have prepared site-specific immunological reagents to study the orientation and surface topography of the integral membrane protein bacteriorhodopsin. Monoclonal and polyclonal antibodies with strong affinity for antigenic determinants on proteolytic and cyanogen bromide fragments of bacteriorhodopsin have been isolated and characterized. Three distinct antibody binding sites have been identified on the cytoplasmic surface of bacteriorhodopsin. The first site is readily accessible in native bacteriorhodopsin and lies close to the COOH terminus. This binding site is lost when only three amino acid residues are removed from the COOH terminus. The second site, which is also near the COOH terminus, is located approximately within the 17 COOH terminal amino acid residues. The third site is in the fragment that comprises Tyr-83 to Met-118 and is probably contained in the short loop connecting the third and fourth helices. The use of COOH terminus-specific antibodies in determination of the orientation of bacteriorhodopsin molecules in the Halobacterium halobium membrane confirms the earlier conclusion that the COOH terminus is on the cytoplasmic side.

Bacteriorhodopsin, the light-driven proton pump in the purple membrane of Halobacterium halobium (1), offers a highly attractive model for studies on the structure and function of energy-transducing proteins. The protein consists of a single polypeptide chain of 248 amino acids and its complete amino acid sequence is now known (2-4) (Fig. 1). A secondary structure model for the bacteriorhodopsin molecule has been developed (5) which is consistent with the amino acid sequence and the diffraction data (6, 7). In this model, rather specific proposals have been made in regard to the amino acids present in each one of the seven helical regions that are embedded in the bilayer, the amino acids that are present in the six loops which must connect the seven helices, as well as those amino acids that are exposed at the NH2 and carboxyl termini (Fig. 2).

Clearly, a variety of experimental approaches will be necessary to test the proposals of the above model in every detail so that ultimately an accurate three-dimensional structure can be put forward for this protein. Of particular importance will be those methods of analysis which provide information on the conformation of the molecule as it exists in a functional state in the phospholipid bilayer. A central question concerns the precise determination of the amino acids which are exposed on one or the other side of the membrane surface. Classically, approaches that are usually applied for studies of this type are accessibility of amino acid residues to different proteolytic enzymes and to different nonpermeant chemical reagents that can label the amino acids at the membrane surface. An alternative general approach involves the use of immunological methods. Thus, an antibody that recognizes a single specific site of the protein molecule can be used as a unique method of identifying the exposed amino acid residues.

In the present study, we demonstrate the utility of site-specific immunological reagents in mapping the surface of bacteriorhodopsin (BR) and in determining its orientation in the cell membrane. Animals immunized with either intact bacteriorhodopsin or defined fragments produced by chemical or proteolytic cleavage were used as sources of specific antisera or for the production of monoclonal antibodies. The antibodies have been rigorously characterized with respect to their binding sites on bacteriorhodopsin. Three distinct antibody binding sites have been identified on the cytoplasmic surface of bacteriorhodopsin; two of these are near the COOH-terminal end of the molecule. The third site is recognized by monoclonal antibodies with reactivity to a fragment extending from Tyr-83 to Met-118 and is most likely confined to the connecting loop centered on Asp-104 (Fig. 2). This observation represents the first evidence that the loop connecting the third and fourth helices is accessible to impermeant probes. Finally,
FIG. 1. The complete amino acid sequence of bacteriorhodopsin and cleavage fragments. The sequence shown is as originally derived by protein sequencing (3) and recently confirmed by the sequence analysis of the bacteriorhodopsin gene (4). Fragments C-2 and C-1, produced by chymotryptic cleavage, are, respectively, from NH-terminal pyroglutamic acid to phenylalanine (amino acid 71) and Gly-82 to Ser-248. The cyanogen bromide fragments (CNBr 1 to CNBr 11) were prepared by cleavage of the C-1 and C-2 fragments with cyanogen bromide (3). The fragment CNBr 10 was cleaved with trypsin to give [CNBr 10]-T2 (Gly-72 to Arg-83) and [CNBr 10]-T1 (Tyr-83 to homoserine 118).

Fig. 2. A model for secondary structure of bacteriorhodopsin derived from the primary structure and electron diffraction data.6

the use of carboxyl terminus-specific antibodies has also provided confirmation of the earlier conclusion (8) that the orientation of BR in the membrane is such that the carboxyl terminus is exposed to the cytoplasmic side.

