

# Chapter 7

## Comprehensive Analysis of the Role of Arrestin Residues in Receptor Binding

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**Abstract** The molecular mechanism of arrestin–G protein-coupled receptor (GPCR) complex formation has been analyzed using inter alia spectroscopic, mutagenesis and structure determination methods. The latest crystal structure elucidating one conformation of the rhodopsin-bound arrestin-1 complex confirmed our structural model of an overall complex conformation and experimentally based assumptions about single residues located at the direct binding interface. Comprehensive analyses of single amino acid contributions in arrestin-1 and other methodologies expand our knowledge of structure–function relationships for receptor recognition and arrestin–GPCR complex formation. With a lot of progress made in the last few years, we review most recent literature covering the roles of essential arrestin-1 residues for pre-activation, receptor recognition and receptor binding. Mechanistic understanding of these processes at the atomic level is key for the identification of receptor conformations triggering given signaling pathways and the creation of novel tools for biased ligand drug discovery.

**Keywords** Arrestin · Rhodopsin · Protein–protein interaction · Mutagenesis · GPCR

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## Introduction: The Role of Arrestin in GPCR Trafficking and Signaling

Arrestins form a family of homologous proteins regulating G protein-coupled receptor (GPCR) signaling. Arrestin-1 and arrestin-4 are expressed in the visual system while arrestin-2 (or  $\beta$ -arrestin1) and arrestin-3 (or  $\beta$ -arrestin2) are expressed ubiquitously in the body (Lohse and Hoffmann 2014). Arrestins were first discovered as desensitizing proteins of G protein signaling, which is induced by ligand binding to GPCRs (Kuhn and Wilden 1987; Benovic et al. 1987). After ligand-induced activation of GPCRs, GPCR kinases (GRKs) phosphorylate specific residues of GPCRs, which leads to the recruitment of arrestins to active phosphorylated receptors. G protein activation is partially inhibited by receptor phosphorylation itself (Benovic et al. 1987). Until recently it was additionally assumed that G protein signaling was completely inhibited after arrestin recruitment to the receptor (Lefkowitz 1998). The notion that sustained G protein signaling can occur for some GPCRs of a certain class after internalization (Ferrandon et al. 2009; Feinstein et al. 2013; Calebiro et al. 2009; Irannejad et al. 2013) stimulated a study that showed for the first time evidence that some GPCRs can form super-complexes composed of G protein and  $\beta$ -arrestin1 explaining sustained G protein signaling (Thomsen et al. 2016).

However, inhibition of G protein activation for the majority of GPCRs is not the only role of arrestins in GPCR signaling.  $\beta$ -arrestin1 and  $\beta$ -arrestin2 also recruit the adaptor protein 2 (AP-2), as well as clathrin, in order to facilitate receptor internalization (Tian et al. 2014). Visual arrestins (arrestin-1 and arrestin-4) only interact marginally with AP-2 and hence do not trigger receptor internalization (Laporte et al. 2000). Internalized receptors are then either dephosphorylated and recycled to the membrane or degraded in lysosomes (Tian et al. 2014). The rate of re-sensitization through recycling or down regulation by degradation determines the amount of receptors available on the membrane and thus affects the G protein signaling response to subsequent stimuli.

For some receptors, such as the  $\beta_2$ -adrenoreceptor, the interaction with  $\beta$ -arrestin is transient.  $\beta$ -arrestins and receptors co-localize in clathrin-coated pits near the cell surface and dissociate before the receptors travel to endosomes. Receptors following this pattern are termed class A receptors and typically undergo rapid recycling after internalization. In contrast, class B receptors, such as the angiotensin receptor AT<sub>1A</sub>, show stronger binding to  $\beta$ -arrestins with co-localization still detectable in endosomes (Oakley et al. 1999, 2000). Receptors of this class typically recycle much slower to the cell surface (DeWire et al. 2007; Pierce and Lefkowitz 2001) and some of them have been reported to promote sustained G protein signaling after receptor internalization (Ferrandon et al. 2009; Feinstein et al. 2013; Calebiro et al. 2009; Thomsen et al. 2016). In addition to their role in desensitization and trafficking, arrestins interact with many signaling proteins.  $\beta$ -arrestins interact with sarcoma family (Src) tyrosine kinases and act as scaffolds for the mitogen-activated protein kinase (MAPK) cascades, including the

extracellular signal-regulated kinase (ERK) cascade and the c-jun N-terminal kinase 3 (JNK3) cascade (Luttrell 2013).

The finding that GPCRs trigger G protein-dependent signaling and  $\beta$ -arrestin-dependent trafficking and signaling opened up new strategies for drug discovery. Searching for ligands preferentially activating G protein or arrestin pathways, so-called biased ligands, might indeed lead to new drugs with reduced side effects, tolerance and toxicity (Luttrell 2013). There is also great potential in looking for ligands preferentially activating given arrestin-dependent signaling pathways or preferentially triggering recycling or degradation of a particular receptor. Indeed, we hypothesize that there might be an even higher complexity of biased signaling for certain class B receptors that internalize via arrestin and subsequently show recycling, degradation and/or simultaneous sustained G protein signaling.

Here we discuss a general framework for arrestin–GPCR complex formation, using the interactions between arrestin-1 and the GPCR rhodopsin as a model, which is so far the most studied. The photoreceptor rhodopsin is comprised of the apo-receptor opsin and its Schiff base-bound natural ligand, retinal. Retinal serves as an inverse agonist in its 11-*cis* conformation and triggers rhodopsin activation subsequently to its light dependent isomerization to all-*trans* retinal. The all-*trans*-retinal-bound activated conformation of rhodopsin induces G protein signaling (Arshavsky et al. 1994) and is susceptible to intracellular phosphorylation (Mendez et al. 2000; Vishnivetskiy et al. 2013a). Only one minute after its activation, the Schiff base-linked all-*trans* retinal dissociates from the receptor, which will further reside in its phosphorylated opsin conformation until dephosphorylation takes place and the binding of another retinal molecule occurs (Hofmann et al. 1992).

