Structure of the Third Cytoplasmic Loop of Bovine Rhodopsin†

Philip L. Yeagle,*,‡ James L. Alderfer,§ and Arlene D. Albert‡

Department of Biochemistry, University at Buffalo, School of Medicine and Biomedical Sciences, Buffalo, New York 14214, and Department of Biophysics, Roswell Park Cancer Institute, Buffalo, New York 14263

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ABSTRACT: The three-dimensional high-resolution structure of rhodopsin is unknown, as is the case for almost all integral membrane proteins. As part of an alternative approach to determination of membrane protein structure, we are pursuing the structure of cytoplasmic domains of this G-protein receptor. A peptide, rhoIII, with the sequence of the third cytoplasmic loop of bovine rhodopsin was synthesized. This soluble peptide was biologically active, inhibiting the light-stimulated activation of the rod cell phosphodiesterase by rhodopsin in rod outer segment disks. Therefore rhoIII likely contains structural elements characteristic of native rhodopsin. The solution structure of rhoIII was determined by ^{1}H nuclear magnetic resonance. A defined structure was obtained for about 70% of rhoIII. A model of a turn−helix−turn motif could then be proposed for the third cytoplasmic loop of rhodopsin, which suggested a molecular switch for activation of the G-protein by the receptor.

Rhodopsin in the rod outer segment (ROS) disk membrane mediates the response of the rod photoreceptor cell to light. When rhodopsin absorbs a photon of light, as part of the resulting photocycle, it enters the Metarhodopsin II state and activates the G-protein, transducin. Rhodopsin is thus a member of the G-protein receptor family. On the basis of circular dichroism (CD) measurements (Albert & Litman, 1978), primary sequence (Hargrave et al., 1983), and recent projection structures (Schertler et al., 1993), a bundle of seven transmembrane helices has been suggested as part of the structure for rhodopsin.

No high-resolution structure is currently available for rhodopsin. In fact, very few high-resolution structures are available for any transmembrane protein because of a lack of crystals suitable for X-ray crystallography. Alternative approaches to membrane protein structure determination are therefore required. A novel approach to the structure of rhodopsin is herein explored in part. The immediate goal is a description of the surface of rhodopsin that interacts with transducin. The specific approach is to study individually the soluble peptides that represent the cytoplasmic portions of the receptor primary sequence. This approach will have merit to the extent that these cytoplasmic domains maintain native structure in the soluble peptides. Biological activity of the peptide is a measure of the presence of native protein structure.

The third cytoplasmic loop of rhodopsin (shaded in Figure 1) is known to have biological activity. The peptide representing this sequence (herein referred to as rhoIII) has been shown to bind to transducin and inhibit the activation of the G-protein by light-stimulated rhodopsin (Konig et al., 1989). In the present study we have confirmed the conclusions from those experiments by studying the effect of rhoIII on the activation of ROS phosphodiesterase (PDEase) by light-stimulated rhodopsin. The rhoIII inhibits the activation of PDEase. These data collectively suggest that crucial structural elements of rhodopsin are retained in the structure of rhoIII.

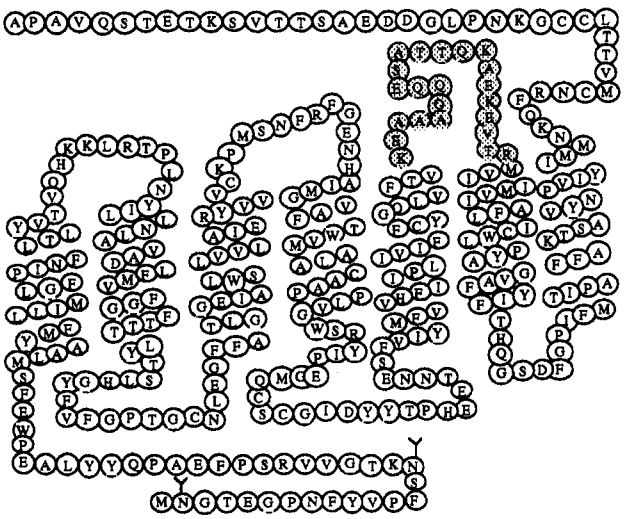

FIGURE 1: Representation of the primary sequence of bovine rhodopsin, after Hargrave et al. (1983). The shaded portion is incorporated into rhoIII.

