Interactions of Metarhodopsin II:

Arrestin Peptides Compete with Arrestin and Transducin

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SUMMARY

Arrestin blocks the interaction of rhodopsin with the G-protein transducin (Gt).
To characterize the sites of arrestin that interact with rhodopsin we have utilized a spectrophotometric peptide competition assay. It is based on the stabilization of the active intermediates, metarhodopsin II (MII) and phosphorylated metarhodopsin II (P-MII), by Gt and arrestin, respectively (extra MII monitor). The protocol involves native disc membranes and three sets of peptides, 10-30 amino acids in length and spanning the arrestin sequence. In the absence of arrestin, none of the peptides by itself has an effect on the amount of MII formed. However, inhibition of arrestin dependent extra MII is found for peptides from residues 11-30 and 51-70 (IC$_{50}$ < 100 µM), and 231-260 (IC$_{50}$ < 200 µM). A similar pattern of inhibition by arrestin peptides is seen when arrestin is replaced by Gt or the Gt$_{\gamma}$-farnesyl C-terminal peptide. Only the peptide Arr(11-30) inhibits Gt-MII less (IC$_{50}$ = 300 µM) than arrestin-P-MII. We interpret the data by competition of the arrestin peptides for interaction sites at rhodopsin, exposed in the MII conformation and specific for both arrestin and Gt. The arrestin sites are located in both the C- and N-terminal domains of the arrestin structure.
INTRODUCTION

G protein-coupled receptors (GPCRs) enable eukaryotic cells to respond to a large variety of extracellular signals including hormones, odorants, and light (1). One of the best studied GPCR-initiated signaling pathways is the visual cascade in retinal rods (2). It is initiated by the absorption of a photon in the visual receptor, rhodopsin, and subsequent isomerization of the 11-cis-retinal, which is covalently attached to Lys\textsuperscript{296} in the seventh transmembrane helix of the receptor. Via a series of transient intermediates, rhodopsin converts in milliseconds into metarhodopsin II (MII), the form of rhodopsin that interacts with the heterotrimeric G-protein transducin (G\textsubscript{t}). This interaction initiates the intermolecular transduction of the light signal by catalyzing GDP/GTP exchange in the nucleotide binding site of the G protein and, in turn activates the G protein-coupled effector, a cyclic GMP-specific phosphodiesterase (PDE). Hydrolysis of cGMP leads to the closure of plasma membrane cation channels and hyperpolarization of the membrane (3).

The deactivation of rhodopsin starts with the binding of a rhodopsin kinase to photoactivated rhodopsin (4). Rapid phosphorylation of the receptor at C-terminal sites (5) increases its affinity for arrestin (6). Biochemical (7) and electrophysiological (8) evidence has been accumulated that visual arrestins deactivate the visual cascade by direct competition with the G-protein for the active receptor. However, the mechanism of interaction underlying the quench is still not well understood.

UV-Vis spectroscopy has shown that, to bind arrestin, the light activation of rhodopsin must proceed up to the MII conformation, in which the Schiff base bond of the retinal to the apoprotein is still intact but deprotonated (9). Only after MII formation and phosphorylation of the receptor, arrestin interacts rapidly with the receptor. Although the catalytic activity of MII towards the G-protein is quenched,
arrestin binding (and phosphorylation) stabilizes MII at the expense of its tautomeric forms (see Ref. (6) and citations therein). A conformational difference in arrestin and/or rhodopsin on interaction was early suggested by the unusually high apparent activation energy of rhodopsin-arrestin interaction (9).Later studies have indeed shown that binding of arrestin to the active receptor protects arrestin against limited proteolysis (10) and Lysine acetylation (11), suggesting that arrestin bound rhodopsin adopts a conformation (Ab) which is different from that of free, inactive (Ai) arrestin.

Investigations employing protein engineering (12,13) and phosphorylated peptides (14) have provided evidence for a sequential mechanism, in which the contact of the negatively charged regions of phosphorylated rhodopsin (P-site) with the cationic region acts as a trigger, switching arrestin into its active conformation and allowing interaction with the rhodopsin binding sites exposed on photoactivation.

