Articles

Structural Properties of Arrestin Studied by Chemical Modification and Circular Dichroism†

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ABSTRACT: A unique conformation of arrestin is crucial for its interaction with phosphorylated photolyzed rhodopsin. Conformational changes in arrestin were investigated using chemical modification and circular dichroism. We studied the kinetics of sulfhydryl modification of bovine arrestin in order to determine whether its conformation is altered by the presence of ligands or salts at different ionic strengths. We found that all three cysteines (stoichiometry was 2.7 ± 0.06 3-carboxy-4-nitrophenyl sulfide (NbS)/arrestin) are accessible for modification by NbS_2. Under pseudo-first-order conditions (30–100-fold excess of NbS_2 over arrestin), the modifications of the 3 cysteines are indistinguishable. At higher concentrations of NbS_2 (150–300-fold excess), the pseudo-first-order plot is not linear, and the reaction can be resolved into two processes that involve two classes of sulfhydryl groups. Addition of $CaCl_2$, $MgCl_2$, inorganic phosphate, MgATP, or MgGTP had little effect on the rate of modification of the cysteine residues; however, heparin and inositol hexakisphosphate, which have been shown to induce conformational changes in arrestin, block modification of one sulfhydryl group of arrestin and accelerate the modification of the remaining two. Analysis of CD spectra revealed that arrestin has virtually no α-helical structure, about 40% β-structure, about 18% β-turns, and about 40% other structure. The CD spectrum for arrestin did not change in the presence of heparin. These studies suggest that arrestin exists in equilibrium between two or more conformational states. However, it is proposed that conversion between these conformations occur without altering significantly the secondary structure of arrestin.

In vertebrate rod photoreceptor cells, light photoisomerizes rhodopsin's chromophore, 11-*cis*-retinal, resulting in a change in the conformation of rhodopsin (Kühn & Hargrave, 1981). As a consequence, photolyzed rhodopsin initiates a cascade of enzymatic reactions that lead to the activation of cyclic GMP phosphodiesterase (Stryer, 1986) and subsequently to the suppression of the dark current through cyclic GMP-sensitive cation channels in the plasma membrane (Chabre & Deterre, 1989). Rhodopsin deactivation is necessary in order to terminate the signal transduction process. Biochemical and electrophysiological evidence suggests that the phosphorylation of rhodopsin and the binding of the regulatory protein arrestin are necessary for deactivation (Kühn, 1984; Wilden et al., 1986; Bennett & Sitaramayya, 1988; Palczewski et al., 1992). Additionally, arrestin acts in the phototransduction process by blocking rapid dephosphorylation of phosphorylated and photolyzed rhodopsin until activated rhodopsin decays (Palczewski et al., 1989). β_2-adrenergic receptor, another G-protein-coupled receptor, is similarly deactivated by phosphorylation and binding of the regulatory protein β-arrestin (Lohse et al., 1990).

Arrestin binds specifically to phosphorylated and photolyzed rhodopsin (Wilden et al., 1986). The binding process is characterized by a high Arrhenius activation energy (Schleicher et al., 1989), which suggests that arrestin undergoes conformational changes during binding. These conformational changes in arrestin between the free and bound form are observed following limited proteolysis (Palczewski et al., 1991a,b). Moreover, heparin induces changes in the conformation of arrestin similar to those produced by rhodopsin (Palczewski et al., 1991a). The dissociation of arrestin from photoactivated rhodopsin occurs when *all-trans*-retinal, the photoisomerized chromophore, is removed and enzymatically reduced to *all-trans*-retinol (Hofmann et al., 1992).

Here, we report studies of arrestin conformation using chemical modification and circular dichroism. We find that changes in the conformation of arrestin do not involve changes in secondary structure; instead, our results suggest that changes in arrestin between the free and bound state of arrestin are due to rearrangement of polypeptide side chains.

Materials and Methods

Purification of Bovine Arrestin. Arrestin was prepared from bovine retinas according to one of two methods described previously (Palczewski & Hargrave, 1990; Palczewski et al., 1991c). Results obtained from the two preparations were identical within experimental error.

Modification of Bovine Arrestin by NbS_2. In kinetic experiments, 500–700 μL of a solution containing arrestin (0.2–0.6 mg/mL) in 20 mM Hepes buffer (pH 7.5) with 100 mM KCl was mixed with 10–20 μL of a solution of NbS_2[1]

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[1] Abbreviations: BTP, 1,3-bis[tris(hydroxylmethyl)methylamino]-propane; CD, circular dichroism spectroscopy; NbS_2, 3-carboxy-4-nitrophenyl disulfide; ROS, rod cell outer segments.