Although rhodopsin, as the dim-light photoreceptor, is characterized by a quite unique activation mechanism, it also shares a multitude of characteristics with other GPCRs. Not only because of these shared attributes, but also because it is the only GPCR that can be isolated and crystallized directly from natural sources (Unger et al. 1997), rhodopsin became a model system for structural analyses of GPCRs early on. Until 2007 it was the only GPCR with a known high resolution crystal structure and the most abundantly used receptor to explain processes, which are shared by the entire GPCR family (Deupi 2014). The first crystal structures of disease-causing GPCR mutants have been obtained from rhodopsin mutants G90D (Singhal et al. 2013) and T94I (Singhal et al. 2016) explaining the structure-function relationship for congenital stationary night blindness. Also the first crystal complex structure of arrestin with a GPCR was elucidated using rhodopsin and arrestin-1 (Kang et al. 2015).

An in-depth understanding of the molecular mechanism by which arrestin is activated and binds a cognate GPCR will enable the identification of receptor conformations leading to specific pathways, as well as to efficient and more precise discovery of biased ligands. Here we present the current knowledge on arrestin recognition of active receptors and the roles of distinct arrestin residues in receptor binding.

## **Molecular Mechanism of Arrestin-1 Engagement with Rhodopsin Revealed by Scanning Mutagenesis**

### ***Scanning Mutagenesis as a Tool for GPCR Pharmacology Elucidation***

Site-directed scanning mutagenesis is known for circa 35 years, but has been recognized more recently as enabling technology to study GPCR pharmacology [reviewed in: Heydenreich et al. (2015)], such as GPCR signaling via G proteins (Flock et al. 2015; Sun et al. 2015), arrestin activation (Ostermaier et al. 2014a, b; Peterhans et al. 2016), or GPCR drug discovery (Congreve et al. 2011, 2014; Robertson et al. 2011; Rich et al. 2011; Chen et al. 2012; Congreve and Marshall 2010). The most frequently seen specialization is alanine scanning, which means the replacement of each amino acid of the native protein individually with alanine (Sun et al. 2013). Depending on the objective of the study the resulting GPCR mutant or signaling protein mutant is tested for its thermostability (Heydenreich et al. 2015; Sun et al. 2015) or altered ability to interact with its corresponding GPCR (Sun et al. 2015; Ostermaier et al. 2014a; Peterhans et al. 2016). Alanine scanning of  $G\alpha_{11}$  and arrestin-1 has led to comprehensive functional maps on top of corresponding 3D protein structures bringing structure and function at single amino acid resolution in direct relationship (Sun et al. 2015; Ostermaier et al. 2014a; Peterhans et al. 2016). In combination with crystal structures of rhodopsin, individual G protein subunits, trimeric G proteins, arrestin-1 or complex structures thereof, key amino acid residue triggers for protein activation and protein–protein interaction have been identified. The high sensitivity of employed assays allows exploration of differences in protein stability or GPCR–protein interaction induced by the presence or absence of GTP or the natural opsin agonist all-trans retinal (Sun et al. 2015; Peterhans et al. 2016). Scanning mutagenesis of arrestin-1 has enabled to ‘read’ ligand-dependent GPCR conformations (Peterhans et al. 2016) similar to FRET-, BRET- or NMR-based methods utilizing arrestin-2 or arrestin-3 as ‘biosensors’ (Nuber et al. 2016; Lee et al. 2016; Yang et al. 2015). Although rational design of arrestin-1 mutants over the course of more than 20 years has finally lead to the structure elucidation of the rhodopsin–arrestin-1 complex (Kang et al. 2015), we would like to point out compelling advantages of scanning mutagenesis versus rational mutant design to reveal molecular mechanisms of arrestin-1 action, such as the unbiased comprehensiveness in the analysis of single residue positions, performance in speed to grasp the larger ‘picture’ of molecular action, and the high detail level regarding quantitative comparison of single residue contributions. Scanning mutagenesis with subsequent combination of further selected single point mutations has been preferred historically (Warne et al. 2008; Lebon and Tate 2011; White et al. 2012; Hollenstein et al. 2013) over evolutionary approaches (Egloff et al. 2014) to obtain crystal structures of GPCRs. We believe that scanning mutagenesis remains an essential and complementary method to

elucidate the pharmacological properties of GPCRs and the first step of arrestin-dependent signaling that is activation and binding of arrestin to its cognate GPCR.

### ***Phosphorylation Defines the Nature of GPCR–Arrestin Interactions***

Arrestin recruitment to the corresponding GPCR is a process comprised of multiple distinct events. Every step influences and, in fact, defines the nature of the ultimately formed complex. The mechanism of receptor activation does not only facilitate the dissociation of the trimeric G protein but may also result in the formation of several different conformational states of the receptor. These distinct conformations affect the affinity towards every intracellular binding partner, such as receptor kinases (GRKs), scaffolding proteins and arrestins (Moore et al. 2007; Mary et al. 2012). Moreover, there is strong evidence that different activated conformations of the GPCR lead to targeted phosphorylation by certain kinases only (Nobles et al. 2011). Thus, a receptor, activated via different processes or ligands, may show different phosphorylation patterns depending on its conformation as well as the affinity towards a distinct set of receptor kinases and particular kinetics of phosphorylation. These so-called “phosphorylation barcodes” as well as the particular receptor conformations have been shown to play a definite role in arrestin recruitment and affect further arrestin functions (Yang et al. 2015).