A highly cationic region near the center of the arrestin sequence, beginning with residue 163, was proposed to mediate the interaction with the P-site, thus enabling the contact with other interaction sites exposed in the MII state (M-sites) (10). Arg175 (within the putative recognition site for the phosphorylated site(s) on rhodopsin) was identified as a key residue for arrestin to distinguish between phosphorylated and unphosphorylated rhodopsin (15,16). Recent structural assignments (17,18) have now identified this residue as part of a “polar core” (18), a central region in arrestin, localized between the N- and C-terminal domains of the molecule. It may act as a fulcrum for the conversion of inactive Ai to active Ab (19).

The conformational switch is additionally controlled by arrestin’s C-terminus (20,21). When it is lacking, as in a splice variant of arrestin, p44, the short arrestin binds both phosphorylated and nonphosphorylated forms of MII and even C-terminally truncated rhodopsin (22,23). Protein engineering (24) and spectroscopic data (25) have suggested that in the Ai state(s), negatively charged C-terminal
residues interact with positively charged residues on the N-terminal region, and that this interaction is broken upon binding to phosphorylated MII.

In the present study we attempt to identify specific sites of arrestin that become exposed by the conformational switch, leading to their interaction with the respective receptor sites in the MII conformation. The technique applied is based on the spectrophotometric MII stabilization assay (9) and on competition with peptides derived from arrestin and Gt. Peptide competition has already been applied in previous studies, to map regions of interaction in the receptor-arrestin complex. Studies with peptides from rhodopsin’s surface exposed sequences suggested a role of different loop structures in the interaction (26). Regarding the interactive domains in arrestin, Hargrave and coworkers have employed a library of peptides covering the entire sequence. Based on phage-display and PDE activation data, they localized one of the principal regions of interaction within residues 109-130 (27) and found indications of additional sites in their data.

We will indeed provide evidence that more than one (namely three) regions in the arrestin molecule are involved in receptor binding. Employing the sensitive MII stabilization assay, and peptide competition not only with arrestin but also with Gt and Gt derived peptides, we can measure with unprecedented specificity. Based on the data from three sets of overlapping synthetic 10 to 30-residue peptides, spanning the arrestin sequence, we propose a model locating the receptor binding regions of arrestin in each of the two domains of the molecule.
EXPERIMENTAL PROCEDURES

Materials. Radioactive \[\gamma^{32}\text{P}]\text{ATP}\] was purchased from NEN Life Science Products. All chemicals were purchased from Merck (Darmstadt, Germany), Boeringer Mannheim or Sigma Chemical Co. (St. Louis, MO). 11-cis-retinal was generously provided by Dr. R. K. Crouch, Medical University of South Carolina.

Isolation of Bovine Rod Outer Segments (ROS). ROS were purified from fresh, dark-adapted bovine retinas obtained from a local slaughterhouse using the discontinuous sucrose gradient method described by Papammer (28). Retinas were dissected, and ROS were isolated, under dim red illumination. All subsequent procedures were performed at 0-5 °C and the ROS were stored frozen at -80°C until use.

Preparation of Washed Membranes. Rhodopsin was prepared by removing the soluble and membrane-associated proteins from the disc membrane by repetitive washes with a low ionic strength buffer (29). All purification steps were performed under dim red illumination and the membrane suspension was stored at -80°C until use.

Preparation of Phosphorylated Opsin. Phosphorylated opsin was prepared from washed disc membranes as described previously by Wilden and Kühn (30). To remove retinaloxime from the membrane-bound phosphorylated opsin the membranes were treated with urea and fatty acid-free bovine serum albumin (31). An average stoichiometry of ~1.5 phosphates per opsin was determined using radioactive \[\gamma^{32}\text{P}]\text{ATP}\] as a tracer.

