Topographic study of arrestin using differential chemical modifications and hydrogen/deuterium exchange

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(RECEIVED August 18, 1994; ACCEPTED October 4, 1994)

Abstract

Arrestin is involved in the quenching of phototransduction by binding to photoactivated and phosphorylated rhodopsin (P-Rho*). To study its conformational changes and regions interacting with P-Rho*, arrestin was subjected to (1) differential acetylation at lysine residues in the presence and absence of P-Rho*, and (2) amide hydrogen/deuterium exchange. Labeled protein was proteolysed and analyzed by mass spectrometry. Three Lys residues, 28, 176, and 211, were protected from acetylation in native arrestin, although they were not located in regions exhibiting slow amide hydrogen exchange rates. The presence of P-Rho* protected lysine 201 from acetylation and partially protected 14 other lysyl residues, including (2, 5), (163, 166, 167), (232, 235, 236, 238), (267, 276), (298, 300), and 367, where parentheses indicate lysine residues found within the same peptide. In contrast, in the C-terminal region of arrestin, lysyl residues (386, 392, 395) were more exposed upon binding to P-Rho*. These data allowed us to identify functional regions in the arrestin molecule.

Keywords: arrestin; chemical modification; G-protein-coupled receptor; mass spectrometry; phototransduction

The arrestin family is a group of monomeric proteins ~40–50-kDa molecular mass that are implicated in the quenching of the activated state of G-protein-coupled receptors. This family of proteins consists of (1) retinal rod arrestin (Shinohara et al., 1987), its spliced variant form, p⁴⁴ (Palczewski et al., 1994; Smith et al., 1994), and retinal cone arrestin (Murakami et al., 1993; Craft et al., 1994); (2) β -arrestins 1 and 2 (Lohse et al., 1990; Attramadal et al., 1992) and their splice forms, β -arr1L and β -arr2L (Pauruti et al., 1993; Sterne-Marr et al., 1993); and (3) invertebrate arrestins (Hyde et al., 1990; Krishnan & Ganguly, 1990; Smith et al., 1990; Yamada et al., 1990). All of these proteins have homologous sequences, although they may be regulated differently. Within the arrestin family, retinal arrestin has been the most extensively characterized (reviewed by Palczewski, 1994).

In vertebrate rod photoreceptor cells, the visual transduction cascade begins with the capture of a photon by rhodopsin, which

leads to the formation of an active intermediate of rhodopsin, metarhodopsin II (Meta II). Meta II activates several thousand molecules of transducin (GTP-binding protein) and is a substrate for rhodopsin kinase (RK) (reviewed by Chabre & Deterre, 1989; Palczewski & Benovic, 1991). RK phosphorylates photolyzed rhodopsin at ³³⁸Ser, ³⁴³Ser, ³⁵⁴Ser, and ³⁵⁶Thr, which are located at the C-terminus of rhodopsin (McDowell et al., 1993; Ohguro et al., 1993, 1994; Papac et al., 1993). Arrestin specifically recognizes and binds to photolyzed and phosphorylated rhodopsin at the phosphorylated C-terminus (Schleicher et al., 1989) and the first and third cytoplasmic loops (Krupnick et al., 1994). As a result, arrestin undergoes a structural rearrangement, which is evident from the Arrhenius activation energy (Schleicher et al., 1989) and an alteration in the reactivity of the sulfhydryl groups (Palczewski et al., 1992). Limited proteolysis studies suggest that the negatively charged C-terminal region of arrestin interacts with a positively charged N-terminal region, but is displaced upon binding to P-Rho* (Palczewski et al., 1991a). Similar conformational changes in arrestin are observed upon binding heparin, which competitively inhibits interactions with P-Rho* (Palczewski et al., 1991c).

Within the native structure of proteins, amide hydrogen exchange rates can vary by several orders of magnitude and these rates are sensitive to changes in protein conformation (reviewed by Englander & Kallenbach, 1983; Gregory & Rosenberg, 1986).

