

# Chapter 8

## How Arrestin Recognizes and Binds Active GPCRs

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**Abstract** Arrestins are structurally flexible and functionally versatile proteins that regulate the activity of hundreds of different G protein-coupled receptors (GPCRs). A hallmark of arrestin function is that these proteins are only activated for binding the active receptor upon interaction with receptor-attached phosphate groups. Recent years have yielded crystal structures of pre-activated arrestins and arrestin in complex with an active receptor, which provide insight into the arrestin activation mechanism. At the same time, functional studies indicate that arrestin employs different binding modes along the path to tight receptor binding, and the structure of the arrestin-receptor complex is modulated by the activation and phosphorylation state of the active receptor. In this chapter we discuss our current understanding of the receptor-binding mechanism of arrestin, from the initial interaction with the phosphorylated receptor to the structural transformation required for tight binding to the active receptor.

**Keywords** Arrestin · Rhodopsin · GPCR · Fluorescence spectroscopy · Mutagenesis

About 800 different G protein-coupled receptors (**GPCRs**) are expressed in the human body and play a central role in multiple sensory and physiological systems. These receptors bind a wide variety of ligands and share a common structure of seven transmembrane (TM) helices. The receptor exists in a conformational equilibrium comprised of multiple states of varying degrees of activity. In the basal inactive state, the helical bundle is bound together by several hydrogen bond networks and electrostatic interactions. The binding of agonist favours an active receptor conformation, in which the cytoplasmic face of the receptor is open for interaction with cytosolic proteins. The binding partners of the receptor are namely G protein, GPCR kinase (GRK), and arrestin.

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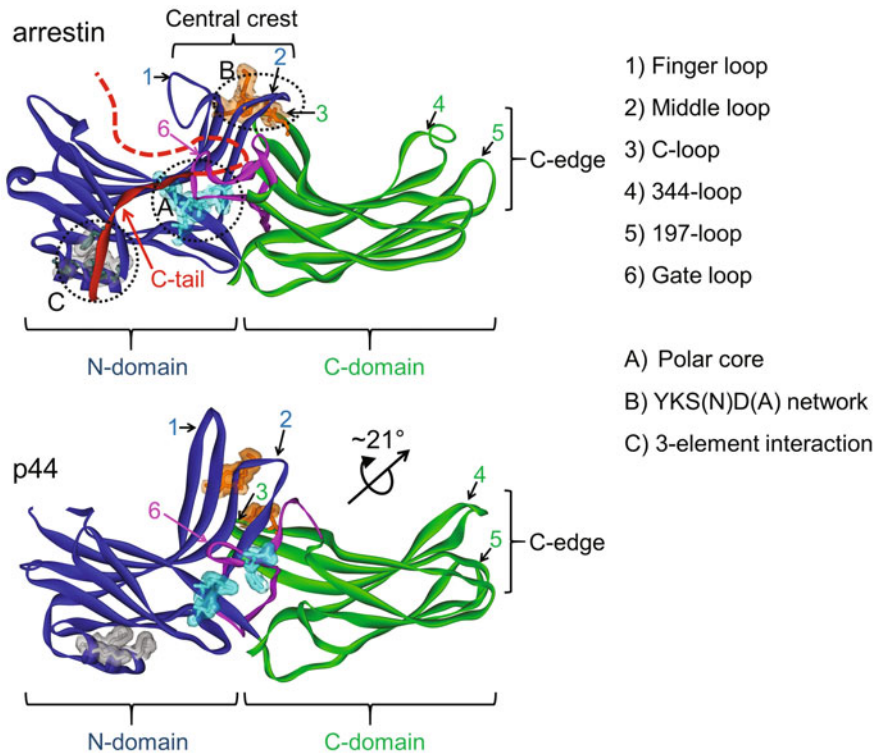
G proteins consist of three subunits, termed  $\alpha$ ,  $\beta$  and  $\gamma$ , and different subunit combinations give rise to about 20 distinct G proteins. Binding of the heterotrimeric G protein to the active receptor induces nucleotide exchange within the G protein and dissociation of the subunits, which then interact with other components of the cell signalling machinery. In essence, the receptor transduces the signal of agonist binding across the cell membrane, and the G protein amplifies this signal within the cytoplasm.

