An arrestin-1 surface opposite of its interface with photo-activated rhodopsin engages with enolase-1

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ABSTRACT

Arrestin-1 is the arrestin-family member responsible for inactivation of the G protein-coupled receptor rhodopsin in photoreceptors. Arrestin-1 is also well known to interact with additional protein partners and to affect other signaling cascades beyond phototransduction. In this study, we investigated one of these alternative arrestin-1 binding partners, the glycolysis enzyme enolase-1, to map the molecular contact sites between these two proteins and investigate how the binding of arrestin-1 affects the catalytic activity of enolase-1. Using fluorescence quench protection of strategically placed fluorophores on the arrestin-1 surface, we observed that arrestin-1 primarily engages enolase-1 along a surface that is opposite of the side of arrestin-1 that binds photo-activated rhodopsin. Using this information, we developed a molecular model of the arrestin-1-enolase-1 complex, which was validated by targeted substitutions of charged-pair interactions. Finally, we identified the likely source of arrestin’s modulation of enolase-1 catalysis, showing that selective substitution of two amino acids in arrestin-1 can completely remove its effect on enolase-1 activity while still remaining bound to enolase-1. These findings open up opportunities for examining the functional effects of arrestin-1 on enolase-1 activity in photoreceptors and their surrounding cells.

INTRODUCTION

The arrestin family of proteins is well established as a key regulator of G protein-coupled receptors, functioning in both desensitization and intracellular trafficking of GPCR’s [see (1) for a recent review]. In the vertebrate visual system, arrestin-1 desensitizes the visual pigment in both rod and cone photoreceptors, binding light-activated and phosphorylated rhodopsin to sterically occlude transducin (2-4). Although there is no evidence that rhodopsin is internalized, and thus no role for arrestin-1 in rhodopsin intracellular trafficking, a number of studies have identified additional interactions for arrestin-1 other than rhodopsin desensitization (5). This list of interactors includes arrestin-1 binding calcium-calmodulin, potentially buffering changes in cytosolic calcium levels in photoreceptors (6); interaction with Src family tyrosine kinases for activation of extracellular signal-regulated kinase 1/2 and E3 ubiquitin ligases (7); and interaction with N-ethylmaleimide sensitive factor (NSF) for regulation of synaptic signaling (8).

In addition to these binding partners for arrestin-1, there is also an interaction between arrestin-1 and enolase-1 (9), one of the key enzymes in the glycolysis pathway that catalyzes the interconversion of 2-phosphoglycerate to phosphoenolpyruvate. This interaction is specific for enolase-1, an observation that is surprising
since enolase-2 is considered to be the “neuronal” enolase (10). Intriguingly, the binding of arrestin-1 to enolase-1 affects the catalytic activity of enolase, reducing its rate of activity by ~25%. Although a reduction of 25% activity may not seem like a large change, photoreceptors require on the order of $10^7$ ATP molecules per second per cell, ranking them as one of the highest energy-consuming cells in the body (11). Furthermore, photoreceptors metabolize 80-96% of available glucose into lactic acid via aerobic glycolysis (12,13), returning the lactate byproduct as an essential metabolic component to the retinal pigmented epithelium and Müller glia. Consequently, small changes in glycolytic efficiency could have a large impact on photoreceptors because of their extreme energetic demands. Because of the impact of arrestin-1 on enolase-1 catalysis, we initiated this study to develop a molecular understanding of the interaction between arrestin-1 and enolase-1 with the goal being to understand how the binding of arrestin-1 could affect the catalytic activity of enolase-1.

RESULTS

In a previous study, we identified that arrestin-1 selectively interacts with enolase-1 in photoreceptors, modulating the catalytic activity of enolase-1 (9). In this study, we investigated the biophysical nature of this interaction between arrestin-1 and enolase-1 with the goal being to understand the mechanism for how the binding of arrestin-1 could affect the enzymatic activity of enolase-1. As a first step towards understanding the interaction of these two proteins, we used targeted fluorescence labeling of arrestin-1 and fluorescence quenching to map the surface on arrestin-1 with which enolase-1 interacts. For this study, 28 cysteine substitutions were introduced individually into arrestin-1 at residues that are positioned across the surface of arrestin-1 (Table 1). Importantly, these cysteine substitutions were introduced into an arrestin-1 in which native Cys-63 and Cys-143 were converted to alanine to remove the endogenous reactive cysteines in arrestin-1. These introduced cysteine residues were labeled with the thiol-reactive fluorophore monobromobimane (mBBr).

We next optimized the conditions for analysis of quenching of the fluorophore by potassium iodide (KI) (14). First, we identified the optimum KI concentration for quenching, performing a titration assay using three different cysteine-substituted arrestin-1 (K53C, S86C, and Y125C) labeled with mBBr, quenching with increasing concentrations of KI. For all three labeled positions, 50% quench of fluorescence of the 3 μM arrestin-bimane was achieved at ~33 mM KI, or with a 10^4 molar excess of KI over the bimane-labeled arrestin.

We next titrated the concentration of enolase-1 that could provide protection of mBBr fluorescence from quenching by the KI, determining that a 90% quench protection could be obtained with a 20-fold molar excess of enolase-1 to arrestin-1 for H10C labeled with mBBr.

Using these parameters of ~50% quench with 10^4 molar excess of KI over arrestin-1 and 20-fold molar excess of enolase-1 over arrestin-1, we scanned the arrestin-1 cysteine point mutations for the potential of enolase-1 to protect the mBBr-labeled arrestin-1 for fluorescence quenching. For this analysis, we measured the fluorescence emission of the mBBr-labeled arrestin-1 with and without potassium iodide to determine the range of quenching by KI, and measured the fluorescence emission quench by KI with and without enolase-1. Three examples of the range of quench protection are shown (Fig 1A), with enolase-1 providing no protection of the mBBr on I72C, approximately 60% protection for S210C, and essentially complete protection for E218C. For each of the 28 labeled arrestin-1 proteins, a quench protection factor was calculated as described in the experimental procedures (Table 1, Figure 1B). This quench protection analysis revealed a range of residues that when labeled by mBBr were highly protected from quenching by enolase. When mapped onto the surface of the arrestin-1 structure, these residues largely mapped to a single surface along the “bottom” of the arrestin-1 protein (Fig 1C).

We then used this information to generate a molecular model of the interaction between arrestin-1 and enolase-1, performing energy minimization docking of arrestin-1 with an enolase-1 dimer using ClusPro 2.0. A dimer of enolase-1 was used for this model since this is the typical physiological state of enolase-1 (15), and our previous study had shown that arrestin-1 crosslinks to a dimer of enolase-1 (9). Based on the
fluorescence quench studies, the interaction sites were constrained to include residues His-10, Asp-183, Glu-218, Glu-302, and Asp-362 of arrestin-1. The resulting model of the complex predicted both N- and C-terminal domains of arrestin-1 to bind to a single unit of the enolase 1 dimer (Figure 2), forming numerous interactions with surface residues, including several paired charged interactions.

Since the surface of the arrestin-1 that is modeled to interact with enolase-1 is on the side opposite of arrestin-1 that engages rhodopsin (16-18), we tested whether the binding of arrestin-1 to light-activated, phosphorylated rhodopsin (pRho*) would exclude enolase-1 binding. For this experiment we used phosphorylated rhodopsin prepared in rod disc membranes, and performed co-sedimentation analysis of arrestin-1 with and without enolase-1/GFP after light activation of the rhodopsin (Fig 3A). Note that for this experiment, we used an enolase-1/GFP fusion so that the molecular masses of arrestin-1 and enolase-1 could be distinguished on gel electrophoresis. In this analysis, enolase-1/GFP co-sedimented with arrestin-1 on membranes with pRho* (Fig 3A, lane 1), but not in mixtures that contained no arrestin-1 (lane 5), or in which the phospho-rhodopsin was not light activated (lanes 2 & 6), indicating arrestin-1 binding to rhodopsin does not exclude the interaction of arrestin-1 with enolase-1. Conversely, quantitative analysis of the arrestin-1 pulled down with pRho* in the presence or absence of enolase-1/GFP indicates that the binding of enolase-1 to arrestin-1 does not affect the binding of arrestin-1 to pRho* (Fig 3B).

