Chapter 3
Initial Crystallographic Studies of Visual Arrestin: Insights and Perspectives

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Abstract Crystallographic studies of visual arrestin in the late nineties built upon fundamental biochemical work that had identified arrestins as proteins central to desensitization of GPCR signaling. The structural findings revealed the arrestin fold that had two related domains and a C-tail, which folded onto the molecule's surface. The structures had a curious "polar core" that sat at the fulcrum of the two domains and which acts as an active site for conformational activation. The structures also served as the basis for elaborating the arrestin fold, found in all the kingdoms of life. Finally, the results suggested that quaternary structure i.e. the oligomeric state played a role in self-regulation.

Keywords Arrestin · Crystal structure · Arrestin fold · Phosphate sensor · Activation · Conformational change

Early Biochemical Studies

The G protein signaling cycle, with the receptor as the focus of signal initiation, was largely worked out in the seventies. The receptors that became paradigmatic for this canonical G protein-mediated signaling were rhodopsin (Rho), the receptor for photons in the retina, and β2-adrenergic receptor (βAR), the receptor for the hormone epinephrine. These receptors were cloned in 1980s and the first glimpses of their molecular structure were obtained (Ovchinnikov 1982; Dixon et al. 1986). The full elucidation of their 3D structure took many decades, as they are integral

This chapter is dedicated to the memory of our colleagues Drs. Serge Pares, Carsten Schubert, and Paul Sigler, who played essential roles in the elucidation of arrestin structures and their implications.

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membrane proteins, which in general have often been refractory to straightforward structure determination. However, the structural biology of the transducing, or in the words of Rodbell, transferring molecules advanced more rapidly with the details of hetero-trimeric G proteins published in the nineties. Subsequent to elucidation of the G-protein cycle in the early eighties, the question of non-agonist specific receptor desensitization was addressed, again in the two aforementioned paradigms. The molecular players for the desensitization system were discovered by the German group of Herman Kuhn (Wilden et al. 1986a), working on Rho and in parallel, the Lefkowitz group in the USA, working on βAR (Benovic et al. 1986). The groups identified a two-step cascade whereby the receptor was phosphorylated by a specific kinase, named G-protein receptor kinase (GRK), on its intracellular C-terminus at multiple sites. The phosphorylation itself slowed coupling with the cognate G protein. A soluble protein with approximate mass of 48 kDa (hence, named by Kuhn p48, also known as S-antigen) terminated the signal by association with Rho (Kuhn et al. 1984; Wilden et al. 1986a). Likewise, a short time thereafter, a protein was identified biochemically by the Lefkowitz group with analogous function in the βAR system (Benovic et al. 1987). These protein "terminators" were dubbed arrestins. Visual arrestin was cloned and published in 1987 by two groups (Shinohara et al. 1987; Yamaki et al. 1987), while β-arrestin, cognate for βAR, was cloned by Lefkowitz and co-workers (Lohse et al. 1990) in 1990. Hoffman's group demonstrated that visual arrestin had a strong affinity for light-activated and phosphorylated Rho (Schleicher et al. 1989). This specific receptor form proved to be the preferred one by a significant ratio, implying that arrestin exquisitely discriminated for the appropriate receptor state when quenching the signal.

What the Structural Biologists Knew

With the cloning of arrestin's cDNA, achieved by biochemical purification of the intact protein, peptide sequencing, and isolation of matching nucleotide sequences from libraries, a primary structure was in hand (Shinohara et al. 1987; Yamaki et al. 1987). Sequence analysis suggested some homology between arrestin and Gα transducin (Gt), a cognate heterotrimeric G protein of rhodopsin. Secondary structure prediction based on the sequence and circular dichroism spectroscopy indicated that the protein was almost complete beta-strand/sheet with quite a small percentage of α-helical structure (Shinohara et al. 1987). Classical biochemical structural probing by limited proteolysis performed by Palczewski and coworkers showed that the protein's N- and C-termini were easily cleaved but a resistant core, spanning residues 6 through 365, was likely to present a stable folded molecule (Palczewski et al. 1991a). An important conclusion was that the protein contained a flexible C-tail of about 40 residues. Concomitant with these biochemical studies, Gurevich and Benovic developed an in vitro translation system for preparation of radiolabeled recombinant arrestin that could be engineered for use in a functional truncation analysis (Gurevich and Benovic 1992). Their salient findings were that a

mini-arrestin, spanning residues 1 through 191, retained significant binding activity to Rho. In addition, they observed that the C-tail, had important impact on arrestin's specificity of receptor state binding (Gurevich and Benovic 1992, 1993). In parallel, Palczewski reported the isolation of a natural splice variant, purified from rod outer segments, called p44 that lacked the C-tail described earlier (Palczewski et al. 1994). Furthermore, p44 binds constitutively to activated but non-phosphorylated Rho. Finally, the polyanionic sugar heparin and phosphorylated Rho C-terminus were shown to bind arrestin (Schleicher et al. 1989; Palczewski et al. 1991b). The conclusion from these studies indicated that the arrestin C-tail was an important self-regulatory element, which held arrestin in a basal, inactive state until it was activated by the activated and phosphorylated receptor.