Seven different GRKs exist, each of which phosphorylates a different subset of GPCRs. The kinase activity of GRKs is stimulated by interaction with activated GPCRs. GRKs phosphorylate several serine and threonine residues within the cytoplasmic C-terminal tail of the receptor. Some receptors are also phosphorylated on their cytoplasmic loops. Only three receptor-attached phosphates are required to stimulate arrestin binding to the active GPCR (Vishnivetskiy et al. 2007), yet most GPCRs contain 7 or more phosphorylation sites. The extra sites confer another level of regulation, as different phosphorylation patterns (also called “barcodes”) control the multiple functions of arrestins [reviewed in Reiter et al. (2012)]. Different phosphorylation barcodes arise from different C-terminal primary sequence, tissue-specific or ligand-selected GRK activity, or sequential rounds of receptor activation.

The arrestin family is composed of only four members. Arrestin-1 and arrestin-4, respectively called rod and cone arrestin, are expressed primarily in the retina and interact with light-sensitive GPCRs called opsins. Arrestin-2 and arrestin-3, respectively called  $\beta$ -arrestin 1 and  $\beta$ -arrestin 2, are expressed ubiquitously and interact with hundreds of different GPCRs. This attribute means the  $\beta$ -arrestins must be versatile and relatively promiscuous binding partners. A primary role of arrestin binding is to block G protein coupling and thereby stop GPCR signalling. The  $\beta$ -arrestins are additionally able to induce receptor internalization by recruiting clathrin and other elements of the cellular internalization machinery, as well as mediate their own signalling cascades by scaffolding signalling kinases. In recent years evidence has emerged that the pattern of receptor phosphorylation affects both the conformation of the bound arrestin (Shenoy et al. 2009; Nobles et al. 2011) as well as the stability of the arrestin-GPCR complex (Zindel et al. 2014; Oakley et al. 2001; Tohgo et al. 2003). These factors presumably control arrestin-mediated receptor internalization and trafficking (Oakley et al. 2001; Zindel et al. 2014) and arrestin-mediated MAP kinase activity (Tohgo et al. 2003; Ren et al. 2005; Nobles et al. 2011) [reviewed in Tobin et al. (2008)].

Arrestins are composed of two cup-like domains composed of  $\beta$ -sheets, called the N- and C-domain (Fig. 8.1). The tips of the domains are capped with flexible loops, which compose the **central crest** (finger loop, middle loop and C-loop) and the **C-edge** (344-loop and 197-loop). The interdomain interface is stabilized by hydrophobic interactions and hydrogen-bond networks. These include the polar core (Han et al. 2001), the YKS(N)D(A) motif (Kim et al. 2013), the middle loop (*a.k.a.* 139-loop) (Vishnivetskiy et al. 2013), and the long Loop 17-18 (Kim et al. 2013; Han et al. 2001), which winds between the two domains and makes multiple contacts with both the N- and C-domains. Loop 17-18 also includes the gate loop,

which is a functional part of the polar core (Fig. 8.1). A third functionally critical domain of arrestin is the auto-inhibitory C-terminal tail (**C-tail**). The *distal C-tail* contains a large number of acidic residues and is highly dynamic, as it is not visualized in any known structure of arrestin. In contrast, the *proximal C-tail* is stably anchored to the body of the N-domain by the hydrophobic 3-element interaction (Vishnivetskiy et al. 2000) and a salt bridge within the polar core. Together, the interdomain interface and the C-tail restrict flexibility within arrestin, namely of the central crest loops and between the N- and C-domains, and thereby prevent interaction with the active receptor.



**Fig. 8.1** Crystal structures of basal arrestin (PDB code 1CF1, molecule D) and pre-activated arrestin p44 (PDB code 4J2Q, molecule B) (Kim et al. 2013; Hirsch et al. 1999). The N-domain is coloured *blue* and the C-domain is coloured *green*. The C-tail of arrestin is *red*, and Loop 17-18 is *magenta*. Note that only the proximal C-tail is resolved in the arrestin crystal structure, and the unresolved dynamic distal portion is illustrated with a *red dashed line*. The loop linking the C-domain to the C-tail is also not resolved in the arrestin crystal structure. Notable loops that are discussed in the text are labelled with numbers, which are defined in the legend to the *right*. Important intramolecular interactions that stabilize the basal state are illustrated by sidechains with Van der Waal surfaces and labelled with capital letters (defined in legend). Note how these networks are broken in the p44 structure, which results in a  $\sim 21^\circ$  rotation of the C-domain compared to its orientation in the basal arrestin

It has long been believed that arrestin activation entails a displacement of the C-tail by the phosphorylated receptor C-terminus (**Rpp**), based on changes in protease susceptibility (Palczewski et al. 1991a, c), distances measured between spin-labels (Hanson et al. 2006; Vishnivetskiy et al. 2010), and changes in the NMR spectrum of arrestin (Zhuang et al. 2010, 2013). This theory is consistent with the phosphorylation-independent receptor binding behaviour of **p44**, a naturally occurring splice variant of arrestin-1 that lacks the entire C-tail (Palczewski et al. 1994; Pulvermüller et al. 1997). Supposedly C-tail displacement by the Rpp breaks the polar core and loosens the intramolecular interactions that hold arrestin in an inactive state. Two crystal structures of pre-active arrestin, p44 (Kim et al. 2013) and arrestin-2 bound to a peptide analogue of the Rpp (Shukla et al. 2013), indicate the conformational changes that occur in arrestin upon full C-tail displacement (Fig. 8.1). These changes include a twisting displacement of the gate loop that breaks the polar core, release of the central crest loops from their restricted basal conformations, and a 21° rotation of the two domains relative to one another.