### **Inactive Arrestin-1 and Its Key Regulation Sites**

The inactive conformation of the arrestin-1 protein is maintained by several stabilizing, intramolecular interactions, which lead to a structural self-inhibition of the protein (Hirsch et al. 1999; Han et al. 2001). First and foremost, the anchoring of the C-terminus at the N-domain of arrestin, held in place by the so-called “three-element” interaction (Gurevich and Gurevich 2013). The “three-element” interaction is formed by hydrophobic residues of  $\beta$ -strand I (H10, V11 and F13) and  $\alpha$ -helix I (L107 and L111), both located in the N-domain of arrestin, in order to form a strong interaction with phenylalanine residues located in the distal C-terminal region (F375, F377 and F380) (Ostermaier et al. 2014a; Peterhans et al. 2016). This entrapment of the rather flexible arrestin-1 C-tail (residues 372–404) is also one of the most important interactions to fall apart during activation, thus enabling the commencement of further structural changes (Gurevich and Gurevich 2013). Mutational studies showed that upon substitution of these residues by alanines the protein exhibits a higher affinity towards unphosphorylated rhodopsin species (like Rh\*) (Peterhans et al. 2016) and higher complex stability in general, if bound to the GPCR (Ostermaier et al. 2014a). Moreover, the phosphorylation-independent rhodopsin-binding of the “triple-A” arrestin-1 mutant (F375A, V376A, F377A)

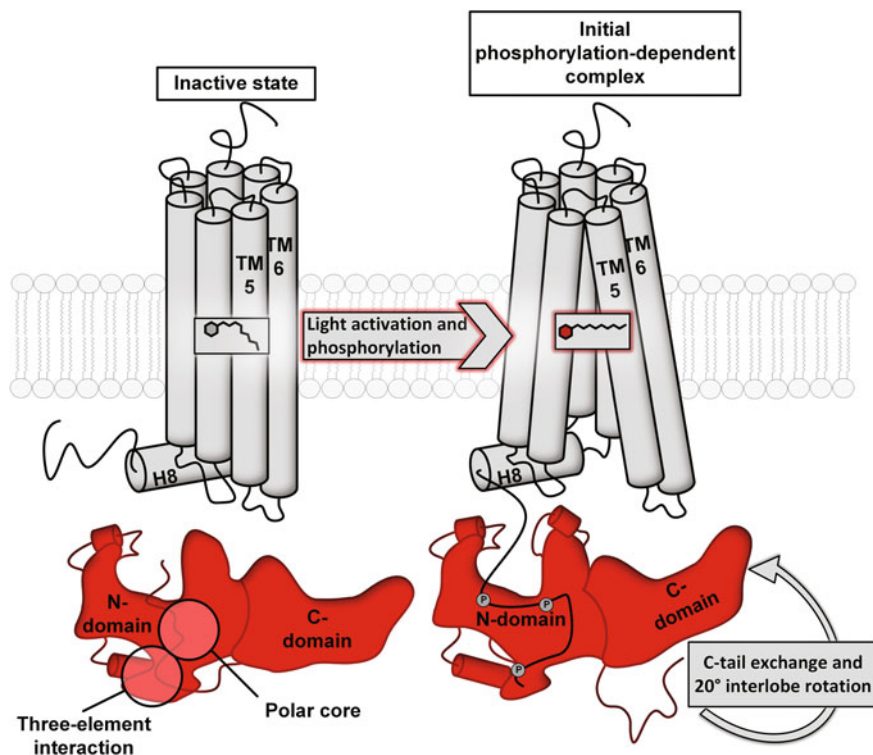
(Gurevich 1998) can be interpreted as a direct consequence of the disturbance of the “three-element” interaction.

The most important interaction stabilizing the orientation of the N- and C-domains towards each other is called the polar core (Hirsch et al. 1999), which is highly conserved in the arrestin family. The term polar core describes a cluster of solvent-excluded, polar amino acids forming an extended hydrogen bond network, embedded between the N- and C-domains of the protein. Due to these interactions, the two domains are held in place as a trademark of the inactive arrestin conformation. The key interactions are formed by two aspartate residues, located in the gate loop (residues 296–305), a functional part of the lariat loop [residues 274–304 (Hirsch et al. 1999)], at position 296 and 303 (Han et al. 2001). In the inactive form those two residues pose as the main counterions for R175, located in  $\beta$ -strand X at the interface between the N- and C-domain (Granzin et al. 2015; Gurevich and Benovic 1995). In a similar orientation, the residue D30 facilitates further interactions between R175 and the crucial entrapment of the C-terminal R382 residue from inside the N-domain, to further fasten the C-tail onto the inactive arrestin conformation (Ostermaier et al. 2014b). The inactive as well as the pre-activated conformation of arrestin-1 are depicted in Fig. 7.1.

