Functionally Discrete Mimics of Light-activated Rhodopsin Identified through Expression of Soluble Cytoplasmic Domains*

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Numerous studies on the seven-helix receptor rhodopsin have implicated the cytoplasmic loops and carboxyl-terminal region in the binding and activation of proteins involved in visual transduction and desensitization. In our continuing studies on rhodopsin folding, assembly, and structure, we have attempted to reconstruct the interacting surface(s) for these proteins by inserting fragments corresponding to the cytoplasmic loops and/or the carboxyl-terminal tail of bovine opsin either singly, or in combination, onto a surface loop in thioredoxin. The purpose of the thioredoxin fusion is to provide a soluble scaffold for the cytoplasmic fragments thereby allowing them sufficient conformational freedom to fold to a structure that mimics the protein-binding sites on light-activated rhodopsin. All of the fusion proteins are expressed to relatively high levels in Escherichia coli and can be purified using a two- or three-step chromatography procedure. Biochemical studies show that some of the fusion proteins effectively mimic the activated conformation(s) of rhodopsin in stimulating G-protein or competing with the light-activated rhodopsin/G-protein interaction, in supporting phosphorylation of the carboxyl-terminal opsin fragment by rhodopsin kinase, and/or phosphopeptide-stimulated arrestin binding. These results suggest that specific segments of the cytoplasmic surface of rhodopsin can adopt functionally discrete conformations in the absence of the connecting transmembrane helices and retinal chromophore.

Rhodopsin is the gateway of vision operated by a light-assisted change in the geometry of the retinal chromophore buried deep within the bundle of seven transmembrane (TM) helices. The light-induced cis → trans isomerization of retinal is thought to induce rigid body movements of the TM helices forcing a transition of rhodopsin to the active metarhodopsin II (MII) state. Formation of the latter, as documented by the combined use of a number of biochemical and biophysical techniques, is accompanied by small but significant tertiary structural changes in the solvent-exposed cytoplasmic interhelical loops thereby opening high affinity sites for the binding and activation of several signaling proteins (1–9).

The sites of interaction for the guanine nucleotide-binding protein transducin (Gt), rhodopsin kinase (RK), and arrestin (Arr) in light-activated rhodopsin have been the subjects of intense investigation. Binding and activation of Gt is believed to involve the second, third, and fourth cytoplasmic loops of rhodopsin (10–21). RK is thought to interact with the second and third cytoplasmic loops prior to phosphorylation of serine and threonine residues in the carboxyl-terminal tail (16, 22–27). Similarly, Arr is believed to bind the first, second, and/or third cytoplasmic loops of MII after interacting with the phosphorylated carboxyl terminus (28–31). Whereas a number of amino acid residues in the cytoplasmic loops that participate in the Gt (13–21, 32–34) and RK (16, 27, 34) interactions have been identified, only a few that interact with Arr have been reported (31). Importantly, however, neither the nature of the conformational changes in the cytoplasmic loops nor how they support consecutive binding and activation of these signaling proteins is known in detail.

Studies on constitutively active rhodopsin mutants have suggested that formation of a signaling-competent cytoplasmic surface can be uncoupled from the process of retinal isomerization and the accompanying changes in the seven-TM bundle (35–42). These results imply that the cytoplasmic surface could function as a distinct entity capable of folding and activating signaling proteins independent of the chromophore and the connecting TM helices. Considering the successful application of the dissection and reconstitution approach to study rhodopsin (43–48) and the difficulties in obtaining high resolution structural information for membrane proteins in general, expression of the cytoplasmic loops of rhodopsin as soluble domain models may facilitate biophysical approaches to the analysis of various receptors and the signaling mechanisms they control. Thus, the goal of this study was to create optimized constructs of the cytoplasmic domain, or its subdomains, and examine their ability to mimic the signal-transducing and deactivation potential of light-activated rhodopsin. For this purpose, defined polypeptide fragments corresponding to the cytoplasmic loops and the carboxyl-terminal region of bovine opsin were inserted onto a surface loop in a mutant form of thioredoxin (HPTRX, histidine patch thioredoxin) (49). The HPTRX fusion provides a soluble scaffold for restraining the ends of the cytoplasmic loops as well as a “histidine patch” for purification by immobilized metal affinity chromatography (IMAC). Some

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§ The abbreviations used are: TM, transmembrane; MI, metarhodopsin I; MII, metarhodopsin II; G-protein, guanine nucleotide-binding protein; Gt, the retinal G-protein transducin; RK, rhodopsin kinase; Arr, arrestin; ROS, rod outer segment; BSA, bovine serum albumin; GPCR, G-protein-coupled receptor; IMAC, immobilized metal affinity chromatography; DTT, dithiothreitol; GTPγS, guanosine 5′-3′-O-(thio)triphosphate; PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid.
of these cytoplasmic domain models effectively mimic the functions of light-activated rhodopsin suggesting that the cytoplasmic loops have the capacity to adopt a signaling state conformation when removed from the constraint imposed by the retinal chromophore and the TM helices.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction endonucleases were from New England Biolabs™ or Roche Molecular Biochemicals, and horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG were from Promega. Complete™ protease inhibitor tablets were from Roche Molecular Biochemicals, and Ni²⁺-NTA resin was from Qiagen. QAE ion exchange resin, ECL and ECL Plus chemiluminescence detection systems were from Amersham Pharmacia Biotech. Centriprep, centron and centrifugal concentrators were from Millipore, and dialysis tubing was from Spectrum. [γ-32P]ATP (3000 Ci/mmol) and [35S]GTPgS (1250 Ci/mmol) were from PerkinElmer Life Sciences. The anti-rhodopsin polyclonal antibody, α, and the anti-arrestin monoclonal antibody C10C10 (50) were gifts from B. Knox (State University of New York at Buffalo). The sources of other materials used in this investigation have been reported (43–45).