As an alternative way to examine the simultaneous interaction of arrestin-1 with both pRho* and enolase-1 we monitored whether the quenching protection provided by enolase-1 for arrestin-1 labeled with mBBr at E218C was affected by arrestin-1 binding to pRho*. When E218C-mBBr is mixed with pRho*, there is no protection of the mBBr fluorophore from quenching by potassium iodide (Fig 3C, orange bars). In contrast, enolase-1 provides nearly complete protection of the fluorophore either without pRho* (blue bars) or with arrestin-1 bound to pRho* (green bars). These studies suggest not only that arrestin-1 can simultaneously engage enolase-1 and pRho*, but also indicate that binding of enolase-1 to arrestin-1 is independent of whether arrestin-1 is in its inactive or receptor-bound conformation.

As a further test of this observation, we also performed a co-purification assay of enolase-1 by immunoprecipitating arrestin-1 with anti-arrestin-1 antibody on Protein G-coated magnetic beads, and measuring pulldown of enolase-1 that was fluorescently labeled with AlexaFluor-546. For this assay, we used either native arrestin-1 (WT) as the inactive form or arrestin-1 with the so called “3A” mutations (i.e., F375A/V376A/F377A) which mobilizes the arrestin-1 C-terminus, allowing it to adopt an active conformation that does not require rhodopsin phosphorylation to bind (19,20). In this assay, there was no distinguishable difference in the pulldown of labeled enolase-1 between wild-type and the pre-activated arrestin-1 (Fig 3D).

Returning to our model of the arrestin-1/enolase-1 complex, we next wanted to further validate this model so that we could then use it for predictive studies. We elected to use the charge-pair interactions to empirically validate the modeled complex. Seven charged-pair interactions (Table 2 and Fig 4A) were identified from the model as having the closest interactions. These seven residues were singly mutated on the arrestin-1 protein to reverse the charge of the amino acid side chain, while approximately preserving side chain size (e.g., Glu to Lys, or Arg to Asp). Following heterologous expression and purification, each arrestin-1 mutant was then assessed for its interaction with enolase-1, using the anti-arrestin-1 immunoprecipitation assay described above to pull down fluorescently labelled enolase-1. In this assay, mutations R29E, E361K, and D362K reduced the pulldown of enolase-1 by ~25% (Fig 4B). Mutations R37D, D183K, and E302K had a smaller, but also significant effect on interaction with enolase-1. The significant effect of each of these amino acid substitutions, with the exception of E36K, on the interaction with enolase-1 suggests that the model of interaction between arrestin-1 and enolase-1 (Fig 2) is correct.

Since each mutation partially disrupted the interaction between arrestin-1 and enolase-1, we reasoned that all seven mutations together would likely have a more significant effect. Accordingly, we assembled all seven point mutations into a single arrestin-1 protein, and also
assembled the corresponding charge reversals in the enolase-1 molecule. When used in our pulldown assay, these mutagenized proteins decreased the binding to their wild-type counterpart by more than 80% (Fig 4C). Importantly, when the combination mutant of arrestin-1 is paired with the combination mutant of enolase-1, binding interaction is restored (Fig 4C, gray bar), providing compelling evidence that these mutations are occurring on complementary pairs of interactions.

Since these binding studies indicated that our model of the interaction between arrestin-1 and enolase-1 is accurate, we next pursued the goal of determining the structural mechanism for how arrestin-1 binding to enolase-1 can affect the catalytic activity of enolase-1. Our previous study demonstrated that the binding of arrestin-1 to enolase-1 diminished the catalytic rate of enolase-1 by ~25% (9). Accordingly, we examined our model of the interaction, focusing on enolase-1 loops L1, L2, and L3 and the magnesium coordinating residues Ser-36, Asp-244, Glu-292, and Asp-317 that comprise the key components of the enolase-1 active site (21,22). In our model, arrestin-1 residues Glu-361 and Asp-362 are the residues most deeply inserted into enolase-1, and these two residues are also in close proximity to all three of the loops that comprise one of the active sites in the enolase-1 dimer, particularly Ser-156 and Gly-159 in loop L2 (Fig 5A). Since we previously demonstrated that the binding of arrestin-1 to enolase-1 diminished the catalytic rate of enolase-1 by ~25%, we speculated that this interaction point might be the source of the perturbation, likely by steric interference. To investigate this idea, we mutagenized both Glu-361 and Asp-362 to glycine, reasoning that if a sterically hindrance effect was occurring that these substitutions would potentially relieve the interference. We first examined whether these mutations affected the binding of arrestin-1 to enolase-1 using the same pulldown assay of fluorescently labelled enolase-1 previously described. In this assay, the pulldown of enolase-1 with the E361G/D362G double mutant of arrestin-1 is indistinguishable from that of native arrestin-1 (Fig 5B). This finding indicates that these two mutations do not significantly disrupt the overall interaction between arrestin-1 and enolase-1. We then moved on to determine if these mutations influenced the impact of arrestin-1 on enolase-1 catalytic activity. In this assay, the catalytic activity of enolase was measured by monitoring the production of ATP from the processing of 2-phosphoglycerate to pyruvate (Fig 5C). Wild-type arrestin-1 inhibited the catalytic activity of enolase-1 by approximately 25%. Significantly, this inhibition of enolase catalysis was completely absent in the E361G/D362G double mutant of arrestin-1, even at 320-fold molar excess. This finding suggests that Glu-361 and Asp-362 are largely responsible for altering the catalytic activity of enolase-1 when the complex forms.

To determine whether the effect of arrestin-1 on enolase catalysis has any potential physiological function, we monitored the glycolytic output of lactate from HEK-293T cells that had been transfected with arrestin-1 (Fig 5D). Because HEK cells use a combination of glycolysis and oxidative phosphorylation for energy production, we biased the cells towards glycolysis by inhibiting oxidative phosphorylation using rotenone and antimycin A to block complex I and complex III of the respiratory chain (23). Measurement of lactate produced by these cells showed a significant reduction in the cells that were transfected with arrestin-1 (Fig 5D & E) compared to cells that were either untransfected or transfected with a plasmid expressing green fluorescent protein (GFP). This result indicates that arrestin-1 can affect glycolytic output in tissue culture context. Consistent with our previous observation, transfection of the modified arrestin-1 with the E361G/D362G mutations (Arr-GG) had no effect on lactate production.

To get a better understanding of how arrestin-1 affects the catalytic activity of enolase-1, we performed an analysis of enolase-1 under Michaelis-Menten conditions. We first determined the linear dependence of the 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP) reaction rate on enolase-1 concentration (Fig 6A). The kinetic parameters for this reaction was then measured using 100 nM enolase-1 with and without arrestin-1 (500 nM), varying the concentration of 2-PGA. A summary of the kinetic parameters (Fig 6C) shows that the only significant effect of arrestin-1 was on the Km. The increase in Km without significantly effects on either Vmax or kcat suggests that arrestin-1 acts as a
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competitive inhibitor, decreasing the access of the 2-PGA substrate for the active site in enolase-1.

DISCUSSION

This study provides several new points of important information regarding the arrestin-1/enolase-1 interaction. First, our quenching studies identified that arrestin-1 primarily engages enolase-1 along the surface opposite the side of the molecule that binds photo-activated rhodopsin. The concomitant binding of both enolase-1 and activated phospho-rhodopsin supports this model. This observation suggests that in photoreceptors, when arrestin-1 translocates to the outer segment to bind pRho*, enolase-1 could be carried with the arrestin-1. However, this light-dependent movement of enolase-1 has not been observed (9), indicating that either the dissociation rate of enolase-1 from arrestin-1 is sufficiently high such that enolase-1 is not be carried with arrestin-1 or that enolase-1 might be interacting with other unidentified binding partners in the inner segment.

Another important conclusion that can be reached by the binding of arrestin-1 to both enolase-1 and pRho* is that the conformation of arrestin-1 that is bound by enolase-1 is independent of the known elements that regulate its transition between the inactive to the receptor-bound state. These regulatory elements include the “finger loop” of the N-terminal domain (24-27) and the phosphate sensors (24,28). Our results showing equivalent binding of enolase-1 to the 3A mutant of arrestin-1 adds further evidence that mobilization of the C-terminus of arrestin-1 is not a critical regulatory element for enolase-1 binding either.