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(50–100 molar excess over arrestin concentration). Solutions were mixed in a disposable polystyrene cuvette, and the cuvette was then placed in a water-jacketed cell holder. Temperature was maintained at 30 ± 0.5 °C. Reaction progress was monitored by the absorption change at 412 nm that accompanies the formation of NbS. For each kinetic run, 100–300 data points were collected. For two classes of sulfhydryl groups differing in reactivity, the time course of the modification of arrestin sulfhydryl groups with NbS_2 can be described by

$$\Delta A^{412nm} = \Delta A_1^{412nm}(1 - \exp(-k_1 t)) + \Delta A_{2,3}^{412nm}(1 - \exp(-k_{2,3} t)) \quad (1)$$

where ΔA_1^{412nm} and $\Delta A_{2,3}^{412nm}$ are amplitudes of the two classes of sulfhydryl groups reacted at time t and k_1 and $k_{2,3}$ are the rate constants for the reaction of NbS_2 with the two classes of sulfhydryl groups. The rate constants and the corresponding amplitudes for the reaction were obtained from the best nonlinear fit of the experimental data to the equation for the pseudo-first-order reactions.

Spectroscopy. An arrestin concentration of 0.65 mg/mL in 0.01 M sodium phosphate buffer (pH 7.5) was used for all measurements. Cells with a 1-cm path length were used in the 280-nm region, and cells with a 100-μm path length were used for 260–178-nm scans. Absorption spectra were measured with a Cary Model 15 spectrophotometer flushed with nitrogen for far-UV transmission. Accurate concentrations of proteins determined by amino acid analysis were correlated with the absorption spectrum. The absorbance of a 0.1% solution at 278 nm was 0.638, provided that scattering from aggregated material (due to the facile denaturation of arrestin) was corrected by matching baseline absorption to the sample at 320 nm and subtracting the baseline. On a per amide basis, the molar extinction coefficients are $\epsilon(278)$ = 71.5 L/(cm·mol·amide) and $\epsilon(190)$ = 10 560 L/(cm·mol·amide).

CD spectra were measured using a vacuum ultraviolet instrument described elsewhere (Johnson, 1971). All measurements were performed over the range of 260–178 nm at a scan rate of 0.5 nm/min. Data were collected by an IBM-compatible PC computer that averaged the spectra for each 0.5-nm interval. Accurate concentrations were obtained by monitoring the absorption of the 190-nm band using its corresponding extinction coefficient. Total optical density of the sample was kept below 1.0 to avoid absorption artifacts in the CD measurements. The instrument was calibrated (Chen & Yang, 1977) with (+)-camphorsulfonic acid (CSA) using $\Delta\epsilon(290.5)$ = 2.36 and $\Delta\epsilon(192.5)$ = −4.9 L/(cm·mol). For arrestin, $\Delta\epsilon$ is expressed on a per amide basis.

The CD spectrum of arrestin was analyzed for five secondary structures: α-helix (H), antiparallel β-strands (A), parallel β-strands (P), β-turns (T), and other structure(s) (O). The method of analysis is a modification of the original Hennessey and Johnson method (1981) that uses a basis set of CD spectra for proteins with known secondary structure, and the singular value decomposition theorem (Noble & Daniel, 1977). Structures were computed by using the generalized inverse (Noble & Daniel, 1977; Compton & Johnson, 1986) and a statistical method called variable selection, which systematically eliminates proteins from the basis set until the CD measurement of the protein being analyzed is well fit and the sum of secondary structures is close to 1.0 (Manavalan & Johnson, 1987). CD measurement over the range of 178–260 nm has an information content equivalent to five equations so it is only possible to solve for five variables (Hennessey & Johnson, 1981; Manavalan & Johnson, 1985). On the other hand, the CD spectrum of a protein depends not only on the five categories of secondary structure listed above but also on prosthetic groups, aromatic side chains, etc. Following the statistical method of variable selection (Mosteller & Tukey, 1977), it is reasonable to remove from the basis set those proteins that have contributions to their CD spectra from factors that are not found in the protein being analyzed, so that the number of variables is reduced to five. We do not know in advance which proteins to remove from the basis set, so calculations are performed for all possible combinations. Criteria for choosing acceptable combinations are the following: (1) the total of all five secondary structures should be between 0.96 and 1.05; (2) negative secondary structures may not be less than −0.05, and these negative numbers are assumed to be zero; (3) the calculated CD spectrum should be reasonable fit to the measured CD spectra (a root-mean-square residual of about 0.2$\Delta\epsilon$ units), but this is a secondary criterion; (4) combinations that meet the first three criteria and eliminate the fewest proteins from the basis set are averaged to give the reported analysis. The error reported in Table II is the standard deviation of these combinations.