**Methods**

**Construction of Opin Fragment Fusion Proteins**—All opsin gene fragments (Table I) were introduced into the pHPTRX expression vector at the unique RsrII site by restriction fragment replacement (49). This strategy results in an additional Gly and Pro residue on the carboxy-terminal end of the insertion. Additional Gly and Pro residues were also introduced between the tandemly linked cytoplasmic fragments to offer a degree of conformational flexibility. The oligonucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer and purified by high pressure liquid chromatography. The sequences of the opsin cytoplasmic fragment insertions were confirmed by the dideoxynucleotide chain termination method of DNA sequencing (51).

**Expression of Opin Fragment Fusion Proteins**—Competent GI934 cells were transformed with the various pHPTRX/opsin fragment plasmids and plated on minimal media plates containing ampicillin (49). An overnight culture of the transformed cells grown in Rich Medium (M9 medium containing 2% (w/v) casamino acids, 0.5% (w/v) glucose, 1 mM MgSO₄, 0.1 mM CaCl₂, and 100 μg/ml ampicillin) was used to inoculate 100–150 ml of IMC/Amp medium (M9 medium containing 0.2% (w/v) casamino acids, 0.5% (w/v) glucose, 1 mM MgSO₄, 0.1 mM CaCl₂, and 100 μg/ml ampicillin) to an A₅₅₀ of 0–0.5. The culture was grown at 30 °C until the A₅₅₀ reached 0–0.5, and then the flask was added to a final concentration of 100 μg/ml, and the culture grown for an additional 4 h at 37 °C. The cells were harvested by centrifugation, and the pellet was processed immediately or stored at −80 °C until use.

**Purification of Soluble HPTRX/Opin Fragment Fusion Proteins**—The cell pellets were resuspended in 9 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, a protease inhibitor tablet, and 1 mM phenylmethylsulfonyl fluoride) and disrupted in a French pressure cell at 900 pounds/square inch with two passages. The concentrations of NaCl and MgCl₂ in the cell lysate were brought to 500 and 1 mM, respectively, and then incubated with 1 μg/ml DNAse for 1 h at 4 °C. The lysates were clarified by centrifugation at 100,000 × g for 20 min. The supernatant was dialysed against 20 mM Tris-HCl, pH 7.5, containing 5 mM MgCl₂, and 2 mM DTT for 30 min at 20 °C. The rhodopsin-containing sample was filtered through nitrocellulose with the aid of a vacuum manifold. The filters were washed four times with 5 ml of 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, and 2 mM EDTA. Bound proteins were eluted with the same buffer containing 100 mM NaCl, 5 mM MgCl₂, and 2 mM DTT. Insoluble material was removed by centrifugation at 100,000 × g for 1 h. The supernatant was diluted into 150 ml of ice-cold 20 mM Tris-HCl, pH 7.5, containing 30 mM NaCl, 0.1 mM EDTA, 0.2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride and incubated overnight at 4 °C. The slightly turbid solution was clarified by centrifugation at 100,000 × g for 1 h, applied to a QAE-Sepharose column (1.5 × 20 cm) attached to the BioCAD Sprint workstation, and washed with 50 column volumes of the same buffer without EDTA. Bound proteins were eluted with the same buffer containing 500 mM NaCl, purified on Ni²⁺-NTA, and chromatographed on the gel filtration column as described above.

**Mass Spectrometric Analysis of Opin Fragment Fusion Proteins**—Molecular masses of the opsin fragment/thioredoxin fusions were determined with a matrix-assisted laser desorption ionization, delayed extraction reflection, time-of-flight instrument (Voyager DE Biospectrometry Workstation, Perseptive Biosystems). The proteins (1–2 pmol) were co-crystallized with equal volumes of matrices consisting of a 30% solution of sinapinic acid in 50% (v/v) trifluoroacetic acid. The protein/matrix mixtures (1 μl) were spotted on the sample plate and allowed to dry for 5 min prior to loading in the spectrometer. The matrix-assisted laser desorption ionization spectra were internally calibrated using myoglobin as a standard.

**Fluorescence Assay of Gα, Activation by Opin Fragment Fusion Proteins**—The fluorescence assay for Gα activation was adapted from previously described methods (52, 53). This assay allows measurement of the rate of GTPγS uptake by Gα, by monitoring the increase in its intrinsic fluorescence. Fluorescence was measured with a FluoroMax-2 spectrofluorimeter in signal/reference mode with excitation at 295 nm (2 nm bandwidth) and emission at 340 nm (12 nm bandwidth). Signal integration time was 2 s, and the temperature was maintained at 20 °C. The 100-μl assay mixture contained 5 mM rod outer segment (ROS) rhodopsin membranes or 100 nM to 1 μM HPTRX/opsin fragment fusion protein and 1 μM Gα in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, and 1 mM DTT. The solution was mixed, and after obtaining a stable base line (~500–1000 s), the reaction was initiated by the addition of 1.5 μM GTPγS and followed for another 2200 s. In some cases, the 100-μl assay mixture initially contained 1 μM Gα, and 1.5 μM GTPγS in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, and 1 mM DTT. After obtaining a stable base line, the reaction was initiated by the addition of 200 nM HPTRX/CDEF and followed for 1000 s. A second aliquot of HPTRX/CDEF (300 nM) was then added bringing the concentration to 500 nM, and the reaction was followed for another 1200 s.