Our molecular model for the arrestin-1/enolase-1 complex, which was validated by targeted mutation of charged-pair interactions, permitted identification of the likely cause for arrestin’s modulation of enolase-1 catalysis. In this model, Glu-361 and Asp-362 of arrestin-1 penetrate deeply into the enolase-1 protein. The proximity of these residues to loops L1, L2, and L3 (7.6 Å, 4.9 Å, and 6.0 Å respectively), which comprise the main elements of the enolase-1 active site (21,22), suggests that Glu-361/Asp-362 likely create a steric interference with the L1, L2, and L3 loops of enolase-1. This conclusion is supported by the results from the kinetic parameters investigation which indicate that arrestin-1 acts as a competitive inhibitor.

The model for how arrestin-1 impacts enolase-1 activity also offers a potential explanation for the previous observation that arrestin-1 has a maximum effect of reducing enolase-1 activity by only ~25% (9). Because arrestin-1 interacts with only one of the active sites in the enolase-1 dimer, the maximum effect that would be expected is only a 50% reduction in enolase activity, even if arrestin-1 completely disrupted the active site.

The functional consequences of the interaction between arrestin-1 and enolase-1 have not yet been elucidated. However, our empirical data from studying the glycolytic potential of kidney cells in tissue culture shows that arrestin-1 can reduce the lactate output. This observation has some intriguing implications for photoreceptors, particularly given the very high glycolytic activity in rods and cones (12,13). In these cells, which are highly modified and polarized sensory cilia, the inner segment portion of rods and cones is responsible for most of the metabolic activity of the photoreceptor, including glycolysis, whereas the outer segment is principally responsible for phototransduction. Accordingly, enolase-1 principally localizes to the inner segment of photoreceptors along with the other glycolytic enzymes (9,29). In contrast, arrestin-1 localization is dynamic, translocating from the inner segment under dark conditions to the outer segment upon light exposure [e.g., (30-33)]. This translocation of arrestin-1 means that arrestin-1 down regulates the activity of enolase-1 during the dark when it localizes to the inner segments, and that enolase-1 activity is upregulated in the light when arrestin-1 moves to the outer segments. Since the energetic demands of photoreceptors are highest in the dark when the cyclic nucleotide-gated channels are open and the sodium-potassium pump is at its maximum rate to maintain ionic equilibrium (34), it is not clear what benefit might be provided by potentially increasing glycolytic activity in the light when enolase-1 quenching is reduced as arrestin-1 translocates to the outer segment. Perhaps an increase in glycolytic activity during light may function to provide more lactate to the RPE or Müller glia for their metabolic demands, rather than increasing the energy supply to photoreceptors. Regardless, this study’s
identification of the mechanism for how arrestin-1 impacts the activity of enolase-1 now opens the opportunity to empirically examine the functional effects of arrestin-1 on enolase-1 activity in photoreceptors and their surrounding cellular neighbors.

EXPERIMENTAL PROCEDURES

Arrestin-1 and enolase-1 mutagenesis. Cysteine substitution mutations, charge reversal mutations, and F375A/V376A/F377A mutations of arrestin-1 were introduced into N-terminally His(6)-tagged bovine arrestin-1 by overlapping PCR amplification of the bovine arrestin-1 cDNA as previously described (35). For the cysteine substitution mutants, the arrestin-1 cDNA template also contained the two cysteine mutations, C63A and C143A, to remove the two reactive cysteines that are endogenously present in arrestin-1 (27). Arrestin-1 containing combinations of mutants was prepared by serial mutagenesis, using the mutagenized cDNA as the template to add the next mutation, until all desired mutations were introduced.

The primers used to introduce these cysteine substitutions and charge reversal mutations are shown in Table 3. The altered cDNA’s were cloned into pPICZ-A at the EcoRI site and heterologously expressed in Pichia pastoris for three days in the presence of 0.5% methanol. Following disruption of the cell wall by French pressing (20,000 psi), the arrestin-1 protein was purified to >95% homogeneity by chromatographic purification over nickel-agarose affinity resin (GE resin) in 50 mM sodium phosphate (pH 8.0) with 300 mM sodium chloride and 10 mM imidazole, eluting with 100 mM ethylenediaminetetraacetic acid (EDTA). Fractions containing purified arrestin-1 protein were pooled, and dialyzed against LAP200 buffer (50 mM HEPES, 200 mM NaCl, 1 mM EGTA, 1 mM MgCl2, 10% glycerol, 0.05% NP-40, pH 7.4).

Enolase-1 was prepared as described for arrestin-1 with the exception that the His(6) tag was added at the C-terminus. Fusion of enolase-1 with green fluorescent protein (GFP) was made by introducing an NheI restriction site prior to the stop codon in the enolase-1 cDNA with an His(6) tag, and then inserting the GFP cDNA flanked with NheI restriction sites. The fusion cDNA was cloned into pPIC-ZA and expressed and purified as described above for arrestin-1.

Quenching assay. Cysteine mutants of arrestin-1 were labeled with monobromobimane (mBBr, ThermoFisher), reacting the arrestin-1 protein with 100-fold molar excess of the fluorescent label for two hours at room temperature. The unreacted label was removed by dialyzing against three sequential changes of 500-volumes of LAP200 buffer. Fluorescence labeling of the arrestin was quantified by measuring the absorbance of mBBr (394 nm, λmax) and arrestin-1 (278 nm, λmax) and calculating the percent labeled (assuming ε_{mBB, 394nm}= 5,300 M⁻¹ cm⁻¹ and ε_{Arr1, 278nm}= 25,200 M⁻¹ cm⁻¹). Fluorescence quenching of the mBBr fluorophore was performed using 0.25 µM mBBr-labeled arrestin-1 with 100 mM potassium iodide in the presence or absence of 5 µM bovine enolase-1 that had been heterologously expressed and purified as previously described (9), monitoring fluorescence emission at 470 nm. To determine the degree to which enolase-1 shielded a residue from quenching by potassium iodide, we calculated a protection factor as:

\[ P_l = \frac{F_{(A+E+K)} - F_{(A+K)}}{F_{(A)} - F_{(A+K)}} \]

where \( F_{(A)} \) is the fluorescence of mBBr-labeled arrestin in solution alone, \( F_{(A+K)} \) is the fluorescence of mBBr-arrestin-1 in the presence of potassium iodide, and \( F_{(A+E+K)} \) is the fluorescence of mBBr-arrestin-1 in the presence of both enolase-1 and potassium iodide. Essentially, this protection quotient looks at the total range of protection offered by enolase normalized to the access of the labeled cysteine to the potassium iodide. Values near zero indicate no protection of the fluorophore-labeled cysteine, whereas the protection factor will approach one if enolase-1 completely shields the mBBr from quenching by the potassium iodide.

For quenching assays done in the presence of pRho*, pRho in disc membranes was sonicated (Sonic Dismembrator, Fisher Scientific) for 5 sec in the dark to make a micellar preparation of the disc membranes. pRho (5 µM) was added to the samples, exposed to light for 1 min to activate the rhodopsin, and then fluorescence quenching measurements made as described above.
Molecular modeling. The interaction of arrestin-1 and enolase-1 was modeled using the crystallographic structure of arrestin-1 [1CF1, Chain A, (36)] and enolase-1 [3B97, (21)], using ClusPro 2.0 (37-39). Initial models were generated with no constraints, using arrestin-1 as the “receptor” molecule and enolase-1 as the “ligand”. In the highest scored reported models, ClusPro 2.0 predicted many of the previously mentioned arrestin-1 residues (His-10, Asp-183, Glu-218, Glu-302, and Asp-362) as potential interface residues. To generate a more accurate prediction, the interaction sites were constrained to include the residues that showed the strongest protection of fluorescence quenching by enolase-1, namely His-10, Asp-183, Glu-218, Glu-302, and Asp-362.

Rhodopsin disc membrane pulldown assay. Phosphorylated rhodopsin (pRh) in rod disc membranes was prepared as previously described (40). For pulldown, 2 µM arrestin-1 with or without 2 µM enolase-1/GFP was mixed with 5 µM pRh under dim red light. Samples with pRh* were activated by exposure to light for 1 min, and then returned to the dark for processing. Samples were centrifuged for 15 min (18,000 x g), and the pellet resuspended in Laemmli sample buffer (41) prior to separation on 12% sodium dodecyl sulfate gel electrophoresis and staining with 0.05% Coomassie R.