EXPERIMENTAL PROCEDURES

Materials

Enzymes and Proteins—Papain was purchased from Worthington, L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin from Miles, and carboxypeptidase L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin from Miles, and carboxypeptidase A, pronase, and rabbit serum albumin from Sigma. Staphylococcus protein A-Sepharose CL-4B was purchased from Pharmacia.

Culture Medium, Cell Lines, and Animals—Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate solution, penicillin-streptomycin solution, t-glutamine solution, fetal calf serum (FCS), and newborn calf serum (NBCS) were purchased from Gibco. Hypoxanthine, aminopterin, thymidine, and 2-mercaptoethanol were from Sigma. Polyethylene glycol 4000 was from Fulka.

The myeloma parent cell lines, X63-Ag8 (9), X63-Ag8.6.5.3. (10), GK/O (11), and Sp2/0-Ag14 (12), were obtained from G. Kohler, Basel, Switzerland.

BALB/c mice were from Jackson Laboratory.

Radioactive Chemicals, Immunoreactive Reagents, and Microtiter Plates—Carrier-free Na125I and 125I-iodinated Bolton-Hunter reagent (132I-iodinated N-succinimidyl 3-(4-hydroxyphenyl) propionate, 200 Ci/mmol) were purchased from New England Nuclear. Staphylococcus aureus cells (Pansorbin) from Calbiochem-Behring, and Staphylococcus protein A from Sigma. Rabbit immunoglobulin was from Amersham. Rabbit immunoglobulin di-thioretiline (DTT) and carrier-free Na125I and 125I-iodinated Bolton-Hunter reagent (132I-iodinated N-succinimidyl 3-(4-hydroxyphenyl) propionate, 200 Ci/mmol) were purchased from New England Nuclear. Staphylococcus aureus cells (Pansorbin) from Calbiochem-Behring, and Staphylococcus protein A from Sigma. Rabbit immunoglobulin was from Amersham. Rabbit immunoglobulin di-thioretiline (DTT) and carrier-free Na125I and 125I-iodinated Bolton-Hunter reagent were all purchased from New England Nuclear.

Method

Preparation of Purple Membrane

H. halobium (strain S9, originally obtained from W. Stoeckenius, University of California, San Francisco) was cultured and the purple membrane was isolated by the method described earlier (13).

D. M. Engelman, personal communication.
Preparation of Bacteriorhodopsin Fragments

Chymotryptic cleavage of apomembrane produced two fragments, C-1 (amino acid residues 1–71) and C-2 (amino acid residues 72–248), which were separated by chromatography on a Sephadex LH-60 column with formic acid-ethanol. The procedure for separation of CNBr fragments 1–10 was carried out as described earlier (14). The fragment CNBr 11 was recovered as an aggregate in the excluded volume after chromatography of the CNBr fragments of C-1 on Sephadex LH-60 (3). This fraction was dried, dissolved in 0.25 ml of anhydrous trifluoroacetic acid, and diluted with 0.5 ml of ethanol and 0.25 ml of 88% formic acid, and the pure CNBr 11 was recovered after chromatography on Sephadex LH-60 as described previously (3).

For tryptic cleavage of CNBr 10, 15 nmol of the fragment were first dissolved in 15 μl of 2% (w/v) SDS and 0.2 mM ammonium formate (pH 8.0) and the solution diluted with 3 ml of 0.2 M ammonium formate (pH 8.0) and 0.15 ml of 0.2 M CaCl₂. Tryptsin was added at a trypsin to fragment ratio of 1:50 (w/w) and digestion was carried out for 16 h at 37 °C. The digestion mixture was lyophilized, the residue was dissolved in 0.15 ml of 88% formic acid and diluted with 0.35 ml of ethanol, and then the products were separated on a Sephadex LH-60 column (Fig. 3).

Cleavage of Bacteriorhodopsin by Papain

PM (50 mg) was resuspended in 25 ml of 0.1 M sodium phosphate, 1 mM cysteine, and 1 mM EDTA (pH 7.5), and the suspension was digested with papain for 6 h at 37 °C. Three volumes of 88% formic acid were added, the solution was adjusted to pH 8.0, and the resulting precipitate was pelleted by centrifugation. The supernatant fluid (approximately 0.8 ml) was removed from each well of the [PM-CT] immunoblot assay using either PM or [PM-CT] as a binding target (see below). The hybridomas in positive wells were expanded as described previously (3).