**Filter-binding Assay of Opin Fragment Fusion Protein-catalyzed GTPγS Uptake by Gi—A Radiouclide Filter-binding assay, which monitors guanine nucleotide exchange by Gα using [35S]GTPγS, was carried out as described previously (43, 54). The filter-binding assay is based on the property that Gα and its bound [35S]GTPγS are retained on nitrocellulose filters, whereas the free [35S]GTPγS passes through the filters. The HPTRX/opsin fragment fusion proteins were evaluated for their ability to activate directly Gi, or compete with the MII/Gi, interaction. For the activation assays (250 μl), 2 mM ROS rhodopsin membranes or 200 mM HPTRX/opsin fragment fusion protein was preincubated with 400 nM Gi, in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, and 2 mM DTT for 30 min at 20 °C. The rhodopsin-containing sample was illuminated (≥495 nm) for 1 min, and then the reactions were initiated by addition of 1 μM [35S]GTPγS. After 30 min at 20 °C, 100 μl of the reaction mixture was removed in duplicate and filtered through nitrocellulose with the aid of a vacuum manifold. The filters were washed four times with 5 ml of 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, and 2 mM DTT, dried, and analyzed for [35S]radioactivity by scintillation counting. For the competition studies, 10–400 mM HPTRX/opsin fragment fusion protein was preincubated with 2 mM ROS rhodopsin membranes or 200–400 nM Gi, in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, and 2 mM DTT for 30 min at 20 °C. The assay mixture (250 μl) was illuminated (≥495 nm) for 1 min, and 1 μM [35S]GTPγS was added to initiate the reaction. After 30 min, 100 μl of the reaction mixture was removed in duplicate, and the amount of bound [35S]GTPγS was quantified as described above.

**Phosphorylation of Rhodopsin by Rk—**Two different procedures
were used to phosphorylate rhodopsin with RK. In the first procedure (55), rhodopsin (100 μM) in ROS membranes containing RK was phosphorylated by addition of 1 mM ATP (spiked with 10 μCi of [γ-32P]ATP for quantitation purposes) in 70 mM NaH2PO4, pH 7.0, containing 5 mM MgCl2, 10 μM GTP, and 150 μM DTT. The total reaction volume was 1 ml. The mixture was illuminated (~495 nm) for 1 min at 20°C, and phosphorylation was allowed to proceed for another 60 min. The reaction was quenched by dilution and addition of 100 mM NH4OH, pH 7.0. The membranes were extensively washed by centrifugation at 100,000 x g for 30 min with 50 mM Tris- HCl, pH 7.5, containing 150 mM NaCl, 2% (w/v) bovine serum albumin (BSA), and 0.1 mM phenylmethylsulfonyl fluoride to remove residual all-trans-retinal oxidase and ATP, and then an additional two times in the same buffer without BSA. The rhodopsin chromophore was regenerated by addition of a 3-fold excess of 11-cis-retinal to the resuspended membranes for 3 h at 4°C. The membranes were washed twice by centrifugation at 100,000 x g for 30 min with 50 mM Tris- HCl, pH 7.5, containing 150 mM NaCl, 2% (w/v) BSA to remove residual 11-cis-retinal followed by two washes with 50 mM Tris- HCl, pH 7.5, containing 800 mM NaCl. Prior to each of the above-mentioned centrifugation steps, the membranes were underlaid with 2 ml of 200 mM sucrose in 50 mM Tris- HCl, pH 7.5, containing 150 mM NaCl. Alternatively, urea-washed membranes were phosphorylated by addition of partially purified RK prepared according to Ref. 56. The reaction mixtures (1.25 ml) contained 10 μM rhodopsin, 30 mM RK, 100 μM [γ-32P]ATP in 70 mM NaH2PO4, pH 7.0, containing 5 mM MgCl2, 10 μM GTP, and 150 μM DTT. The assay mixtures were illuminated (~495 nm) for 1 min. At different time points (2–60 min), 100 μl was removed, and the proteins were precipitated with 900 μl of 5 mM phosphoric acid containing 7% (w/v) trichloroacetic acid and 25 μg of BSA. After 2 h at 4°C, the samples were centrifuged at 20,200 x g for 30 min, and the precipitate was washed five times with 1 ml of the 7% trichloroacetic acid solution. Cerenkov counting was used to analyze the pellets for 32P radioactivity. Alternatively, the samples were analyzed by SDS-PAGE, and the 32P in the protein bands was quantitated by phosphorimaging (Molecular Dynamics Storm PhosphorImager).

**Phosphorylation of HPTRX/CT by RK—HPTRX/CT was also phosphorylated by addition of partially purified RK. For the time course reaction, the assay mixtures (1.25 ml) contained 10 μM rhodopsin, 30 mM RK, 100 μM [γ-32P]ATP in 70 mM NaH2PO4, pH 7.0, containing 10 mM MgCl2, 10 μM GTP, and 150 μM DTT. At different time points (2–60 min), 100 μl of the reaction mixture was removed, and the proteins were precipitated with trichloroacetic acid and analyzed for 32P radioactivity as described above for ROS rhodopsin. The interaction between HPTRX/ABCDEF and RK was also examined by phosphorylation of HPTRX/CT. The assay mixtures (0.5 μl) contained 1 mM HPTRX/CT (or 1 mM HPTRX), 200 mM HPTRX/ABCDEF, 100 mM RK, and 5 mM [γ-32P]ATP in 70 mM NaH2PO4, pH 7.0, containing 10 mM MgCl2, 10 μM GTP, and 150 μM DTT. After 60 min, the proteins were precipitated with trichloroacetic acid and 32P in the pellet quantitated by Cerenkov counting.