The high-resolution solution structure of rhoIII was therefore investigated by NMR. About 70% of the peptide produced useful constraints in the NMR studies, so the solution structure of that portion of the peptide could be determined. The structure consisted of an helical section in the middle third and a turn in the carboxyl-terminal third. Since the amino-terminal of rhoIII must connect to the fifth transmembrane helix and the carboxyl-terminal of rhoIII must connect to the sixth transmembrane helix, a model could be proposed for the structure of this portion of rhodopsin. If it becomes possible to determine the structure of all of the cytoplasmic loops of rhodopsin, in a biologically relevant

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* To whom correspondence should be addressed.
‡ University at Buffalo.
§ Roswell Park Cancer Institute.

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manner, then perhaps the entire surface of rhodopsin involved in interactions with the α-subunit of the G-protein, transducin, could be defined. In principle, the results of this study support this new approach to the determination of the structure of rhodopsin.

MATERIALS AND METHODS

Phosphodiesterase Assays. Rod outer segment disk membranes were obtained from bovine retinas. The hypotonic extract containing the phosphodiesterase and the G-protein, transducin, was prepared, and the PDEase assay was carried out as described (Boesze-Battaglia & Albert, 1990). Transducin and PDEase were prepared from ROS by washing them with a hypotonic buffer. The soluble enzymes were added to disk membranes with cGMP and GTP. Upon exposure to light, the hydrolysis of cGMP was monitored by the change in pH (Liebman & Evanczuk, 1982; Miller et al., 1986). The activity in the presence of rhoIII was determined by incubation of the peptide with the hypotonic extract prior to initiating the assay. The assay contained 0.5 mg of rhodopsin and from 0 to 1 mg of rhoIII.

Peptide Synthesis. The polypeptide constituting the 22 amino acid residues of the third cytoplasmic loop of bovine rhodopsin, rhoIII, was synthesized in the Biopolymer Facility of Roswell Park Cancer Institute, using FMOC chemistry. It was purified by HPLC, the amino acid composition was analyzed, and the primary sequence was determined. Figure 4 shows the sequence with both the internal nomenclature for the peptide and the sequence assignment from the primary structure of rhodopsin (Hargrave et al., 1983).

NMR Spectroscopy. All NMR spectra were recorded on a Bruker AMX-600 spectrometer at 5 °C. Standard pulse sequences and phase cycling were employed to record the following: in D_2O, COSY (Nagayama et al., 1980; Rance et al., 1983); in H_2O, double-quantum-filtered (DQF) COSY, HOHAHA (Bax & Davis, 1985; Braunschweiler & Ernst, 1983), and NOESY (200 and 400 ms mixing time) (Kumar et al., 1980). All spectra were accumulated in a phase-sensitive manner using time-proportional phase incrementation for quadrature detection in F1. Chemical shifts were referenced to internal methanol.

Structure Refinement. The sequence-specific assignment of the ^1H NMR spectrum was carried out using standard methods. Assigned NOE cross peaks were segmented using a statistical segmentation function and characterized as strong medium and weak corresponding to upper bounds distance range constraints of 2.7, 3.5, and 5.0 Å, respectively. Lower bounds between nonbonded atoms were set to the sum of their van der Waals radii (approximately 1.8 Å). Pseudoatom corrections were added to interproton distance restraints where necessary (Wüthrich et al., 1983). Distance geometry calculations were carried out using the program DIANA (Guntert et al., 1991) within the SYBYL 6.1 package (Tripos Software Inc., St. Louis, MO). First-generation DIANA structures, 150 in total, were optimized from step 1 to step 22 with the inclusion of three REDAC cycles. Energy refinement calculations (restrained minimizations/dynamics) were carried out on the best distance geometry structures using the SYBYL program implementing the Kollman all-atom force field. All calculations were performed on a Silicon Graphics 4D/440 computer.

Imaging of the resulting structures and construction of the transmembrane helices of rhodopsin were performed on a PowerMac with MacImdad (Molecular Applications Group, Palo Alto, CA). Statistics on structures were obtained from XPLOR.

