A General Method for Mapping Tertiary Contacts between Amino Acid Residues in Membrane-Embedded Proteins†

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ABSTRACT: A general method for mapping tertiary interactions in membrane proteins using the visual pigment rhodopsin as a model is presented. In this approach, the protein is first assembled from two separately expressed gene fragments encoding nonoverlapping segments of the full-length polypeptide. Cys residues are then introduced into each of the two fragments such that juxtaposed residues are able to form disulfide cross-links in the protein either spontaneously or with the assistance of a Cu^{2+}-(phenanthroline)$_3$ oxidant. The cross-linked polypeptides are identified from a characteristic mobility shift on sodium dodecyl sulfate (SDS) gels as detected by Western blot analysis where the covalently bound heterodimer migrates with a mobility essentially identical to that of the native, full-length protein. Three different split rhodopsin mutants were prepared: one with a split in the loop connecting helices 3 and 4 (the 3/4 loop), one with a split in the 4/5 loop, and one with a split in the 5/6 loop. Each of these proteins when purified from transfected COS cells bound 11-*cis*-retinal, had a native absorption maximum at 500 nm, and activated transducin in a light-dependent manner. The cross-linking assay was tested with the rhodopsin mutant split in the 5/6 loop using the rho-1D4 antibody (which recognizes the carboxy terminal eight amino acids of rhodopsin) to detect the proteins on Western blots of SDS gels. Cys residues were substituted for Val-204 in the amino terminal fragment and Phe-276 in the carboxy terminal fragment of the rhodopsin mutant because Schwartz and co-workers [Elling et al. (1995) *Nature 374*, 74−77] have shown that these two amino acids are close to each other in the tertiary structure of the related tachykinin NK-1 receptor by engineering a metal ion binding site into the protein. Cys-204 and Cys-276 were found to cross-link in the presence of Cu^{2+}(phenanthroline)$_3$ oxidant as judged by the fact that the mutant split proteins comigrate with full-length rhodopsin on SDS gels. This method of using Cys mutagenesis and disulfide cross-linking with split proteins to map tertiary interactions should be generally applicable to a number of different membrane proteins.

Elucidation of a high-resolution, three-dimensional structure for rhodopsin and other G protein-coupled receptors is a goal that has generated intense interest in the field but relatively little progress due to the difficulties inherent in working with membrane proteins. To date, the best effort has been from the pioneering efforts made by Henderson and Schertler and co-workers to establish a map of rhodopsin at 7 Å resolution using electron cryomicroscopy of two-dimensional crystalline arrays of the protein (Schertler et al., 1993, 1995; Unger & Schertler, 1995). At this resolution, the seven transmembrane helical segments in the protein are clearly delineated but amino acid side chains are not visible, and although theoretical structures have been proposed (Baldwin, 1993; Alkorta & Du, 1994; Donnelly et al., 1994; Donnelly & Findlay, 1994), experimental data to resolve differences among the models are lacking. Therefore, a general procedure for mapping tertiary contacts in these membrane proteins is greatly needed.

Cys-scanning mutagenesis has been combined with oxidative cross-linking to characterize the intramembranous helical region of the aspartate (Falke & Koshland, 1987; Lynch & Koshland, 1991), Tar (Pakula & Simon, 1992), and Trg (Lee et al., 1994) bacterial chemotactic receptors. In this approach, amino acids of the helical regions are systematically replaced by Cys residues, the proteins exposed to chemical oxidant to cross-link juxtaposed Cys residues, and the disulfide cross-linked homodimers detected by a shift in the electrophoretic mobility of the protein on sodium dodecyl sulfate (SDS) gels. A key to the success of this approach is that the bacterial receptors form homodimers in their native state which can be cross-linked to form a product that is readily identifiable by a routine assay. In contrast, the native structure of rhodopsin is a monomer with a single polypeptide chain, and there would be no large molecular mass change upon disulfide formation between proximal residues within the protein.

To utilize the disulfide cross-linking approach, we have explored the possibility of using split receptor constructs in which fully functional rhodopsin mutants are produced from two separately expressed and assembled fragments of the protein. Many different membrane proteins have been shown to be capable of assembly in functional form from two or more protein fragments, including bacteriorhodopsin (Huang et al., 1981; Kahn & Engelman, 1992; Hansen et al., 1994),

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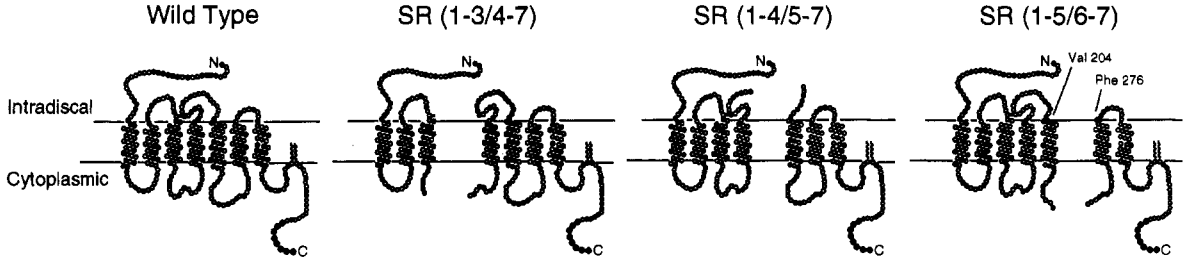