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Abbreviations: ES/MS, electrospray mass spectrometry; LC/MS, liquid chromatography mass spectrometry; MS/MS, tandem mass spectrometry; Rho, rhodopsin; Rho*, photolyzed rhodopsin; P-Rho, phosphorylated rhodopsin; P-Rho*, phosphorylated and photolyzed rhodopsin; TFA, trifluoroacetic acid.

Typically, amide hydrogen exchange kinetics are studied by NMR; however, a more sensitive technique takes advantage of the fact that hydrogen exchange is considerably reduced at low pH and temperature (Englander et al., 1985; Rosa & Richards, 1979). This procedure employs peptic cleavage of partially tritiated proteins at 0 °C, followed by HPLC separation of the peptides. The separated peptides are identified either by N-terminal sequence analysis or by amino acid composition, and the extent of tritium incorporation is determined by scintillation counting. A conceptually similar approach employs LC/MS for the identification of peptic peptides and their level of deuterium incorporation (Zhang & Smith, 1993). Using this latter technique, amide hydrogen exchange measurements were made for free arrestin.

In the present study, arrestin binding was studied by differential chemical modification and hydrogen exchange labeling, analyzing the results by ES/MS (Fenn et al., 1989), LC/MS, and MS/MS (Biemann, 1990a; Hunt et al., 1986). A number of methods for chemically modifying proteins have been developed where the aim of such studies has been to infer a protein structure–function relationship (Glazer et al., 1975). These techniques can be effectively adapted to mass spectrometric studies (Qin et al., 1992; Suckau et al., 1992), thereby providing considerable advantages such as high sensitivity, the ability to work with complex mixtures, and increased speed of analysis. Here we examine changes in differential acetylation of lysine residues in response to binding to either heparin or P-Rho*.

Results

Effect of selective chemical modifications on arrestin binding to P-Rho*

To identify residues in arrestin that are critical for the interaction between arrestin and P-Rho*, native arrestin was partially modified at lysine (50–70% acetylated), arginine (40–60% blocked with 2,3-butanedione), and cysteine (2 of 3 residues carboxymethylated). Arrestin modified at either arginine or cysteine residues was still able to bind to P-Rho* (Fig. 1, samples 1, 2), but acetylated arrestin showed no binding activity to P-Rho* (Fig. 1, sample 3). To determine if acetylation prevented the formation of a suitable conformation for binding to P-Rho*, partially modified arrestin was produced by acetylation of the arrestin/P-Rho* complex, quenching the reaction, and separation of arrestin and P-Rho*. Binding of the partially modified arrestin to P-Rho* was observed, although to a lesser extent (Fig. 1, sample 4). These data demonstrate that positively charged lysine residues are important for binding of arrestin to P-Rho*, and suggest that critical lysyl residues may be partially protected from acetylation in the complex. Thus, differential acetylation could be employed for the identification of the lysine residues involved in this interaction.

Arrestin topology by amino acetylation and hydrogen/deuterium exchange of arrestin

Arrestin topology was investigated by probing lysine residues with deuterated acetic anhydride at different concentrations (0.5, 1.0 or 2.0, and 10 mM) for 10 min on ice. The partially deuterio-acetylated arrestin was then fully acetylated by undeuterated acetic anhydride in the presence of 6 M guanidine hydrochloride.

Fig. 1. Effect of selective chemical modifications of native arrestin on the binding to P-Rho*. Arg, Cys, or Lys residues of arrestin were modified by either 2,3-butanedione (sample 1), iodoacetic acid (sample 2), or acetic anhydride (sample 3). Partially modified arrestin was produced by acetylating the arrestin/P-Rho* complex with 2 mM acetic anhydride. The modified arrestin was separated from P-Rho* and used for a subsequent binding experiment (sample 4). The binding assay for arrestin to P-Rho* was performed as described by Hofmann et al. (1992). Under dim red light, P-Rho was mixed with chemically modified arrestin in 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl. The reaction was performed with (+) or without (–) illumination for 10 min at 30 °C. The arrestin/P-Rho* complex was centrifuged at 200,000 × g for 5 min, and the supernatant was subjected to SDS-PAGE (upper panel). The pellet was washed with 1 mL of the Hepes buffer, centrifuged for 5 min at 200,000 × g, dissolved in 55 µL of 1% SDS containing 0.1% β-mercaptoethanol and subjected to SDS-PAGE (lower panel).