Preparation of Bacteriorhodopsin Vesicles

Cell envelope vesicles were prepared from H. halobium cells by sonication as described previously (17, 18). These vesicles were approximately 95% right side out as indicated by the latency of NADH oxidation at 8000 g for 15 min. The vesicles were reconstituted with 0.137 M NaCl, 5.37 mM KCl, 5 mM NaH₂PO₄, 0.2% (w/v) glucose, and 0.001% (w/v) phenol red. After allowing large particle matter to settle out, the vesicles were centrifuged at 200,000 g for 2 h at 4 °C. The supernatant was dialyzed against 0.15 M NaCl, 0.02% (w/v) NaN₃, and 0.01 M Tris-HCl, and then dialyzed against 0.15 M NaCl, 0.02% (w/v) NaN₃ at 4 °C for 24 h.

Immunization of Mice

Mice were immunized for three separate fusion experiments. In the first, three female C57BL mice were injected with bacteriorhodopsin which had been purified by preparative SDS-polyacrylamide gel electrophoresis. Each mouse received a total of approximately 25 μg of protein in one subcutaneous and one intraperitoneal injection with complete Freund's adjuvant. For the second and third fusions, female Balb/c mice were injected subcutaneously with 0.1 mg of heat-denatured [BR-CT] in complete Freund's adjuvant. In all cases, the mice received 3–4 intraperitoneal booster injections containing 10–20 μg of the protein at 4-week intervals until high levels of antibody were detected in the serum. Fusion experiments were performed 3 days after the last booster injection.

Cell Fusion

In the first fusion experiment, spleen cells from the C57BL mice which had been immunized with SDS-denatured bacteriorhodopsin were fused with X63-Ag8 a azaguanine-resistant myeloma cells (9, 20). In later experiments, nonproducing myeloma cells were used as fusion partners. Spleen cells from BALB/c mice were grown to early logarithmic phase in Dulbecco's modified Eagle's medium with 16% (v/v) fetal calf serum (DMEM-FCS), harvested by centrifugation, and washed once with cold DMEM solution. The myeloma cells were grown to early logarithmic phase in DMEM-FCS, harvested by centrifugation, and washed once with cold DMEM solution. Typically, 8 × 10⁶ spleen cells were mixed with 3–5 × 10⁵ myeloma cells and centrifuged at 200 × g for 10 min. To the cell pellet, 0.75 ml of 50% (v/v) polyethylene glycol 4000 in GKN solution was added dropwise with mixing at room temperature over 60 s. The suspension was incubated at 37 °C for 90 s with gentle shaking and was then diluted by gradually adding 1 ml of DMEM-FCS at room temperature during 30 s, then 3 ml in 30 s, and finally 16 ml in 60 s. The diluted suspension was incubated at room temperature for 5 min and then added dropwise with mixing at room temperature for 5 min at 37 °C. The cells were resuspended in 48 ml of DMEM-FCS containing 1 × 10⁻⁵ M hypoxanthine, 4 × 10⁻⁷ M aminopterin, and 1.6 × 10⁻³ M thymidine (DMEM-HAT), and mouse peritoneal macrophages were added to a concentration of 1 × 10⁷ cells/ml. This cell suspension was distributed in 1-ml aliquots to the wells of two Costar-24 plates. The plates were kept at 37 °C in a 5% CO₂ and 95% air atmosphere at 85% humidity. The plates were fed every 2 days by adding 1 ml of DMEM-HAT and, thereafter, at 3-day intervals by replacing one-half of the medium (1 ml) with fresh DMEM-HAT.

Cloning and the Production of High Titer Ascitic Fluid

Ten to fourteen days after the fusion, an aliquot of the culture supernatant fluid (approximately 0.8 ml) was removed from each well and tested for the presence of relevant antibody in a solid phase antibody-binding assay using either PM or [PM-CT] as a binding target (see below). The hybridomas in positive wells were expanded in 20 ml of DMEM-HAT medium containing 1 × 10⁻⁴ M hypoxanthine and 1.6 × 10⁻³ M thymidine and cloned by the limiting dilution technique as described (22).

