Conformational changes in the phosphorylated C-terminal domain of rhodopsin during rhodopsin arrestin interactions.

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Phosphorylation of activated G-protein coupled receptors and the subsequent binding of arrestin mark major molecular events of homologous desensitization. In the visual system, interactions between arrestin and the phosphorylated rhodopsin are pivotal for proper termination of visual signals. Using high-resolution proton Nuclear Magnetic Resonance spectroscopy of the phosphorylated C-terminus of rhodopsin, represented by a synthetic seven-phospho-polypeptide, we show that the arrestin-bound conformation is a well-ordered helix-loop structure connected to rhodopsin via a flexible linker. In a model of the rhodopsin-arrestin complex the phosphates point in the direction of arrestin and form a continuous negatively charged surface, which is stabilized by a number of positively charged lysine and arginine residues of arrestin. Opposite to the mostly extended structure of the unphosphorylated C-terminal domain of rhodopsin, the arrestin-bound C-terminal helix is a compact domain that occupies a central position between the cytoplasmic loops, and occludes the key binding sites of transducin. In conjunction with other binding sites, the helix-loop structure provides a mechanism of shielding phosphates in the center of the rhodopsin-arrestin complex, and appears critical in guiding arrestin for high-affinity binding with rhodopsin.
INTRODUCTION

Following activation by a variety of sensory stimuli, such as hormones, neurotransmitters or light, G-protein coupled receptors (GPCRs) are deactivated by multiple phosphorylations and subsequent binding of a regulatory protein arrestin (1, 2). Deactivation of the active receptor is obligatory and insures the quantum character of the response to an extracellular signal. GPCR kinases (GRKs) and arrestin proteins are receptor specific but share universal mechanisms of action, with prototypical rhodopsin kinase and visual arrestin involved in the termination of visual signal transduction (3, 4). Quenching of the photoresponse in the retinal photoreceptor cells proceeds through phosphorylation of multiple serine and threonine residues at the carboxy-terminus of light-activated rhodopsin by GRK1 and high-affinity binding of visual arrestin. In vitro studies have implicated all seven serine and threonine residues within the Rh(334-343) C-terminal stretch as possible substrates of the phosphorylation reaction. The question of the exact number and position of residues phosphorylated in vivo, however, remains uncertain possibly due to the inherent difficulties of controlling dephosphorylation, different kinetics of phosphorylation/dephosphorylation at individual residues, possible contribution of kinases other than GRK1, and other secondary factors. Either Ser 334 (5) or Ser 343 (6) are phosphorylated initially. The majority of studies provide strong evidence, however, that multiple phosphorylation is required for reproducible deactivation (5-8). In order to circumvent the current uncertainty about the exact sequence of phosphorylation events, we have used a model synthetic peptide phosphorylated at all seven positions, which mimics the major biological properties of phosphorylated rhodopsin.
Conceptually it is thought that arrestin directly competes for the binding site of transducin (9-11), a heterotrimeric GTP-binding protein responsible for relaying the signal to the cyclic GMP phosphodiesterase, an enzyme of the intracellular second messenger system. The mechanism of arrestin binding and signal shut-off at the molecular level, however, is not understood. The C-terminal region of rhodopsin is resolved poorly in available crystal structures. Phosphorylation of multiple residues can potentially have significant impact on the conformation of the C-terminus, and prearrange the Rh(330-348) domain for effective interactions with arrestin. NMR studies using model peptides with various numbers of phosphates (12), and full protein (13) showed little ordering of this region of rhodopsin upon phosphorylation. Whether the phospho-Rh(330-348) assumes a defined conformation in the rhodopsin-arrestin complex remains unclear. We posed the question whether the phosphorylated C-terminus of rhodopsin is simply passive bait for arrestin, or if it is directly involved in shaping the cytoplasmic surface of the receptor into a conformation associated with termination of a signaling state. We used high-resolution proton NMR to study the dynamics of the model synthetic peptide, representing the fully phosphorylated region of rhodopsin Rh(330-348), 7PP, in solution and in an arrestin-bound state. Rigid body docking of rhodopsin, containing the C-terminal region in a conformation determined from this experimental study, to arrestin, allowed us to propose a model of the rhodopsin-arrestin complex.
MEHTODS

Arrestin and phosphorylated peptide:

Arrestin was prepared by the method of Buczyłko and Palczewski with modifications as described earlier (14). Peptide Rh(330-348) from the sequence of bovine rhodopsin was synthesized by standard Fmoc chemistry on an Applied Biosystem model 431A peptide synthesizer. Multiply phosphorylated peptide 7-phospho-Rh(330-348), 7PP, was synthesized on phenylacetamidomethyl polystyrene resin using Boc-O-(diphenylphosphono)-serine and -threonine as described (15). The peptides were homogeneous by HPLC and showed the expected mass when examined by MALDI-TOF mass spectrometry (15).