Immunoprecipitation/enolase pulldown assay. The interaction between arrestin-1 and enolase-1 was assessed by using anti-arrestin-1 antibody to immunoprecipitate the arrestin-1-enolase-1 complex in which the enolase-1 was fluorescently labeled with AlexaFluor546. For this assay, 1.5 mg of Protein G-coated magnetic beads (DynaBeads, ThermoFisher), were coated with 10 µg of purified C10C10 anti-arrestin-1 monoclonal antibody (42). Enolase-1 was labeled on its reactive cysteines with AlexaFluor-546 maleimide (ThermoFisher), reacting the enolase-1 with 100-fold molar excess of the fluorescent label. The fluorescently labeled enolase-1 was then sequentially dialyzed in LAP200N buffer to remove any unreacted label. For the immunoprecipitation of the arrestin-1-enolase-1 complex, 5 µM arrestin-1 (or arrestin-1 mutant) was mixed with 5 µM enolase-1-Alexa546 in LAP200N in a 200 µL final volume for 2 h at 4°C, to which 10 µg of anti-arrestin-1 antibody on magnetic beads was subsequently added for 16 h with gentle rotation. The beads were magnetically captured, washed ten times with LAP200N buffer, and then eluted with 0.1 M glycine (pH 2.5). After neutralizing the pH with 0.1 volume 1.5 M Tris base (pH 8.5), the fluorescence of the captured enolase-1-Alexa546 was measured, exciting fluorescence at 530 nm, and measuring average emission at 570-575 nm with a dual-detector fluorimeter (QM-1 steady state fluorescence spectrophotometer, Photon Technologies Inc).

Enolase activity assay. The catalytic activity of enolase-1 was measured in reconstitution assays by monitoring ATP production from the processing of phosphoenolpyruvate produced by enolase catalysis of 2-phosphoglycerate. For the reaction, 2 mM 2-phospho-glycerate (Sigma), 2 mM adenosine diphosphate (Sigma), and 0.3 U/mL pyruvate kinase (MP Biochemicals) was mixed with 50 nM enolase-1 with 0-1.6 µM arrestin-1 or arrestin-1 mutant. ATP production was monitored using a luciferase luminescence assay, mixing equal volumes of the reaction mixture and firefly luciferase (CellTiter-Glo 2.0 Cell Viability Assay, Promega). Luminescence was monitored at 550-570 nm at 5 min intervals for 40 minutes, calculating the rate of luminescence production from a linear regression of luminescence as a measure of enolase catalytic activity.

For measuring glycolytic production of lactate in tissue culture, arrestin-1 (Arr-WT), arrestin-1 with E361/D362 (Arr-GG), and GFP were cloned behind the ubiquitous small chicken beta actin (smCBA) promoter in the pTR-smCBA vector at the NotI sites (43). Human embryonic kidney cells (HEK-293T; 6 x 10⁶ cells) (44) were electroporated (Celectrix Biotechnologies), with 10 µg of each plasmid at 600 V for 3 msec and plated in six-well tissue-culture plates in Dulbecco’s modified eagle medium (DMEM) with 5% fetal bovine serum. After 24 hours, the media was removed and replaced with fresh DMEM supplemented with 10 mM glucose and 1 µM rotenone and 1 µM antimycin A to block the respiratory chain (23). Media samples were removed at 10 minute intervals and lactate measured by luminescence according to the
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manufacturer’s recommendation (Lactate-Glo, Promega).

For measuring the kinetic parameters of enolase-1 catalysis, enolase-1 activity was monitored by the increase in phosphoenolpyruvate (PEP) absorbance at 240 nm using a ClarioStar plate reader (BMG Labtech) and UV-transparent 96-well plates. A linear dependence on the reaction was first determined using 0-300 nM enolase-1 with 2 mM 2-phosphoglycerate (2-PGA; Sigma-Aldrich) in LAP200N buffer. The turnover number was calculated using the extinction coefficient of 1520 M⁻¹ for PEP. Kinetic parameters under Michaelis-Menten conditions were then measured using 100 nM enolase-1 with 0-6 mM 2-PGA in LAP200N buffer. Kinetic parameters $K_M$, $V_{max}$, and $k_{cat}$ were estimated by GraphPad Prism (v 8.4; GraphPad Software).

Statistical comparisons. Statistical comparisons were performed with GraphPad Prism. For multiple comparisons, one-way analysis of variance was conducted with Sidak’s post hoc multiple comparisons.

For enolase catalysis, the effect of arrestin-1 on enolase-1 activity was curve fit by non-linear regression analysis (inhibitor vs. response). Rate of lactate production from HEK-293T cells was determined from the slope of a best fit linear regression.

In all cases, differences were considered significant if $p<0.05$.

DATA AVAILABILITY
All data for this publication are either included in the manuscript or are available from the corresponding author upon request (W. Clay Smith, University of Florida, wcsmith@ufl.edu).

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.
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Table 1. Fluorescence quenching of mBBr-labeled arrestin-1 cysteine mutants by potassium iodide with protection by enolase-1.

| Cysteine Substitution | F(A)    | F(A+K)  | F(A+E+K) | P_f |
|-----------------------|---------|---------|----------|-----|
| H10C                  | 12595.7 | 1449.61 | 10279.5  | 0.792 |
| R18C                  | 40060.9 | 1148.8  | 963.2    | -0.005 |
| Y25C                  | 17932.1 | 789.91  | 599.8    | -0.011 |
| K28C                  | 23275.8 | 557.8   | 17195.5  | 0.732 |
| R37C                  | 28962.6 | 682.3   | 16642.6  | 0.564 |
| E50C                  | 25319.4 | 891.0   | 8143.2   | 0.297 |
| K53C                  | 34263.2 | 421.3   | 11031.5  | 0.314 |
| I72C                  | 40304.8 | 549.0   | 630.0    | 0.002 |
| S86C                  | 20069.2 | 5060.5  | 6159.6   | 0.073 |
| V94C                  | 16472.6 | 935.5   | 1901.7   | 0.062 |
| A113C                 | 25323.9 | 750.9   | 3465.0   | 0.109 |
| Y125C                 | 44782.9 | 13617.9 | 13978.0  | 0.012 |
| V139C                 | 27086.9 | 813.6   | 778.3    | -0.001 |
| K166C                 | 24572.6 | 404.6   | 1041.5   | 0.026 |
| D183C                 | 24307.5 | 538.9   | 19234.9  | 0.787 |
| R189C                 | 23296.8 | 490.8   | 11133.8  | 0.467 |
| W194C                 | 44782.9 | 13617.9 | 13978.0  | 0.012 |
| S199C                 | 23297.2 | 662.5   | 636.1    | -0.001 |
| S210C                 | 13660.6 | 724.1   | 8717.0   | 0.618 |
| E218C                 | 8846.4  | 953.6   | 9498.0   | 1.083 |
| E231C                 | 6973.8  | 682.0   | 694.6    | 0.002 |
| S251C                 | 6123.7  | 674.5   | 730.5    | 0.011 |
| K267C                 | 5523.2  | 350.3   | 2851.8   | 0.484 |
| V281C                 | 6002.9  | 1167.2  | 2439.3   | 0.269 |
| E302C                 | 4562.3  | 686.6   | 3676.4   | 0.772 |
| D317C                 | 33509.8 | 11518.1 | 11514.0  | 0.002 |
| D362C                 | 4735.3  | 950.7   | 5491.7   | 1.200 |
| E393C                 | 3604.6  | 542.6   | 952.8    | 0.134 |

Table 2. Charged-pair interactions between arrestin-1 and enolase-1 in the energy minimized docking model shown in Figure 3. The amino acid change made for charge reversal in the various arrestin-1 and enolase-1 mutants are indicated in parentheses after each amino acid.

| Arr1 residue (charge reversal) | Enolase-1 residue (charge reversal) | Molecular Distance (Å) |
|--------------------------------|------------------------------------|------------------------|
| Arg-29 (Glu)                   | Asp-265 (Lys)                      | 2.8                    |
| Glu-36 (Lys)                   | Lys-53 (Glu)                       | 2.7                    |
| Arg-37 (Asp)                   | Glu-197 (Lys)                      | 1.9                    |
| Asp-183 (Lys)                  | Lys-59 (Asp)                       | 2.3                    |
| Glu-302 (Lys)                  | Lys-255 (Asp)                      | 1.8                    |
| Gu-361 (Lys)                   | Lys-196 (Glu)                      | 2.6                    |
| Asp-362 (Lys)                  | Lys-192 (Glu)                      | 2.5                    |
Table 3. Targeted mutations and primer pairs used for site-directed mutagenesis in arrestin-1*.