**Arr Binding to Phosphorylated Rhodopsin and Opsin Fragment Fusion Proteins—** Arr was prepared from bovine retina according to the method of Puig et al. (29). The purified Arr (20 μM) was preincubated with 200 nM HPTRX/CT or HPTRX/ABCDEF for 60 min at 4°C and then incubated with 80 nM phosphorylated, regenerated ROS membranes in 50 mM Tris- HCl, pH 7.5, containing 0.5 mM MgCl2, 1.5 mM DTT, and 50 mM potassium acetate for 5 min at 37°C in the dark. The reaction mixture (50 μl) was diluted with an equal volume of ice-cold 50 mM Tris- HCl, pH 7.5, containing 0.5 mM MgCl2, 1.5 mM DTT, and 50 mM potassium acetate and underlaid with 2 ml of 200 mM sucrose in the same buffer. The sample was centrifuged at 80,000 x g for 1 h, and the membrane pellet was resuspended and washed in the same buffer without potassium acetate. The pellets were solubilized in 20 μl of concentrated (4X) SDS-PAGE sample buffer, diluted to 80 μl with 50 mM Tris- HCl, pH 7.5, containing 0.5 mM MgCl2, 1.5 mM DTT, and an aliquot analyzed by SDS-PAGE. The amount of Arr bound to the phosphorylated rhodopsin membranes was determined by immunoblotting with the C10C10 anti-arrestin monoclonal antibody. The immunoreactive protein bands were visualized by chemiluminescence and quantitated by phosphorimaging.

**SDS-PAGE Analysis of Opsin Fragment Fusion Proteins—** Protein samples were analyzed by reducing SDS-PAGE (57) with a 5% stacking and a 15 or 16% resolving gel. Proteins were visualized by Coomassie Blue staining or electrophroblotted onto poly(vinylidene difluoride) membranes and probed with the opsin antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG as the second antibody. The immunoreactive protein bands were visualized by chemiluminescence.

**Other Methods—** Protein determinations were done using the method of Peterson (58) with BSA as the standard.

**RESULTS**

**Design of the Soluble Cytoplasmic Domain Polypeptides**

**The Opsin Fragment HPTRX Fusion Proteins**—The main goal of this work was to reconstruct the interacting surfaces for Gα and carboxyl-terminal tail domain insertions, or in combination, onto a surface loop in HPTRX are shown (see Table I). The numbers indicate the amino acid position in the full-length opsin polypeptide, and the letters A–G denote the designations for the seven transmembrane segments. Opsin fragment/HPTRX fusion proteins that contain more than one cytoplasmic loop have Gly/Pro linkers (underlined) inserted between the different segments. The zigzag lines represent a continuation of the HPTRX polypeptide chain.

**Soluble Mimics of Rhodopsin Functions**

**Protein Determinations**—Protein determinations were done using the method of Peterson (58) with BSA as the standard.

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expression levels for the fusion proteins were estimated by densitometry of SDS-PAGE and mass spectrometry. In virtually all cases, greater than 10 mg of protein was obtained from a 100–150-ml shake flask culture. Surprisingly, only the opsin fragments were inserted singly, and in combination, onto a surface loop in a mutant form of thioredoxin.

Thioredoxin has previously been shown to tolerate fairly large peptide insertions between Pro-34 and Cys-35 and has been effectively used as a soluble scaffold for expressing otherwise insoluble proteins (69). Furthermore, the thioredoxin mutant, HPTRX (Fig. 2), contains four strategically placed histidine residues that form a metal binding histidine patch only upon correct assembly of the protein (49). If the opsin cytoplasmic fragment(s) inserted into HPTRX perturb the folding and/or structure of thioredoxin, the protein will not be retained on IMAC. For these reasons, HPTRX appeared to be a suitable soluble scaffold for expressing the opsin cytoplasmic fragments as well as a “folding indicator” of the overall structure of the fusion protein.

Expression of the Cytoplasmic Domain Polypeptides in E. coli

The Opsin Fragment Fusion Proteins with Single Loop Insertions—Cellular expression of the opsin fragment fusion proteins (Table I) was examined by SDS-PAGE of whole cell extracts. All of the fusion proteins containing a single cytoplasmic fragment insertion were stably produced in GI934 cells (Fig. 3A). Importantly, all of these fusion proteins migrated slower than HPTRX and close to their calculated molecular mass. The expression levels for the fusion proteins were estimated by comparing the intensity of the Coomassie Blue-stained bands with that of known amounts of purified HPTRX. In virtually all cases, greater than 10 mg of protein was obtained from a 100–150-ml shake flask culture. Surprisingly, only the opsin fragment fusion proteins containing the third cytoplasmic loop (HPTRX/EF) and carboxyl-terminal tail (HPTRX/CT) were expressed as a completely soluble form. The HPTRX/AB, HPTRX/CD, and HPTRX/GP proteins, which contain fragments corresponding to the first, second, and fourth cytoplasmic loops (Table I), respectively, were expressed in a primarily insoluble form.

The Opsin Fragment Fusion Proteins with Multiple Loop Insertions—Stable expression of the opsin fragment fusion proteins containing different combinations of the cytoplasmic loops (Table I) were also examined by SDS-PAGE following cell disruption. As shown in Fig. 3B, all of these opsin fragment fusion proteins were stably produced in GI934 cells as well. Similarly, the levels of expressed protein approached and solubilized in a saturated solution of guanidinium hydrochloride containing high concentrations of reducing agent. Refolding was initiated by diluting the protein samples into large volumes of buffers with and without redox reagents (e.g. GSH and GSSG). Final purification of the refolded proteins was achieved by ion exchange and IMAC chromatography. The preparations were estimated to be greater than 95% pure based on SDS-PAGE and mass spectrometry.