In the recently published crystal structure of arrestin-1 in complex with an active GPCR (Kang et al. 2015), it is clear how these structural changes facilitate arrestin binding of the active receptor (discussed in more detail below). The complex consists of a constitutively active mutant of human rhodopsin fused on its N-terminus to T4 lysozyme and fused on its C-terminus to a constitutively active mutant of mouse arrestin-1 via a 15 amino-acid linker. Although the receptor in this structure lacks ligand (opsin), it is obviously in an active state (Ops\*). Likewise, the arrestin in this structure shows all the hallmarks of activation seen in the pre-activated arrestin structures (Kim et al. 2013; Shukla et al. 2013), even though the receptor in the complex is not phosphorylated. Despite the artificial nature of the Ops\*/arrestin-1 fusion complex structure, it provides a first glimpse of arrestin bound tightly to an active receptor.

This chapter focuses on the molecular mechanisms by which arrestin couples to an active, phosphorylated GPCR. We discuss the initial interaction of arrestin with the phosphorylated receptor, which we call the pre-complex, and the conformational changes in arrestin involved in formation of the high-affinity complex. We have gained insight into these functionally distinct complexes using the long-studied GPCR rhodopsin and its binding partner arrestin-1, which are expressed in the rod cells of the retina and mediate dim-light vision. Dark-state inactive rhodopsin consists of the aporeceptor opsin and a covalently attached inverse agonist, 11-*cis*-retinal. Light absorption converts the ligand to the agonist all-*trans*-retinal, which results in the active receptor form Metarhodopsin II (**Meta II**). Meta II is phosphorylated by GRK1 on its cytoplasmic C-terminal tail. As an experimental model of the pre-complex, we study the interaction of arrestin-1 with phosphorylated inactive forms of the receptor, specifically dark-state phosphorylated rhodopsin (**Rho-P**) and the phosphorylated aporeceptor opsin (**Ops-P**). Using these receptor forms, we can experimentally probe arrestin-receptor interactions that occur before full arrestin activation and formation of the high-affinity complex. Transition to the high-affinity complex is induced by irradiation of the sample with visible light, which converts Rho-P to Meta II-P. Methods we have used to study

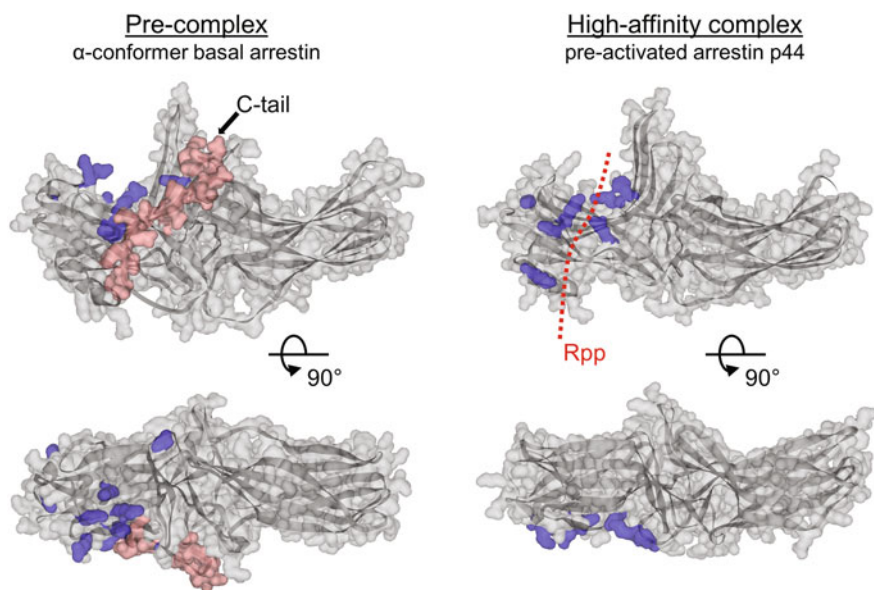
arrestin-receptor interactions include site-directed fluorescence spectroscopy, Fourier transform infrared spectroscopy (FTIR), and alanine scan mutagenesis. The functional insights we gain from these studies are interpreted within the context of the crystal structures of different arrestins in basal (Hirsch et al. 1999; Granzin et al. 1998; Han et al. 2001; Granzin et al. 2012), pre-activated (Granzin et al. 2015; Kim et al. 2013; Shukla et al. 2013), and fully activated states (Kang et al. 2015). This approach has yielded valuable insights that allow us to propose a mechanistic model of arrestin activation and receptor coupling.