Preparation of Phosphorylated Rhodopsin. Phosphorylated rhodopsin was prepared by regeneration of phosphorylated opsin with 11-cis-retinal (32). Phosphorylated opsin was suspended in 10 mM BTP buffer, pH 7.5 containing 100 mM NaCl. A 3-fold molar excess of 11-cis-retinal was added in the dark to the sample,
followed by incubation for 1 h at room temperature, and then overnight at 4 °C. After regeneration, phosphorylated membranes were centrifugated (45,000g for 20 min) and washed four times with 10 mM BTP buffer, pH 7.5 containing 100 mM NaCl to remove excess 11-cis-retinal and stored at -80°C.

**Preparation of arrestin.** Arrestin was purified from frozen dark-adapted bovine retinas as described by Heck et al. (33).

**Preparation of transducin.** Transducin was isolated from frozen dark-adapted bovine retinas according to Heck and Hofmann (34).

**Peptide synthesis, purification and characterization.** Peptides were synthesized using the Fmoc (N-(9-fluorenylmethoxycarbonyl) strategy with HBTU activation (Fastmoc – 0.1 mmol small-scale cycles) on an ABI Model 433A peptide synthesizer. The peptides were purified by reverse phase high performance liquid chromatography (HPLC), lyophilized and stored at -20°C. Immediately before the experiments, the peptides were dissolved in deionized water to obtain stock solutions of 2 mM and pH was adjusted to 7.0 with NaOH. Farnesylation of Gtγ-derived peptides was carried out as described (35).

**Protein Determinations.** The concentration of rhodopsin and phosphorylated rhodopsin was determined spectrophotometrically at 498 nm as previously described (23). Purified arrestin was determined spectrophotometrically at 278 nm, assuming a molar absorption coefficient of $E^{0.1\%} = 0.638$ (36) and a molecular mass of 45,300 Da. Purified transducin (Gt) concentration was determined using the Bradford method (37).

**UV/Vis Spectroscopy.** Formation of the photoproduct metarhodopsin II (MII, $\lambda_{\text{max}} = 380$ nm) was assayed using the two-wavelength technique (9,38). This technique minimizes scattering artifacts by comparing the flash-induced changes in the absorbance at 380 and 417 nm. The absorbance change at 417 nm (MI isosbestic to MII) serves as a reference for determining the level of MII. The two-
A wavelength spectrophotometer (UV 300, Shimadzu Scientific Instruments, Inc., Japan; 2 nm slit) is equipped with thermostated cuvettes (2 mm path), temperature regulation (Circulator G/D8, Haake GmbH, Karlsruhe, Germany), and a green photoflash (filtered to 500 ± 20 nm).

When photolysed rhodopsin in its native disc membrane is cooled to temperatures at which the equilibrium is on the MI side (below 5°C and pH 8.0) (39), any specific binding of protein or peptide to MII causes enhanced formation of MII (extra-MII). Extra MII provides a kinetic and stoichiometric measure for the complex between photoactivated rhodopsin and the interactive polypeptide (40).
RESULTS

Light induced Interaction between Rhodopsin and Arrestin is Influenced by Synthetic Arrestin Peptides. The enhancement by arrestin of the MII photoprodutct formed after a flash of light ("extra MII") is specific for prephosphorylated rhodopsin and not seen with unphosphorylated rhodopsin (9,23). Fig. 1 shows two examples how synthetic peptides derived from the arrestin sequence influence the formation of extra MII from prephosphorylated samples. Arrestin peptide comprising residues 11-30 (Arr(11-30)) reduces the amount of extra MII formed in a concentration-dependent manner (Fig. 1A, compare traces b and c); at 1000 µM, Arr(11-30) abolishes extra MII nearly to the control level without arrestin (Fig. 1A, compare traces c and e). This effect is not observed with the arrestin peptide Arr(291-310); even at 1000 µM peptide, extra MII is formed as with arrestin alone (Fig. 1B, traces c and a). None of the peptides investigated in this study stabilized MII in the absence of arrestin (for example, Fig.1 traces d), in contrast to transducin C-terminal peptides (Gtα(340-350), Gtγ(50–71)-farnesyl; see (41)).