Rhodopsin binding:

Various amounts of the Gtβγ were reconstituted with 3 μg of Gtα and 30 μg of urea-washed ROS membranes, UM, in 100 ml of buffer ROS-ISO (10mM Tris pH 7.4, 100mM NaCl, 5mM MgCl₂, 1mM DTT, 0.1mM PMSF) on ice in the absence or presence of 5μg of bovine arrestin and 100 μM of 7PP. The reaction was initiated by exposure to light. UM were centrifuged at 109,000 x g, 4°C for 10 min in a TLA-100.3 rotor on a Beckman TL-100 Ultracentrifuge. The pellet was washed twice with buffer ROS-ISO. UM with Gt bound was resuspended in buffer ROS-Hypo (10 mM Tris-HCl pH 7.4, 0.5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF) and 250 μM GTPγS, incubated on ice for 30 min, and centrifuged. The supernatant was analyzed for the presence of G-protein subunits by immunoblotting.
NMR and structure calculations:

NMR sample preparation and data processing was essentially as we described (16). NMR samples contained 0.16 mM purified arrestin, 1.77 mM 7PP, 7-phospho-Rh(330-348) or unphosphorylated Rh(330-348) in sodium phosphate buffer, 0.1 M, pH 6.5 and 10% D₂O in a total volume of 0.6 ml. Two-dimensional high-resolution proton Transferred Nuclear Overhauser Effect Spectra (TrNOESY) were acquired at 4°C on the Varian Unity-600 spectrophotometer as described earlier (17). Data were processed off-line using VNMR 5.2. Total Correlation Spectroscopy (18) (2D TOCSY, MLEV-17 mixing sequence of 120 ms, flanked by two 2 ms trim pulses, 0.5 s preacquisition delay and 1.0 s presaturation), and 2D NOESY(19) (Tmix=250 ms, 2x280x2049 data matrix with 16 scans per t₁, using WATERGATE water suppression protocol) were used for sequence-specific and stereo-specific assignments. NOEs were classified into weak, medium and strong based on the peak volume and translated to corresponding interproton distances of 1.9-5.0 Å, 1.9-3.5 Å, and 1.9-2.7 Å respectively. One hundred ninety eight constraints were used for structure calculations, which involved distance geometry (DISTGEOM of TINKER 3.9 (20)), 1000K restrained molecular dynamics and simulated annealing (ANNEAL, 15 ps total time) and structure refinement (NEWTON, 0.001 RMS gradient). Calculations utilized CHARMM forcefield. In order to identify biologically relevant conformations of 7PP in the arrestin-bound state, the structure calculations based on the NMR-derived constraints were performed on the full set of X-ray coordinates of rhodopsin (21) with unresolved parts of loop C3 and C-terminal region rebuilt in Insight II. The starting conformation of a rebuilt C-terminus was mostly extended. Only region Rh(324-348) was allowed to move during calculations. For the final energy refinements, restrained molecular dynamics, simulated annealing and energy minimization protocols were applied to Rh(330-348) separate from rhodopsin coordinates. One hundred
structures were generated independently. Fifteen models were chosen based on the lowest energy and best local geometry and superimposed using main chain atoms. Coordinates of the ensemble and the NMR constraints file were deposited to PDB, #1TQK. Phosphates were added to a representative model in Insight II. Molecular rigid body docking was done manually in Insight II using surface complementation, electrostatic and hydrophobic interactions as guidance. The final model was chosen based on the strongest charge-charge interactions that avoided steric clashes. Calculation of electrostatic potential, molecular surfaces, and graphics were in MOLMOL (22).
RESULTS AND DISCUSSION

Seven-phospho-Rh(330-348) peptide mimics phosphorylated C-terminus of rhodopsin.