For production of large quantities of monoclonal antibodies, mice were injected intraperitoneally with 0.5 ml of 2,6,10,14-tetramethylpentadecane and 7 days later with 5 × 10⁶ hybridoma cells. The mice showed a prominent accumulation of ascitic fluid 10 days later. The ascitic fluid was collected and centrifuged at 65,000 × g for 30 min and the immunoglobulin fraction was precipitated at 40% (w/v) ammonium sulphate (pH 7.4) at 4 °C for 16 h. The precipitate was washed once with 40% ammonium sulphate, dissolved in a minimum volume of water, and dialyzed against 0.15 M NaCl, 10 mM Tris-HCl, and 0.02% (v/v) NaN₃ at 4 °C for 24 h.
Rabbit Antiserum to the CNBr 6 Fragment

The CNBr 6 fragment (30 amino acids, Fig. 1) was conjugated to rabbit serum albumin to enhance its antigenicity. For cross-linking, 0.5 mg of CNBr 6 and 1.5 mg of rabbit serum albumin were dissolved in 0.5 ml of 0.15 M NaCl and 20 mM sodium phosphate buffer (pH 7.4), glutaraldehyde was added dropwise with mixing to a final concentration of 1% (w/v), and the reaction mixture was incubated at room temperature for 1 h with mixing. The conjugated protein was injected with complete Freund's adjuvant into the dorsal skin of five New Zealand White rabbits. Three weeks later, a booster dose of 0.3 mg of CNBr 6 in 0.15 M NaCl and 20 mM sodium phosphate buffer (pH 7.4) was given intravenously to each rabbit. Serum was collected at weekly intervals and stored at 4 °C.

Three of five rabbits gave a high titer antiserum against the CNBr 6 fragment. The immunoglobulin G fraction from 3 ml of high titer antiserum from one rabbit (binding capacity 9.28 nmol of CNBr 6/d) was purified by affinity chromatography on a Staphylococcus protein A-Sepharose column. The column was washed with 10 mM sodium phosphate and 0.025% (w/v) NaN₃ and kept at 4 °C until the absorbance at 280 nm of the eluate was less than 0.01. The immunoglobulin G fraction was eluted with 1 M acetic acid and 0.036% (w/v) NaN₃ and kept at 4 °C.

Iodination of Proteins

Antibodies were iodinated with 125I using the chloramine-T method (23). Bolton-Hunter reagent (24). Bolton-Hunter reagent (1 mL) and 0.03 mg of CNBr 6 were dissolved in 0.1 M sodium phosphate buffer (pH 8.0) at 0 °C and the solution incubated at 4 °C for 16 h. 125I-labeled CNBr 6 was separated from the unreacted reagent by chromatography on a Sephadex LH-20 in formic acid/ethanol (3:7). The radioactive CNBr 6 (125I) was dried and then dissolved in 10 mM sodium phosphate and 0.036% (w/v) NaN₃ and kept at 4 °C.

Radioimmunoassays

Liquid-Phase Competitive Radioimmunoassays—Samples of reconstituted vesicles or of purple membrane were incubated in 0.2 ml of 0.15 M NaCl, 0.02 M sodium phosphate, 0.025% (w/v) NaN₃, and 0.1% (w/v) bovine serum albumin (ph 7.4) (RIA buffer) for 30 min at room temperature with 0.48 pg of rabbit anti-CNBr 6 IgG at 4 °C. Then, 125I-labeled CNBr 6 (20,000 cpm) was added and the mixture incubated for 2 h. S. aureus cells (1 mg) were added and the mixture incubated for 20 min. After adding 2 ml of RIA buffer containing 0.5% (w/v) Tween 80, the mixture was centrifuged at 1,000 × g for 30 min. The pellet was washed once with RIA buffer and counted in a Beckman 4000 γ counter. Reactions without anti-CNBr 6 IgG or with 0.48 pg of nonimmune rabbit IgG were used as controls.