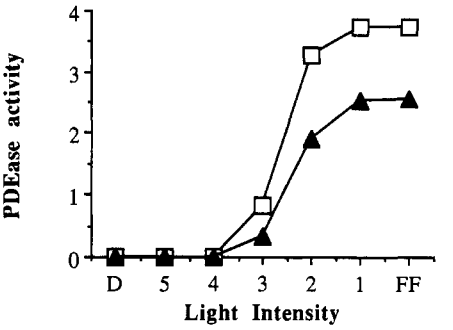

FIGURE 2: Light-stimulated phosphodiesterase activity measured as described in the text: ▲, with 0.75 mg of rhoIII; □, control.

Table 1: Inhibition of PDEase Activity by rhoIII

mg of rhoIII	n^a	% control[b]
0.25	2	93 ± 1
0.5	3	88 ± 7
0.75	3	67 ± 6
1.0	2	60 ± 3

[a] Number of independent experiments of the kind presented in Figure 2. [b] "±" indicates range of values.

RESULTS

Inhibition of PDEase Activity by rhoIII. To determine whether rhoIII, the peptide containing the sequence of the third cytoplasmic loop of bovine rhodopsin, contained biologically relevant structure, the influence of rhoIII on the activation of bovine ROS PDEase by light stimulation of rhodopsin was measured. A typical experiment appears in Figure 2. PDEase activity was measured in the presence of bovine ROS disk membranes as a function of light exposure. In the control, light-stimulated PDEase activity increased with an increase in intensity of light stimulation. At the higher levels of light stimulation, a plateau in activity was reached. In the presence of rhoIII, light-stimulated PDEase activity was less than the control at all light levels. The PDEase activity in the presence of rhoIII can be expressed as a percent of the PDEase activity in the absence of rhoIII, in the plateau region. Table 1 presents these results, as a function of the amount of rhoIII in the assay for several concentrations of rhoIII. There is a dose response, with increasing concentrations of rhoIII leading to decreasing levels of the PDEase response to light stimulation of rhodopsin. Therefore, rhoIII inhibits the ability of rhodopsin to initiate the events that lead to activation of PDEase.

These results agree with previous studies on this peptide. Activated rhodopsin binds transducin which is activated and in turn activates the PDEase. A peptide with the sequence of rhoIII, plus one additional amino acid, was reported to inhibit the binding of transducin to rhodopsin (Konig et al., 1989). Without binding of transducin to rhodopsin, transducin cannot be activated and thus transducin cannot activate PDEase. Therefore the data on transducin binding to rhodopsin, and the inhibition of PDEase activity reported here, together suggest that rhoIII in solution retains structure characteristic of rhodopsin in the disk membrane (while an induction of an alternate conformation in rhoIII through

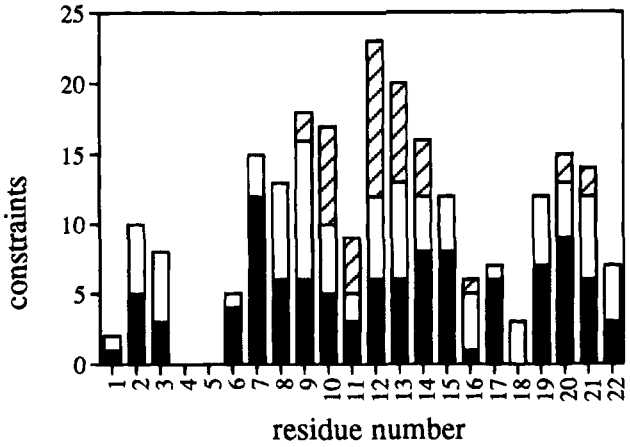

FIGURE 3: Distribution of the NOE-derived constraints, as a function of amino acid in the primary sequence of rhoIII: ■, intraresidue; □, sequential; cross-hatched bars, long range.

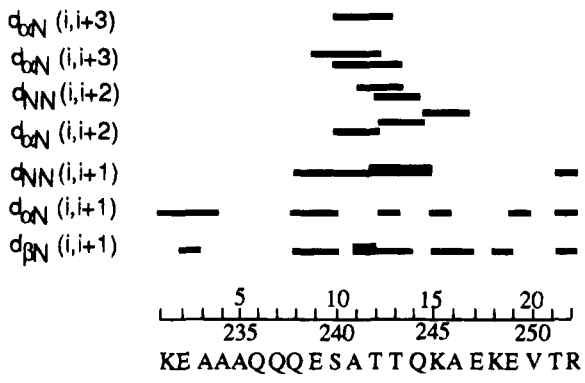

FIGURE 4: Connectivities among the constraints derived from the NOE data. The sequence of rhoIII is presented both with the internal labeling scheme (1−22), and with the corresponding residue numbers from the primary sequence of rhodopsin.

binding to transducin cannot be ruled out, there is at present no data in support of such an hypothesis).