Crystallographic Studies

Due to the biological and biomedical significance of the GPCR signaling systems, elucidation of the components' 3D structures became an immediate goal in several laboratories. Ongoing successful efforts to understand the mechanism of the hetero-trimeric G protein cycle elicited the crystallographic structures of the visual and inhibitory G protein complexes in various nucleotide states. The prerequisite for these studies was abundant and homogeneous protein preparations. Due to the nature of the visual system, it was possible to purify from bovine rod outer segments (ROS) copious amounts of Rho, Gt, and arrestin. In parallel, recombinant expression was pursued, using both bacterial and baculovirus/insect cell culture systems. In the end, both approaches proved to be productive in the hands of the competing laboratories, which included the Sigler group at Yale, the Sprang and Gilman groups at Southwestern, and the German groups, which worked on arrestin.

For the arrestin work, ROS were the starting material and the membranes themselves were used to purify arrestin by adsorption and subsequent chromatography (Wilden et al. 1986b). Wilden and colleagues provided this preparation for crystallization, which was achieved at room temperature (Wilden et al. 1997). In contrast, our group pursued recombinant bacterial expression (Schubert et al. 1999), employing a modified preparation protocol based on Gurevich and coworkers' work, published in 1997 (Gurevich and Benovic 1997). The system did not employ any real protein engineering so that no affinity tags were used. Hence, we used column chromatography, in particular heparin Sepharose to obtain the requisite protein (Hirsch et al. 1999). In our hands, crystals were only obtained at 4 °C, although there was considerable polymorphism. Some of these forms did exhibit reasonable diffraction. Many did not. The crystal forms that were the basis of structure determination for both research groups were related and had in common an asymmetric unit comprised of a dimer of dimers with the same configuration, discussed below. These forms had high solvent content ($\sim$70%), confounding efforts to measure quality data sets. In our hands, crystals were generally non-isomorphic, further challenging phasing efforts. With synchrotron radiation,

diffraction limits were pushed to 3.3 Å for the Julich group led by Granzin et al. (1998) and to 2.8 Å for the Yale group (Hirsch et al. 1999). Experimental phasing was obtained by isomorphous replacement (Julich) or anomalous diffraction and isomorphous replacement (Yale).

The tertiary structure of arrestin from these studies revealed a protein with two clear domains, labeled the N- and C-domains, connected by a linker lacking secondary structure. Both domains are β-sandwiches i.e. each comprises two stacked β-sheets, each bearing four strands. Each domain is autonomous, lacking any interleaving elements from the other domain. Indeed, the N-domain has the protein's single α-helix, formed from a linker connecting two strands. The β-sandwiches have significant curvature, so that both appear as cupped palms (Fig. 3.1). These β-sandwiches have been classified as members in the immunoglobulin superfamily by projects such as SCOP. The third structural element found in the structures of the Yale group was the C-tail. This element spans residue 363 through the C-terminus. Poorly defined electron density characterized residues 363–371 and 395–402, likely due to high mobility of these polypeptide sections. Importantly, the segment from 372 to 395 was clearly modeled. Indeed, it forms, in part, a β-strand that interacts with the β-sheet of the N-domain. This C-tail has been observed in several arrestin family crystal forms and its location and conformation provide essential functional insights, in addition to being consistent with a large body of biochemical findings.