FIGURE 1: Schematic representation of the structure of the wild-type and split rhodopsins used in this study. The nomenclature used to identify each protein is adopted from Kobilka et al. (1988) and is described in Experimental Procedures. The carboxy terminal eight amino acids in each structure are highlighted by filled circles to indicate the location of the epitope recognized by the rho-1D4 monoclonal antibody (Molday & MacKenzie, 1983). SR(1−3/4−7) is split between Pro-142 and Met-143 in the second cytoplasmic loop separating transmembrane segments 3 and 4. SR(1−4/5−7) is split between Pro-194 and His-195 in the second intradiscal loop separating transmembrane segments 4 and 5. SR(1−5/6−7) is split between Ser-240 and Ala-241 in the third cytoplasmic loop separating transmembrane segments 5 and 6. The carboxy terminal fragments of SR(1−4/5−7) and SR(1−5/6−7) each have an additional Met residue at the amino terminus to allow for initiation of translation. The two residues (Val-204 and Phe-276) identified in the structure of SR(1−5/6−7) are the residues used in the cross-linking studies.

β_2-adrenergic receptor (Kobilka et al., 1988), the sodium channel (Stühmer et al., 1989), adenylylcyclase (Tang et al., 1991), muscarinic acetylcholine receptor (Maggio et al., 1993; Schoneberg et al., 1995), lactose permease (Bibi & Kaback, 1990; Wrubel et al., 1990, 1994; Zen et al., 1994), the yeast a-factor transporter (Berkower & Michaelis, 1991), and, recently, rhodopsin (Ridge et al., 1995a). If Cys-scanning mutagenesis is used in combination with the split receptor constructs, then disulfide cross-linking between juxtaposed Cys residues located on different fragments of the protein would result in a large increase in molecular mass and a readily apparent decrease in electrophoretic mobility on SDS gels.

Rhodopsin was used as a model for these studies because mutants of the protein can be assayed spectrophotometrically and functionally in a straightforward manner for native-like structure and function. Rhodopsin is composed of an apoprotein opsin and an 11-*cis*-retinal chromophore covalently bound by means of a protonated Schiff base linkage to the ϵ-amino group of Lys-296 of the seventh transmembrane helix of the protein. As a result, rhodopsin has an absorption maximum at 500 nm which is diagnostic for the chromophore in a properly folded protein environment. In addition, rhodopsin undergoes a characteristic photoreaction in which the Schiff base deprotonates to form the active intermediate metarhodopsin II (MII). MII has a blue-shifted absorption maximum at 380 nm (Matthews et al., 1963) and is the intermediate that activates the G protein transducin (Emeis et al., 1982; Kibelbek et al., 1991).

We report here the successful use of split rhodopsin constructs with Cys mutagenesis and disulfide cross-linking to demonstrate the proximal location of amino acid residues in the three-dimensional structure of rhodopsin. This is a completely general procedure that should find application with many different proteins.

EXPERIMENTAL PROCEDURES

Materials. 11-*cis*-Retinal was the generous gift of Peter Sorter and Hoffman-La Roche (Nutley, NJ). Bovine retinae were obtained from J. A. Lawson Co. (Lincoln, NE). DE-52, CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate], 1,10-phenanthroline, and Sepharose 4B were from Sigma (St. Louis, MO). [^{35}S]GTPγS (1156 Ci/mmol) was from NEN. Nonradiolabeled GTPγS (tetralithium salt) was from Boehringer Mannheim. NBT (nitro blue tetrazolium), BCIP (5-bromo-4-chloro-3-indolylphosphate), and the alkaline phosphatase-conjugated goat anti-mouse antibody used for Western blot analysis were from Promega (Madison, WI).

The anti-rhodopsin monoclonal antibody 1D4 (Molday & MacKenzie, 1983; MacKenzie et al., 1984) was purified from hybridoma culture medium (National Cell Culture Center, Minneapolis, MN) by $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography on DE-52 according to standard protocols as has been described (Oprian et al., 1987). The antibody was then coupled to the Sepharose 4B solid support by the method of Cuatrecasas (1970). Peptide I (AspGlu-AlaSerThrThrValSerLysThrGluThrSerGlnValAlaProAla), used for elution of opsin from the 1D4-Sepharose 4B matrix, was purchased from American Peptide Co., Inc. (Santa Clara, CA).

Mutagenesis and Expression of the Rhodopsin Gene. The synthetic rhodopsin gene used in these studies was designed to contain about 30 unique restriction sites to facilitate cassette mutagenesis (Ferretti et al., 1986). All procedures for DNA manipulation, mutation of the rhodopsin gene, DNA sequence analysis, and transient expression of the rhodopsin gene and the rhodopsin gene fragments in the COS cell system were performed as previously described (Ferretti et al., 1986; Oprian et al., 1987, 1991; Oprian, 1993; Zhukovsky & Oprian, 1989; Zhukovsky et al., 1991).