Arrestin Peptides Compete with Arrestin and Transducin. The inhibition of extra MII formation by arrestin peptides indicates that they compete with their parent protein for rhodopsin binding sites. To analyze this more quantitatively, overlapping sets of arrestin peptides with different length were synthesized, and their effect in the extra MII assay measured. Each of the peptides was titrated up to a final concentration of 1000 µM. Fig. 2 shows the data for two arrestin peptides, from stretches 51-70 and 231-250 (Arr(51-70) and Arr(231-250), respectively). Peptides with the original sequence, but not with a scrambled sequence, exhibit the dose-dependent effect on MII formation (Fig. 2A). The hyperbolic fits to the data are
consistent with a direct competitive effect of both peptides, although with different efficiency. Three sets of arrestin peptides, covering the entire arrestin sequence, were tested. Their IC\textsubscript{50} values, calculated dissociation constants (K\textsubscript{D}, see Appendix) and apparent Hill coefficients (n), are listed in Table I. High values of n may be due to a tendency of the peptide to aggregate and do therefore not enter the calculation in the Appendix.

Transducin forms enhanced MII complexes with photoactivated nonphosphorylated rhodopsin. It is further known that arrestin inhibits the activity of photoactivated rhodopsin towards the G-protein (7). We therefore investigated whether arrestin peptides can inhibit the transducin dependent formation of extra MII. As shown in Fig. 2B, the two peptides that compete with arrestin also compete with G\textsubscript{i} with similar relative efficiency, providing a control for direct interaction of the peptides with rhodopsin (see Discussion).

**Arrestin Peptides Compete with G\textsubscript{i}γ-farnesyl but not G\textsubscript{i}α C-terminal Peptides.** The question was whether arrestin peptides interfere with G\textsubscript{i} derived peptides in their interaction with photoexcited rhodopsin. One may expect that this can be tested by direct competition, because C-terminal peptides from G\textsubscript{i} stabilize the MII state like the holoprotein (41,42), but arrestin peptides do not (see above, Fig. 2). Fig. 3 shows for two examples that arrestin peptides can indeed competitively inhibit the formation of extra MII induced by G\textsubscript{i} derived peptides. The effect is specifically seen with the G\textsubscript{i}γ-farnesyl peptide, indicating an overlap of the respective binding sites at rhodopsin. No such inhibition was measured for the interaction with the G\textsubscript{i}α C-terminal peptide (native and high affinity analog).

As is seen in Fig. 4, almost all the peptides that compete with arrestin do so with G\textsubscript{i} and the G\textsubscript{i}γ C-terminal peptide as well. An exception is the region near the N-
terminus, which shows reduced competition with Gt and Gtγ-farnesyl peptide, as compared to arrestin (see Discussion).
DISCUSSION

Peptide competition as a monitor of arrestin-receptor interaction sites.
The data provide new information on the receptor binding sites of S-antigen, the arrestin responsible for termination of visual transduction in rods. Using overlapping sets of synthetic arrestin-derived peptides and their competition with the parent protein for the flash-activated phosphorylated receptor, we identified peptides from three regions of the arrestin sequence which competed with values lower than 200 µM. The respective parts of the sequence are located in both the N-terminal- and C-terminal domains of the protein structure.

The regions of peptide competition mark possible candidates for interaction sites with active phosphorylated rhodopsin. However, it is clear that peptide competition by itself is not specific. For example, the peptides could affect sites of intramolecular interaction, involved in conversions necessary to reach the active conformation. Since we know that such a conformational switch in arrestin occurs (see below), this latter possibility is quite realistic. A criterion was found in the comparison of arrestin with Gt and Gt peptides. The data in Fig. 4 have shown that most of the peptides that compete with arrestin, also compete with Gt and the farnesylated Gtγ C-terminal peptide. The loss of competition between position 65 and 83 is also reproduced. Because intramolecular switches in arrestin and Gt are unlikely to involve homologous domains, this similarity can be taken as a strong evidence for binding of arrestin and Gt or its γ-farnesyl peptide to rhodopsin’s surface with sufficient overlap (Fig. 5).