The chemically modified arrestin was digested by trypsin, chymotrypsin, and/or *Staphylococcus aureus* V8 protease, peptides were separated by HPLC (C8), and fractions were analyzed by ES/MS and MS/MS. In some cases, proteolytic mixtures were analyzed by LC/MS. Eighteen peptides containing acetylated lysine residues (representing a total of 32 out of 35 lysines) were identified by ES/MS, MS/MS, and LC/MS (see Fig. 2). Data were not obtained for Lys residues at positions 141, 330, and 332. The N-terminal peptide was found with an acetylated N-terminus and deuterio-acetylated ²Lys and ⁵Lys, showing that the N-terminus is acetylated in native arrestin (Fig. 3).

As an example, Figure 4A shows doubly charged parent ions corresponding to ³⁰³DTNLASSTII(Ac-K)E from arrestin deuterio-acetylated under native conditions and acetylated under denaturing conditions. The identification of the sequence was confirmed by MS/MS as shown in the Figure 4B. Because deuterio-acetyl- and acetyl-peptides are chemically identical, the electrospray ionization efficiencies are the same. Thus, this approach allowed us to estimate individual lysine reactivities in arrestin by simply comparing the relative abundances of ions corresponding to deuterated and undeuterated peptides. Several peptides were tested for the reproducibility of the method and no more than 5% variability between experiments was found.

When native arrestin was treated with 0.5, 1, or 10 mM deuterated acetic anhydride, lysine residues 28 (peptide CV26–31), 176 (TV172–182), and 211 (TV205–211) were completely unreactive in native conditions (Fig. 2). Two other residues, Lys 257 (CV256–263) and Lys 313 (TV303–314), were less reactive than the rest. These differences in reactivity could be a result of sev-

Fig. 2. Extent of acetylation of native arrestin in 3 concentrations of deuterio-acetic anhydride in the presence or absence of P-Rho. The analysis of deuterio-acetylation of arrestin at specific residues was performed as described in the legend to Figure 4 in the presence of 1 mM (native arrestin) or 2 mM (in the presence of P-Rho) deuterio-acetic anhydride. Peptides were generated by trypsin (T), trypsin and *S. aureus* V8 protease (TV), or chymotrypsin and *S. aureus* V8 protease (CV). Peptides were identified by MS/MS. Experiments were performed in the presence (diagonal cross-hatched bars) or absence (solid bars) of P-Rho. Experiments were performed in duplicate.

eral factors, including (1) relative exposure to solvent and (2) chemical effects such as protection by salt bridge formation.

To further investigate the native arrestin structure, amide hydrogen/deuterium exchange of arrestin was studied. After incubation of arrestin with D_2O for 5 min at $20^\circ C$, the reaction was quenched by the addition of 1 M potassium phosphate buffer, pH 2.6 (low pH slows the amide exchange), and arrestin was digested with pepsin. Peptic peptides were separated at low pH and analyzed by LC/MS (Fig. 5). Two of the 3 unreactive Lys residues (28 and 176) and the 2 slowly reactive Lys residues (257 and 313) are not located in regions of arrestin that undergo unusually slow amide hydrogen/deuterium exchange. One of the unreactive residues, Lys 211, was not recovered among the peptic peptides. These data suggest that at least 4 of these 5 Lys residues are located in regions of arrestin exposed

Fig. 3. MS/MS spectrum of the NH_2 -terminal tryptic fragment from bovine arrestin. After deuterio-acetylation (10 mM), arrestin was digested with chymotrypsin and V8 protease, and the N -terminal peptide was isolated on a C8 column and subjected to MS/MS analysis. The mass (M_r calculated: 1,101.5/observed: 1,102.2), and fragmentation pattern is consistent with an acetylated N -terminus and deuterio-acetylated 2Lys and 3Lys . The fragment ion nomenclature is that proposed by Biemann (1990b).