**TABLE I**

| Fusion protein | Amino acids inserted
|----------------|----------------------|
| HPTRX         |                      |
| HPTRX/AB      | 61–75                |
| HPTRX/CD      | 132–154              |
| HPTRX/EF      | 231–252              |
| HPTRX/GP      | 311–321              |
| HPTRX/CT      | 330–348              |
| HPTRX/ABCD    | 61–75; 132–154       |
| HPTRX/ABEF    | 61–75; 231–252       |
| HPTRX/ABGP    | 61–75; 311–321       |
| HPTRX/CDEF    | 132–154; 231–252     |
| HPTRX/ABCDEFGP| 61–75; 132–154; 231–252; 311–321 |

a A Gly/Pro linker(s) is inserted between the different cytoplasmic loops(s) and HPTRX.

b One of several HPTRX/CDEF derivatives constructed.

Fusion protein Amino acids inserted

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**FIG. 2.** A three-dimensional structural model of HPTRX. The model was generated from the x-ray structure of E. coli thioredoxin (88) using the Protein Data Bank accession number 2TRX and the molecular-rendering package *Ras ter3D* (88). The histidine residues at positions 2, 6, 30, and 62 are highlighted in blue, and the site of opsin fragment insertion between Pro-34 and Cys-35 is indicated in red.

**Purification of the Soluble Cytoplasmic Domain Polypeptides**

**The Soluble Opsin Fragment Fusion Proteins**—Since the mutant thioredoxin scaffold contains strategically placed histidine residues capable of coordinating metal ions, facile purification of HPTRX, HPTRX/EF, HPTRX/CT, HPTRX/ABEF, and HPTRX/CDEF was achieved using a two-step procedure involving IMAC and gel filtration. First, the crude clarified cell extracts were directly applied to the Ni2⁺-NTA agarose resin. After extensive washing with high ionic strength buffers supplemented with low concentrations of imidazole, the HPTRX opsin fragment fusion proteins were eluted with high concentrations of imidazole. However, despite extensive washing, the eluate, as demonstrated by SDS-PAGE, still contained several high molecular weight proteins (data not shown). These contaminants were successfully removed using gel filtration chromatography, and the resulting proteins were shown to be relatively pure by mass spectrometry. Furthermore, the molecular masses were in agreement with the calculated values.

**The Insoluble Fusion Proteins**—As many of the opsin fragment fusion proteins were expressed in an insoluble form, a reliable and reproducible refolding protocol was developed based on the methods of Stern and colleagues (70) for the class II major histocompatibility complex. Briefly, the cell extracts were centrifuged, and the pellets were washed several times with buffer supplemented with sodium deoxycholate in order to remove membranes and membrane-bound proteins. The pellet was finally washed in the same buffer without deoxycholate and solubilized in a saturated solution of guanidinium hydrochloride containing high concentrations of reducing agent. Refolding was initiated by diluting the protein samples into large volumes of buffers with and without redox reagents (e.g. GSH and GSSG). Final purification of the refolded proteins was achieved by ion exchange and IMAC chromatography. The preparations were estimated to be greater than 95% pure based on SDS-PAGE and mass spectrometry.
Functional Characterization of the Cytoplasmic Domain

Polyptides

Binding and Activation of G<sub>α</sub>—The ability of the opsin cytoplasmic fragment fusion proteins to bind and activate G<sub>α</sub> was investigated using both radioligand filter binding and fluorescence assays. Table II shows some of the HPTRX/CDEF derivatives that were constructed and their corresponding levels of G<sub>α</sub> activation relative to ROS rhodopsin. In all cases, the HPTRX/CDEF derivatives are present at a 100-fold higher concentration than rhodopsin. For clarity, the HPTRX fusion containing the inserted amino acids 132–154 from the CD loop region and 231–252 from the EF loop region will be referred to as HPTRX/CDEF (as indicated in Table I). All deviations from these two sequences have been designated with a subscript (e.g., CD<sub>1</sub> or EF<sub>2</sub>). Analysis of the G<sub>α</sub> activating potentials of the various constructs (Table II) revealed that whereas extending the initial EF loop sequence up to Arg-252 resulted in a modest increase in activity (HPTRX/CD<sub>1</sub>EF<sub>1</sub> versus HPTRX/CD<sub>2</sub>EF), extending the CD loop sequence up to Ala-132 proved to be a more critical adjustment (HPTRX/CD<sub>2</sub>EF versus HPTRX/CDEF). Extending the CD loop sequence to include Leu-131 resulted in no major change in the level of G<sub>α</sub> activation (HPTRX/CD<sub>2</sub>EF versus HPTRX/CDEF). Quite strikingly, however, a construct (HPTRX/CDEF<sub>2</sub>) that contains the “optimized” CD loop sequence (Ala-132 to Ile-154) but lacks Arg-252 in the EF sequence shows considerably less activity when compared with HPTRX/CDEF. Thus, although the length and/or composition of the CD loop sequence inserted into HPTRX appears to be most important for effective G<sub>α</sub> activation, it is clear that specific amino acids in both sequences are required to achieve the highest level of observed activity.

The activation of G<sub>α</sub> by HPTRX/CDEF was also compared with ROS rhodopsin using a fluorescence assay. As shown in Fig. 4A, addition of GTP<sub>S</sub> to the reaction mixture containing light-activated ROS rhodopsin results in an increase in GTP<sub>S</sub> fluorescence. Quite remarkably, virtually the same kinetics and level of G-protein activation can be achieved using an 80–120-fold excess of HPTRX/CDEF over photoexcited rhodopsin. These findings suggest that the CD and EF loop insertions in HPTRX/CDEF present a structure(s) to the G-protein that is recognized as being similar to the light-activated conformation of rhodopsin. Surprisingly, the HPTRX/ABCDEF/GP fusion protein, which contains all four cytoplasmic loops, showed strikingly different kinetics and levels of G<sub>α</sub> activation in the same concentration range (400–600 nM) as HPTRX/CDEF (Fig. 4B).