Structure Determination of rhoIII. The structure of rhoIII was determined by homonuclear ^{1}H NMR as described in Materials and Methods. In total, 171 unique constraints were obtained: 107 intraresidue constraints, 44 sequential constraints, and 20 long-range constraints (no useful dihedral angle information could be obtained from the coupling constants). Figure 3 shows the number of constraints per residue. The middle third of the peptide was well constrained, while the carboxyl-terminal third had only a modest number of constraints. In contrast, the amino-terminal third of rhoIII produced almost no useful constraints. Therefore, as observed below, no structure could be obtained for the amino-terminal third of rhoIII. The temperature of these experiments was 5 °C, in an attempt to stabilize a single structure for the peptide.

Analysis of the connectivity in the set of usable constraints is presented in Figure 4. There is extensive connectivity in the middle third of the peptide, with much less in the carboxyl-terminal third and almost none in the amino-terminal third (due to the general lack of constraints in that region). The connectivity in the middle third suggests the presence of some helical structure. The CD spectrum of rhoIII also suggested a small amount of helical structure in the peptide (data not shown).

Using these constraints, DIANA was used to generate compatible structures, and the best structures were minimized, as described in Materials and Methods. An overlay of six of the best structures appears in Figure 5. Further refinement by simulated annealing and another energy minimization did not produce much change in overall structure. The six amino-terminal residues of rhoIII are not shown in Figure 5, since no structural information was available for that portion of the molecule. The middle third of the molecule is well defined, and the carboxyl-terminal third, though less well defined, nevertheless shows some structure. Table 2 shows the statistics for this ensemble of structures, as well as the energies.

DISCUSSION

The structural information from this study contributes significantly to an understanding of the structure of the surface of the G-protein receptor, rhodopsin, that interacts with the G-protein, transducin in the ROS. About 70% of the third cytoplasmic loop of rhodopsin can be defined. The structure that was obtained could be related to the active form of the receptor, since the ability of rhodopsin to activate the cGMP cascade in response to light was inhibited by rhoIII.

The structure of rhoIII contains a short helical section of about one turn in the middle of the peptide. The attachment of both ends of this loop to transmembrane helices of rhodopsin provides a further constraint on the native structure. A model incorporating both the NMR structure and this additional constraint is presented in Figure 6. To build this model, the fifth and sixth transmembrane α-helices of rhodopsin were constructed separately. These helices were oriented parallel to each other, according to the model of Baldwin (1993). A structure was chosen from the ensemble of rhoIII structures that could be connected to these two helices. Figure 6 suggests that the third cytoplasmic loop is most likely a turn−helix−turn motif. This structure presents the middle third of the loop to the surface of protein, outside of the membrane, where it can presumably interact with the G-protein, transducin.

Lys245$_r$ and glu239$_r$ extend from the same side of the rhoIII helix in the middle of the loop and are likely available for interaction with transducin. This matched set of opposite charges on the receptor suggests an interesting speculation. Two peptides from transducin, encompassing residues 316$_t$−323$_t$ and residues 340$_t$−350$_t$, were reported to inhibit transducin binding to rhodopsin (Hamm et al., 1988). Reference to the crystal structure (Noel et al., 1993) of transducin (the former peptide) and NMR (Dratz et al., 1993) studies (the latter peptide) show that in each peptide there is a matched pair of oppositely charged amino acids, and in each case the pair extends from the same side of the structure. In the peptide 340$_t$−350$_t$ from transducin, there is a pair of oppositely charged amino acids, glu342$_t$ and lys345$_t$, which the authors found in an intramolecular salt bridge when bound to the unactivated receptor. When bound to the activated receptor, the salt bridge was broken, though the two charges were still found on the same side of the molecule. The pair, lys245$_r$ and glu239$_r$, on rhodopsin could link with the pair, glu342$_t$ and lys345$_t$, on transducin in the active state, substituting an intermolecular salt bridge for an intramolecular salt bridge. Such a structure could be the switch converting transducin from the inactive form to the active form.