| Targeted Substitution | Synthetic overlapping oligonucleotide pairs for mutagenesis |
|-----------------------|------------------------------------------------------------|
| H10C                  | 5'-AAGCCGCACAAACTGTTATTTATCCAGAGAAGAGAGAGAGGCTT |
| R18C                  | 5'-TTCAGAAGATCCCTCTGCTGATAAACATCGGTGACC  
                      | 5'-GGTCACCCGATTTATCACAGAGATCTTCTGAA |
| Y25C                  | 5'-GATAAACCGGTGACCATCTGCTGCTGGAGAGAGAGATTAC  
                      | 5'-GTAATCTTCTCTTCCCCAGACAGATGTCACCGATTTATC |
| K28C                  | 5'-GACCATCCACTGAGGGAATGAGGACTGACATGACAG  
                      | 5'-GTCTATGTAATCTTACACCCAGTAGATGTC |
| R37C                  | 5'-ATAGACCACGGTGAATGAGGACCTGTGAGTTGA  
                      | 5'-CATACACAGGCTACACTACATCAAGGAG |
| E50C                  | 5'-GTCTGTGTTGGAATCGACGATGAGGAGGCAAG  
                      | 5'-CTTGGGCTCTACAGACAGATGACAGGGACAC |
| K53C                  | 5'-GATCCTGAGCTGTTGAGGAAGAGGAGTTAC  
                      | 5'-GTACACTTCTCTGCAACAGAGACGTAGGATG |
| I72C                  | 5'-TACGGGCAAGAAGACTGTGATGAGGAGTG  
                      | 5'-GCCCATACGTCAGCTTTCCTGGCCGTA |
| S86C                  | 5'-CAGGGGACTCTTCTTCTGCAATGAGGTGTTCC  
                      | 5'-GGAACAGCTGAACTGAGAGGAGGAG |
| V94C                  | 5'-CAGGTGTCTCTCAGGCTGAGGAGGAGTCG  
                      | 5'-GGCGGCGGCGGCGGCAAGGAGACACCTG |
| A113C                 | 5'-ATCAAGAGCTGAGCTGTAACACCTACCCCTTC  
                      | 5'-GAAGGGGTAGGTTACAGGACAGGATC |
| Y125C                 | 5'-TCACCAGTTTCTGACTGTTTGGCCCTTGTTGAGTG  
                      | 5'-CAACGGAAGGGAACACGTAGGAAAGCTGAG |
| V139C                 | 5'-CCACGCTCCGGAAGATTGTGGAAGAGGAGCGAGG  
                      | 5'-CCCCGCTGGCTCTGCAACATCTTGGGAG |
| K166C                 | 5'-GAGGACAAATATTCTGTTGAGACCTCGTGGC  
                      | 5'-CGCACGAGAGTCTTACAGGAAATTTITTGCTC |
| D183C                 | 5'-CAGCACGCGCAGAGATGATGATGGTCCCAAGC  
                      | 5'-CGGGGCTGCGTGAGCAAGCCAGCAGCTG |
| R189C                 | 5'-GATATGGGCTCCGAGCCCTGGGAGGCTCCTGGG  
                      | 5'-CCAGGAGCCGCCCTCGAGCAAGGCTGGAG |
| W194C                 | 5'-TGGCAGTTCCTCTGCTGCTGACTGAGTAGCTCATGGC  
                      | 5'-CGACATGAAAGAAAGCTGAGAGGAGCGCTGCTC |
| S199C                 | 5'-TGGCCAGTTCCTCTGCTGCTGACTGAGTAGCTCATGGC  
                      | 5'-CGGAGGGGTAGGGAGCAAGGAGGACTG |
| S210C                 | 5'-CTGCGGCTCCGCTGCTGCTGACTGAGTAGCTCATGGC  
                      | 5'-GTATAGATGATTTTACAGAGCGAGCAGGAG |
| E218C                 | 5'-ATCTATTACACGGGTGCTCCATCTGTCGACC  
                      | 5'-GGTCACAGGAGTGAGGACACCACGTGAAATGAG |
| S251C                 | 5'-CTTCTACGCTGGTCTGCTGCTGACTGAGTAGCTCATGGC  
                      | 5'-CGTGGTTTCTACTGAGTAGGATTATTAC |
### Arrestin-enolase interaction

| Mutation | 5’-Sequence |
|----------|-------------|
| K267C    | 5’-GAGGGAAAGCAGAGAATGTGCGGCTCCAAACAGC |
|          | 5’-GCTGTTTGCCGGCACAATCTCTGTGCCTTCCTC |
| V281C    | 5’-AGAGACGCTAGTGGTCCTCCTTCGGCCACAAC |
|          | 5’-GTGGCAGCAAGAGGAGACACAGTGCAGGCTT |
| E302C    | 5’-GGGAAAATCAAGCAGACCAACCGCAGC |
|          | 5’-GGGAGGTTCGCTGTCACAGTGCAGGCTT |
| D317C    | 5’-CATAAAGGAGAATATGTAAGACGCTCATGGGG |
|          | 5’-CCCATGACGGTCTAACATATCCCTCCTTATG |
| D362C    | 5’-CATCCCCAGCGAGATGATCCCGATCCGCAAG |
|          | 5’-CTTGGCGGGATCTGGGTACCTTGGGGATG |
| E393C    | 5’-GCAGGAGAATATAATGAGTGAAGACGACCAG |
|          | 5’-GTGGTGCTATGTAATCTTCCGTTGGGGATG |
| R29E     | 5’-CATCTACCTGGGAGGAAGGAGATACATAGACCAC |
|          | 5’-GTGGTCTATGTGTAATCTCTCCGCCAGGAGATG |
| E36K     | 5’-GATTACATAAGACCAGCTTTAAACAGGATAGCGCTGTG |
|          | 5’-CACAGGCTCTACTGCTTAAAACGTGCTATAGTACG |
| R37D     | 5’-CATAGACCACGTGGAGATGATGAGCCTGTGATG |
|          | 5’-CATCCCACAGCTCATACCATACCATACAGCTG |
| D183K    | 5’-CAGCACCGCCAGCAAGGATGATCGCCAGCAG |
|          | 5’-GGCTGGGAGCAACATCTCTGCTGCGGCTG |
| E302K    | 5’-GGGAAAATCAAGCAGACCAACCGCAGC |
|          | 5’-GGGAGGTTCGCTGTCACAGTGCAGGCTT |
| E361K    | 5’-CATGCATCCCCAGCCAAAGGACCCAGATACCGC |
|          | 5’-GTGGTGCTATGTAATCTTCCGCCAGGAGATG |
| D362K    | 5’-CATCCCCAGCCAGAAGGACAGATACCCACAG |
|          | 5’-CTTGGCGGGATCTGGGTACCTTGGGGATG |
| E361G/D362G | 5’-CATGCATCCCCAGCCAGGAGGATCGCCAGATACCCACAG |
|          | 5’-CTTGGCGGGATCTGGGTACCTTGGGGATG |

*The arrestin-1 cDNA utilized contained an N-terminal His(6) tag introduced after the initiating methionine and with C63A and C143A mutations to remove the two reactive cysteines.*
REFERENCES

1. Gurevich, V. V., and Gurevich, E. V. (2019) GPCR signaling regulation: The role of GRKs and arrestins. *Front Pharmacol* **10**, doi:10.3389

2. Wilden, U., Hall, S. W., and Köhn, H. (1986) Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc Natl Acad Sci USA* **83**, 1174-1178

3. Krupnick, J. G., Gurevich, V. V., and Benovic, J. L. (1997) Mechanism of quenching of phototransduction: binding competition between arrestin and transducin for phosphorhodopsin. *J Biol Chem* **272**, 18125-18131