Solid Phase Binding Assay with Immobilized Antigen—Polyvinyl chloride microtiter wells were coated with antigen for 30 min at room temperature using 60 μl of 3.5 mg/ml of PM or [PM-CT] or with bacteriorhodopin fragments at 20 nmol/ml in 0.2% (w/v) SDS, 0.5% (w/v) Triton X-100, and 10 mM sodium phosphate buffer (pH 8.0). After the coating solution was recovered, the wells were washed four times with 0.15 M NaCl, 10 mM sodium phosphate, and 0.025% (w/v) NaN₃ (pH 7.4) (PBS). The wells were then filled up with PBS containing 5% (v/v) newborn calf serum (NBCS-PBS) and incubated for 20 min at room temperature in order to block unfilled protein-binding sites. The blocking solution was discarded and the wells received 80-μl aliquots of either hybridoma culture supernatants or the diluted immunoglobulin fraction obtained from hybridomas-induced ascitic fluid. After incubation at 4 °C for 16 h, the wells were washed four times with NBCS-PBS, filled with 60 μl of 125I-labeled rabbit immunoglobulin directed against mouse immunoglobulins (250,000 cpm/well, 15 Ci/g), and incubated for 4 °C for 16 h. The wells were washed four times with NBCS-PBS and dried, and the radioactivity in each well was determined in a Beckman 4000 γ counter.

In some binding assays, the microtiter wells were coated by drying down a formic acid-ethanol solution of bacteriorhodopsin fragment directly in the wells. We have also used a modification in which the monoclonal antibodies were labeled with 125I, thereby eliminating the incubation with 125I-labeled second antibody.

Solid Phase Binding Assay with Immobilized Antibody—The microtiter wells were coated with the Ig fraction from high titer ascitic fluid. After blocking excess protein-binding sites with NBCS-PBS, either 125I-labeled [CNBr 10]-T2 (350 Ci/mmol, 350,000 cpm/well) or 125I-labeled [CNBr 10]-T2 (490 Ci/mmol, 400,000 cpm/well) was added with NBCS-PBS to the wells. Because of the low solubility of [CNBr 10]T1, 0.1% SDS was also added to the wells receiving this fragment. After incubation for 16 h at 4 °C, the wells were washed four times with NBCS-PBS and then counted in a γ counter.

RESULTS

Peptide Sequence Specificity of Rabbit Anti-CNBr 6 IgG

The binding specificity of the rabbit anti-CNBr 6 IgG preparation was determined by liquid phase competitive radioimmunoassays in which the binding of 125I-labeled CNBr 6 to the anti-CNBr 6 antibodies was measured in the presence of PM, [PM-CT], delipidated BR, [BR-CT], and CNBr 6 as competing species (Fig. 4). As seen, the antibody membrane, delipidated BR, and the CNBr 6 fragment competed effectively, while [PM-CT] and [BR-CT] were not recognized by the antibody. The papain-cleaved COOH terminal region, comprising about 17 amino acids, was, therefore, important for the binding of the anti-CNBr 6 antibodies. This conclusion was further supported by the finding that the 125I-labeled delipidated BR but not 125I-labeled [BR-CT] was effectively precipitated by anti-CNBr 6 IgG in the presence of formalin-fixed S. aureus cells (data not shown).

In order to further characterize the antigenic determinant...
anti-mouse subclass antisera, and 125I-labeled protein A lines were observed with ascitic fluid, the immunoglobulin class was hybridoma-induced ascitic fluid. In cases where multiple precipitin lines were observed with reaction was terminated by the addition of 4 volumes of 0.2 M sodium citrate (pH 2.2), and aliquots of the supernatant solution were subjected to amino acid analysis. The recovery was corrected by using norleucine as an internal standard. The digested PM samples were washed three times with 0.25 M sodium phosphate buffer (pH 7.5) and aliquots (38 pmol) were incubated with 0.48 µg of anti-CNBr 6 IgG and 125I-CNBr 6 (20,000 cpm) and 1 mg of S. aureus cells as described under "Methods". A, the release of terminal amino acids on digestion with carboxypeptidase A, B, effect of digested PM on the competitive binding of 125I-CNBr 6 to anti-CNBr 6 IgG.