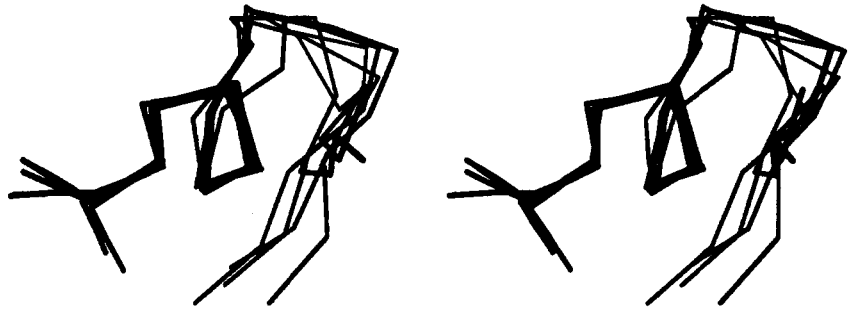

FIGURE 5: Stereoview of the superposition of the six best structures for rhoIII, obtained as described in the text. Only residues 7−22 are shown, because no structural information could be obtained for residues 1−6 of rhoIII from the NMR data.

Table 2: Structural Statistics of rhoIII

constraints	
intraresidue	107
sequential	44
long range	20
total	171
rmsd[a]	
heavy atoms	1.9 (res 8−21), 1.6 (res 8−15)
backbone	1.2 (res 8−21), 1.0 (res 8−15)
energies (av)[b]	
bond stretching	7.4
angle bending	54
torsional	57
improper torsional	4.5
van der Waals	−42
electrostatic	−580
H−bond	−5.5
fixed distance range	51
total	−140

[a] rmsd from mean structure (in angstroms). [b] In kcal/mol.

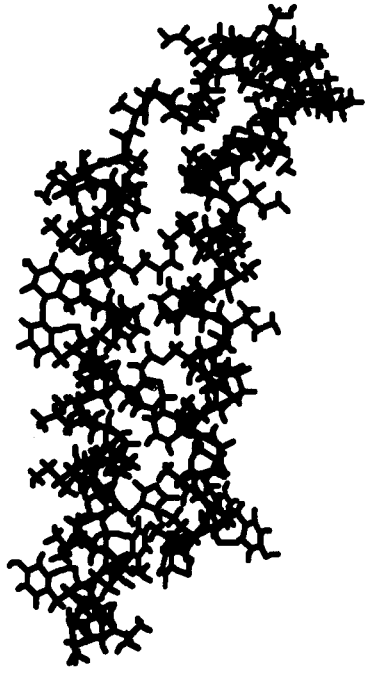

FIGURE 6: Model for the third cytoplasmic loop as it might connect with the transmembrane helices 5 and 6 of bovine rhodopsin. The geometry of the connection of rhoIII to the two helices is presently speculative.

The observation of structure in the rhoIII peptide may shed some light on the problem of insertion of membrane proteins into membranes during biosynthesis. One question that has arisen concerns the formation of the loops that connect the transmembrane helices of integral membrane proteins. One hypothesis suggests that loop formation is driven by the packing and transmembrane location of the transmembrane helices, which in turn force the loop conformation. Another hypothesis suggests initially that a substructure forms with two transmembrane helices connected by a loop, which is then inserted into the membrane (Engelman & Steitz, 1981). The former hypothesis implies that the conformation of the loop is primarily determined by the folding of the ensemble of transmembrane helices, and the latter hypothesis implies that the amino acid sequence of the loop itself should direct the conformation of the loop. The structural information obtained here is more consistent with the latter hypothesis than the former. Much of the sequence of rhoIII is incorporated into a defined structure in the absence of the transmembrane helices and in the absence of the membrane. Since that defined structure is biologically active, it is reasonable to presume that the structure of rhoIII observed here is very much like the structure of the third cytoplasmic loop of rhodopsin in the biological membrane. The portion of rhoIII which is not structured in this study will take on structure when covalently connected to transmembrane helices 5 and 6 of the receptor. The orientation of the transmembrane helices may thus influence the ability of the third cytoplasmic loop, and in particular the helical region, to interact with transducin.

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