Fig. 4. Identification of acetylated arrestin peptides by ES/MS and MS/MS. **A:** Native arrestin was partially modified using 10 mM $(CD_3CO)_2O$, then denatured and fully acetylated with $(CH_3CO)_2O$. The product was digested with trypsin and *S. aureus* V8 protease. The ES/MS spectrum of the arrestin peptide, $^{303}DTNLSASTII(Ac-K)E$, exhibits 2 doubly charged ions corresponding to the deuterio-acetylated (m/z 669.1, D-peptide) and the acetylated (m/z 667.6, H-peptide) peptides. **B:** MS/MS spectrum of the doubly charged precursor ion at m/z 669.1 provides sequence identification. The ion nomenclature is that proposed by Biemann (1990b).

Fig. 5. Distributions of amide hydrogen/deuterium exchange in arrestin. Deuterium incorporation into different segments of arrestin was performed after 5-min incubation. Identification of all peptides shown in this figure was determined by ES/MS and MS/MS. Experiments were performed in triplicate.

to solvent, and that the side chains of Lys 28, 176, 257, and 313 may be involved in side-chain interactions with other residues in native arrestin.

Arrestin/P-Rho* interactions

The deuterio-acetylation experiments were repeated with 10 mM deuterated acetic anhydride in the presence of P-Rho under both dark and light conditions. Again, no deuterio-acetylation was observed at lysines 28, 176, and 211 (data not shown). At lower concentrations of deuterio-acetic anhydride (0.5 and 2 mM), selective differences between light and dark conditions for each peptide are seen (Fig. 6). In the light, where P-Rho* is known to bind arrestin, the largest difference was seen to be a protection of Lys 201. Smaller effects were found in clusters of lysines between residues 2–5, 163–167, 232–238, 267–276, and 298–300, and at lysines 150 and 367. Conversely, acetylation of 3 lysines (386, 392, 395) by 2 mM deuterio-acetic anhydride was slightly enhanced upon binding to P-Rho*, suggesting that the C-terminal region is more exposed when arrestin binds P-Rho*. It should be noted that acetylation at all concentrations of deuterio-acetic anhydride is complicated by the addition of P-Rho-containing membranes because amino-containing lipids also undergo modification and decrease the concentration of $(\text{CD}_3\text{CO})_2\text{O}$. This effect can be seen in Figure 2, where the solid bars indicate that the level of deuterio-acetylation in the absence of P-Rho is higher

than that observed in the presence of P-Rho plus membranes (cross-hatched bars). To control for the unknown concentrations of deuterio-acetic anhydride in the P-Rho*-binding experiments, the same experiment was performed in the absence of light. At high deuterio-acetic anhydride concentrations, the interaction between arrestin and P-Rho* could also be altered due to modification of peripheral lysines residues.

When the deuterio-acetylation experiments were repeated in the presence of heparin, small decreases (<10%) in acetylation were observed at 6 lysines with the greatest protection of highly polycationic peptide TV232–242 and peptide TV292–302 (data not shown), regions that were weakly protected during interaction with P-Rho*. These data are consistent with an overlap of binding regions for heparin and P-Rho* and for the reported competition between P-Rho* and heparin for the binding to arrestin (Palczewski et al., 1991b, 1991c).