**TABLE I**

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Antigen</th>
<th>Fusion partner</th>
<th>Immunoglobulin class</th>
<th>Specific binding to fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR114</td>
<td>BR</td>
<td>X63-Ags</td>
<td>IgG&lt;sub&gt;K&lt;/sub&gt;</td>
<td>CNBr-6</td>
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<td>[PM-CT]&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>[PM-CT]&lt;sup&gt;*&lt;/sup&gt;</td>
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<sup>*</sup> Immunoglobulin class was determined by double immunodiffusion in agar plates using goat anti-mouse Ig subclass antisera and the hybridoma-induced ascitic fluid. In cases where multiple precipitin lines were observed with ascitic fluid, the immunoglobulin class was determined in a binding assay with immobilized [PM-CT] followed by the addition in sequence of hybridoma supernatant, goat anti-mouse subclass antisera, and 125I-labeled protein A.

<sup>+</sup> There was no binding to C-1, C-2, or any of the CNBr fragments.

for the anti-CNBr 6 IgG, the binding of this antibody to carboxypeptidase A-treated PM was studied. As shown in Fig. 5A (cf. Ref 6), carboxypeptidase removes sequentially Ser-248, Thr-247, and Ala-246, and 245. The binding of anti-CNBr 6 IgG was studied as a function of the release of these terminal amino acids (Fig. 5B). As shown, the removal of Ser-248 and Thr-247 did not significantly affect antibody binding. However, there was a dramatic loss of immunoreactivity following the removal of Ala-246, suggesting that this preparation of antibodies is highly specific for a site which includes Ala-246 as an essential component.

**Monoclonal Antibodies to Bacteriorhodopsin**

A total of 12 stable hybridoma cell lines was derived from the hybrids produced in three independent cell fusions. The monoclonal antibodies secreted by these cells bind specifically to either PM or papain-digested PM and have been characterized with respect to immunoglobulin class and specificity for binding to cyanogen bromide fragments (Table I).

**Binding Specificity of Monoclonal Antibodies to Bacteriorhodopsin Fragments**

The CNBr and proteolytic fragments of BR were used to determine the specificities of different monoclonal antibodies. Since protein A does not bind tightly to all of the monoclonal antibodies and since most CNBr fragments are insoluble in water, a solid phase antibody-binding assay with antigen immobilized in polyvinyl chloride microtiters was used instead of the liquid phase competitive radioimmunoassay.

**Monoclonal Antibody to the COOH-terminal Region of Bacteriorhodopsin**—The hybridoma BR114 was the only clone derived from mice immunized with denatured bacteriorhodopsin. Like the rabbit anti-CNBr 6 antibodies described above, the BR114 monoclonal antibody did not bind to [PM-CT] (Table II) and did bind to CNBr 6 (data not shown). Although the monoclonal antibody BR114 and the rabbit anti-CNBr 6 antibodies clearly recognize sequences at or near the carboxyl terminus of BR, the sites do not appear to be identical. Thus, in contrast with the loss of binding of anti-CNBr 6 IgG to carboxypeptidase A-treated PM, the binding of BR114 to the latter was not affected (Table II).

**Monoclonal Antibodies to a Site(s) on CNBr 10—Except for BR114, all of the hybridoma cell lines were generated from mice immunized with delipidated [BR-CT] and, therefore, were not expected to bind to sites at the COOH terminus. In fact, all of the antibodies from these clones bound very well to [PM-CT] and five of them were specific for the CNBr 10 fragment.

Reactivity with bacteriorhodopsin in PM clearly indicated that the binding sites for the CNBr 10 monoclones are exposed at the surface of the PM. However, since it is likely that a greater part of the CNBr 10 fragment is embedded in the membrane (Fig. 2), two antibody binding sites can be envisaged. One of these would comprise the extracellular surface including residues Gly-72 to Asn-76, the region where the antibodies bind.
chymotrypsin cleavage occurs (14). The second could be on the cytoplasmic surface containing the sequence Asp-102 and Gly-106. To delineate the binding site(s) further, the fragments [CNBr 10]-T1 and [CNBr 10]-T2 ("Methods") were tested. These tryptic fragments were prepared and separated on a Sephadex LH-60 column as shown in Fig. 3. The different column fractions were tested for their capacity to bind to [125I]-labeled monoclonal antibodies. The binding profile for hybridoma B131-D3 is shown in Fig. 6. High levels of binding were detected in fractions containing undigested CNBr 10 and [CNBr 10]-T1; there was no binding in fractions corresponding to [CNBr 10]-T2. The results shown in the inset of Fig. 6 for fractions obtained by further HPLC separation of pooled [CNBr 10]-T1 fractions further confirmed the above results. Similar results we obtained with hybridomas B214-C1 and B242-H9. Thus, it appears that these antibodies bind to determinants in a segment of the polypeptide delimited by Tyr-59 and Met-118.