Multiple interaction sites. Our approach is similar to the one taken by Smith et al. (27), who employed overlapping synthetic peptides to determine regions of
arrestin that bind to rhodopsin. Using a phage-display technique of arrestin fragments and Gt binding and activation assays, these authors identified a stretch comprising residues 109-130 as a site involved in the interaction with rhodopsin. Because of the low affinity of this region (IC$_{50}$ of 1.1 mM) it was suggested that this portion of arrestin may be only one of several binding sites for rhodopsin (27). In the present study, using the fundamentally different extra MII assay, we find indeed peptide competition with similar IC$_{50}$ in this region (Table I), but stronger competition in other regions, namely residues 11-30 and 51-70 (IC$_{50}$ < 100 µM) and 231-250/241-260 (IC$_{50}$ < 200 µM). These regions and others with lower IC$_{50}$ are located in arrestin’s C- and N-terminal domains (Table I, Fig. 4). The reason for detection of these sites by our assay is probably the capturing of the interactions as they occur within seconds after the activating light flash.

The region near the N-terminus (11-30) shows strong competition but is only conserved in the visual arrestins, or S-antigens (43). This is also the region where the only significant difference between arrestin and Gt competition arises. Possible explanations include a role both in arrestin-rhodopsin interaction and in the switch mechanism (see below), in agreement with the conclusions drawn from mutational studies (19). In any case, the lack of conservation in this region would argue for a specific role in visual arrestins.

**Conformational switch.** S-antigen, and presumably all arrestins share with the G-proteins a conformational switch, operated by the contact with the active receptor (3,9,10,12,13). Based on structural assignments, Sigler and coworkers (18,19) have specified a trigger mechanism of the conformational switch, in which the phosphorylated C-terminus of the receptor interacts with the “polar core”, embedded between the N- and C-terminal domains in the fulcrum of the molecule. Upon this
interaction, intramolecular interactions, including a hydrogen-bonded network of buried ion pairs and salt bridges between charged side chains are disrupted, leading to structural changes, possibly involving an en bloc rearrangement of the N- and C-terminal domains (18).

Although the two published structures of arrestin are not in full agreement with each other in important details such as the location of the N- and C-termini, one may attempt to assign the identified binding sites to the available structural elements. The structures used in Fig. 6 (17) and (18) are both likely to represent the inactive conformation of the molecule (Hui-Woog Choe, personal communication). The sites identified in the present study are distant and do not form a flat surface. A conformational switch may thus be required to allow their simultaneous interaction with the relevant receptor loop structures. The situation is similar for the G-protein Gt, where two distant sites (the C-termini of the α- and the γ-subunits, which are 45 Å apart) are involved in the signal transfer (41). For arrestin and Gt (see Refs. (23) and (39), respectively), the simultaneous binding of two receptors at one molecule is unlikely because the titration of the complexes yields 1:1 ratios.

In spite of the similarity in conformational interlocking, arrestin and Gt appear to use different mechanisms of microscopic (i.e. site to site) recognition. For the G-protein, it is known that the Gtα and farnesylated Gtγ C-terminal sequences have the capacity to recognize the MII species and to distinguish it from the other intermediates (41,44). None of the numerous arrestin peptides examined displayed such specificity for the stabilization of MII (see Figs.1 and 2 for examples). Although we took advantage of this behavior when measuring competition with the stabilizing Gt peptides, we do not yet understand its molecular basis. All we can say is that the parent arrestin protein reserves the capacity to recognize the MII conformation with high selectivity.
Physiological function of the binding sites. We have identified some of the sites involved in the stable protein-protein interaction between arrestin and its receptor. However, it should be noted that this interaction may be short-lived in vivo; recent experiments in mice indicate that MII-arrestin complexes dissociate, to allow the reduction of all-trans-retinal (45). It remains to be studied how the necessary release of arrestin is induced and how it depends on the interaction sites identified here.
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Abbreviations: ROS: rod outer segment(s); GPCR: G protein-coupled receptor; MI: metarhodopsin I; MII: metarhodopsin II; P-MII: phosphorylated metarhodopsin II; Gt: retinal G-protein transducin; G-protein: guanine nucleotide-binding regulatory protein; Gtα: α subunit of transducin; Gtγ: γ subunit of transducin; Gtα(340-350): peptide corresponding to residues 340-350 of Gtα; Gtα(340-350)HAA: high-affinity analog of Gtα(340-350); Gtγ(50-71)-farnesyl: farnesylated peptide corresponding to residues 50-71 of Gtγ; BTP: 1,3-bis[tris(hydroxymethyl)-methylamino]propane; HEPES: 4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid.
**FIGURE LEGENDS**