On a functional level, the C-terminus of bovine rhodopsin, the region spanning amino acids 330-348, which contains three serine and four threonine residues, acts as an independent domain with characteristics closely linked to the signal shut-off. Ablation of this region by genetic methods in mice leads to a phenotype in which photo-signal termination is dramatically compromised (23). The soluble polypeptide Rh(330-348) has been synthesized chemically in fully phosphorylated form (seven-phospho-peptide, 7PP (15)), and shown to duplicate major functions of the native phosphorylated rhodopsin. The fully phosphorylated form was chosen because multiple phosphorylation was observed in vivo (6, 8), and both serine and threonine residues have been shown to be required for activation of arrestin’s action (5, 24-26). 7PP inhibited phototransduction from photoactivated unphosphorylated rhodopsin in the presence of arrestin, based on the phosphodiesterase assay (27). It also induced conformational changes in arrestin typically seen upon interaction of arrestin with native phosphorylated rhodopsin (14). As predicted from the previous data, we show here that in direct competition experiments, binding of transducin to light activated unphosphorylated rhodopsin in membranes is inhibited by addition of arrestin and 7PP together, but not arrestin or 7PP alone (Fig.1). These experiments demonstrate close functional approximation between the effects of rhodopsin phosphorylation in vivo, and the effects of the model phospho-peptide in the presence of unphosphorylated rhodopsin in vitro. They also indicate that major structural features of the phosphorylated C-terminus of rhodopsin are preserved in 7PP, and that it
can be used to study the structural dynamics of the rhodopsin-arrestin interface and the mechanism of signal shut-off.

**NMR structures of 7PP in the arrestin-bound state.**

To identify the structural basis of an inhibitory effect of arrestin in the arrestin-rhodopsin complex, high-resolution proton Nuclear Magnetic Resonance (NMR) Spectroscopy was used to determine the solution structure of 7PP and the changes in 7PP conformation upon binding to arrestin. As we reported, no secondary structure elements can be recognized in 7PP in solution, indicating high flexibility and a disordered state of the molecule (16). The result is not surprising, because of the mostly extended conformation of the C-terminal region in available X-ray structures of rhodopsin with the average B-factor values for the Rh(330-348) region of 70.4±8.8 (PDB#: 1HZX) (21). The disorder of 7PP in solution is also consistent with previous solution NMR experiments of model peptides (12), and a recent study combining solution and solid state NMR of native phosphorylated rhodopsin (13).

Addition of arrestin under the same experimental conditions resulted in striking changes in 7PP conformation, as evident from the NOESY spectra before and after addition of arrestin, Fig 2a (16). One hundred ninety eight constraints were identified and used in generation of a first subset of structures consistent with the NMR data by distance geometry and high temperature simulated annealing. The summary of the experimental constraints is shown in Fig. 2b. The analysis of a family of 7PP conformations in an arrestin-bound state showed a well-ordered C-terminal helix for residues Glu 341 to Ala 346, and a fairly disordered loop for residues Asp 330 to Thr 340 (Fig. 2). Energy refinements lead to significant improvements of the local geometry and the overall
quality of structures, compared to the initial set of models reported. The latest models have more than 83% of residues in the most favored regions of the Ramachandran plot and about 17% of residues in additionally allowed regions (Fig. 3), compared to 71% and 29% respectively for the first generation of structures (16).

Restricted mobility and tighter structural organization of the C-terminal region, evident from small RMSD values for residues 340-348 (Fig. 3), point to a more prominent role of the C-terminal helix in arrestin binding. Because any structuring is absent in an unphosphorylated peptide or 7PP without arrestin, it appears the C-terminal helix containing phosphates at Thr 342 and Ser 343 is ultimately involved in binding and, thus, is more constrained by the geometry of the binding site on arrestin. This observation correlates well with the proposed sequence of phosphorylation events progressing from Ser 343 to Ser 334 (6). The first phosphates added are known to have the most effect on the affinity of binding. We predict that the structural elements of the helix loop structure of 7PP would be detectable with only Ser 343 phosphorylated. In support of this prediction, Ser 343 was shown to be phosphorylated most rapidly in vivo to produce the dominant monophosphorylated form of rhodopsin within the first seconds after light activation (6).

A prominent feature of the arrestin-bound structure is the position of seven phosphates that are clustered on the loop connecting the disordered N-terminal region and helical C-terminus. Remarkably, in the models calculated in the context of the whole rhodopsin, the phosphate groups are facing in one direction, away from rhodopsin, forming a unified negatively charged surface (Fig. 4). Carboxy groups of Asp 330, Glu 332 and Glu 341 additionally contribute to this surface. Such orientation of phosphates is not observed for
the peptide in the absence of arrestin and would be highly unlikely in aqueous environment for the 7PP polypeptide alone, because of the strong negative charge repulsion. It appears possible because of the interactions of the phosphate groups with the complementary positively charged binding site of arrestin.