## The Pre-complex

The initial interaction of the phosphorylated receptor and arrestin recruits arrestin to the active receptor and activates arrestin for binding the helical bundle of the receptor. Currently very little is known regarding the structure and organization of the pre-complex. Experimental evidence suggests the primary sites of interaction are the phosphorylated receptor C-terminus (Gurevich and Benovic 1993, 1995; Vishnivetskiy et al. 2000; Hanson and Gurevich 2006; Peterhans et al. 2016) and the membrane (Lally et al. 2017). The pre-complex was first proposed by Alexander Pulvermüller, Klaus Peter Hofmann and colleagues (IMPB Charité, Berlin) based on kinetic binding experiments comparing arrestin-1 and p44 (Schröder et al. 2002). This proposal was based on the conclusion that p44 can bypass the initial binding step, which serves normally to displace the C-tail of arrestin. Subsequently two independent studies have presented time-resolved fluorescence data supporting a multi-step binding mechanism, in which arrestin is recruited to the receptor before undergoing a significant conformational change (Kirchberg et al. 2011; Nuber et al. 2016). We interpret these two stages to represent, first formation of the pre-complex, and second, transition to the high-affinity complex.

It has been long known that arrestins intrinsically bind poly-anions, including heparin and inositol phosphate (Palczewski et al. 1991b), as well as receptor-attached phosphate groups [but not simple anions like phosphate (Wilson and Copeland 1997)]. The concave surfaces of all four arrestin subtypes are studded with basic residues that could serve as binding sites for poly-anions. Crystallographic analysis indicated that two molecules of IP6 bind arrestin-2, one each within the concave surfaces of the N- and C-domains (Milano et al. 2006). IP6 binding facilitates homo- and hetero-dimerization of arrestin-2 and arrestin-3. IP6 also binds arrestin-1, although with much lower affinity [ $K_D$  in the range of 0.2–160  $\mu\text{M}$  (Palczewski et al. 1991b; Wilson and Copeland 1997; Zhuang et al. 2010)]. NMR analysis indicated that IP6 binds within the N-domain of arrestin-1 (Zhuang et al. 2010). This NMR analysis did not detect any binding of IP6 within the C-domain, although site-directed fluorescence experiments suggest a second low-affinity IP6 binding site, most likely in the C-domain (Sommer, unpublished work).

Mutagenesis has long been applied to arrestin to identify potential receptor-interacting sites, and to differentiate which sites are sensitive to the phosphorylation and activation states of the receptor [reviewed in (Gurevich et al. 2011)]. Alanine scan mutagenesis is an unbiased and comprehensive method that has been recently applied to arrestin-1 by the group of Joerg Standfuss (PSI, Switzerland) to identify sites of interaction and functionally important areas that undergo structural changes during arrestin activation and receptor binding (Ostermaier et al. 2014a; Peterhans et al. 2016). For the most part, the alanine-scan studies confirmed the findings from previous mutagenesis studies (Ostermaier et al. 2014a, b). In collaboration with the Standfuss group, we found evidence that the phosphorylated receptor C-terminus binds arrestin differently in the pre-complex and the high-affinity complex (Fig. 8.2). For the interaction of arrestin-1 with Ops-P, which mirrors the pre-complex, mutation of ten different basic residues within the cup of the N-domain significantly decreased arrestin-1 binding to Ops-P.



**Fig. 8.2** Different “Rpp-binding footprints” for the pre-complex and the high-affinity complex. Alanine scan mutagenesis identified different sets of positively charged residues used in binding the phosphorylated receptor C-terminus (Rpp) in binding inactive Ops-P (pre-complex, *left*) and light-activated Meta II-P (high-affinity complex, *right*) (Peterhans et al. 2016). The implicated residues are plotted in *blue* on the structures of basal arrestin ( $\alpha$ -conformer, PDB code 1CF1) for the pre-complex and p44 (PDB code 4J2Q). Note that for basal arrestin, the distal C-tail (*rose*) does not block access to the implicated phosphate-binding residues, which is consistent with our hypothesis that only the proximal C-tail is displaced in the pre-complex. The putative Rpp-binding crevice that was identified in the crystal structure of activated arrestin-2 bound to a peptide analogue of the Rpp (Shukla et al. 2013) is indicated by a *dashed red line*. Lateral views of arrestin and p44 are shown in the *top panels*, and top-down views (showing receptor binding interface) are presented in the *bottom panels*