Fig. 1

**Flash induced arrestin dependent formation of extra metarhodopsin II (extra MII) in the presence of synthetic arrestin peptides.** Prephosphorylated disc membranes were reconstituted with purified arrestin and various amounts of synthetic peptide. The signals represent the absorbance change at 380 nm minus the absorbance change at 417 nm; the abrupt decrease after the flash is due to rapid formation of MII precursors. In both A and B, trace (a) represents the sample without peptide, (b) with 100 µM peptide, (c) with 1000 µM peptide, (d) control without arrestin plus 1000 µM peptide, and (e) the control without arrestin and without peptide. A. Effect of arrestin peptide Arr(11-30) on extra MII formation. B. Effect of arrestin peptide Arr(291-310) on extra MII formation. In all measurements the final concentrations in the samples were 5 µM phosphorylated rhodopsin and 1 µM arrestin (except d, e). Experimental conditions: 100 mM HEPES pH 8.0 at 1°C, sample volume 200 µL, cuvette path length 2 mm, 12% of rhodopsin was photolyzed per flash.

Fig. 2

**Competition between synthetic arrestin peptides and arrestin or transducin for binding to photoactivated rhodopsin.** A. Inhibition by synthetic arrestin peptides of arrestin binding to phosphorylated metarhodopsin II. Extra MII assay as in Fig. 1. Experiments were performed with arrestin peptide Arr(51-70) (*top*) and Arr(231-250)
(bottom). B. Inhibition of transducin binding to metarhodopsin II with the same arrestin peptides as in A. Data points represent the normalized level of extra MII formed minus the control MII, as a function of added peptide, in the presence (circles) and absence (squares) of arrestin or Gi. In each panel, triangles represent the data for scrambled arrestin peptides, with the sequences (LVCQVYAEKVRGSLYKRTG) and (EIQLKKVYTVNVLVVKKEV) replacing arrestin sequences Arr(51-70) and Arr(231-250), respectively. Filled and empty symbols indicate the results obtained from different sets of experiments. Solid lines represent fits according to the hyperbolic equation \( f = (a-d)/(1+(x/c)^n) \) where \( a \) and \( d \) are the asymptotic maximum and minimum, \( x \) stays for peptide concentration, \( c \) for IC50, and \( n \) for the Hill coefficient. The experiments were otherwise performed as in Fig. 1.

Fig. 3

**Competition between synthetic arrestin peptides and synthetic C-terminal G\(_i\alpha\)-and G\(_i\gamma\) peptides for binding to photoactivated rhodopsin.** Inhibition of G\(_i\gamma\)(50-71)-farnesyl (cycles), G\(_i\alpha\)(340-350) native peptide (squares) and high-affinity analog peptide (VLEDLKSCGLF) (triangles) binding to unphosphorylated metarhodopsin by arrestin peptides. Extra MII assay as in Figs. 1 and 2. A. Inhibition by synthetic arrestin peptide Arr(51-70). B. Inhibition by peptide Arr(231-260), symbols as in A. Conditions as in Fig. 1, representation of the data and fits as in Fig. 2. Concentrations of G\(_i\) peptides are 2 µM, 500 µM and 200 µM for G\(_i\alpha\)(340-350)HAA, G\(_i\alpha\)(340-350) and G\(_i\gamma\)(50-71)-farnesyl, respectively.
Fig. 4

Inhibition of arrestin, G\textsubscript{i} and G\textsubscript{t}-peptide binding to metarhodopsin II by arrestin peptides. Bars represent IC\textsubscript{50} values of arrestin peptide inhibition of binding to metarhodopsin II, for arrestin (white), G\textsubscript{i} (black), and farnesylated G\textsubscript{t}\gamma C-terminal peptide (grey, expanded scale at the right). Rhodopsin was prephosphorylated in the case of arrestin. The ordinate cuts off at 500 µM. For better resolution of the binding domains, overlapping peptides (10-30 amino acids in length) were used.