Design and Construction of the Split Rhodopsin Mutants. We have adopted with minor modification the nomenclature of Kobilka et al. (1988) to describe the split receptors. Thus, SR(1−3) refers to an N-terminal fragment of the protein containing the first three transmembrane segments. SR(4−7) refers to the C-terminal complement containing the last four transmembrane helices. SR(1−3/4−7) refers to a rhodopsin mutant that has been assembled from the two separately expressed fragments SR(1−3) and SR(4−7). Point mutations are indicated by beginning with the name of the fragment, followed by a colon, followed by the single letter code for the wild-type amino acid, followed by the number of the amino acid, followed by the single letter code for the new amino acid. For example, the double mutant in which Val-204 is changed to Cys in SR(1−5) and Phe-276 is changed to Cys in SR(6−7) is designated as SR(1−5:V204C/6−7:F276C).

There are three split rhodopsin constructs (six individual fragments) presented in this report: SR(1−3/4−7), SR(1−4/5−7), and SR(1−5/6−7) (Figure 1). In each case, the N-terminal and C-terminal fragments are encoded on separate

pMT3 (Franke et al., 1988) expression vectors so that the fragments can be expressed individually in COS cells or they can be coexpressed. Each gene fragment has been cloned into the vector as an *Eco*RI-*Not*I cassette, with a Kozak consensus sequence immediately 5′ of the initiator Met codon, exactly as has been described for the full-length, wild-type rhodopsin gene (Franke et al., 1988). The six individual fragments have the following composition: SR(1−3), Met-1 to Pro-142; SR(4−7), Met-143 to Ala-348; SR(1−4), Met-1 to Pro-194; SR(5−7), begins with an added initiator Met followed by His-195 to Ala-348; SR(1−5), Met-1 to Ser-240; and SR(6−7), begins with an added initiator Met followed by Ala-241 to Ala-348.

Reconstitution and Purification of the Proteins. Transfected COS cells were harvested 72 h after initial exposure to DEAE−dextran and DNA. Procedures for reconstitution of the pigments with 11-*cis*-retinal, solubilization of the COS cell membranes with 1% (w/v) CHAPS or β-D-dodecyl maltoside, and purification of the proteins by immunoaffinity chromatography on the 1D4-Sepharose 4B matrix have been described (Oprian et al., 1987, 1991; Oprian, 1993; Zhukovsky & Oprian, 1989; Zhukovsky et al., 1991, 1992).

Absorption Spectroscopy. UV/visible absorption spectra were recorded on samples eluted from the anti-rhodopsin 1D4-Sepharose 4B immunoaffinity matrix using a Hitachi Model U-3210 spectrophotometer that was specifically modified by the manufacturer for use in a dark room. Data were acquired with the aid of an Everex System 1700 microcomputer using Spectra Calc software from Galactic Industries Corp. (Salem, NH). All spectra were recorded on samples of 1.0 cm path length.

Assay for Activation of Transducin. Transducin was purified from bovine retinae according to the procedure of Wessling-Resnick and Johnson (1987) and then subjected to ion-exchange chromatography on DE-52 as described by Baehr et al. (1982). As a final step, the protein was dialyzed against 10 mM Tris buffer (pH 7.5) containing 50% (w/v) glycerol, 2 mM $MgCl_2$, and 1 mM dithiothreitol and stored at −20 °C. The wild-type and mutant rhodopsins were assayed for their ability to catalytically activate transducin by following the binding of [^{35}S]GTPγS as has been described previously (Zhukovsky et al., 1991) using purified receptors at 5 nM concentration in detergent solution (0.01% dodecyl maltoside). The wild-type and mutant rhodopsins were assayed in detergent solution so that an absorption spectrum could be recorded on the same sample that was used for the transducin assays. Assuming that the molar extinction coefficients of the wild-type and mutant rhodopsins are identical, we could make a direct determination of the specific activities for these samples.

Disulfide Cross-Linking. Disulfide cross-links were induced to form in the split receptor constructs by treatment of the purified samples with 3 mM $CuSO_4$, 9 mM 1,10-phenanthroline, and 3.3% (v/v) glycerol in 10 mM sodium phosphate buffer (pH 7) for 20 min at 37 °C as described by Lee et al. (1994). The reactions were conducted in the dark until terminated by addition of (final concentrations) 12.5 mM *N*-ethylmaleimide and 12.5 mM EDTA in Laemmli load buffer [60 mM Tris buffer (pH 6.8) containing 2% (w/v) SDS, 6% (w/v) sucrose, and 0.005% (w/v) bromophenol blue]. When desired, the stop solution also contained 5% (v/v) β-mercaptoethanol.

Western Blot Analysis. Protein samples were separated by electrophoresis on 10 or 15% polyacrylamide gels according to the procedure of Laemmli (1970). The separated peptides were then transferred to nitrocellulose for Western blot analysis as described by Burnette (1981) with 1D4 as the primary antibody and an alkaline phosphatase-conjugated anti-mouse IgG as the secondary antibody. Protein bands were visualized by reaction with NBT and BCIP according to directions supplied by the manufacturer (Promega). Unless noted otherwise in the text, the sample buffer for electrophoresis did not contain β-mercaptoethanol.