The large number of implicated phospho-sensing residues is congruent with the fact that the affinity of arrestin-1 for Ops-P is directly proportional to the degree of receptor phosphorylation (i.e. the more phosphates per receptor, the higher the affinity) (Vishnivetskiy et al. 2007). This binding mode stands in contrast to that used for Meta II-P, in which fewer residues along the lateral side of the N-domain are implicated (Fig. 8.2). The Rpp binding mode utilized by Meta II-P is discussed in more detail in the section below.

If Rpp binds only within the cup of the N-domain, then it is possible that only the distal C-tail of arrestin is displaced in the pre-complex. This assumption is supported by site-directed fluorescence experiments using a mutant designed to monitor the relative position of the gate loop and, by extension, the state of the polar core (Kim et al. 2013; Peterhans et al. 2016). These experiments suggest that the polar core is not broken and thus the proximal C-tail is likely not displaced when arrestin is bound to Ops-P in a pre-complex. Only upon activation of the receptor and transition to the high-affinity complex is the polar core broken. Furthermore, site-directed fluorescence experiments employing mutants that report on other conformational changes associated with activation (e.g. release of central crest loops and interdomain rotation) suggest that arrestin is bound to the receptor in a conformational state resembling the basal state in the pre-complex (Lally and Sommer, unpublished work). This supposition is consistent with differences seen in the functional maps for pre-complex and high-affinity complex derived from alanine-scan mutagenesis (Peterhans et al. 2016). Collectively the fluorescence and mutagenesis data argue against the common assumption that Rpp binding in the pre-complex displaces the entire C-tail and induces activating conformational changes in arrestin (Shukla et al. 2013; Kang et al. 2015; Schröder et al. 2002).

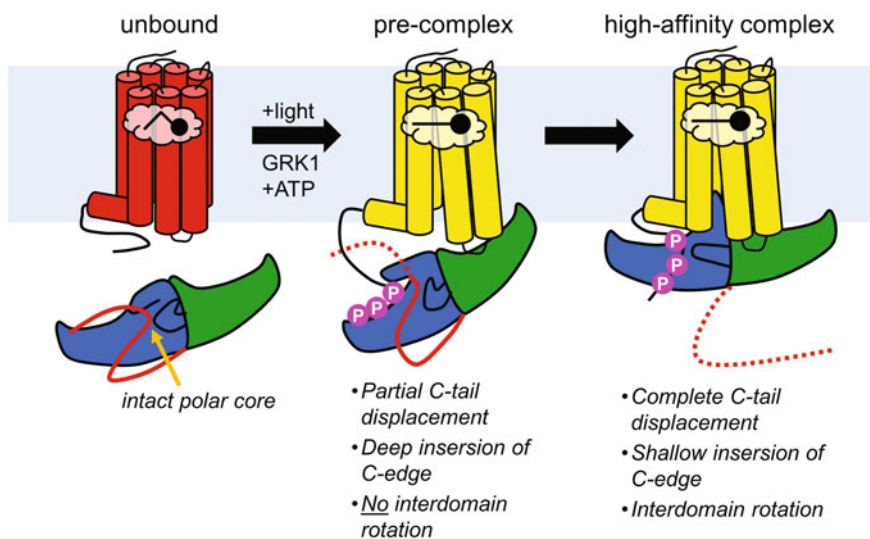
The other site of interaction in the pre-complex is the anchoring of the C-edge of arrestin in the membrane. This interaction has recently been characterized by our group using site-directed fluorescence spectroscopy (Lally et al. 2017). Briefly, the proximity of bimane fluorophores placed at specific sites on loops within the C-edge to the membrane was probed using spin-labelled fatty acids, which were incorporated into native rod outer segment membranes containing phosphorylated rhodopsin. Spin-labels quench bimane fluorescence when in close proximity, and fatty acids with spin label at different positions on the acyl chain allowed the differentiation of deep and shallow membrane anchoring. Our results clearly show that the 344-loop embeds deep within the hydrophobic layer of the membrane when arrestin is bound to dark-state Rho-P in a pre-complex. Furthermore, membrane anchoring is dependent on the presence of phosphorylated receptor, suggesting that Rpp binding either activates the C-edge of arrestin for membrane anchoring or is required to recruit arrestin to the membrane. The relative levels of quenching for neighbouring sites suggest an extended 344-loop conformation (Lally et al. 2017), similar as in the “ $\alpha$ -conformer” of the basal arrestin-1 structure (Hirsch et al. 1999).