Structural studies of arrestin-1 mutants, lacking polar amino acids in this cluster, and activated  $\beta$ -arrestin1 show that, upon breakdown of the polar core interactions, a substantial rotation of the N- and C-domain relative to each other takes place. With this interlobe rotation of  $20^\circ$ – $21^\circ$  (Kim et al. 2013; Shukla et al. 2013), alongside further rearrangements of receptor interacting loops located in the central crest of the arrestin molecule, arrestin becomes capable of high affinity GPCR binding (Kang et al. 2015). The driving force responsible for these dramatic conformational changes is the breakdown of the polar core, initiated by releasing the C-tail from the described intramolecular interactions, namely the “three-element” interaction as well as the polar core itself. For this process to take place, an association of the inactive arrestin and a phosphorylated GPCR C-tail is needed, in order to establish high-affinity interactions that are able to force open the C-tail restraint inside arrestin (Vishnivetskiy et al. 2000). The manner in which this so-called “C-tail exchange” takes place is highly dependent on the number and location of phosphorylated residues in the C-terminal domain of the GPCR (Yang et al. 2015; Moore et al. 2007), defining a conformational need for distinct phosphorylation patterns.

### ***Pre-activation of Arrestin-1 by C-Tail Exchange Mechanism***

Phosphorylation of intracellular elements of activated GPCRs occurs after dissociation of the primary signaling adaptor protein—the trimeric G protein. The discovery of the GPCR kinase 1 (GRK1), formerly known as rhodopsin kinase, was supplying the first evidence that phosphorylation of the GPCR may affect further processing, as well as signaling (Weller et al. 1975). This initial discovery is now



**Fig. 7.1** Comparison of the inactive and pre-activated arrestin-1 conformation. In the inactive conformation, the two tight intramolecular interactions, namely the polar core and the “three-element” interaction, stabilize arrestin-1. Upon light activation and phosphorylation of rhodopsin, arrestin-1 engages the phosphorylated C-tail of the receptor. By disruption of the polar core as well as the “three-element” interaction, the C-tail of arrestin-1 is dislocated and sterically replaced by the phosphorylated GPCR C-tail. Arrestin-1 changes its conformation while performing an interlobe rotation of 20°. Arrestin-1 is now pre-activated and capable of high affinity binding

extended by the realization that not only seven different GRKs may alter the phosphorylation state of a given GPCR, but also different other cytoplasmic kinases, such as protein kinase A (PKA). Interestingly, each kinase generates a distinct phosphorylation pattern at the intracellular surface of the GPCR, leading to major differences between phosphorylation patterns generated by either GRKs or PKA (Tran et al. 2004) but also between patterns created by different members of the GRK family (Nobles et al. 2011). GPCR phosphorylation is essential for structural rearrangement of the arrestin protein, in order to reach a conformation suitable for high-affinity receptor binding. Thus, differences in receptor phosphorylation have been hypothesized to affect the affinity of the GPCR–arrestin complex and might even lead to differences in arrestin-mediated receptor trafficking and processing. Latest studies revealed strong evidence for the existence of so-called

“phosphorylation barcodes” generated by a variety of kinases and the ability of arrestins to decipher this phosphorylation inscribed information, in order to facilitate different signaling outcomes (Yang et al. 2015).

### **Establishment of an Initial Phosphorylation-Dependent Binding**

The unique ability of the arrestin protein to detect and embrace a phosphorylated GPCR C-tail is mediated by certain phosphate-sensing residues. A multitude of solvent-exposed and positively charged residues is located in the N-domain, which are likely predominant interaction sites with the phosphorylated GPCR C-tail residues. In the inactive conformation, these seem not to be accessible for intermolecular charge-charge interactions due to the steric positioning of the gate loop and the C-tail. Especially the gate loop positioning in the inactive form hides R175, a residue which was previously considered crucial for phosphate detection (Kovoor et al. 1999), due to the interactions of the polar core network. Nevertheless, when arrestin engages the phosphorylated GPCR C-tail, this region is the first to respond. Structural analysis of pre-activated arrestin species (Kim et al. 2013) and arrestin–GPCR (or C-terminal phosphopeptide) complexes (Kang et al. 2015; Shukla et al. 2013) reveal a shift of the gate loop. With phosphorylated GPCR C-terminal residues in close proximity to it, residue K300 recognizes this change of charges as part of the loop. This interaction might then be sufficient to dislocate the gate loop (Shukla et al. 2013). Due to this rearrangement, the charge-charge network of the polar core collapses, while the gate loop is undergoing a dramatic reorientation of up to 180°. The alanine mutant K300A is characterized as one of the arrestin-1 mutants with the lowest affinity towards phosphorylated and light-activated rhodopsin (Ostermaier et al. 2014a), underlining the importance of this single residue for receptor recognition. The new positioning of the gate loop is stabilized by further charged interactions, established between D303 and R288 (located in the lariat loop) as well as R291 and D138 [located in the middle loop (residues 133–142)]. The gate loop is now clipped between the lariat and middle loop and reveals the polar core. The latest structures of receptor- or phosphopeptide-bound arrestins reveal that there is in fact no interaction to be detected between R175 and phosphate moieties of the GPCR C-tail (Kang et al. 2015; Shukla et al. 2013), it becomes clear that R175 serves as an important anchor of the polar core, stabilizing the inactive arrestin-1 conformation, but not as a phosphate-interacting residue per se.