This difference in the level of activating potential between these two constructs and photoexcited rhodopsin could be due to multiple factors including the possibility that the interaction with G<sub>α</sub> is not catalytic. This likelihood is further suggested by the effect of HPTRX/CDEF concentration on G-protein activation (Fig. 4C). None of the opsin fragment fusion proteins containing single cytoplasmic loop insertions (Fig. 4, A and B) or those containing the ABCD, ABEF, ABGP, and ABCDEF loops linked in tandem showed appreciable G<sub>α</sub> activation in the concentration ranges tested (up to 1 μM).

The ability of the latter opsin fragment fusion proteins to compete with the MI/G<sub>α</sub> interaction was also tested using the radioligand filter-binding assay. In the concentration range tested (10–400 nM), only the HPTRX/ABCDEF fragment showed a significant degree of inhibition (Fig. 5). At 100 nM HPTRX/ABCDEF, the ability of light-activated rhodopsin to stimulate GDP-GTP<sub>S</sub> exchange by G<sub>α</sub> was reduced by ~60%. Higher concentrations (100–400 nM) of HPTRX/ABCDEF resulted in only a modest further inhibition of the MI/G<sub>α</sub> interaction, to ~70% at 400 nM protein. Although the reason(s) for this rather surprising decrease in inhibitory potency are not clear at the present time, it is evident that in the presence of the AB loop, the activating potential of the CD and EF loops toward G<sub>α</sub> appears to be compromised. However, as shown in Fig. 4B, this can be partially restored by addition of the fourth cytoplasmic loop (GP).

Interactions with RK—It has been shown that peptides derived from the carboxyl terminus of rhodopsin and containing all known phosphorylation sites could serve as substrates for RK provided that rhodopsin devoid of the carboxyl terminus was also present in the reaction mixture, and the proteins were exposed to light (26, 39). These observations have suggested
that phosphorylation of rhodopsin first requires activation and/or sequestration of RK through binding to the cytoplasmic loop segments of light-activated rhodopsin prior to phosphorylating the serine and threonine residues in the carboxyl-terminal region. To test these possibilities, we adapted our system to examine the phosphorylation potential of RK toward the same carboxyl-terminal peptide incorporated into HPTRX (HPTRX/CT) and to analyze the possible stimulatory effect of the opsins cytoplasmic fragment fusion proteins on the phosphorylation reaction.

Fig. 6 shows a time course of HPTRX/CT and ROS rhodopsin phosphorylation by RK. Although the kinetics of HPTRX/CT phosphorylation is similar to that of rhodopsin and the phosphorylation reaction is essentially complete after 60 min, a relatively high concentration of HPTRX/CT (1 mM) compared with rhodopsin (10 μM) was required to generate such a profile. To examine whether an opsin fragment fusion protein could substitute for light-activated rhodopsin in activating RK, HPTRX/ABCDEF was included in the HPTRX/CT phosphorylation assay. Quantitative analysis of the results showed a modest 2-fold increase in 32P incorporation into HPTRX/CT in the presence of HPTRX/ABCDEF (Table III). These findings suggest that the AB, CD, and/or EF loop segments incorporated into HPTRX adopt a conformation that has only a marginal capacity to interact functionally with RK.

Binding of Arr—The mechanism of Arr-mediated visual desensitization initially involves binding of the protein to the phosphorylated carboxyl-terminal region of MII (71). After binding, Arr is thought to undergo an "activating" conformational change that facilitates the recruitment of the guanine nucleotide exchange factor Gt (2.5 mM GTP, 2.5 mM GTPγS) to the Arr-RK complex (10–400 nM) (71). After recruitment, Gt catalyzes the exchange of GDP by GTP and therefore is the driving force behind the association of Arr and RK. To test this hypothesis, we adapted our system to examine the dependence of RK phosphorylation on the concentration of Gt.

The concentration of Gt was varied between 500 nM and 1 μM, and the phosphorylation reaction was followed for 2200 s. The final concentrations of the different HPTRX proteins for the results shown were 500 nM for HPTRX, HPTRX/AB, HPTRX/CD, HPTRX/EF, and HPTRX/GP, 400 nM for HPTRX/ABCDEF, and 550 nM for HPTRX/ABCDDEF. The assay mixture (100 μl) contained 1 μM Gt, 1 μM GTPγS, and 1 μM DTT, and was then added bringing the concentration to 500 nM, and the reaction was followed for another 1200 s. Differences in base-line intensity between 5 nM ROS rhodopsin and 400–600 nM of the HPTRX/opsin fragment fusion proteins were in the range of 12–17% but do not reflect the increase in base-line drift that occurs in the rhodopsin sample upon MII decay.
Soluble Mimics of Rhodopsin Functions

After a 60-min phosphorylation reaction, the proteins were precipitated with trichloroacetic acid, and \(^{32}\)P was quantified by Cerenkov counting. The results are averages from three separate experiments. The background level of \(^{32}\)P observed in the HPTRX and HPTRX/ABCD EF samples is due to phosphorylation of RK.

| Fusion protein          | mol \(^{32}\)P/mol protein |
|------------------------|---------------------------|
| HPTRX                  | 0.24 ± 0.11               |
| HPTRX/CT               | 0.97 ± 0.19               |
| HPTRX/ABCD EF          | 0.17 ± 0.06               |
| HPTRX/CT + HPTRX/ABCD EF | 2.11 ± 0.17              |

**DISCUSSION**

Although a variety of experimental strategies can be employed to gain valuable information about the structure of rhodopsin and the conformational changes accompanying receptor activation, we have utilized the approach of expressing fragments of the opsin polypeptide to study these processes. Previous work from our laboratory and other laboratories (43–48) has shown that many two and three helix membrane domain fragments from bovine opsin contain sufficient information to fold independently, insert into a membrane, and assemble with a complementary fragment(s) in vivo to form a rhodopsin-like pigment. In the present work, we have extended these studies to focus on whether the solvent-exposed cytoplasmic loop segments can fold independent of the TM helices and functionally mimic the cytoplasmic surface of light-activated rhodopsin by interacting with various protein targets involved in visual transduction and desensitization. For this purpose, defined fragments encoding the cytoplasmic loop segments and/or the carboxyl-terminal region were inserted into a surface loop on HPTRX, a mutant form of thioredoxin. The expressed and purified fusion proteins were then tested for their ability to interact with \(G_\text{q}\), RK, and Arr.