RESULTS

The aim of this study is to develop a general, rapid, and easy assay for the routine determination of tertiary contacts in integral membrane proteins. We have used for this purpose the visual pigment rhodopsin as a model. Our approach is to use disulfide cross-linking of Cys mutations to demonstrate the proximal location of amino acid side chains within the protein. To facilitate the identification of cross-linked residues, we have engineered the Cys mutations into split receptor constructs of the protein such that cross-linking can be readily detected by a mobility shift on SDS gels.

To demonstrate the feasibility of this approach, we need to accomplish three goals. (1) We need to show that split receptor mutants of rhodopsin can be generated from coexpressed gene fragments and that these split receptors fold into a native conformation as judged by wild-type spectral properties of the purified pigments and wild-type ability to activate the G protein transducin. (2) We need to show that the small carboxy terminal fragments which contain the 1D4 epitope (Molday & MacKenzie, 1983) can be visualized by Western blot analysis after separation by SDS gel electrophoresis. (3) We need to show that two proximal Cys mutations in the split receptor can be cross-linked and that this cross-linking can be detected on Western blots of SDS gels by a shift in the electrophoretic mobility of the carboxy terminal fragment.

(1) Expression of Split Rhodopsins

We have expressed and purified three different split rhodopsin mutants in this study: SR(1−3/4−7), SR(1−4/5−7), and SR(1−5/6−7).

SR(1−3/4−7). The rhodopsin mutant SR(1−3/4−7) is composed of two rhodopsin fragments that are split in the cytoplasmic loop connecting transmembrane segments 3 and 4. The reconstituted pigment was purified from transfected COS cells in about 30% yield compared to the wild-type pigment. The spectral properties of the purified mutant were highly characteristic of the wild-type pigment, with an absorption maximum at 500 nm (Figure 2). Upon exposure to light, the maximum shifted to 380 nm, characteristic of the photointermediate MII (Figure 2). The specific activity of SR(1−3/4−7) for the light-dependent activation of transducin is essentially identical to that of the wild-type protein (Figure 2).

SR(1−4/5−7). The rhodopsin mutant SR(1−4/5−7) is composed of two rhodopsin fragments that are split in the extracellular loop connecting transmembrane segments 4 and 5. The reconstituted pigment was purified from transfected COS cells in essentially the same yield as the wild-type pigment. The spectral properties of the purified mutant were highly characteristic of the wild-type pigment, with an absorption maximum at 500 nm (Figure 2). Upon exposure