Discussion

Differential acetylation revealed a number of changes in lysine reactivities upon binding of arrestin to P-Rho*. The most significant decrease in acetylation was observed at residues (2, 5), (163, 166, 167), 201, (232, 235, 236, 238), (267, 276), (298, 300), and 367, where parentheses indicate lysine residues found within the same peptide. Increased acetylation was also observed at lysines (386, 392, 395), which is consistent with earlier work demonstrating enhanced proteolysis of the C-terminal tail of arrestin in the presence of P-Rho* (Palczewski et al., 1991a, 1991c). The polybasic region containing Lys (232, 235, 236, 238) and another region (298, 300) also appears to be involved in the competitive interaction with heparin (Palczewski et al., 1991c). Several lysine residues—(2, 5), (163, 166, 167), 201, and (267, 276)—show a considerable decrease in deuterio-acetylation upon binding to P-Rho*, but not to heparin. This is not surprising because arrestin stabilizes Meta II (Schleicher et al., 1989) and this interaction may involve multiple attachment sites between arrestin and the receptor. Interaction between arrestin and heparin may be much simpler. The changes in acetylation may be due to either steric protection by P-Rho* and heparin, or conformational changes within arrestin that alter the reactivity of individual lysine residues. In either case, these results provide a basis for further studies of the interaction of arrestin and P-Rho* using site-specific mutagenesis. From limited proteolysis data (Palczewski et al., 1991a), regions at the N-terminus (Lys 2 and 5) and in the mid-portion (Lys 201) in native arrestin appear to be solvent exposed. Because arrestin does not bind to P-Rho, binding of arrestin to P-Rho* may involve a steric trigger that is probably related to conformational changes in the C-terminus of arrestin. We have proposed that the acidic C-terminus forms a salt bridge with a polybasic region within the arrestin molecule (reviewed by Palczewski, 1994). This C-terminal region is pushed away by the phosphorylated C-terminus of P-Rho*, thus leading to multi-site interactions between both proteins and the stabilization of the complex. By this mechanism, arrestin would recognize only photolyzed and phosphorylated rhodopsin, as observed experimentally (Schleicher et al., 1989). This model predicts that when the C-terminus of arrestin is eliminated (as in the splice variant form, p⁴⁴), such truncated forms would bind to Rho* without phosphorylation. Again, this prediction was observed experimentally (Palczewski et al., 1994).

Fig. 6. Percent change in deuterio-acetylation of arrestin in the presence of P-Rho*. The percent of deuterio-acetylation of arrestin at specific residues in the presence of P-Rho* was compared with that of arrestin in the presence of P-Rho (data from Fig. 2) and expressed as a percent of change between light and dark conditions. Peptides were generated by trypsin (T), trypsin and *S. aureus* V8 protease (TV), or chymotrypsin and *S. aureus* V8 protease (CV). Peptides were identified by MS/MS. Experiments were performed in duplicate, and both results are shown as differently shaded bars.

Recently, Gurevich and Benovic (1993) speculated that the binding of arrestin to P-Rho* was sequential at multiple sites: (1) phosphorylation recognition sites (2–16, 158–185); (2) receptor activation recognition sites (17–145, 145–191); (3) regulatory sites (C-terminus); and (4) hydrophobic interaction sites (191–365). Our model of arrestin structure and its interactions with P-Rho* is generally in accordance with that of Gurevich and Benovic (1992, 1993), Gurevich et al. (1993, 1994), and Krupnick et al. (1994); however, there are inconsistencies relating to the sequential binding steps, the role of the C-terminal region, and the specific regions involved in the interaction with P-Rho*. This could be attributed to differences in the experimental approaches. For instance, the interactions between arrestin and different forms of rhodopsin were evaluated by Gurevich and Benovic (1993) using deletion mutants whose integrity was not investigated. Furthermore, their binding assay employed a form of P-Rho that was produced using a physiologically irrelevant kinase (β -adrenergic receptor kinase), which has a different initial phosphorylation site compared to rhodopsin kinase (K. Palczewski, H. Ohguro, R. Premont, & J. Inglese, unpubl.).

Materials and methods

Modifications of arrestin

Acetylation of lysine residues was carried out as described by Buechler and Taylor (1988) with modifications. Purified arrestin (10 μ M) (Buczylko & Palczewski, 1993) was mixed with heparin (0.5 mg/mL) or P-Rho (10-fold molar excess to arrestin) in 100 mM sodium borate buffer, pH 8.5, containing 100 mM NaCl. The arrestin/P-Rho mixture was preincubated for 5 min: (1) under dark conditions or (2) illuminated under white light (a 150-W lamp from a distance of 10 cm) at 30 °C. Arrestin, arrestin/heparin, arrestin/P-Rho, and arrestin/P-Rho* were each incubated with deuterated acetic anhydride at various concentrations (0.1–10 mM) on ice. At 10 min, the reaction was mixed with β -mercaptoethanol (50 mM) and then with NaCl (500 mM).