4. Wilden, U. (1995) Duration and amplitude of the light-induced cGMP hydrolysis in vertebrate photoreceptors are regulated by multiple phosphorylation of rhodopsin and by arrestin binding. *Biochem* **34**, 1446-1454

5. Peterson, Y. K., and Luttrell, L. M. (2017) The diverse roles of arrestin scaffolds in G protein-coupled receptor signaling. *Pharmacol Rev* **69**, 256-297

6. Wu, N., Hanson, S. M., Francis, D. J., Vishnivetskiy, S. A., Thibonnier, M., Klug, C. S., Shoham, M., and Gurevich, V. V. (2006) Arrestin binding to calmodulin: a direct interaction between two ubiquitous signaling proteins. *J Mol Biol* **364**, 955-963

7. Hanson, S. M., Cleghorn, W. M., Francis, D. J., Vishnivetskiy, S. A., Raman, D., Song, X., Nair, K. S., Slepak, V. Z., Klug, C. S., and Gurevich, V. V. (2007) Arrestin mobilizes signaling proteins to the cytoskeleton and redirects their activity. *J Mol Biol* **368**, 375-387

8. Huang, S.-P., Brown, B. M., and Craft, C. M. (2010) Visual arrestin 1 acts as a modulator for N-ethylmaleimide-sensitive factor in the photoreceptor synapse. *J Neurosci* **30**, 9381-9391

9. Smith, W. C., Bolch, S., Dugger, D. R., Li, J., Esquenazi, I., Arendt, A., Benzenhafer, D., and McDowell, J. H. (2011) Interaction of arrestin with enolase1 in photoreceptors. *Invest Ophthalmol Vis Sci* **52**, 1832-1840

10. Haque, A., Polcyn, R., Matzelle, D., and Banik, N. L. (2018) New insights into the role of neuron-specific enolase in neuro-inflammation, neurodegeneration, and neuroprotection. *Brain Sci* **8**, 33

11. Kanow, M. A., Giarmarco, M. M., Jankowski, C. S. R., Tsantilas, K., Engel, A. L., Du, J., Linton, J. D., Farnsworth, C. C., Sloat, S. R., Rountree, A., Sweet, I. R., Lindsay, K. J., Parker, E. D., Brockerhoff, S. E., Sadilek, M., Chao, J. R., and Hurley, J. B. (2017) Biochemical adaptations of the retina and retinal pigment epithelium support a metabolic ecosystem in the vertebrate eye. *eLife* **6**, e28899

12. Hurley, J. B., Lindsay, K. J., and Du, J. (2015) Glucose, lactate, and shuttling of metabolites in vertebrate retinas. *J Neurosci Res* **93**, 1079-1092

13. Lehrer, S. (1971) Solute perturbation of protein fluorescence. Quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochem* **10**, 3254-3263

14. Wold, F. (1971) Enolase in *The enzymes, 3rd edn* (Boyer, P. D. ed.), Academic Press, New York. pp 499-538

15. Kang, Y., Zhou, X. E., Gao, X., He, Y., Liu, W., Ishchenko, A., Barty, A., White, T. A., Yefanov, O., Han, G. W., Xu, Q., de Waal, P. W., Ke, J., Tan, M. H. E., Zhang, C., Moeller, A., West, G. M., Pascal, B. D., Van Eps, N., Caro, L. N., Vishnivetskii, S. A., Lee, R. J., Suino-Powell, K. M., Gu, X., Pal, K., Ma, J., Zhi, X., Boutet, S., Williams, G. J., Messerschmidt, M., Gati, C., Zatsepin, N. A., Wang, D., James, D., Basu, S., Roy-Chowdhury, S., Conrad, C. E., Coe, J., Liu, H., Lisova, S., Kupitz, C., Grotjohann, I., Fromme, R., Jiang, Y., Tan, M., Yang, H., Li, J., Wang, M., Zheng, Z., Li, D., Howe, N., Zhao, Y., Standfuss, J., Diederichs, K., Dong, Y., Potter, C. S.,
Arrestin-enolase interaction

Carragher, B., Caffrey, M., Jiang, H., Chapman, H. N., Spence, J. C. H., Fromme, P., Weierstall, U., Ernst, O. P., Katritch, V., Gurevich, V. V., Griffin, P. R., Hubbell, W. L., Stevens, R. C., Cherezov, V., Melcher, K., and Xu, H. E. (2015) Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. Nature 523, 561-567

17. Zhou, X. E., Gao, X., Barty, A., Kang, Y., He, Y., Liu, W., Ishchenko, A., White, T. A., Yefanov, O., Han, G. W., Xu, Q., de Waal, P. W., Suino-Powell, K. M., Boutet, S., Williams, G. J., Wang, M., Li, D., Caffrey, M., Chapman, H. N., Spence, J. C. H., Fromme, P., Weierstall, U., Stevens, R. C., Cherezov, V., Melcher, K., and Xu, H. E. (2016) X-ray laser diffraction for structure determination of the rhodopsin-arrestin complex. Sci Data 3, 160021

18. Kang, Y., Gao, X., Zhou, X. E., He, Y., Melcher, K., and Xu, H. E. (2016) A structural snapshot of the rhodopsin–arrestin complex. FEBS J 283, 816-821

19. Gurevich, V. V. (1998) The selectivity of visual arrestin for light-activated phosphorhodopsin is controlled by multiple nonredundant mechanisms. J Biol Chem 273, 15501-15506

20. Vishnivetskii, S. A., Chen, Q., Palazzo, M. C., Brooks, E. K., Altenbach, C., Iverson, T. M., Hubbell, W. L., and Gurevich, V. V. (2013) Engineering visual arrestin-1 with special functional characteristics. J Biol Chem 288, 3394-3405

21. Kang, H. J., Jung, S.-K., Kim, S. J., and Chung, S. J. (2008) Structure of human alpha-enolase (hENO1), a multifunctional glycolytic enzyme. Acta Crystallogr D Biol Crystallogr 64, 651-657

22. Schreier, B., and Höcker, B. (2010) Engineering the enolase magnesium II binding site: Implications for its evolution. Biochem 49, 7582-7589

23. Mookerjee, S. A., Nicholls, D. G., and Brand, M. D. (2016) Determining maximum glycolytic capacity using extracellular flux measurements. PloS ONE 11, e0152016-e0152016

24. Sente, A., Peer, R., Srivastava, A., Baidya, M., Lesk, A. M., Balaji, S., Shukla, A. K., Babu, M. M., and Flock, T. (2018) Molecular mechanism of modulating arrestin conformation by GPCR phosphorylation. Nature Struct Mol Biol 25, 538-545

25. Ostermaier, M. K., Peterhans, C., Jaussi, R., Deupi, X., and Standfuss, J. (2014) Functional map of arrestin-1 at single amino acid resolution. Proc Natl Acad Sci USA 111, 1825-1830

26. Gurevich, V. V., and Benovic, J. L. (1997) Mechanism of phosphorylation-recognition by visual arrestin and the transition of arrestin into a high-affinity binding state. Mol Pharmacol 51, 161-169

27. Sommer, M. E., Farrens, D. L., McDowell, J. H., Weber, L. A., and Smith, W. C. (2007) Dynamics of arrestin-rhodopsin interactions: Loop movement is involved in arrestin activation and receptor binding. J Biol Chem 282, 25560-25568

28. Vishnivetskii, S. A., Schubert, C., Climaco, G. C., Gurevich, Y. V., Velez, M. G., and Gurevich, V. V. (2000) An additional phosphate-binding element in arrestin molecule - Implications for the mechanism of arrestin activation. J Biol Chem 275, 41049-41057

29. Hsu, S. C., and Molday, R. S. (1991) Glycolytic enzymes and a GLUT-1 glucose transporter in the outer segments of rod and cone photoreceptor cells. J Biol Chem 266, 21745-21752

30. Broekhuyse, R. M., Tolhuizen, E. F. J., Janssen, A. P. M., and Winkens, H. J. (1985) Light induced shift and binding of S-antigen in retinal rods. Curr Eye Res 4, 613-618

31. Philp, N. J., Chang, W., and Long, K. (1987) Light-stimulated protein movement in rod photoreceptor cells of the rat retina. FEBS Lett 225, 127-132

32. Whelan, J. P., and McGinnis, J. F. (1988) Light-dependent subcellular movement of photoreceptor proteins. J Neurosci Res 20, 263-270

33. Zhu, X., Li, A., Brown, B., Weiss, E. R., Osawa, S., and Craft, C. M. (2002) Mouse cone arrestin expression pattern: light induced translocation in cone photoreceptors. Mol Vis 8, 462-471

34. Kaupp, U. B., and Seifert, R. (2002) Cyclic nucleotide-gated ion channels. Physiol Rev 82, 769-824

35. McDowell, J. H., Smith, W. C., Miller, R. L., Popp, M. P., Arendt, A., Abdulaeva, G., and Hargrave, P. A. (1999) Sulfhydryl reactivity demonstrates different conformational states for
arrestin, arrestin activated by a synthetic phosphopeptide, and constitutively active arrestin. 