Membrane anchoring in the pre-complex involves the embedding of leucine residues on the 344-loop within the hydrophobic interior of the membrane. Intriguingly, rhodopsin flips negatively charged acidic phospholipids to the cytoplasmic side upon activation (Hessel et al. 2000, 2001), and acidic phospholipids



have been shown by us and others to be necessary for arrestin-1 binding of light-activated phosphorylated rhodopsin (Sommer et al. 2006; Bayburt et al. 2011). Furthermore, we have failed to detect any pre-complex formation of arrestin-1 with dark-state Rho-P in the absence of acidic phospholipids (Sommer, unpublished work). These data indicate that acidic phospholipids are required for arrestin to interact with the phosphorylated receptor. Notably, several basic residues line the C-domain (e.g. K235, K236, K238, K267, K330, and K332 in bovine arrestin-1), which we hypothesize help attract the C-edge of arrestin for membrane anchoring.

To summarize the experimental evidence, in the pre-complex arrestin is bound by the Rpp within the cup of the N-domain and by the membrane at the C-edge (Fig. 8.3). Given the relatively deep insertion of the membrane anchor, the peripheral engagement of the Rpp, and the fact that the central crest loops do not engage the receptor, arrestin is likely loosely associated with the receptor in the pre-complex. A great degree of rotational freedom and a highly dynamic complex is expected. Interestingly, a “hanging” interaction mode of arrestin-2 with a chimeric phosphorylated GPCR was observed by negative-stain electron microscopy (Shukla et al. 2014). The authors of this study hypothesized this complex represents tethering of arrestin solely by the Rpp. Due to the artificial nature of the Fab-stabilized



**Fig. 8.3** Model of the pre-complex and high-affinity complex. Binding of arrestin to rhodopsin is illustrated. Basal arrestin (blue N-domain and green C-domain) has an intact polar core and does not interact with dark-state rhodopsin (red). Upon receptor light-activation (Meta II, yellow) and phosphorylation by GRK, arrestin interacts in a pre-complex that displaces the distal C-tail and embeds the C-edge membrane deep in the membrane. However, no significant activating conformational changes (e.g. interdomain rotation) take place. Upon transition to the high-affinity complex, the Rpp moves from the cup of the N-domain to the putative Rpp-binding trench located along the side of the N-domain. This movement displaces the entire C-tail and results in significant conformational changes linked to interdomain rotation



complexes and the absence of membrane (receptors were solubilized in a neutral detergent), we postulate the visualized complexes represent the pre-complex without the benefit of membrane anchoring. Hence future investigations of the overall organization and structure of the pre-complex should include membrane or a membrane mimic in order to engage the C-edge of arrestin.

Notably, pre-complex formation as the initial interaction of arrestin and the receptor is fairly nonspecific and likely similar for all GPCRs. The interaction with the Rpp and the membrane is primarily electrostatic and hydrophobic, respectively. The nonspecific nature of these interactions allow arrestins to interact with a wide variety of GPCRs, since active receptors are normally phosphorylated in their active state, and all receptors reside in a membrane. This attribute makes sense for the  $\beta$ -arrestins (arrestin-2 and arrestin-3), which couple to hundreds of different GPCRs.

However, the functional relevance of the pre-complex is not limited to being an obligate step before forming the high-affinity complex. Arrestin also forms complexes with phosphorylated inactive receptors, which occur when the receptor returns to an inactive state after ligand dissociation and before receptor dephosphorylation occurs (Lee et al. 2010; Sommer et al. 2014; Zhuang et al. 2013). Inactive receptors in the vicinity of active receptors can also be phosphorylated by activated GRKs, a phenomenon called high-gain phosphorylation (Shi et al. 2005; Binder et al. 1990, 1996). The interaction of arrestin with inactive phosphorylated receptors could play a significant physiological role. For example, in the visual system binding of arrestin-1 to Ops-P could quench the residual G protein-activating ability of opsin (Sommer et al. 2014; Zhuang et al. 2013). In addition, our group discovered that arrestin-1 facilitates the uptake of all-*trans*-retinal in Ops-P (Sommer et al. 2012), which could limit the build-up of potentially toxic levels of retinal in rod cells in bright continuous light (Sommer et al. 2014). The fact that most GPCRs have seven or more phosphorylation sites suggests the interaction of arrestins with highly phosphorylated aporeceptors might be prevalent within the larger GPCR family and could play a role in regulating ligand binding affinity (Gurevich et al. 1997).