**A model of rhodopsin-arrestin interactions.**

Modeling of the cytoplasmic surface of rhodopsin based on the NMR-derived constraints shows that, in the arrestin-bound state, the C-terminal helix occupies a central position between three cytoplasmic loops of rhodopsin. This crevice is thought to open up after rhodopsin photoactivation to form a site for transducin docking (28, 29). The arrestin-bound conformation also completely occludes the N-terminus of helix eight, one of the major sites of transducin interaction (30). Formation of a compact helical structure at the C-terminus of rhodopsin upon arrestin binding may facilitate interactions between arrestin and additional sites of arrestin interactions on loops two and three. As mentioned above, the concerted position of phosphates in an arrestin-bound conformation of 7PP shows that the phosphates must be completely shielded from solvent in the arrestin-rhodopsin complex. This conclusion is consistent with data demonstrating that arrestin prevents de-phosphorylation of rhodopsin by phosphatase (31). To visualize the position of the phosphates, and to estimate the overall geometric plausibility of the rhodopsin-arrestin complex, we performed molecular rigid body docking of available crystal structures of arrestin (PDB # 1CF1) with phosphorylated rhodopsin models produced by this study. The model of the complex does not take into consideration global conformational changes in arrestin, which are expected based on the biochemical studies (32), but rather reflects initial stages of the contact between the two proteins. A prominent saddle on arrestin with the highest concentration of positively charged lysine
and arginine residues was chosen as a complimentary site for the phosphates on the rhodopsin C-terminal domain (Fig. 5). The polar core of this site contains Lys 14, Lys 15, and Arg 175 which are proposed to interact with phosphates on rhodopsin (33). Previous data have shown that interaction of the phosphate groups with Arg 175 in the region 166-179 triggers the electrostatic switch in arrestin (33). The sequence of molecular events that follows can be described most closely as the induced fit mechanism of interactions, with both the rhodopsin C-terminus and arrestin changing conformations for the most precise fit. In fact, conformational changes and repositioning of the rhodopsin C-terminus may help to direct arrestin for the most specific docking with rhodopsin (34), making possible interactions with other regions of arrestin, such as the 109-130 stretch (35) or other regions, that become available due to local and global rearrangements in arrestin.

**Analogies with the ball-and-chain mechanism of inactivation of potassium channels.**

Involvement of a built-in domain of a transmembrane signaling protein in the mechanism of inactivation struck us as remarkably similar in concept to the ball-and-chain mechanism of inactivation of voltage-gated potassium channels, described originally for the Drosophila Shaker B channel (36), and then for a variety of other channels (37). The inactivation domain, the ball, blocks the channel pore reversibly, and is connected to the channel via a flexible linker, the chain. In both cases, inactivation occurs by a cytoplasmic terminal domain of a transmembrane protein, the N-terminal inactivation gate in the case of the channels, and the C-terminal domain in the case of GPCRs. In both instances, the inactivation properties of the domain can be regulated by phosphorylation (38), and the domains function fairly independently of the rest of the protein. Their removal proteolytically or by genetic methods leads to a complete loss of inactivation. The inactivation is restored when the domains are added as independently synthesized
polypeptides (14, 39). What makes the mechanism of GPCR inactivation distinct is the obligatory requirement for an auxiliary protein, arrestin, which induces the active “ball” conformation of the phosphorylated C-terminus, as we show in this study. In fact, arrestin binding appears to enhance the original nucleation effect of the rhodopsin C-terminus in a snowball manner, which completely excludes interactions with transducin.

Inactivation gates of several potassium channels have been studied by NMR. While fairly diverse in their three-dimensional structure, the ball-and-chain domains of the voltage-dependent potassium channel RCK4 (Kv1.4) (40) and the β2-subunit of a large conductance (BK) Ca\(^{2+}\) and voltage dependent potassium channel (41) show striking resemblance to the arrestin-bound structures of the rhodopsin C-terminus. Despite little primary sequence similarities, and obvious structural variations, the helix-loop hairpin motif can be readily recognized (Fig. 6). There is no reason to suspect an evolutionary link between channels and GPCRs. More likely, the remarkable similarity between the two systems in concept and structural detail represents a fascinating example of evolutionary convergence of the molecular domains evolved to perform similar regulatory functions.

Conclusions.