**Biochem** 38, 6119-6125

36. Hirsch, J. A., Schubert, C., Gurevich, V. V., and Sigler, P. B. (1999) The 2.8 angstrom crystal structure of visual arrestin: A model for arrestin's regulation. **Cell** 97, 257-269

37. Kozakov, D., Beglov, D., Bohnud, T., Mottarella, S. E., Xia, B., Hall, D. R., and Vajda, S. (2013) How good is automated protein docking? **Proteins: Structure, Function, and Bioinformatics** 81, 2159-2166

38. Kozakov, D., Hall, D. R., Xia, B., Porter, K. A., Padhorny, D., Yueh, C., Beglov, D., and Vajda, S. (2017) The ClusPro web server for protein–protein docking. **Nature Protoc** 12, 255-278

39. Vajda, S., Yueh, C., Beglov, D., Bohnud, T., Mottarella, S. E., Xia, B., Hall, D. R., and Kozakov, D. (2017) New additions to the ClusPro server motivated by CAPRI. **Proteins** 85, 435-444

40. McDowell, J. H. (1993) Preparing rod outer segment membranes, regenerating rhodopsin, and determining rhodopsin concentration. **Meth Neurosci** 15, 123-130

41. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature** 227, 680-685

42. Donoso, L., Gregerson, D., Smith, L., Robertson, S., Knosp, e. V., Vrabec, T., and Kalsow, C. (1990) S-antigen: preparation and characterization of site-specific monoclonal antibodies. **Curr Eye Res** 9, 343-355

43. Haire, S. E., Pang, J., Boye, S. L., Sokal, I., Craft, C. M., Palczewski, K., Hauswirth, W. W., and Semple-Rowland, S. L. (2006) Light-driven cone arrestin translocation in cones of postnatal guanylate cyclase-1 knockout mouse retina treated with AAV-GC1. **Invest Ophthalmol Vis Sci** 47, 3745-3753

44. DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P. M., Miller, J. H., and Calos, M. P. (1987) Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. **Mol Cell Biol** 7, 379-387
Figure Captions

**Figure 1. Protection of fluorescence quenching of bimane-labeled arrestin-1 by enolase-1.** A) Fluorescence emission spectra from examples of three arrestin-1 mutants labeled with bimane (green traces) and the quenching of fluorescence caused by potassium iodide in the presence (blue traces) or absence (red traces) of 20-fold molar excess of enolase-1; enolase-1 provides no protection of the bimane fluorophore for I72C, intermediate protection for S210C, and full protection for S218C. B) Summary of the quenching protection provided by enolase-1 for twenty-eight cysteine-substituted mutants of arrestin-1 (as indicated) labeled with mBBr (each bar shows mean +/- SD; n=4). Mutants for which enolase provided >75% protection of the KI quenching are shown in red, 50-75% protection in orange, 25-49% protection in yellow, and 0-24% protection in pale blue. C) The various mutants indicated in “B” are shown plotted on a three-dimensional rendering of arrestin-1, retaining the same color coding for amino acids as in “B”; the four models show four views of the same rendering (two sides, top, and bottom).

**Figure 2. Molecular model of arrestin-1 (green) docked with a dimer of enolase-1 (cyan).** A) Docking model showing opposite sides of the same model, with arrestin-1 residues His-10, Asp-183, Glu-218, Glu-302, and Asp-362 indicated in red. B) Magnified view of the interface area as shown in panel A.

**Figure 3. Enolase-1 binding is not affected by arrestin-1 conformational changes induced by binding to rhodopsin or by mutations that mobilize the C-terminus of arrestin-1.** A) Arrestin-1 was pulled down by phosphorylated rhodopsin kept in the dark (pRho) or exposed to light (pRho*) in rod photoreceptor disc membranes, either in the presence (lanes 1&2) or absence of enolase-1/GFP (lanes 3&4). Enolase-1/GFP pulled down with arrestin-1 only when arrestin-1 pulled down with pRho* (lane 1) and not when arrestin-1 was absent (lanes 5&6). Lanes 7&8 show aliquots of the purified arrestin-1 (Arr1) and enolase-1/GFP (Eno1-GFP), respectively, used in the pulldown assay. Gel shows proteins samples separated by 12% SDS-PAGE and stained with coomassie blue; molecular mass markers are shown in kilodaltons. B) Quantitative summary of arrestin-1 pulled down with phospho-rhodopsin kept in the dark (pRho) or activated by exposure to light (pRho*) in the presence of equimolar enolase-1 (hatched bars) or without enolase-1 (unfilled bars); bars show mean +/- SD (n=3); ns indicates no significant change. C) E218C-mBBr-labeled arrestin-1 quenching by potassium iodide (gray bars) is not changed by binding of arrestin-1 to pRho* (orange bars); similarly, protection of bimane quenching provided by enolase-1 (blue bars) is also not affected by arrestin-1 binding to pRho* (green bars); bars show mean +/- SD (n=4); ns indicates no significant change. D) Wild-type arrestin-1 (green curve) or “3A” arrestin-1 (F375A/V376A/F377A; blue curves) was immunoprecipitated with anti-arrestin1 antibody, pulling down enolase-1 fluorescently labeled with AlexaFluor-546. The curves show the fluorescent profile of the captured enolase-1 in replicate experiments, compared to the background of enolase-1 capture when no arrestin-1 is present (red curves); inset shows quantitative summary of pulldown assay, normalized to the WT arrestin-1; bars show mean +/- SD (n=6); ns indicates no significant change.

**Figure 4. Enolase-1 pulldown by mutants of arrestin-1 designed to disrupt charge-pair interactions with enolase-1.** A) Molecular model of the arrestin-1/enolase-1 complex showing the charged residues on arrestin-1 (ball and stick) that were selected for reversal, and their proximity to enolase-1 (cyan, surface representation, with enolase-1 charged pair shown in
pink). B) Arrestin-1 with the indicated point mutations was immunoprecipitated with an anti-arrestin monoclonal antibody attached to magnetic beads, pulling down fluorescently labelled enolase-1. The captured enolase-1 is normalized to the pulldown of enolase-1 by arrestin-1 with no mutations (WT); the bar indicated as “no Arr” shows the background pulldown of labeled enolase-1 in the absence of any arrestin-1; inset shows examples of raw emission spectra collected for D362K mutant. C) enolase-1 pulldown with arrestin-1 with all seven point mutations (SDM-Arr; blue bar) or arrestin-1 pulldown of enolase-1 with all seven charge-pair mutation (SDM-Eno; green bar) showed essentially no pulldown. Combining SDM-Arr with SDM-Eno restored pulldown of enolase (gray bar); enolase-1 pulldown by the mutant arrestins that are significantly different than WT are indicated with an asterisk (p<0.05).

**Figure 5. Arr-E361G/D362G binds enolase-1, but does not affect enolase catalytic activity.**
A) Molecular model of the Arr1-Eno1 complex showing the proximity of Glu-361/Asp-362 on arrestin-1 (red spheres) relative to active site loops L1 (magenta dots), L2 (orange dots), and L3 (yellow dots) on enolase-1. B) Immunoprecipitation pulldown of fluorescently labelled enolase-1 was performed using native arrestin-1 (Arr-WT) or arrestin-1 with E361G and D362G mutations (Arr-GG). The binding of enolase-1 was not significantly changed by the double mutations E361G/D362G; bars show means +/- SD (n=8). C) The influence of these same two mutations on enolase-1 catalytic activity was assessed, monitoring the production of ATP from the processing of 2-phosphoglycerate to pyruvate. The E361G/D362G arrestin-1 (closed circles) did not show inhibition of enolase-1 activity that is evident with native arrestin-1 (open squares); each point shows mean +/- SD (n=3). D) Lactate production from HEK-293T cells transfected with plasmids expressing arrestin-1 (Arr-WT, blue squares), arrestin-1 with E361G/D362G (Arr-GG, open circles), green fluorescent protein (GFP, green triangles), or untransfected (no DNA, inverted triangles); lines show linear regression through mean +/- SD (n=3). E) Rates of lactate production determined from “D”; bars show mean +/- SD (n=3); significantly different rates are indicated with an asterisk (p<0.05).