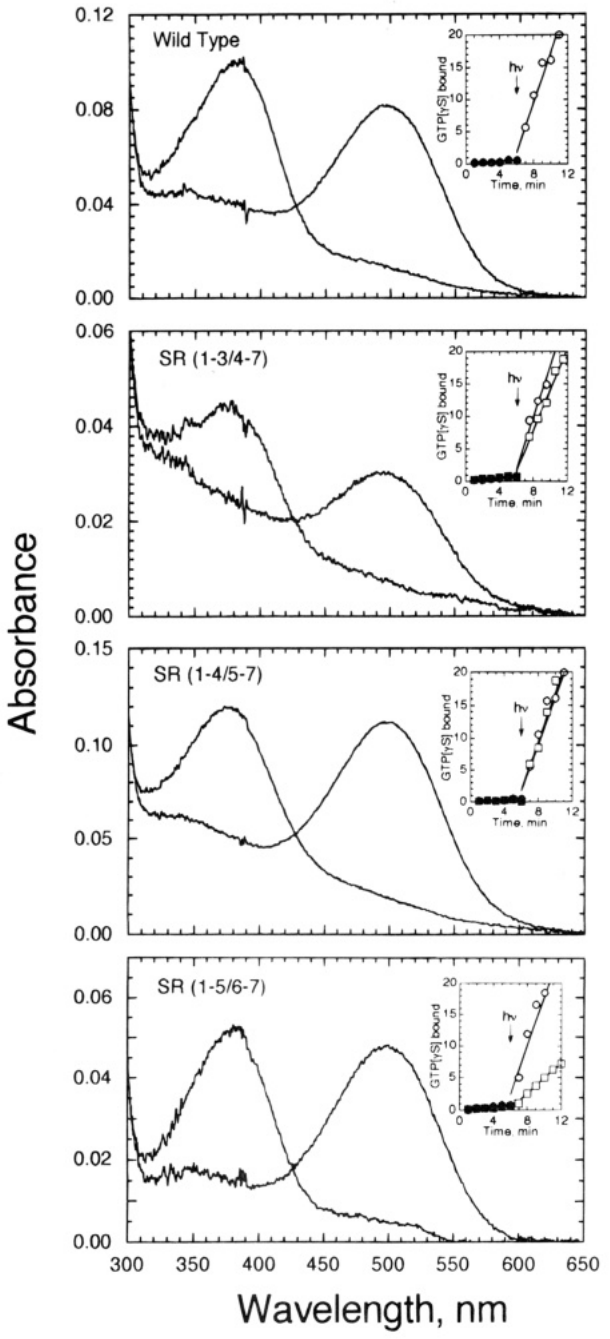

FIGURE 2: Absorption spectra and activity of wild-type, SR(1−3/4−7), SR(1−4/5−7), and SR(1−5/6−7) rhodopsins purified from transfected COS cells. There are two spectra shown for each pigment: one with a maximum at 500 nm and one with a maximum at about 380 nm. The spectra were obtained from the purified pigments as isolated from COS cells in 0.1% dodecyl maltoside according to the protocol described in Experimental Procedures. Following purification, a small aliquot of each sample was removed for activity assays and an initial absorption spectrum recorded on the remainder. In each case, the spectrum had a 500 nm absorption maximum, characteristic of the wild-type pigment. After this initial spectrum was recorded, the sample was exposed to light and another spectrum recorded immediately. In each case, the spectrum had changed to one with a maximum at about 380 nm, characteristic of the active intermediate MII. That this new species was indeed MII and not free retinal was confirmed by trapping the intermediate in acid solution to generate an absorption spectrum with a maximum at 440 nm, characteristic of a protonated Schiff base of *all-trans*-retinal (not shown). (inset), Light-dependent activation of transducin. The activation of transducin was measured as described in Experimental Procedures: (closed symbols) activity in the dark, (open symbols) activity after exposure to light, (circles) activity of wild-type rhodopsin, and (squares) activity of the split rhodopsin mutants.

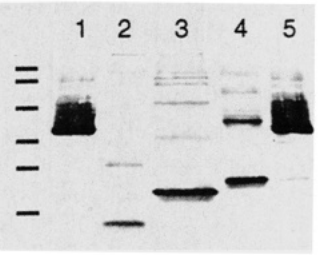

FIGURE 3: Western blot analysis of individually expressed and immunoaffinity-purified C-terminal split rhodopsin fragments. The fragments were purified in 1% (w/v) CHAPS from 10 plates of transfected COS cells using the 1D4-Sepharose 4B matrix essentially as has been described for the wild-type and split rhodopsins (Experimental Procedures). Each sample (2.5−5.0 μL) was then combined with load buffer and separated on a 15% polyacrylamide gel. The peptides were then transferred to nitrocellulose for Western blot analysis using the 1D4 antibody: lanes 1 and 5, wild-type rhodopsin from COS cells; lane 2, SR(6−7); lane 3, SR(5−7); and lane 4, SR(4−7). Western blots of the coexpressed split rhodopsin constructs looked essentially identical to that shown here for the separately expressed C-terminal fragments. Horizontal marks to the immediate left of the Western blot indicate the position of molecular mass standards run in the same gel. From top to bottom, the marks correspond to 107, 76, 52, 36.8, 27.2, and 19 kDa.

to light, the maximum shifted to 380 nm, characteristic of the photointermediate MII (Figure 2). The specific activity of SR(1−4/5−7) for the light-dependent activation of transducin is essentially identical to that of the wild-type protein (Figure 2).

SR(1−5/6−7). The rhodopsin mutant SR(1−5/6−7) is composed of two rhodopsin fragments that are split in the cytoplasmic loop connecting transmembrane segments 5 and 6. The reconstituted pigment was purified from transfected COS cells in about 50% of the yield of the wild-type pigment. The spectral properties of the purified mutant were highly characteristic of the wild-type pigment, with an absorption maximum at 500 nm (Figure 2). Upon exposure to light, the maximum shifted to 380 nm, characteristic of the photointermediate MII (Figure 2). The specific activity of SR(1−5/6−7) for the light-dependent activation of transducin is about 25% of that of the wild-type protein (Figure 2).

(2) Detection of C-Terminal Fragments on Western Blots

As can be seen in Figure 3, the carboxy terminal fragment of each split rhodopsin mutant is readily observed by Western blot analysis of SDS gels using the 1D4 monoclonal antibody. The carboxy terminal fragments migrate with greater mobility than the wild-type protein, consistent with their smaller molecular masses, and they migrate with mobility decreasing in order of their increasing molecular size (Figure 3, lanes 2−4). Each lane contains a single major band corresponding to the fragment monomer along with more faintly staining additional bands of higher molecular mass. These additional bands appear to be multimers of the individual fragments. The multimers are observed when the carboxy terminal fragments are expressed alone, as in Figure 3, and when they are coexpressed with the amino terminal fragments (not shown). In fact, we noted no differences in the Western blots of the individually expressed and coexpressed fragments when probed with the 1D4 antibody. It is noteworthy in this context that the carboxy terminal fragment of the purified SR(1−3/4−7) mutant migrates with a mobility identical to that of the isolated fragment and different from that of the wild-type pigment. This was a surprise since we

expected SR(1−3) and SR(4−7) to be covalently bound by the disulfide bond between Cys-110 and Cys-187 (Karnik & Khorana, 1990). We can offer no explanation at this time for why the disulfide bond is not formed in SR(1−3/4−7) but note that failure to form this bond was also observed by Ridge et al. (1995a) for a nearly identical split rhodopsin mutant. Despite the fact that the Cys-110−Cys-187 disulfide bond is not formed, it is important to emphasize that the SR(1−3/4−7) mutant displays wild-type spectral properties and specific activity for the light-dependent activation of transducin (Figure 2).