The disruption of the polar core, facilitated by the rotation of the gate loop, triggers several other structural changes, which lead to pre-activation of the arrestin protein. Due to a withdrawal of negatively charged residues (D303 and D296) from the polar core region as the gate loop rotates towards the middle loop, the major C-tail anchoring residue R382 is also freed from the polar core. Furthermore, it was shown that R29, a residue located in the N-domain between the polar core and “three-element” sites, interacts with other residues of the GPCR C-tail. These interactions lead to a collapse of further hydrogen bonds to C-terminal residues, namely F380, E379 and F378 (Granzin et al. 2015). Characterized not only as a

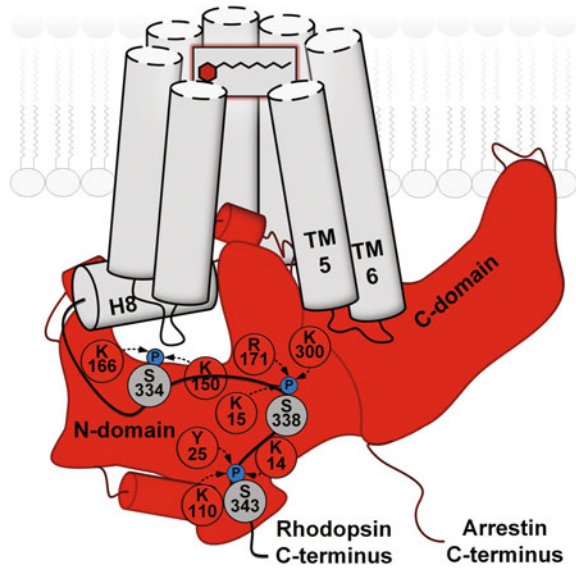


highly important residue for receptor recognition via scanning mutagenesis, but in fact as the residue with the most negative impact on receptor binding when substituted with alanine (Ostermaier et al. 2014a; Peterhans et al. 2016), R29 is one of the most critical residues controlling the arrestin C-tail release as a phosphate sensor.

As the arrestin C-tail gains flexibility with every collapsed interaction, more and more positively charged residues inside the arrestin N-domain become solvent-exposed. In its phosphorylated and light-activated state, rhodopsin usually exhibits three phosphorylated residues, namely S334, S338 and S343 (Ohguro et al. 1996). Now the computational model of arrestin with the rhodopsin C-tail, relying on the latest crystal structure, is showing the phosphate interacting residues of arrestin in the final complex. According to this model, the phosphorylated S334 residue of the receptor interacts with K150 and K166, two lysine residues located at the tip of the N-domain. Beside the gate loop residue K300, K15, and R171 are assumed to interact with the phosphorylated S338 residue, whereas K14, Y25 and K110 are in reach to interact with the phosphorylated S343 (all residue positions have been adjusted to the corresponding positions of bovine arrestin-1 and rhodopsin) (Kang et al. 2015). Similar conclusions, regarding the layout of possible phosphate interacting residue clusters, but for a different species of both rhodopsin and arrestin-1, were drawn in a previously published review article (Ostermaier et al. 2014b). With the positioning of these residues, a positively charged basin is formed inside the N-domain, in which the phosphorylated GPCR C-tail can neatly be embraced (as depicted in Fig. 7.2). These interactions lead to the disruption of the “three-element” interaction, ultimately replacing the arrestin C-tail with the phosphorylated GPCR C-tail. The substitution of any residues involved in the hydrophobic “three-element” interaction with alanine strongly enhances the stability of the GPCR–arrestin complex. With H10A, V11A, F13A, L107A and L111A exhibiting very high values for receptor complex stability, it is interesting that the alanine mutants F375A and F377A as well as F380A enhance receptor binding to an even larger extent (Ostermaier et al. 2014a). This again underlines the importance of the “three-element” interaction site in anchoring the arrestin C-tail and stabilizing the inactive conformation of the protein. With the collapse of both intramolecular interaction sites, the polar core and the “three-element” site, facilitating the dislocation of the arrestin C-tail, the protein reorganizes and enters a pre-activated conformation, now capable of high-affinity complex formation with the receptor.

We propose the described process of initial complex formation between arrestin-1 and rhodopsin via the C-tail exchange mechanism as a framework for initial phosphorylation-dependent interactions of other arrestins with their GPCR (Ostermaier et al. 2014b). One more recent example supporting this hypothesis is the structural architecture of a chimeric  $\beta_2$ -adrenoreceptor/vasopressin type 2 receptor in complex with a “hanging”  $\beta$ -arrestin1, which has been derived by single-particle electron microscopy (Shukla et al. 2014). We would like to point out the relevance of slight differences in arrestin–GPCR interactions for subsequent cellular processes even if we assume at least one fundamentally conserved process

**Fig. 7.2** Arrestin-1 in its rhodopsin bound conformation. The cytoplasmic interface of rhodopsin as well as arrestin-1 in its receptor-bound conformation are depicted. The phosphorylated residues of the rhodopsin C-tail as well as the interacting arrestin-1 residues are *highlighted*. Residue positions have been adjusted for bovine arrestin-1 and rhodopsin

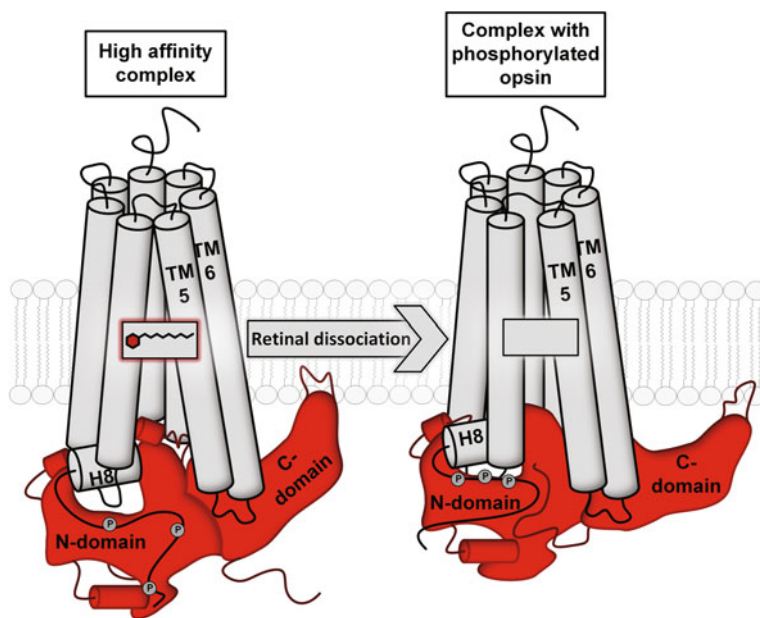


for complex formation across the four arrestins with phosphorylated GPCRs that consists of “fishing” arrestin via C-tail exchange, initial engagement with receptor and subsequent formation of a low- or high-affinity complex.