Fig. 5

Reaction scheme of arrestin peptide binding and competition. After light excitation, metarhodopsin I (MI) or metarhodopsin II (MII) can bind the arrestin peptide. Arrestin (in its binding conformation A\textsubscript{b}) or transducin (G\textsubscript{i}) bind and stabilize MII at the expense of MI. Arrestin peptide competes with both proteins, thus reducing the level of extra MII formed.

Fig. 6

Arrestin rhodopsin interaction – binding sites of arrestin from peptide competition. Data are taken from Table I and Fig. 4. Structures in A and B are from Refs. (17) and (18), respectively; in each case, molecule B of the unit cell is shown in a (ribbon drawing) and b (space filling model). The approximate affinities of the sites, as estimated from IC\textsubscript{50} of peptide competition are color coded, as indicated. The
picture in the center of the Figure represents the rhodopsin structure in the ground state (46), with the cytoplasmic loops in green and the retinal in purple. The distance bar refers to both the arrestin and rhodopsin structures.
Table I: **Inhibition of arrestin binding to phosphorylated metarhodopsin II by synthetic arrestin peptides**

| Peptide (amino acids) | IC$_{50}$ (µM) | n  | KD$_{3}$ (µM) | Peptide (amino acids) | IC$_{50}$ (µM) | n  | KD$_{3}$ (µM) | Peptide (amino acids) | IC$_{50}$ (µM) | n  | KD$_{3}$ (µM) |
|-----------------------|----------------|----|---------------|-----------------------|----------------|----|---------------|-----------------------|----------------|----|---------------|
| 1-20                  | >500           | ND*| >200          | 11-30                 | 76             | 3.9| 30           | 1-30                  | 39             | 3.1| 16           |
| 21-40                 | ND*            | ND*| ND*          | 31-50                 | 270            | 0.9| 108          | 65-83                 | >500           | ND*| >200         |
| 41-60                 | 198            | 2.0| 79           | 51-70                 | 25             | 8.6| 10           | 109-130               | >500           | ND*| >200         |
| 61-80                 | ND*            | ND*| ND*          | 71-90                 | ND*           | ND*| ND*         | 231-260               | 99             | 2.0| 40           |
| 81-100                | >500           | ND*| >200         | 91-110                | >500           | ND*| >200        | 301-310               | >500           | ND*| >200         |
| 101-120               | >500           | ND*| >200         | 111-130               | >500           | ND*| >200        | 311-320               | >500           | ND*| >200         |
| 121-140               | >500           | ND*| >200         | 131-150               | >500           | ND*| >200        | 321-330               | >500           | ND*| >200         |
| 141-160               | >500           | ND*| >200         | 151-170               | 374            | 1.8| 150          |                       |                |    |              |
| 161-180               | >500           | ND*| >200         | 171-190               | >500           | ND*| >200        |                       |                |    |              |
| 181-200               | >500           | ND*| >200         | 191-210               | ND*           | ND*| ND*         |                       |                |    |              |
| 201-220               | >500           | ND*| >200         | 211-230               | ND*           | ND*| ND*         |                       |                |    |              |
| 221-240               | >500           | ND*| >200         | 231-250               | 135            | 2.2| 54           |                       |                |    |              |
| 241-260               | 167            | 1.9| 67           | 251-270               | >500           | ND*| >200        |                       |                |    |              |
| 261-280               | ND*            | ND*| ND*         | 271-290               | >500           | ND*| >200        |                       |                |    |              |
| 281-300               | >500           | ND*| >200         | 291-310               | >500           | ND*| >200        |                       |                |    |              |
| 301-320               | 363            | 1   | 145        | 311-330               | >500           | ND*| >200        |                       |                |    |              |
| 321-341               | ND*            | ND*| ND*         | 331-351               | >500           | ND*| >200        |                       |                |    |              |
| 342-362               | >500           | ND*| >200         | 352-372               | >500           | ND*| >200        |                       |                |    |              |
| 363-383               | ND*            | ND*| ND*         | 373-393               | >500           | ND*| >200        |                       |                |    |              |
| 384-404               | >500           | ND*| >200         |                       |                |    |              |                       |                |    |              |