The structure revealed what had been discerned early on with publication of the primary sequence, namely that the two primary domains were organized in a tandem fashion and had some level of sequence similarity, albeit rather low (about 19%). The tertiary structure clearly exhibits an intra-molecular dyad, relating the two β-sandwiches (Fig. 3.1). Perhaps even more striking is the topology of the sandwiches, in particular the connecting turns and loops, which is conserved between domains. The sequence and topology similarities are exhibited at the level of superpositions such that a majority of the Ca atoms have 1.8 Å RMSD with a pseudo two-fold axis (∼160°) (Hirsch et al. 1999). And while the fold classifiers

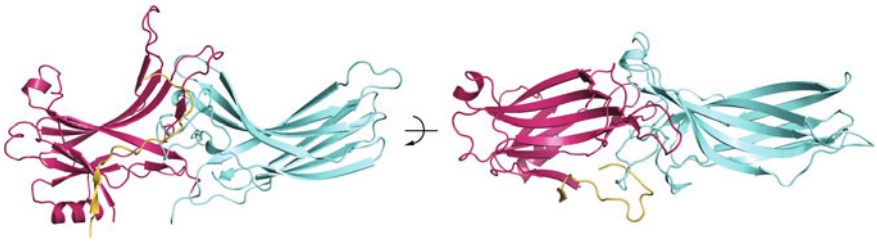

Fig. 3.1 The crystallographic 3D structure of bovine visual arrestin at 2.8 Å resolution. A protomer is shown, depicting the secondary structure elements. A *side* perspective is shown at *left* and a *top* perspective is displayed at *right*. Note the pseudo twofold axis perpendicular to the plane in the *right panel* that relates the two domains. The N-domain is depicted in magenta while the C-domain is *cyan*. The C-tail is *colored yellow*. The illustration is based on coordinates of PDB entry 1CF1 and was prepared using PyMol

have created unique motifs for each domain (N and C), most known arrestin family sequences have the tandem domain architecture (Alvarez 2008). This empirical observation can be readily justified by inspection of the structure. The two domains abut each other at the respective ends and that interface is largely hydrophobic in nature with a caveat described below. So while each domain is topologically independent, the domains are unlikely to be stable or soluble individually in solution.

Close examination of the structure, directed by earlier mutagenesis and functional characterization (Gurevich and Benovic 1995), brought focus to an unusual and concentrated constellation of charged residues situated in the fulcrum of the molecule, i.e. between the N- and C-domains. These residues were largely buried, with the attendant energetic penalties involved, posing a riddle to any thoughtful biophysical chemist. Indeed, Mayo and coworkers have outlined three categories for the underlying rationale of buried polar residues: active sites, thermodynamic stability, and folding/structure specificity (Bolon and Mayo 2001). We named this constellation of buried salt bridges the polar core. The earlier structure-function studies had revealed that a single point mutant, Arg175Gln or Arg175Glu, could render visual arrestin constitutively active i.e. it was able to bind with avidity to activated Rho without need for phosphorylation of the receptor (Gurevich and Benovic 1995, 1997). This behavior was akin to the p44 splice variant described above. These previous structure-function studies had sought to map the phosphate sensor in arrestin with the assumption that the Rho phospho-C-tail activated arrestin into a receptor binding-competent state. Hence, the positively charged residues buried in the polar core could attract the phosphopeptide, triggering and inducing a conformational change in arrestin. Importantly, the crystallographic studies elegantly provided the atomic details for arrestin's charged residues that acted as an "active site" for catalyzing the protein's conformational changes. Specifically, Asp296 served as the counter-ion for Arg175, holding the two arrestin domains in the basal orientation. Other charged residues contributed to the polar core, in particular Arg382 from the C-tail. Structure-guided mutagenesis of this polar core and functional characterization validated and reinforced the crystallographic insights, and taken together resulted in a model of arrestin activation by disruption of the polar core upon association with light-activated phospho-Rho (Hirsch et al. 1999; Vishnivetskiy et al. 1999). The model included the idea that the C-tail maintained the basal state by its interactions with the polar core and neighboring surfaces. At activation, the C-tail is released, the polar core is exposed to the receptor's phosphopeptide and arrestin undergoes a conformational change. Strikingly, the polar core could be remodeled by reciprocally swapping charged residues. In addition, the protein's electrostatic properties were consistent with the notion that basic surfaces of arrestin attract the flexible receptor phosphopeptide and guide it to the polar core. The centrality of the polar core has been borne out in later structural work which includes the structure determination of p44, Arg175Glu, and the most recent Rho*-Arr complex (Kim et al. 2013; Granzin et al. 2015; Kang et al. 2015).

One of the curious observations we made about these initial structures was the finding that several regions encoded chameleon sequences. Chameleon sequences are polypeptide stretches, which can adopt multiple conformations including

switches of secondary structure [for a recent review see Li et al. (2015)]. We noted in our original study [see Fig. 5 in Hirsch et al. (1999)] that residues 68–79, later called the finger loop (Hanson et al. 2006), undergo a transition between loop and β-strand/sheet conformations in the two different protomer copies of arrestin. In the recently solved Rho-arrestin complex crystal structure, the finger loop is one of three important patches of the protein-protein interface. Remarkably, in the receptor complex the loop forms an α-helix. It seems likely that chameleon sequences are well suited for serving as molecular recognition features and may be related to some of the general properties of intrinsically disordered proteins (Cumberworth et al. 2013).