### Activation and Phosphorylation States of the GPCR Influence the Conformation of the Final Complex

Phylogenetic analyses have revealed that the human genome harbors more than 800 genes encoding GPCRs (Fredriksson et al. 2003). As every single receptor is characterized by its own mode of activation and intracellular receptor phosphorylation, arrestins have to be very versatile to be able to interact with even a majority of GPCRs. Especially the phosphorylation of GPCR C-tails may differ widely, as it is not only highly dependent on the kinases which are co-expressed by the receptor-bearing cell type but also due to sequence variations of the receptor-specific C-terminal residues. Interestingly, phosphate-interacting residues in arrestin-1 seem to vary if arrestin-1 is bound to light-activated and phosphorylated rhodopsin or if bound to phosphorylated opsin (Peterhans et al. 2016) (Fig. 7.3). We hypothesize that the phosphorylated opsin state of the receptor forms after light activation of the retinal-bound rhodopsin, as the subsequent hydrolysis of the Schiff-base linkage enables the retinal molecule to leave the binding cavity inside the GPCR (Hofmann et al. 1992). As phosphate-sensing residues, which interact with the phosphorylated C-tail of light-activated rhodopsin, were already discussed above, it was shown by scanning mutagenesis that the residues K300, K14 and K15 are important for arrestin-1 binding to either receptor state. The complex stability of arrestin-1 and

phosphorylated opsin, on the other hand, is heavily decreased if the positive charges on residues R18, K55, R56, R81, K150, K166 or R171 are removed. Residues, which are shown to be important for the binding to phosphorylated opsin, mark a distinctly different pattern of positive charges in the arrestin-1 protein, if compared to light-activated and phosphorylated rhodopsin binding. Whereas both clusters of phosphate-interacting residues are located in the N-domain of arrestin-1, it seems that the conformational change of the receptor, which goes in hand with the dissociation of retinal, may lead to a translocation of the GPCR C-tail, which still stays attached to arrestin-1. As the phosphorylated opsin C-tail rather interacts with residues along the cup-shaped region on top of the arrestin-1 N-domain, instead of associating with residues across it (including residues, which are located near the N-terminus, like K110), these findings indicate that the activation state of the receptor may influence the conformation, orientation and the positioning of interacting residues in the GPCR–arrestin complex. A comparison of the activated and phosphorylated rhodopsin and putative phosphorylated opsin-bound states is shown in Fig. 7.3.



**Fig. 7.3** Comparison of arrestin-1 bound to either activated and phosphorylated rhodopsin or to phosphorylated opsin. As scanning mutagenesis revealed different phosphate-interacting arrestin-1 residues for the binding of activated and phosphorylated rhodopsin as well as phosphorylated opsin, the putative conformational states of receptor binding were depicted. As the affinity of arrestin-1 for phosphorylated opsin is generally weaker if compared to phosphorylated and activated rhodopsin, the illustration shows one of many possible layouts, rather than a definite conformation

Similar conclusions were also drawn based on site-directed fluorescence studies of arrestin-1 binding to different receptor states (Sommer et al. 2012). One substantial difference between arrestin-1 binding to light-activated and phosphorylated rhodopsin and the phosphorylated apo-receptor might be the rotation of the gate loop, which only seems necessary for arrestin-1 association with ligand-bound receptor states. Without this intramolecular reorientation, it is likely that the arrestin-1 C-tail stays attached to the polar core and “three-element” interaction in a basal state-like fashion, while being bound to phosphorylated opsin. This distinct conformation would then hinder phosphate-interacting residues across the arrestin-1 N-domain to interact with the phosphorylated opsin C-tail and provoke a different binding pattern (Peterhans et al. 2016). The concealment of these main phosphate-interacting arrestin-1 residues may also explain earlier findings, indicating that arrestin-1 binding to the apo-form of the receptor is more dependent on higher phosphorylation levels of the C-terminus as compared to arrestin-1 binding to light-activated rhodopsin (Vishnivetskiy et al. 2007).

With tremendous efforts being made to unravel the mechanism of biased arrestin signaling and the various functions and pathways in which arrestin may serve as a mediator, NMR studies now revealed the existence of a variety of distinct arrestin conformations induced by different GPCR phosphorylation patterns (Yang et al. 2015). As already discussed, phosphorylation patterns of GPCR C-tails gain their diversity through the particular activation state, phosphorylation by different intracellular kinases, as well as GPCR-specific sequence variances. With every phosphorylation pattern attracting a distinct set of arrestin phosphorylation-sensing residues (Nobles et al. 2011), the GPCR-arrestin complex materializes in a unique conformation, characterized also by the affinity towards intracellular binding partners (Yang et al. 2015). The fashion in which activated GPCR trafficking and non-canonical GPCR signaling occurs, may even be much more defined by slight differences in GPCR phosphorylation than assumed before (Nuber et al. 2016).

The culminating conclusion can only be that interactions between arrestins and phosphorylated GPCR residues highly depend on a multitude of parameters defined by the receptor and its environment. It now becomes clear that there is no such thing as a universal pre-activated or active arrestin conformation, but rather distinct intramolecular arrangements, which organize according to the particular receptor sequence, activation state and C-terminal phosphorylation pattern.