IC$_{50}$ and the Hill coefficient (n) were determined employing the extra MII assay (Fig. 2). KD$_{3}$ values were estimated assuming the reaction scheme as described in the Appendix.

* Not determined
APPENDIX

The following reaction scheme was used to determine the $K_{D3}$ values:

\[
 K_{A1} \\
 MI \rightleftharpoons MII \\
 K_{A2} \\
 MII + A \rightleftharpoons MII \cdot A \\
 K_{A3} \\
 R^* + P \rightleftharpoons R^* \cdot P \quad (R^* = MI + MII)
\]

where MI and MII are metarhodopsin I and II, A is arrestin and P represent different arrestin peptides, respectively.

The solution for $K_{D3}$ requires three equilibrium equations:

\[
 K_{A1} = \frac{[MII]}{[MI]} \quad (1) \\
 K_{A2} = \frac{[MII \cdot A]}{[MII] \cdot [A]} \quad (2)
\]
\[ K_{A3} = \frac{[R^*P]}{[R^*][P]} \]  

and three conservation equations:

\[ [R^*]_0 = [MI] + [MII] + [MII A] + [MIP] + [MII P] \]  

\[ [A]_0 = [A] + [MII A] \]  

\[ [P]_0 = [P] + [MIP] + [MII P] \]  

[MI], [MII], [A], and [P] represent the molar concentration of MI, MII, A and P, respectively. The MI-MII equilibrium constant \( K_{A1} \) was calculated according to Parkes et al. (39), \( K_{A2} \) was determined using the \( K_D \) from the arrestin rhodopsin complex (23). \([R^*]_0, [A]_0 \) and \([P]_0 \) are the total concentration of photolyzed rhodopsin, arrestin and arrestin peptides, respectively. By use of eqs 1 to 6, \( K_{D3} \) can be expressed as:

\[ K_{D3} = \left( \frac{K_{A1} \cdot K_{A2}}{K_{A1} + 1} \right) \cdot \left( [A]_0 - \frac{[R^*]_0}{2} - 1 \right)^{-1} \cdot IC_{50} \]  

The \( IC_{50} \) value represent the arrestin peptide concentration where the extra MII signal is inhibited to 50%.

Note: Peptide binding to R or \( R^* \) led to very similar \( K_{D3} \) values (errors < 1%).
Arr(11-30)

\[ \Delta A = 0.002 \]

Arr(291-310)

\[ \Delta A = 0.001 \]

Fig. 1
Figure 2

(A) Arrestin

(B) Transducin

Peptide Concentration (M) vs. extra meta II (%)

Arr(51-70) vs. scrambled vs. native

Arr(231-250) vs. scrambled vs. native

Fig. 2B Transducin
Fig. 3

Peptide Concentration (M)

A

extra meta II (%)

G_t-\alpha peptides

G_t-\gamma-far peptide

Arr(51-70)

B

extra meta II (%)

G_t-\alpha peptides

G_t-\gamma-far peptide

Arr(231-260)
Fig. 4

IC₅₀ (M)

Arrestin Peptides

IC₅₀ (M)
Fig. 5

M I

M II

Arrestin Peptide

Gt

α

βγ

M II

A_b

M II

→ Arrestin Peptide
Fig. 6

IC50 < 100 M
IC50 < 200 M
IC50 < 300 M
polar core
C-terminus

A

B

20 Å