Subsequent to the initial structures of visual arrestin, other arrestin family structures were determined (Han et al. 2001; Milano et al. 2002; Sutton et al. 2005), demonstrating the conserved arrestin fold fold. Other structural work revealed that Vps26, a protein important for cellular trafficking and part of the retromer, a protein complex that directs transmembrane cargo from endosomes to the Golgi, surprisingly had an arrestin fold, despite no apparent sequence similarity (Shi et al. 2006). Moreover, Vps26 had something resembling a polar core, a constellation of charged residues holding the N- and C-domains together, albeit in a different configuration than that found in the arrestins. The function of this polar core, though, may not be analogous to that of arrestin as based on recent structural work of the retromer complex (Lucas et al. 2016). Alvarez performed a comprehensive bioinformatic analysis of the arrestin super-family, incorporating molecular phylogenetics on the basis of expanded genomic data (Alvarez 2008). These structural findings and sequence observations have led to the conclusion that there is an arrestin fold, found throughout the eukaryotic kingdom, extant in fungi and metazoa. Indeed, fungi have a wide variety of arrestins, containing homologs to both the N- and C-domain, but including other protein-protein interaction or protein phospholipid motifs such as PY or FYVE (Aubry et al. 2009). Using phylogenetic analysis, Alvarez delineated two subfamilies, α and β. The former (traditionally called arrestin domain-containing proteins, ARRDCs) has many representatives in the fungal and metazoan world while the latter is found only in metazoa, with *Hydra* being the most primal species. The β subfamily includes arrestin1-4, i.e. visual/β-arrestins that have been characterized so extensively in vertebrates. Notably, no species has exclusively single domain arrestins, indicating that the gene duplication responsible for the tandem N- and C-domains is very ancient and consistent with our understanding of the fold structure. Vps26 is more closely related to the α arrestins but is clearly ancient also, as it is found in all eukaryotes including plants, which lack the β arrestin subfamily. Interestingly, Spso0M proteins, related to the α arrestins have been found in archaea and bacteria (Alvarez 2008).

Quaternary Structure

As mentioned earlier, the asymmetric unit for both crystal structures comprised a dimer of dimers, with two distinct protomer conformations. These tetrameric complexes had the same exact quaternary structure despite having been crystallized under vastly different conditions and forming different crystallographic habits. The Yale group noted this conserved molecular symmetry and asked if such a particle was found in solution or restricted to the crystalline form and therefore irrelevant to molecular physiology. In the first reports of biochemical isolation, an oligomeric state had been detected (Wacker et al. 1977). To address this question rigorously, we used a sedimentation equilibrium (Schubert et al. 1999). Our conclusions were that indeed tetrameric arrestin is found in solution at high concentrations but that there is a monomer-dimer-tetramer equilibrium and this mixture may be a relevant quaternary state in photoreceptor cells as based on the very high concentration of arrestin in these cells (Strissel et al. 2006; Song et al. 2011; Hanson et al. 2007a). At the time, we proposed that self-association engenders an inactive storage form, rapidly converted to the monomer for proper and rapid quenching of signal. In elegant subsequent studies reviewed in Chen et al. (2014), this proposal was modified but the basic idea remains valid; that arrestin self-association plays an important role in arrestin function by enabling the correct concentration for the monomeric species capable of receptor binding (Hanson et al. 2007b) while at the same time maintaining a large dynamic range of signal amplitude. Further structural and biochemical work demonstrated that the solution tetramer was configured differently than the crystalline form, although certain molecular interfaces were the same (Hanson et al. 2008). The solution tetramer model utilizes all potential molecular interfaces in a closed ring, explaining why it is stable, whereas the crystalline tetramer (asymmetric unit) does not, enabling lattice formation. It remains possible that the solution tetramer structure was actually crystallized by the Yale group in the gamut of polymorphic crystals obtained, but due to poor diffraction of these crystals, no structure was determined.

Thus, the first crystal structures of visual arrestin and subsequent structure-based mutagenesis and biophysical studies yielded the first glimpse into molecular mechanism of arrestin activation and arrestin regulation by self-association. The structures showed that arrestin is a metastable protein, perfectly designed for a global conformational change. Moreover, the structures revealed a novel arrestin fold, which was subsequently shown to be shared by all receptor-binding arrestins and at least some arrestin-domain containing proteins.

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