Partially modified arrestin was extensively dialyzed against 0.5 M NaCl and water and treated with 20 mM acetic anhydride (undeuterated) in 100 mM sodium borate buffer, pH 9.0, containing 6 M guanidine hydrochloride for 10 min at room temperature to acetylate remaining lysine residues. To the reaction mixture, 50 mM β -mercaptoethanol was added and the fully modified arrestin was dialyzed against water, lyophilized, and subjected to enzymatic digestion.

Modification of arginine residues by 2,3-butanedione was performed as described by Riordan (1973). Native arrestin (1 nmol) was treated with 10 mM 2,3-butanedione in 100 mM borate buffer, pH 9.0, for 10 min at 37 °C.

Proteolysis of acetylated arrestin

Acetylated arrestin (5 nmol) in 50 μ L of 100 mM Tris-HCl buffer, pH 8.0, containing 4 M urea was treated with trypsin-TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone; 5 μ g; Worthington) or α -chymotrypsin (5 μ g; Worthington) at 30 °C for 6 h. Trypsin or α -chymotrypsin activity was quenched by incubating with 10 mM phenylmethylsulfonyl fluoride at room temperature for 30 min, and subsequently subdigested with *S. aureus* protease V8 (1 μ g; Worthington) at 30 °C for 6 h.

Amide hydrogen exchange

Arrestin (0.2 nmol) in 10 mM Hepes buffer, pH 7.5, containing 100 mM NaCl and 95% (v/v) D₂O was incubated at 20 °C. At 5 min, the amide hydrogen exchange reaction was quenched by dropping the pH to 2.6 by the addition of 1 M phosphate buffer, pH 2.5, and was stored at –80 °C. The sample was thawed on ice, digested with pepsin using an enzyme substrate ratio of 1:1 for 5 min on ice, and subjected to LC/MS analysis. The injector valve, column, and transfer lines were cooled in ice water to limit back exchange.

Electrospray and tandem mass spectrometry

ES/MS and MS/MS data were acquired as described by Ohguro et al. (1993). Proteolytic products of acetylated arrestin were applied to a 2×100 mm C8 column (Applied Biosystems, Aquapore) operating at a flow rate of 0.2 mL/min using a 30-min linear gradient of 0–80% acetonitrile in 0.05% TFA. Peptic peptides of partially deuterated arrestin were applied to a 1×30 mm C8 column (Applied Biosystems) operating at a flow rate of 75 μ L/min using a 10-min linear gradient of 0–80% acetonitrile in 0.03% TFA. A post-column splitting T was used to divert 10–20% of the column effluent to the mass spectrometer (API-III, PE Sciex Thornhill, Ontario, Quebec), and the remaining 80–90% was directed to a UV detector monitoring 214 nm. Fractions were collected by hand. The mass spectrometer was operated at unit resolution using a nebulization-assisted electrospray ion source, typically scanning from m/z 300 to 1,800 at a rate of 3–5 s/scan (dwell time of 0.4–0.5 ms and a step size of 0.2 or 0.25 Da).

Acknowledgments

We thank Profs. John C. Saari and Paul A. Hargrave for helpful comments on the manuscript and J. Preston Van Hooser for technical assistance. This study was supported by USPHS grant EY09339 and EY01730 (K.P.), the Royalty Research Fund (R.S.J.), an award from Research to Prevent Blindness, Inc. to the Department of Ophthalmology at the University of Washington, and a Human Frontiers in Science Program grant (O.H., K.P.). K.P. is the recipient of a Jules and Doris Stein RPB Professorship.

Note added in proof

Recently, Kieselbach et al. (1994) showed that a peptide corresponding to 170–182 of arrestin is capable of binding to both photolyzed and bleached rhodopsin.

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