## Transition to the High-Affinity Complex

Pre-complex formation serves to recruit arrestin to the receptor, where arrestin is anchored at the N-domain by the Rpp and at the C-edge by the membrane (Fig. 8.3). Although arrestin is not fully activated in the pre-complex, the displacement of the distal C-tail increases flexibility and mobility of the central crest loops. Importantly, pre-complex formation brings the receptor-binding elements of arrestin close to the helical core of the receptor. Hence the two proteins can interact with one another as they sample their individual conformational spaces. Transition from the pre-complex to the high-affinity complex involves a substantial intramolecular conversion in arrestin, which mutually stabilizes the active form of

the receptor. Agonist binding favours an active conformation of the receptor, and engagement by arrestin further stabilizes this active conformation. These conclusions are supported by spectroscopic studies of arrestin-1 binding to light-activated phosphorylated rhodopsin, which are briefly summarized below.

Over twenty five years ago, Klaus Peter Hofmann and co-workers developed an absorbance-based assay called “extra Meta II” to observe the formation of the high-affinity arrestin-1/Meta II-P complex (Schleicher et al. 1989). Light-activation of rhodopsin results in an equilibrium of two species, the active state Meta II and its inactive precursor Meta I. At low temperature and high pH (e.g. 2 °C and pH 8), the Meta I photoproduct is heavily favoured. When the receptor is phosphorylated and arrestin-1 interacts, Meta II is stabilized at the expense of Meta I. In other words, arrestin-1 binding shifts the Meta I  $\leftrightarrow$  Meta II equilibrium in favour of Meta II. Importantly, the extra Meta II assay can be used to quantitatively describe the formation of the high-affinity arrestin-receptor complex. Arrestin-1 activation and formation of the high-affinity complex entails a high activation energy (140 kJ/mol). For p44, the energy required for Meta II-P binding is halved (70 kJ/mol) (Pulvermüller et al. 1997), suggesting that about half the energy of activation is involved in releasing the intramolecular restrictions of the C-tail. At sufficiently high concentrations, Meta II-P binding by arrestin-1 and p44 occur at the same rate, which suggests that both arrestin-1 and p44 undergo the same intramolecular conversion, and that this step is rate-limiting for formation of the high-affinity complex (Schröder et al. 2002). Our group has recently characterized this interaction using FTIR spectroscopy (Beyriere et al. 2015). The observed spectral signatures confirmed the stabilization of the active Meta II species by arrestin-1 and additionally indicated a loss of beta-sheet, most likely arising from changes in the secondary structure of arrestin-1. Importantly, Meta II stabilization and beta-sheet loss in arrestin occurred at the same rate, indicating that both proteins mutually stabilize one another in their active states during formation of the high-affinity complex. Moreover, p44 and arrestin showed the same rates of Meta II binding and spectral signatures of beta-sheet loss, indicating that they undergo the same structural transition during high-affinity complex formation (Beyriere et al. 2015).

The comparison of the structure of basal arrestin to that in the Ops\*/arrestin-1 fusion complex indicates the intramolecular conversions that arrestin undergoes during tight receptor binding. Below we summarize the major conformational changes, and how these changes facilitate coupling to the active phosphorylated receptor:

*Displacement of the proximal C-tail*—In the pre-complex, the Rpp is bound within the cup of the N-domain, and transition to the high-affinity complex is accompanied by a movement of the Rpp to a positively charged binding trench on the lateral side of the N-domain (Fig. 8.3). This trench is normally obscured by the proximal C-tail, and the Rpp presumably displaces the C-tail. A peptide analogue of the Rpp has been observed by X-ray protein crystallography to bind within this trench (Shukla et al. 2013). This crystal structure shows that peptide-attached phosphate groups interact with many of the residues identified as phospho-sensors

for Meta II-P binding (e.g. K14, K15, R29, K110, K300) (Fig. 8.2) (Peterhans et al. 2016). Displacement of the C-tail and binding of the Rpp induces a forward twisting movement of the gate loop, which breaks the polar core and brings a phosphate-binding lysine residue on the gate loop (K300 in arrestin-1, K294 in arrestin-2) into the Rpp-binding trench.