A major challenge in the present investigation was to define effectively the amino acid sequence and/or length of the loops and adjoining linker(s) to be inserted into the HPTRX scaffold. This turned out to be a rather critical issue since many of our initial constructs containing the CD and EF loops showed little or no biological activity in the \(G_\text{q}\) assays. Although the approximate membrane/aqueous boundaries for the CD and EF loops as well as many of the amino acid residues important for function are known with a high degree of certainty, both segments required several rounds of modification in order to achieve a functional protein. For example, a comparison between the levels of \(G_\text{q}\) activation for the different HPTRX/CDEF derivatives (Table II) shows that placing two additional amino acids before the highly conserved EKY (erythroid) sequence (amino acids 134–136) in the CD loop in conjunction with extending the EF sequence to Arg-252 has a significant impact. Clearly, interactions between key residues in these two segments and/or their overall disposition relative to each other in the HPTRX scaffold is important for effective G-protein signaling. Although it is likely that the CD and EF segments in the different HPTRX/CDEF constructs extend beyond the loop regions and into the TM helices of the full-length opsin, it provided us with a minimal construct that was readily amenable to the design of additional cytoplasmic domain polypeptides containing the AB and/or GP loops. Importantly, these latter segments have not undergone extensive modifications to the same degree as CD and EF, and it is likely that the current sequences extend into the TM helices as well.

The second but equally important consideration was the choice of linker sequence to be placed between the individual cytoplasmic loops (and HPTRX) in order to afford some degree of conformational flexibility. Previous work by Erni and coworkers (72) on the design of a fusion protein comprising the four subunits of the glucose phosphotransferase system of *Escherichia coli* found that Ala-Pro-rich linkers were most effective for this purpose. Similarly, Gouaux and colleagues (73, 74) tested several different linkers to join the extracellular S1 and S2 loops of the ligand-binding region of the ionotropic glutamate receptor and found that relatively short linker sequences afforded the most desirable properties. Since the NMR data (59, 61–63) on the synthetic opsin cytoplasmic loop peptides showed that they adopt ordered structures with relatively close amino and carboxyl termini, we opted to use simple Gly-Pro linkers and, as mentioned above, focus on optimizing the insertions through changes within the opsin sequence.

**Interactions with G-protein**—Several lines of evidence suggest that some of the opsin cytoplasmic fragments in HPTRX adopt a conformation that is recognized by \(G_\text{q}\). First, the HPTRX/CDEF protein shows similar kinetics and levels of \(G_\text{q}\) activation when present at an 80–120-fold higher concentration than light-activated rhodopsin (Fig. 4A). This relatively large difference in activating potential between HPTRX/CDEF and HPTRX/CFDE.
and photoexcited rhodopsin can be partially explained by the observation that interaction of the former with Gt does not appear to be catalytic (Fig. 4C). It is also possible that the inserted CD and EF loops possess a high degree of flexibility and may only transiently interact to form a functional binding site for Gt. In this case, the activating conformation of the loops may actually be more similar to that of bovine opsin (75, 76). Since the single loop insertions do not activate Gt to any appreciable level (Fig. 4A), this would suggest that both the CD and EF loops are necessary for a productive interaction. These findings are in agreement with an earlier mutagenesis study by Karnik and co-workers (33) showing that both loops are strictly required for interaction with Gtα. In this context, it should be emphasized that synthetic peptides corresponding to the second, third, and/or fourth cytoplasmic loops of rhodopsin have been shown to inhibit the rhodopsin-mediated stimulation of the GTPase activity of Gt (10), compete with MII for binding to Gt (12), or in the case of the third loop peptide, to also inhibit Gt-mediated cGMP phosphodiesterase activity (59). To our knowledge, the ability of individual cytoplasmic surface peptides of rhodopsin to activate directly Gt has not been demonstrated. However, some GPCR-based peptides have been shown to stimulate various G-protein subtypes. In particular, individual synthetic peptides corresponding to the distal portion of the third cytoplasmic loop of the β2-adrenergic receptor mimic the agonist-activated receptor by directly stimulating Gt at nanomolar concentrations (77, 78). Similarly, synthetic peptides corresponding to the second and/or third cytoplasmic loops of the α2-adrenergic and M4 muscarinic cholinergic receptors effectively mimic the activated receptors by stimulating their cognate G-protein(s) (79).

The HPTRX/ABCDFGP protein, which contains all four cytoplasmic loop segments, shows significantly less activating potential than HPTRX/CDEF in the fluorescence assay (Fig. 4B). Furthermore, the kinetics of activation shows a significant lag when compared with HPTRX/CDEF. Such a difference may be due to the fact that addition of the AB and/or GP loop segments, which results in a very large insertion, has a destabilizing effect on the CD/EF interaction thereby rendering a functionally less active conformation. Importantly, since HPTRX/ABCDFGP can be purified by IMAC, the overall fold of the thioredoxin portion of the fusion protein does not appear to be severely compromised. The HPTRX/ABCDF protein, which lacks the fourth cytoplasmic loop, does not show any activating potential toward Gt but rather inhibits the MII/Gt interaction (Fig. 5). Thus, the interactions between CD and EF in this fusion protein must be drastically perturbed by the loss of the GP loop such that they no longer activate G-protein. This result was not entirely unexpected since the overall contribution of the fourth cytoplasmic loop to the MII/Gt interaction as well the importance of several amino acids therein have been reported (10, 12, 19–21). Notably, it has been suggested that a family of toxin peptides called mastoparans, which activate some G-proteins in a manner similar to those of GPCRs (80, 81), may in fact mimic the structure of the fourth cytoplasmic loop (82). Although these amphiphilic α-helical tetradecapeptides do not activate Gt, in the recently reported crystal structure of bovine rhodopsin (83) a portion of the C-IV (fourth) loop forms a short amphipathic α-helix.