(3) Cross-Linking of Cysteine Mutants

To test the cross-linking assay, we tried to engineer a disulfide bond between residues 204 and 276 in the split receptor SR(1−5/6−7). Residue 204 is a Val in the native protein located near the extracellular surface of transmembrane segment 5. Residue 276 is a Phe in the native protein located near the extracellular surface of transmembrane segment 6. Schwartz and co-workers have shown that, when the analogous residues in the tachykinin NK-1 receptor are both His, they form a metal ion binding site in the protein that was detected by the ability of Zn^{2+} to inhibit binding of antagonists to the receptor (Elling et al., 1995). Thus, these two residues are located close to each other in the tertiary structure of the NK-1 receptor, and we speculated that they may also be close to each other in the tertiary structure of rhodopsin. Therefore, we used these residues to test the cross-linking assay.

The Cys-204 mutant and wild-type SR(1−5) fragments were coexpressed with the Cys-276 mutant and wild-type SR(6−7) fragments. The three purified pigments, SR(1-5:V204C/6−7), SR(1−5/6−7:F276C), and SR(1−5:V204C/6−7:F276C), bound 11-*cis*-retinal to form a pigment with the 500 nm absorption maximum characteristic of native rhodopsin (Figure 4), and all formed the 380 nm maximum characteristic of MII upon exposure to light, which could be trapped with acid to form the 440 nm maximum of a protonated Schiff base (not shown). Finally, all of the mutants activated transducin in a light-dependent manner with specific activities similar to that of the SR(1−5/6−7) parent (Figure 4, inset). In conclusion, the substitution of Cys for the native residues at positions 204 and 276 does not appear to greatly perturb the structure of the SR(1−5/6−7) mutant.

Western blot analysis of SR(1−5:V204C/6−7:F276C) shows that the 1D4-staining, carboxy terminal SR(6−7) fragment comigrates with the wild-type SR(6−7) fragment in the absence of Cu^{2+}(phenanthroline)$_3$, but it comigrates with full-length wild-type rhodopsin upon treatment with the Cu^{2+}(phenanthroline)$_3$ oxidant; subsequent treatment with 5% (v/v) β-mercaptoethanol in the load buffer causes the migration pattern to return to that of wild-type SR(1−5/6−7) (Figure 5a). In contrast, the SR(6−7) fragments of the two single mutants, SR(1−5:V204C/6−7) and SR(1−5/6−7:F276C), comigrate with the isolated wild-type SR(6−7) fragment in both the presence and absence of the Cu^{2+}-(phenanthroline)$_3$ oxidant (Figure 5a). These data suggest that SR(1−5) and SR(6−7) are bound covalently in the double mutant upon treatment with Cu^{2+}(phenanthroline)$_3$ by a disulfide bond between Cys-204 in transmembrane helix 5 and Cys-276 in transmembrane helix 6. As is shown in Figure 5b, wild-type SR(1−5/6−7) does not cross-link upon

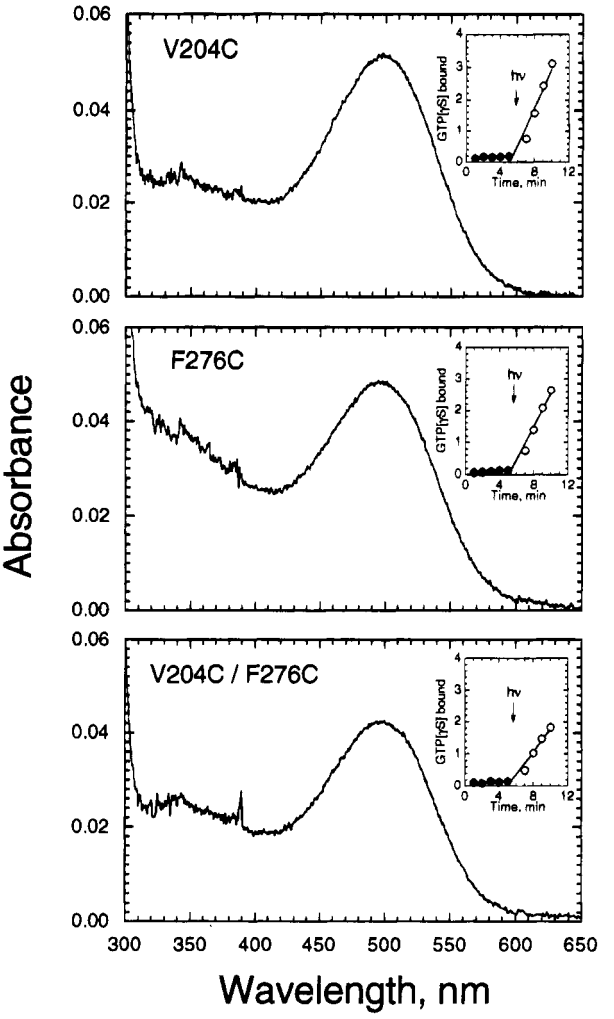

FIGURE 4: Absorption spectra and transducin activity of SR(1−5/6−7) mutants. Procedures are essentially identical to those described in the legend to Figure 2. Absorption spectra are for SR(1−5:V204C/6−7), SR(1−5/6−7:F276C), and SR(1−5:V204C/6-7:F276C), as indicated in each panel. (inset) Light-dependent activation of transducin: (closed circles) reaction in the dark, (open circles) reaction in the light. The specific activity of each mutant relative to SR(1-5/6−7) is as follows: SR(1−5:V204C/6−7), 0.79; SR(1−5/6−7:F276C), 0.67; and SR(1−5:V204C/6−7:F276C), 0.46.