Overall principles of rhodopsin inactivation, including multiple phosphorylation of the C-terminal serine and threonine residues by rhodopsin kinase, and the binding of arrestin, are understood fairly well conceptually. Mechanistic details of rhodopsin-arrestin interactions at the atomic level, however, are lacking. Previous studies have focused on the mechanisms of arrestin activation and conformational changes in arrestin
accompanying its binding to phosphorylated rhodopsin. We show in this study, that binding of the fully phosphorylated polypeptide, representing the last nineteen residues of bovine rhodopsin, to arrestin, leads to the peptide folding into a helix-loop structure. When modeled in the context of the whole rhodopsin, this compact structure occupies a central position between cytoplasmic loops of rhodopsin. This unique orientation of the rhodopsin C-terminus and the bound arrestin shields phosphates in the center of the complex, occludes major sites of transducin binding and is associated with termination of a signaling state. Because both GPCRs and arrestins are families of proteins with conserved functions, we predict that diverse C-terminal regions of other GPCRs should exhibit structural properties similar to those identified in this study.

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**Abbreviations footnote:**

Data deposition footnote: Atomic coordinates and NMR constraints file for the arrestin-bound C-terminus of rhodopsin have been deposited in the Protein Data Bank (accession code 1TQK).
Figure 1. 7PP and arrestin inhibit light- and GTP-dependent binding of transducin to rhodopsin membranes. (a). Dose-dependent binding of transducin to rhodopsin membrane. The arrow indicates the concentration of transducin Gtα and Gtβγ subunits at which competition experiments in panel (b) were done. Quantitation results of an immuno blot using Gtβ1 specific antibodies represent five independent experiments. (b). Inhibition of transducin binding to rhodopsin membranes in the presence of different combinations of arrestin (Arr), seven-phospho-Rh(330-348) peptide (7PP), and unphosphorylated Rh(330-348) peptide (unP). The bars represent average data and standard errors from five independent experiments.

Figure 2. NMR experiment and structure determination. (a) A representative view of the 1H-Tr-NOESY spectra of 7PP in the presence of bovine arrestin. The NH-αH region is shown. Solid line shows sequential connectivities. (b). Summary of the experimental constraints. (c). A cross-eye view of an ensemble of fifteen NMR structures consistent with the NMR data.

Figure 3. Ramachandran plot and structure statistics for the refined ensemble of arrestin-bound 7PP.

Figure 4. (a) Ribbon model of bovine rhodopsin in blue with phosphorylated C-terminal domain shown in yellow in arrestin-bound conformation. Side chains of phosphorylated serines and threonines are shown as ball-and-stick models. Semi-transparent solvent-accessible surface of the phosphate groups is in red. Position of the transmembrane helices of rhodopsin is labeled. (b) van der Waals surface of rhodopsin (cytoplasmic side) as seen by arrestin with the electrostatic potential mapped. White - neutral, red –
negatively charged, blue - positively charged. The phospho-groups on Ser 334, Thr 335, Thr 336, Ser 338, Thr 340, Thr 342 and Ser 343 together with carboxy groups of Glu 332 and Glu 341 form a continuous negatively charged surface. C2 and C3 show position of cytoplasmic loops two and three.

**Figure 5.** A model of rhodopsin-arrestin complex. Docking of arrestin to rhodopsin with phosphorylated Rh(330-348) region in arrestin-bound state was manual, assuming interaction of the phosphate groups with region 166-179 of arrestin, a site of rhodopsin interactions with the most concentration of arginine and lysine residues. β-strands containing the region 166-179 and segments determining receptor specificity are in purple. Inset highlights the interaction site. Phosphate groups of rhodopsin are shown in red and labeled according to the phosphorylated residue number. Lysine and arginine side chains positioned for possible interactions with phosphates are in blue and labeled.

**Figure 6.** Arrestin bound C-terminus of rhodopsin and ball-and-chain domains (inactivation gates) of some potassium channels. (a) Phosphorylated C-terminus of rhodopsin, arrestin-bound state, PDB # 1NZS. (b) β2-subunit of a BK large conductance Ca2+ and voltage-dependent potassium channel, PDB # 1JO6. (c) RCK4 (Kv1.4), a voltage-gated potassium channel, PDB # 1ZTO.
References:

Figure 1
Residues in most favored regions [A,B,L] 83.3%
Residues in additional allowed regions [a,b,l,p] 16.7%
Residues in generously allowed regions [~a,~b,~l,~p] 0.0%
Residues in disallowed regions 0.0%

Geometrical and stereochemical features:
Close contacts none
All covalent bonds lie within a 6.0*RMSD range about the standard dictionary values yes
All covalent bond angles lie within a 6.0*RMSD range about the standard dictionary values yes
Stereochemical violations none

PROCHECK overall G-factor -0.46

RMSD to the mean for backbone atoms (Å)
Residues 330-339 1.16±0.26
Residues 340-348 0.13±0.12

RMSD to the mean for side chain atoms (Å)
Residues 330-339 1.52±0.31
Residues 340-348 0.48±0.38
Figure 5