The ability of HPTRX/ABCDF to compete with the MII/Gt interaction (Fig. 5) in the range of 10–100 nM is rather impressive when compared with the millimolar concentrations of some synthetic opsin cytoplasmic loop peptides that are required to inhibit the Gt-mediated shift in the metarhodopsin I (MI) → MII equilibrium (12). However, above ~100 nM, the potency of HPTRX/ABCDF is significantly less pronounced and shows only a further ~10% inhibition in MII-mediated Gt activation up to 400 nM. This behavior was observed at three different concentrations of Gt (200, 300, and 400 nM) suggesting that HPTRX/ABCDF itself may undergo some destabilizing change above ~100 nM. Whether this is due to aggregation of HPTRX/ABCDF in our assay system or reflects an intrinsic property of the protein deserves further study.

Interaction with Rhodopsin Kinase—Like many of the synthetic carboxy-terminal opsin peptides (24), HPTRX/CT serves as a substrate for phosphorylation by RK in the millimolar concentration range (Fig. 6). The relatively high levels of HPTRX/CT relative to light-activated rhodopsin that are required for effective phosphorylation are likely due to multiple factors. Foremost among these is the observation that activation of RK by the light-exposed cytoplasmic loops may be a prerequisite for phosphorylation of Ser and Thr residues in the carboxy-terminal region (27). Furthermore, the length of the peptide containing the phosphorylation sites is likely to be important for the RK/light-activated rhodopsin interaction (24). Our findings that the addition of HPTRX/ABCDF to the phosphorylation reaction has at best a 2-fold stimulatory effect on the phosphorylation of HPTRX/CT by RK (Table III) does not allow us to differentiate between these possibilities at the present time. However, one of the more interesting aspects of this result is that it demonstrates that noncontiguous segments of the opsin cytoplasmic surface can be added in trans to achieve a constructive effect. Notably, the HPTRX/ABCDF protein was the only cytoplasmic domain polypeptide to inhibit the MII/Gt interaction. These findings would also suggest that the CD and EF loops in this fusion protein possess a functionally discrete conformation from those in HPTRX/CDEF. Furthermore, the absence of a large stimulatory effect by HPTRX/ABCDF on the phosphorylation of HPTRX/CT may actually reflect differences in the ability HPTRX/ABCDF to interact with Gt and RK especially since some studies have indicated that activation of RK may occur at the MI, not MII, stage (84, 85). In fact, Bourne (86) has suggested that seven-helical receptors may adopt conformationally discrete cytoplasmic surfaces depending on whether the activated receptor is poised for transduction or desensitization.

Interaction with Arrestin—The binding of arrestin to HPTRX/ABCDF was also tested by forming an arrestin/phosphorylated “dark” rhodopsin complex and then adding the cytoplasmic domain polypeptide to the preformed complex. Here, the experimental design was patterned on the observation that arrestin is activated upon binding to the phosphorylated carboxy-terminal region and subsequently binds to the cytoplasmic loops of MII (29). Presumably, the activated conformation of arrestin is “primed” for interacting with the cytoplasmic loops (87). Like Gt, arrestin also interacts with HPTRX/ABCDEF (Fig. 7), suggesting that the cytoplasmic loops in this particular construct also adopt a conformation that is recognized by a component of the desensitization pathway. Moreover, the interaction between arrestin and HPTRX/ABCDEF is significantly more pronounced than that for RK, suggesting that this cytoplasmic domain polypeptide may have a higher affinity for arrestin. In fact, preliminary binding studies with a constitutively active mutant of arrestin, R175E, suggest that this is indeed the case.3

Conclusions—The results reported here show that specific segments of the cytoplasmic surface of bovine opsin can be fused onto a soluble protein scaffold and functionally interact with various target proteins of the visual cascade. These findings suggest that the inhibitory constraint imposed on the

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3 N. G. Abdulaev and K. D. Ridge, unpublished observations.
clayopastic loops by the TM helices and the retinal chromophore in the ground state rhodopsin is absent in the HIPTRX fusion protein. Presumably, this constraint is relaxed in rhodopsin following cis $\rightarrow$ trans isomerization of retinal and subsequent MII formation. Clearly, the soluble clathrinoid domain models are not as efficient as MII in binding and/or activation of G$_R$, RK, and Arr. One possible explanation for this is that although the thioredoxin scaffold provides a degree of stability to the loop insertions, it may deprive them of the finely tuned structural flexibility that the TM segments have to offer. Alternatively, the presence of a phospholipid membrane or additional key amino acid residues that are required for a more productive interaction may be necessary. Whether these clathrinoid polypeptide binds to the same interacting surfaces on G$_R$, RK, and Arr, as MII also deserves further study. Synthetic G$_R$, RK, and Arr peptides that have been mapped to the rhodopsin surface may prove useful for this purpose.

The approach described here and in a similar forthcoming study from Karnik$^4$ for the clathrinoid surface of rhodopsin offers a novel strategy for studying protein-protein interactions in seven TM receptors as well. Of particular interest is designing soluble mimics for a class of GPCRs that bind their ligand(s) on the extracellular surface as well as for those receptors that are poorly expressed or are available in minute quantities or are only known at the gene sequence level.

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