treatment with Cu^{2+}(phenanthroline)$_3$ as judged by the fact that the SR(6−7) fragment comigrates with the isolated SR-(6−7) fragment and not with full-length rhodopsin.

DISCUSSION

The goal of this study was to develop a procedure for mapping tertiary contacts between amino acid residues in membrane-embedded receptors as a general method for characterization of the three-dimensional structure of membrane proteins. To accomplish this goal, we began with the disulfide cross-linking procedure pioneered by Falke and Koshland (1987), and then elaborated by others (Lynch & Koshland, 1991; Pakula & Simon, 1992; Lee et al., 1994), to map quaternary contacts in the bacterial chemotaxis receptors. This procedure exploits the fact that the native structure of the chemotaxis receptors is a noncovalently bound homodimer and makes use of SDS gels to detect disulfide bonds formed between proximal Cys residues located on different subunits in the receptor complex. Thus, when a disulfide forms between subunits, the covalently bound dimer migrates with a much slower electrophoretic

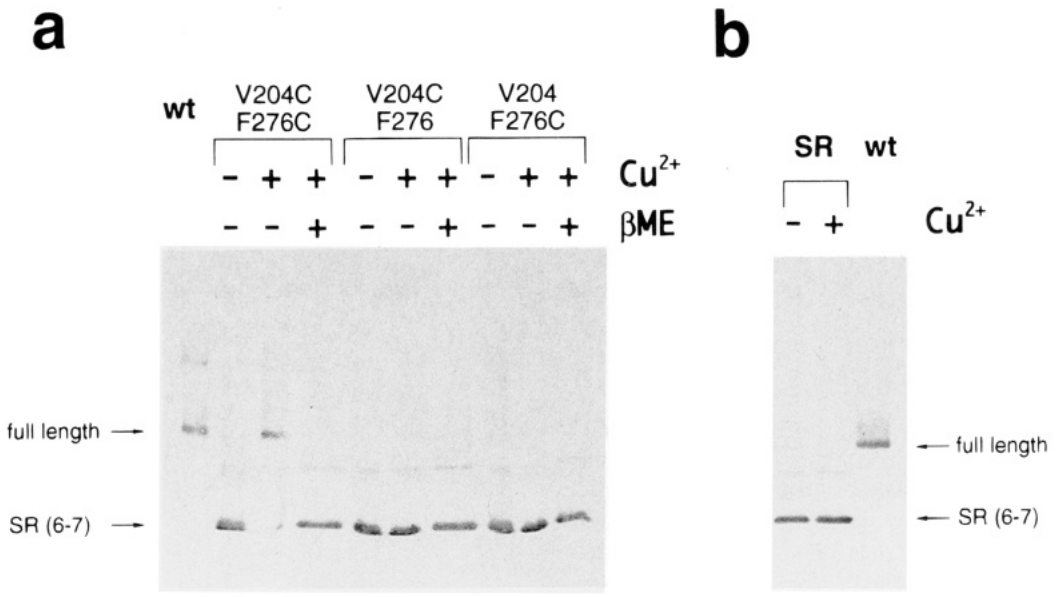

FIGURE 5: Western blot analysis of cross-linked SR(1−5/6−7) mutants. Details of the cross-linking reactions, electrophoresis on 10% polyacrylamide gels, and Western blot analysis using the 1D4 antibody were as described in Experimental Procedures. (a) Cross-linking of Cys-204 to Cys-276. From left to right, the lanes contain wild-type rhodopsin, SR(1−5:V204C/6−7:F276C), SR(1−5:V204C/6−7), and SR(1−5/6−7:F276C), as indicated above each column by the designation wt for wild-type rhodopsin or by the single letter code for amino acid at positions 204 and 276. For each mutant, there are three lanes: before treatment with copper (−), after treatment with copper (+), and after treatment with β-mercaptoethanol (βME). The positions of the full-length rhodopsin and the carboxy terminal SR(6−7) fragment are indicated by arrows to the left of the figure. (b) Effect of Cu^{2+}(phenanthroline)$_3$ treatment on SR(1−5/6−7). This panel shows a Western blot analysis for SR(1−5/6−7) in the presence and absence of the Cu^{2+}(phenanthroline)$_3$ oxidant, as indicated at the top of each column.

mobility than the subunit monomers of the wild-type receptor. Despite the elegance and great appeal of this procedure, it has found limited application because it is restricted to proteins composed of multiple subunits.