**FIG. 6.** Assay of fractions from Sephadex LH-60 chromatography of trypsin-digested CNBr 10 for binding to [125I]-labeled CNBr 10 monoclonal antibody, B131-D3. An aliquot of the eluted fractions (0.1 ml) (Fig. 3) was placed directly in the microtiter wells and dried at room temperature. After washing the plate four times with PBS, the plate was filled with NBCS-PBS and incubated for 30 min. Then, NBCS-PBS was replaced by 60 ml of [125I]-labeled CNBr 10 monoclonal antibody, B131-D3 (270,000 cpm/well). The plate was incubated at 4 °C for 16 h. After washing the plate four times with NBCS-PBS, the plate was filled with NBCS-PBS and incubated for 20 min. Then, NBCS-PBS was replaced by 60 ml of [125I]-labeled CNBr 10 monoclonal antibody, B131-D3 (270,000 cpm/well). The plate was incubated at 4 °C for 16 h. After washing the plate four times with NBCS-PBS, the wells were cut out and counted in a γ counter.

**FIG. 7.** Competitive binding of CNBr 10 monoclonal antibodies, BR114 and B131-D3, respectively, to PM. The microtiter wells were coated with 60 μl of 8 mg/ml of PM. Then [125I]-labeled BR114 (192,000 cpm/well) was mixed with varying concentrations of monoclonal antibodies, BR114 and B131-D3. These mixtures were added to the wells and incubated at 4 °C for 16 h. The wells were then washed four times with NBCS-PBS, cut out, and counted in a γ counter.

The COOH-terminal Region of Bacteriorhodopsin Inhibits the Binding of CNBr 10—Early experiments showed that the binding of the CNBr 10 antibodies was much more effective when papain-digested purple membrane was used to coat the microtiter wells instead of native purple membrane. One possible explanation could be that the COOH terminus interferes with the binding of these antibodies. Alternatively, there could be the trivial explanation that papain-digested purple membrane might simply be more efficient in coating the wells than the PM itself. To avoid the latter possibility, the wells were first coated with PM and then treated with papain. The rate of removal of the COOH terminus was monitored by the loss of the binding site for [125I]-labeled BR114, the above described monoclonal antibody specific for the COOH terminus. During the same time course, there was an increase in the binding of the three CNBr 10 monoclonal antibodies tested (Fig. 8). The response of the B242-H9 antibody was striking in that its binding was completely blocked prior to cleavage by papain. Apparently, the COOH terminus of BR hinders the approach of antibodies to the surface of the protein. It is interesting to note that even though all of the CNBr 10 antibodies are of the IgM class, and presumably bind to a very limited region of bacteriorhodopsin near Asp-104, the presence of the COOH terminus has a clear differential effect on the binding of these antibodies.

**Orientation of BR in H. halobium Cells and in Reconstituted Vesicles**

Although alternative evidence has been adduced for the orientation of BR in natural membrane and in reconstituted vesicles (8), it was of interest to apply the present immunological reagents to a further study of this question. Cell envelope vesicles (17, 18) as prepared from the *H. halobium* cells pump protons from inside to outside like the whole cells and, therefore, have the right side out orientation. On the other hand, vesicles reconstituted with a variety of phospholipids from PM or delipidated BR (16, 25) pump protons from outside to inside, and the protein molecules are uniformly oriented with the COOH terminus exposed to the cytoplasmic side. We have now prepared the cell envelope (right side out orientation >95%) vesicles as well as the reconstituted vesicles and have measured the availability of the COOH terminus of BR for binding to rabbit anti-CNBr 6 antibodies in a competitive radioimmunoassay.