**Figure 6. Kinetic parameters for the effect of arrestin-1 on enolase-1 catalysis.** A) Influence of enolase-1 concentration on turnover number for the catalysis of 2-PGA to PEP; curve shows linear regression fit for replicate samples (n=3). B) Kinetics of the enolase-1 reaction for 2-PGA to PEP with 100 nM enolase-1 without arrestin-1 (●) or with 500 nM arrestin-1 (▼) in Michaelis-Menten plots; points show means +/- SD (n=5). Curves show non-linear regression fit to Michaelis-Menten function. C) Kinetic properties of enolase-1 with and without arrestin-1; bars represent mean +/- SD (n=5) with statistically significant differences indicated with an asterisk (p<0.05).
A

Fluorescence (cps)

Wavelength (nm)

Protection Factor

B

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Figure 1. Protection of fluorescence quenching of bimane-labeled arrestin-1 by enolase-1. A) Fluorescence emission spectra from examples of three arrestin-1 mutants labeled with bimane (green traces) and the quenching of fluorescence caused by potassium iodide in the presence (blue traces) or absence (red traces) of 20-fold molar excess of enolase-1; enolase-1 provides no protection of the bimane fluorophore for I72C, intermediate protection for S210C, and full protection for S218C. B) Summary of the quenching protection provided by enolase-1 for twenty-eight cysteine-substituted mutants of arrestin-1 (as indicated) labeled with mBBr (each bar shows mean +/-SD; n=4). Mutants for which enolase provided >75% protection of the KI quenching are shown in red, 50-75% protection in orange, 25-49% protection in yellow, and 0-24% protection in pale blue. C) The various mutants indicated in “B” are shown plotted on a three-dimensional rendering of arrestin-1, retaining the same color coding for amino acids as in “B”; the four models show four views of the same rendering (two sides, top, and bottom).
Figure 2. Molecular model of arrestin-1 (green) docked with a dimer of enolase-1 (cyan). A) Docking model showing opposite sides of the same model, with arrestin-1 residues His-10, Asp-183, Glu-218, Glu-302, and Asp-362 indicated in red. B) Magnified view of the interface area as shown in panel A.
Figure 3. Enolase-1 binding is not affected by arrestin-1 conformational changes induced by binding to rhodopsin or by mutations that mobilize the C-terminus of arrestin-1. A) Arrestin-1 was pulled down by phosphorylated rhodopsin kept in the dark (pRho) or exposed to light (pRho*) in rod photoreceptor disc membranes, either in the presence (lanes 1&2) or absence of enolase-1/GFP (lanes 3&4). Enolase-1/GFP pulled down with arrestin-1 only when arrestin-1 pulled down with pRho* (lane 1) and not when arrestin-1 was absent (lanes 5&6). Lanes 7&8 show aliquots of the purified arrestin-1 (Arr1) and enolase-1/GFP (Eno1-GFP), respectively, used in the pulldown assay. Gel shows proteins samples separated by 12% SDS-PAGE and stained with coomassie blue; molecular mass markers are shown in kilodaltons. B) Quantitative summary of arrestin-1 pulled down with phospho-rhodopsin kept in the dark (pRho) or activated by exposure to light (pRho*) in the presence of equimolar enolase-1 (hatched bars) or without enolase-1 (unfilled bars); bars show mean +/- SD (n=3); ns indicates no significant change. C) E218C-mBBr-labeled arrestin-1 quenching by potassium iodide (gray bars) is not changed by binding of arrestin-1 to pRho* (orange bars); similarly, protection of bimane quenching provided by enolase-1 (blue bars) is also not affected by arrestin-1 binding to pRho* (green bars); bars show mean +/- SD (n=4); ns indicates no significant change. D) Wild-type arrestin-1 (green curve) or “3A” arrestin-1 (F375A/V376A/F377A; blue curves) was immunoprecipitated with anti-arrestin1 antibody, pulling down enolase-1 fluorescently labeled with AlexaFluor-546. The curves show the fluorescent profile of the captured enolase-1 in replicate experiments, compared to the background of enolase-1 capture when no arrestin-1 is present (red curves); inset shows quantitative summary of pulldown assay, normalized to the WT arrestin-1; bars show mean +/- SD (n=6); ns indicates no significant change.
Figure 4. Enolase-1 pulldown by mutants of arrestin-1 designed to disrupt charge-pair interactions with enolase-1. A) Molecular model of the arrestin-1/enolase-1 complex showing the charged residues on arrestin-1 (ball and stick) that were selected for reversal, and their proximity to enolase-1 (cyan, surface representation, with enolase-1 charged pair shown in pink). B) Arrestin-1 with the indicated point mutations was immunoprecipitated with an anti-arrestin monoclonal antibody attached to magnetic beads, pulling down fluorescently labelled enolase-1. The captured enolase-1 is normalized to the pulldown of enolase-1 by arrestin-1 with no mutations (WT); the bar indicated as “no Arr” shows the background pulldown of labeled enolase-1 in the absence of any arrestin-1; inset shows examples of raw emission spectra collected for D362K mutant. C) enolase-1 pulldown with arrestin-1 with all seven point mutations (SDM-Arr; blue bar) or arrestin-1 pulldown of enolase-1 with all seven charge-pair mutation (SDM-Eno; green bar) showed essentially no pulldown. Combining SDM-Arr with SDM-Eno restored pulldown of enolase (gray bar); enolase-1 pulldown by the mutant arrestins that are significantly different than WT are indicated with an asterisk (p<0.05).
**Figure 5.** *Arr-E361G/D362G binds enolase-1, but does not affect enolase catalytic activity.*

A) Molecular model of the Arr1-Enol complex showing the proximity of Glu-361/Asp-362 on arrestin-1 (red spheres) relative to active site loops L1 (magenta dots), L2 (orange dots), and L3 (yellow dots) on enolase-1. B) Immunoprecipitation pulldown of fluorescently labelled enolase-1 was performed using native arrestin-1 (Arr-WT) or arrestin-1 with E361G and D362G mutations (Arr-GG). The binding of enolase-1 was not significantly changed by the double mutations E361G/D362G; bars show means +/- SD (n=8). C) The influence of these same two mutations on enolase-1 catalytic activity was assessed, monitoring the production of ATP from the processing of 2-phosphoglycerate to pyruvate. The E361G/D362G arrestin-1 (closed circles) did not show inhibition of enolase-1 activity that is evident with native arrestin-1 (open squares); each point shows mean +/- SD (n=3). D) Lactate production from HEK-293T cells transfected with plasmids expressing arrestin-1 (Arr-WT, blue squares), arrestin-1 with E361G/D362G (Arr-GG, open circles), green fluorescent protein (GFP, green triangles), or untransfected (no DNA, inverted triangles); lines show linear regression through mean +/- SD (n=3). E) Rates of lactate production determined from “D”; bars show mean +/- SD (n=3); significantly different rates are indicated with an asterisk (p<0.05).
Figure 6. Kinetic parameters for the effect of arrestin-1 on enolase-1 catalysis. A) Influence of enolase-1 concentration on turnover number for the catalysis of 2-PGA to PEP; curve shows linear regression fit for replicate samples (n=3). B) Kinetics of the enolase-1 reaction for 2-PGA to PEP with 100 nM enolase-1 without arrestin-1 (●) or with 500 nM arrestin-1 (▼) in Michaelis-Menten plots; points show means +/- SD (n=5). Curves show non-linear regression fit to Michaelis-Menten function. C) Kinetic properties of enolase-1 with and without arrestin-1; bars represent mean +/- SD (n=5) with statistically significant differences indicated with an asterisk (p<0.05).
An arrestin-1 surface opposite of its interface with photo-activated rhodopsin engages with enolase-1

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