To generalize this approach for use with single subunit receptors, we have combined Cys mutagenesis and disulfide cross-linking with split receptor constructs of the proteins. In a sense, we have engineered the receptor to be a heterodimer for this purpose. We have used rhodopsin as a model to develop this system and have constructed three split receptor mutants: SR(1−3/4−7), SR(1−4/5−7), and SR(1−5/6−7). Each of these mutants bound 11-*cis*-retinal to form a pigment with a 500 nm absorption maximum, indicative of a properly folded protein with wild-type conformation. In addition, each mutant activated transducin in a light-dependent manner. SR(1−3/4−7) and SR(1−4/5−7) activated transducin with wild-type specific activity, while SR(1−5/6−7) activated transducin with about 25% of the specific activity of wild-type rhodopsin. Similar results have recently been reported by Ridge et al. (1995a). The low activity of SR(1−5/6−7) is consistent with the decreased adenylylcyclase activity observed for a comparable mutation in the β-adrenergic receptor (Kobilka et al., 1988) and with the fact that the location of the split in SR(1−5/6−7) is in a region of the protein where mutations are known to disrupt interaction with transducin (Franke et al., 1992; Resek et al., 1994; Shi et al., 1995). The observation of wild-type spectral properties and specific activity for activation of transducin in the SR(1−4/5−7) mutant might be a surprise at first glance since mutations in the extracellular loops of rhodopsin are known to disrupt the native conformation of the protein (Doi et al., 1990; Anukanth & Khorana, 1994; Ridge et al., 1995b). However, in selecting a site for the split in the 4/5 loop, we were careful to restrict our search to those regions of the polypeptide chain known to be tolerant of mutagenic changes (Doi et al., 1990).

We have taken one of these split rhodopsin mutants, SR(1−5/6−7), and shown that the two fragments can be cross-linked by a disulfide bond if Cys is substituted for the naturally occurring residues at positions 204 and 276 in the fifth and sixth helices of the protein, respectively. These data clearly demonstrate the success of the approach. The data also demonstrate a high degree of structural similarity between rhodopsin and the tachykinin NK-1 receptor since we selected residues 204 and 276 on the basis of the studies of Schwartz and co-workers with the NK-1 receptor (Elling et al., 1995). The similarity was observed despite the fact that these two proteins share less than 30% amino acid identity (Hershey & Krause, 1990). We find further support of this conclusion if we examine residue 200. A His at position 200 in the NK-1 receptor (rhodopsin-numbering system) forms a higher affinity metal ion binding site with 276 than does a His at position 204 (Elling et al., 1995). Correspondingly, we note that a Cys substituted for Asn-200 in rhodopsin cross-links to Cys-276 spontaneously [without addition of the Cu^{2+}(phenanthroline)$_3$ oxidant], suggesting that it is closer to 276 in the tertiary structure of rhodopsin than is 204 (Yu and Oprian, unpublished).

The cross-linking of Cys-204 and Cys-276 clearly demonstrates that these two residues can come close enough together to form a covalent bond in the protein. However, further interpretation of the data in terms of protein structure should be undertaken with considerable caution, as has been discussed extensively by Falke and Koshland (1987). A major concern is that the observed cross-linking may result from the trapping of a random conformational fluctuation in the protein. For this reason, it is essential to analyze a more global data set from a Cys scan of the protein so that a pattern of self-consistency in the data may be demonstrated. In addition, it is very important to functionally characterize the cross-linked proteins. These experiments are currently in progress. In this context, we do note that the cross-linking

reactions display a high degree of specificity since we have retained in all of the split rhodopsin mutants the wild-type complement of 10 naturally occurring Cys residues. Despite retention of the naturally occurring Cys residues, the two fragments cross-link only after introduction of a Cys at both positions 204 and 276.

Several other techniques have been used recently to map tertiary interactions in membrane proteins, including the use of electron paramagnetic resonance (EPR) spin-labeled probes in rhodopsin (Hubbell et al., 1995), site-specific eximer fluorescence in lac permease (Jung et al., 1993), and engineered metal ion binding sites in the tachykinin NK-1 receptor (Elling et al., 1995) and lac permease (Jung et al., 1995). One advantage of the disulfide cross-linking approach presented here is that the tertiary contacts are detected by gel electrophoresis and Western blot analysis which is very rapid, universally available, and highly amenable to large scale screening that could be performed in combination with Cys-scanning mutagenesis of the protein. In anticipation of such a large scale screen, we recently tried the cross-linking procedure in crude cell lysates and found that it works as well as with the purified protein (Kono & Oprian, unpublished). Thus, we anticipate that the method of disulfide cross-linking for mapping tertiary contacts in rhodopsin will be complementary to the cryoelectron microscopy studies of Henderson and Schertler and others (Schertler et al., 1993; Unger & Schertler, 1995) for determination of the three-dimensional structure of the protein and will provide a means to experimentally evaluate theoretical models for the structure of rhodopsin and other members of the large class of G protein-linked receptors (Donnelly & Findlay, 1994; Baldwin, 1993; Alkorta & Du, 1994; Donnelly et al., 1994).

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