The binding site on the COOH terminus was not accessible in the cell envelope vesicles; however, when the vesicles were lysed, either by dialysis against water or by treatment with Triton X-100, effective binding was observed (Fig. 9). (Surprisingly, the high concentration of salt necessary to maintain the integrity of cell envelope vesicles did not significantly affect antibody-antigen interactions in this assay.)
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**Fig. 8.** Binding of CNBr 6 and CNBr 10 monoclonal antibodies to papain-digested PM. The microtiter wells were coated with 0.1 ml of 8 mg/ml of PM at room temperature for 30 min. After removal of the excess of the PM solution and washing the plates with PBS four times, the wells were incubated with 0.1 ml of 8 pg/ml of papain in 0.1 M sodium phosphate, 1 mM cysteine, and 1 mM EDTA (pH 7.5) at room temperature. At different time intervals, the papain solution was removed from the wells and the reactions were terminated by adding 0.1 ml of 25 mM iodoacetic acid in 0.1 M sodium phosphate (pH 8.0) to the wells. The plates containing digested PM were washed with PBS four times and used for the binding assay with 125I-labeled monoclonal antibodies, BR114, B242-H9, B214-C1, and B131-D3. Binding values (100%) in different experiments were as follows: A, BR114, 23,200 cpm; B, B242-H9, 117,700 cpm; C, B214-C1, 4,100 cpm; D, B131-D3, 2,600 cpm.

An experiment with rabbit anti-CNBr 6 antibodies and reconstituted vesicles showed clearly that the binding sites are exposed in these vesicles (Fig. 10). However, relative to the extent of binding observed with a comparable amount of bacteriorhodopsin in purple membranes, only about 25% of the total sites present in the vesicles appear to be reactive.

Control experiments showed that these vesicles were not leaky to either 14C-inulin or 32P, and that all of the bacteriorhodopsin molecules were oriented with the COOH terminus on the outside, accessible to pronase.

**DISCUSSION**

Immunological approaches have been widely used to study the orientation and surface topography of membrane proteins (see Ref. 26 for review). However, the usefulness of antibodies as probes of fine structure is often limited by the heterogeneity...
of an antiserum which may contain a complex mixture of antibodies with reactivity to several antigenic determinants on a protein. Furthermore, the use of site-specific antibodies in the mapping of surface topology may be hampered by the lack of adequate structural information on the membrane proteins. Bacteriorhodopsin is an ideal subject for the present approach since it is a highly characterized protein and specific models (e.g., Fig. 2) for its structure have been proposed. Therefore, there is a reasonable likelihood of pinpointing the binding sites for both monoclonal and polyclonal antibodies.

In this initial immunological approach to the study of the bacteriorhodopsin surface, we have concentrated on antibodies with strong affinity for determinants on isolated proteolytic and CNBr cleavage fragments of the protein. Two classes of antibodies have been characterized; those which react to determinants at or near the COOH terminus of the molecule and those which bind to determinants on a trypic fragment of CNBr 10. Both classes of antibody have been clearly demonstrated to react with bacteriorhodopsin on the cytoplasmic surface of the purple membrane. Antibodies to other antigenic sites on B5 were also isolated in the course of this study, but these failed to bind to specific fragments and were not characterized further. Apparently, some antibodies recognized determinants that were not retained in isolated polypeptide fragments, perhaps because the antigenic site was lost after proteolytic cleavage and fragment isolation. For example, the loop connecting helices 2 and 3 contains both a CNBr and likely to disrupt antigenic sites in this region of the poly-a chymotryptic cleavage site and, therefore, fragmentation is peptide chain. It is also possible that some small cleavage chloride in an orientation that masked the antigenic site. fragments lost immunoreactivity in solid phase binding assays after proteolytic cleavage and fragment isolation. For example, the binding of B242-H9 is totally blocked in intact purple membranes, whereas the binding of B214-C1 and B131-D3 is only inhibited by 50% when the COOH terminus is intact.

The results presented here demonstrate the potential of site-specific immunological reagents for probing the surface domains of integral membrane proteins. While only two regions of the protein were examined in this study, it should be possible to isolate antibodies specific for most, if not all, of bacteriorhodopsin regions exposed out of the bilayer. Indeed, the potential of immunological results for the mapping of bacteriorhodopsin may be greater than those of chemical and enzymatic probes which have only limited reactivity toward bacteriorhodopsin in native purple membrane. This is illustrated by the binding of monoclonal antibodies B242-H9, B214-C1, and B131-D3 to a region of bacteriorhodopsin which had not been shown previously to be accessible to impermeant reagents. In addition, it should be possible to further define the binding sites for antibodies through use of chemically synthesized peptides.

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