### *Active GPCR Recognition on a Single Residue Scale*

As the arrestin phosphate sensors engage the phosphorylated C-tail of the GPCR, a second set of putative GPCR-interacting residues establishes the formation of a high-affinity complex. With the crystal structure of the rhodopsin-arrestin-1 complex at our disposal (Kang et al. 2015), it is now possible to precisely describe the interface of the complex on a single residue scale.

## GPCR Activation-Sensing Residues of Arrestin-1

Pre-activation of the arrestin molecule induces several significant structural re-arrangements affecting not only the C-tail of arrestin but also the positioning of intramolecular loops and a substantial inter-domain rotation. These changes of the tertiary structure of arrestin are thought to expose distinct activation-sensing residues and enable the protein to engage the activated GPCR. Beside the movement of the gate loop as one of the hallmark processes in arrestin pre-activation, especially the displacement of the middle loop as well as the finger loop (residues 68–78) are of major importance to the recognition of receptor activation (Kang et al. 2015). Already shown in the crystal structure of the truncated and pre-active arrestin mutant p44 (Granzin et al. 2012), the middle loop undergoes a shift upon arrestin pre-activation, thus moving away from the receptor binding interface and towards the N-domain (Vishnivetskiy et al. 2013b). This shift might be a direct result of interactions between the dislocated gate loop and the middle loop itself.

In the inactive conformation, the middle loop is held in place by a salt bridge between K141 and D71, the latter located in the finger loop. This interaction is disrupted for high-affinity receptor binding, which explains the enhanced receptor binding capabilities of the K141A mutant (Ostermaier et al. 2014a; Peterhans et al. 2016). While mutations in the middle loop affect the binding of arrestin to activated and phosphorylated rhodopsin, more drastic changes have been observed for the affinity towards non-preferred receptor species (Vishnivetskiy et al. 2013b). Thus, alanine substitutions in the middle loop region of arrestin generally enhance the binding to phosphorylated opsin (Peterhans et al. 2016), indicating that a functional middle loop determines the specificity of arrestin towards the agonist-bound and activated receptor conformation. Whereas rhodopsin in its activated and phosphorylated conformation is able to interact with the middle loop in order to facilitate its rotation away from the binding interface, other receptor conformations might fail to provoke a necessary rotation of the gate loop which would enable such a structural re-arrangement. These findings mark the middle loop, in its entirety, as an activation-sensing component of arrestin. While binding of activated and phosphorylated rhodopsin, the intracellular loop 2 (ICL 2) of rhodopsin can neatly enter a cleft between the middle loop and the lariat loop, which is generated due to the interlobe rotation of arrestin and the relocation of the two loops (Kang et al. 2015).

Pre-activation of the arrestin molecule also influences the secondary and tertiary structure of the finger loop. The  $\beta$ -strands bordering the loop extend upon re-arrangement of the arrestin molecule to enable a stretching of its conformation towards the receptor (Granzin et al. 2012). The arrestin-1 finger loop is able to engage rhodopsin at a cavity, which opens up due to the conformational change upon receptor activation. In this sense, the shape of the finger loop acts as an indirect activation sensor, as it is only establishing high-affinity binding upon activation-dependent opening of the cytoplasmic receptor crevice. When bound to the receptor, the finger loop adapts a short  $\alpha$ -helical conformation (Feuerstein et al. 2009; Kim et al. 2012) in order to interact with the C-terminus of transmembrane (TM) helix 7 as well as the N-terminus of helix 8 of the receptor (Kang et al. 2015).

The residues which are involved in these interactions as well as the structural integrity of the finger loop (Q70, V75, M76, G77 and L78) are crucial for receptor binding and heavily decrease the affinity towards different receptor species upon alanine substitution (Peterhans et al. 2016). The structural fit of the finger loop inside the positively charged cytoplasmic cavity of rhodopsin is additionally supported by a charge complementation effect, as the finger loop features three negatively charged residues, namely E71, D72 and D74 (Kang et al. 2015).

The  $\beta$ -strand following the finger loop (residues 79–86), on the other hand, establishes an additional binding interface between arrestin and the receptor, as it interacts with TM5, TM6 and the connective ICL3. X-ray crystallography validates the positioning of residues F86 and D83 in close proximity to the receptor, thus they are thought to enable these interactions (Kang et al. 2015). Furthermore, the cytoplasmic side of TM5 seems to be enclosed in the central loop area of arrestin, as it is also interacting with residues located in the lariat loop of arrestin [especially Y251 (Ostermaier et al. 2014a)]. These interactions are, again, dependent on the characteristic outward movements of TM5 and TM6, which happen upon rhodopsin activation. In the case of  $\beta$ -arrestin1, hydrogen-deuterium exchange mass spectrometry and chemical cross-linking studies are consistent with a very similar engagement of the corresponding finger, middle and lariat loop for the binding of the  $\beta_2$ -adrenoreceptor chimera (Shukla et al. 2014).

Whereas arrestin pre-activation is highly dependent upon the establishment and location of distinct phosphorylation patterns at the GPCR C-tail, activation-sensing is rather governed by steric barriers, which have to be overcome for the formation of a high-affinity complex. Both, the pre-activation of arrestin as well as the activation of the GPCR, are prerequisites, which prime the two proteins by arranging the essential binding interface.