*Interdomain rotation*—The binding of the Rpp within the putative binding trench is associated with a movement of the gate loop. This conformational change is transmitted along the entire length of Loop 17-18, which winds between the N- and C-domains and forms multiple contacts that stabilize the basal-state interdomain interface (Fig. 8.1). Loop 17-18 is pulled down and away from the central crest region, thereby causing a lateral displacement of the middle loop away from the body of arrestin (Kim et al. 2012; Kang et al. 2015) and a movement of the C-loop (Y247-Y254 in arrestin-1) down and away from the central crest. These movements break the YKS(N)D(A) network, which stabilizes basal arrestin by linking the C-loop (Y247-Y254 in arrestin-1) within the C-domain to the middle and finger loops within the N-domain. Along with the breaking of the polar core, these conformational rearrangements dramatically increase flexibility between the two domains of arrestin. Hence a 21° rotation of the C-domain is observed in the crystal structures of pre-activated and receptor-bound arrestin (Kim et al. 2013; Shukla et al. 2013; Kang et al. 2015). Interdomain rotation has two major effects on the arrestin structure: a crevice opens within the central crest, and the C-edge adopts a different orientation and conformation. These changes facilitate arrestin binding to the receptor and the adjacent membrane.

*Opening of receptor-binding crevice*—The lateral displacement of the middle loop and the downward movement of the C-loop opens a cleft that accommodates intercellular loop 2 (CL2) of the receptor. In the Ops\*/arrestin-1 fusion complex structure, CL2 forms a short helix that makes hydrophobic and van der Waals contacts with the middle loop and C-loops of arrestin. In addition to this interaction, the downward displacement Loop 17-18 opens space for the TM5/6 bundle. In the crystal structure of the complex, a few hydrogen bond contacts are present between TM5/6 and arrestin. In essence, coupling to the helical bundle of the receptor is a direct result of interdomain rotation that allows a deformation of the flexible loops of arrestin. Furthermore, the sparseness of specific hydrogen bonds or salt bridges within the arrestin-receptor interface hints at how the  $\beta$ -arrestins are able to bind so many different GPCRs. Notably, the interdomain hydrogen bond networks of arrestin-2 and arrestin-3 are much weaker than in arrestin-1 (Kim et al. 2013), meaning these arrestins are already “half-way on” (Gurevich et al. 2011) and explains their much lower dependence on receptor phosphorylation for activation and receptor binding.

*Engagement of the finger loop*—In the pre-complex, the displacement of the distal C-tail by the Rpp likely increases mobility of the finger loop (Hanson et al. 2008). The flexible finger loop is positioned close to the open cytoplasmic face of the receptor, where it can explore the binding cavity. It is possible that binding of the finger loop and stabilization of the active state of the receptor follows a similar

stepwise and mutual reduction of conformational space as recently described for G protein (Elgeti et al. 2013). In the Ops\*/arrestin-1 fusion complex structure, the finger loop is observed to adopt a helical conformation within the cytoplasmic crevice of the active receptor (Kang et al. 2015). A similar binding mode was observed in a crystal structure of Ops\* bound to a peptide analogue of the finger loop (Szczepek et al. 2014). In this structure, specific hydrogen bonding is observed between the finger loop and critical functional motifs on the receptor (e.g. NPxxY (x)<sub>5,6</sub>F and E(D)RY), which are highly conserved among GPCRs. Consistently, the finger loop sequence is highly conserved among all four arrestins. Engagement of the finger loop is crucial for stabilizing the active form of the receptor (Sommer et al. 2012) and hence formation of the high affinity complex.

*Membrane anchoring of the C-edge*—Rotation of the C-domain results in a different alignment of the C-edge that changes how this functional domain interacts with the membrane as compared to the pre-complex. In the pre-complex, the 344-loop adopts an extended conformation and is deeply inserted into the hydrophobic layer of the membrane. In the high-affinity complex, this loop adopts a folded conformation as seen in the p44 structure and interacts more shallowly with the membrane. In addition, the rotation of the C-domain allows the 197-loop to engage the membrane in the high-affinity complex. These differences in C-edge membrane engagement in the pre-complex and high-affinity complex were clearly observed in site-directed fluorescence experiments (Lally et al. 2017) and are consistent with differences in functional maps for Meta II-P binding and Ops-P binding derived from alanine scan mutagenesis (Peterhans et al. 2016).

In summary, formation of the arrestin-receptor complex occurs by a multistep mechanism (Fig. 8.3). Initial electrostatic attraction of arrestin to the Rpp and the negatively charged membrane surface recruits arrestin to the membrane, where the N-domain binds the Rpp and the C-edge anchors within the membrane. This interaction brings the central crest of arrestin within close proximity of the receptor helical bundle, so that the two proteins can interact and mutually affect one another. Transition to the high-affinity complex involves multiple conformational rearrangements in arrestin, which allow arrestin to both specifically stabilize the active form of the receptor as well as flexibly accommodate the cytoplasmic face of the receptor. This flexibility is likely critical for making the  $\beta$ -arrestins versatile binding partners for so many different GPCRs.

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