## Membrane Interactions of Arrestin-1 and Binding Stoichiometry

The C-edge is another region of arrestin-1, which is thought to equip the protein with characteristics crucial for high-affinity receptor binding (Ostermaier et al. 2014a). Although it is not located in close proximity to any receptor residues of the putative binding interface between arrestin-1 and rhodopsin, alanine substitutions in the solvent-exposed loops of the C-edge strongly influence binding affinities towards various receptor species (Peterhans et al. 2016). A cluster of uncharged or hydrophobic residues, which are comprised within the 344-loop (L338, L339, L342 and S345) as well as the 200-loop (F197, M198 and S199), were shown to influence arrestin-1 binding to the activated and phosphorylated receptor. Due to the recent elucidation of the complex crystal structure and the reported interlobe rotation of arrestin-1, as well as the asymmetric nature of the rhodopsin–arrestin-1 interaction, it is now possible to associate the location of these residues with the hydrophobic interior of the phospholipid membrane. Arrestin-1 in its receptor-bound conformation might thus be able to penetrate the cell membrane and enable an additional anchoring of the complex in the phospholipid bilayer, adjacent to the receptor

(Kang et al. 2015). Interestingly, for phosphorylated opsin binding of arrestin-1, alanine mutations of the 344-loop increase the affinity towards the receptor. Similar findings show, that the residues V268, N271, S272 and L274, located within the 270-loop of the C-edge, strongly influence the specificity of arrestin-1 towards different receptor species. These residues have been shown to increase the affinity of arrestin for the activated and phosphorylated receptor upon alanine substitution while decreasing its affinity towards phosphorylated opsin (Peterhans et al. 2016). As the re-positioning of the C-edge is dependent on the rotation of the N- and C-domain against each other, these findings again imply the existence of highly different arrestin-1 binding conformations for particular receptor activation states.

Still, the exact function of the C-edge and other structures at the outline of the arrestin-1 molecule are not completely understood. There are several hypotheses, which explain the influence of these regions on receptor binding with the existence of further binding interfaces or varying binding stoichiometry. Some studies suggest that the C-edge of arrestin in its receptor-bound conformation might interact with an adjacent receptor implying a process in which arrestin is able to bind to rhodopsin in a two-to-one stoichiometry (Sommer et al. 2012). Furthermore, the 160-loop of arrestin-1 might be seen as an additional binding interface for rhodopsin, as it was shown that it interacts with the central cytoplasmic cavity of rhodopsin (Sinha et al. 2014).

## Pharmacological Relevance of Phosphobarcode for Arrestin-Dependent Signaling

How arrestin-1 interacts with rhodopsin is by far most in-depth studied compared to any other arrestin–GPCR interactions. On top of that studies on the interaction of arrestin-1 with disease-causing rhodopsin mutants have revealed that G90D rhodopsin, which causes congenital stationary night blindness, is phosphorylated to a slightly higher degree than wild type rhodopsin, but demonstrated reduced ability to bind arrestin-1, especially in its phosphorylated opsin state (Vishnivetskiy et al. 2013a; Singhal et al. 2013). Thus the arrestin-1–rhodopsin interaction serves as a framework and guidance towards elucidation of the pharmacologically impactful, diverse and ubiquitous  $\beta$ -arrestin–GPCR complexes. Research on arrestin-1 had a considerable lead in time over  $\beta$ -arrestins dating back to the mid 1970s with purification and characterization of the protein (Wacker et al. 1977; Dorey and Faure 1977). In the early 1990s Vsevolod Gurevich has started mutagenesis studies on arrestin-1 and continued in his group until key elements in the activation mechanism of arrestin-1 have been revealed [reviewed in (Gurevich et al. 2011)]. At the same time one of the two  $\beta$ -arrestins has been discovered as G protein-inhibiting factor (Lohse et al. 1990).  $\beta$ -arrestins were first considered to only play a role in receptor desensitization and internalization (Ferguson et al. 1996). It was then discovered that they can also activate G protein-independent signaling pathways (Luttrell and Lefkowitz 2002; Charest and Bouvier 2003)

and that some ligands activate preferentially one or the other pathway, which resulted in the concept of functional selectivity or biased signaling (Azzi et al. 2003; Shenoy and Lefkowitz 2005). Now, recent evidence suggests that there is much more diversity than a simple dichotomy between G protein-dependent and  $\beta$ -arrestin-dependent signaling. An analysis of SII and TRV027, two angiotensin AT<sub>1</sub> receptor ligands biased towards  $\beta$ -arrestin signaling, showed that they lead to distinct patterns of kinase phosphorylation and gene expression (Santos et al. 2015). While it is possible that this diversity is the result of yet unknown effectors, it was also shown that SII and TRV027 lead to distinct conformational changes of  $\beta$ -arrestins and that this could account for the observed dissimilarity in signaling patterns. In addition, a study using fluorescein arsenical hairpin bioluminescence resonance energy transfer reporters to monitor conformational changes in  $\beta$ -arrestin2 showed that information about ligand-induced receptor conformation is encoded in changes in  $\beta$ -arrestin2 conformation (Lee et al. 2016). This provides further insights into how receptors can use  $\beta$ -arrestins for different purposes and highlights the role of  $\beta$ -arrestins in reading receptor states and in consecutively encoding this information as distinct  $\beta$ -arrestin conformations. Moreover, it was shown in recent years that  $\beta$ -arrestin1 and  $\beta$ -arrestin2 have non-overlapping and sometimes antagonistic functions and that they can be differentially recruited depending on receptor, ligand and receptor phosphorylation (Srivastava et al. 2015). Together, these findings show the diversity and pharmacological potential of arrestin-dependent signaling and the need of further work in understanding arrestin-receptor interaction that could lead to novel tools for the discovery of more functional selective ligands.

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