Reagent Concentration. NbS concentration was measured spectrophotometrically at 412 nm using a molar absorption coefficient of 13 600 (Ellman, 1959), or 14 900 if the reaction was carried out in 2–5 M urea (Gething & Davidson, 1972). The concentration of NbS_2 was determined from its absorbance at 412 nm after reduction with an excess of 2-mercaptoethanol.

Results

Chemical Modification of Arrestin. The primary structure of bovine arrestin deduced from its cDNA nucleotide sequence has three cysteines (Shinohara et al., 1987). By following the kinetics of cysteine modification, we hope to learn more about the conformational properties of the protein and whether they are altered upon binding of a ligand.

We found that all three cysteines are available for NbS_2 modification. The stoichiometry of the NbS_2 modification in 10 mM Hepes buffer, pH 7.5, containing 100 mM KCl is 2.7 ± 0.06, since modification of 13 μM arrestin caused a change in absorption of ΔA^{412nm} = 0.477. The stoichiometry for the reaction of arrestin with NbS_2 obtained from nonlinear fits to the experimental data was 3.1 ± 0.2, and it is similar to that obtained for arrestin in 6 M urea (data not shown). Under pseudo-first-order conditions (30–100-fold excess of the reagent over each of the three sulfhydryl groups of arrestin), the modifications of the three cysteines are indistinguishable, since $\ln[(\Delta A_\infty - A_0)/\Delta A_\infty]$ versus time is linear up to 95% of the modification (data not shown). The overall pseudo-first-order k_{obs} is in the range 0.000 31 to 0.000 43 s^{-1}. At higher concentrations of the reagent (150–300-fold excess), the pseudo-first-order plot is not linear (Figure 1) and the reaction can be resolved into two processes described by eq 1. This scheme was chosen because fitting the data to eq 1 improves χ^2 significantly over the value obtained for a single-exponential fit. Here, $\chi^2 = [\Sigma(A_{obs} - A_{exp})^2]/(N - p)$, A_{obs} is the observed change in absorption, A_{exp} (ΔA^{412nm}) is obtained from 1, N is the number of the data points, and p is the number of calculated parameters. Adding a third exponential does not change the χ^2 significantly. As shown in Figure 2, the faster reaction is dependent on the concentration of NbS_2 (k_{obs} = 0.000 58 to 0.000 94 s^{-1}) with a second-order rate constant of 6.35 M^{-1} s^{-1} (calculated as described in Materials and Methods), and the slower reaction is independent of the reagent concentration (k_{obs} = 0.000 45 s^{-1}).

Potassium fluoride destabilizes arrestin, causing more rapid modification of one sulfhydryl group but a slower rate of modification for the two remaining sulfhydryl groups (Table

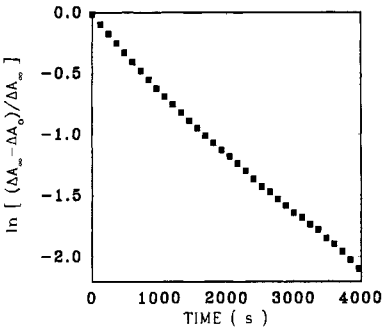

FIGURE 1: Pseudo-first-order plot of arrestin modification by NbS_2. Arrestin (13 μM) in 20 mM Hepes buffer, pH 7.5, containing 100 mM KCl was mixed with NbS_2 (6.34 mM), and the progress of the reaction was monitored spectrophotometrically at 412 nm. In the control sample without arrestin, the minimal changes at 412 nm (less than 0.010) due to the residual decomposition of NbS_2 were subtracted. The reaction was temperature controlled at 30 °C.

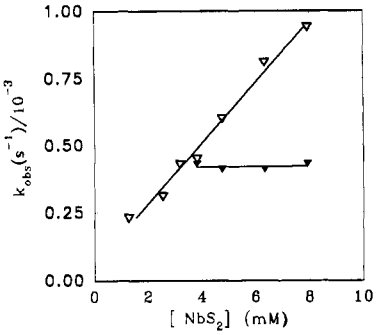

FIGURE 2: Dependence of pseudo-first-order rate constants of the sulfhydryl modification reaction versus NbS_2 concentration. Arrestin (13 μM) in 20 mM Hepes buffer, pH 7.5, containing 100 mM KCl was mixed with different concentrations of NbS_2 (1.27–7.92 mM), and the progress of the reaction was monitored spectrophotometrically at 412 nm as described in the legend to Figure 1. The rate constants were calculated as described in Results. Open triangles represent the [NbS_2]-dependent modification of the sulfhydryl groups of arrestin, and filled triangles represent [NbS_2]-independent modification.

I). Potassium chloride has a similar effect, when applied at twice the concentration of potassium fluoride. In contrast, ammonium sulfate has only a slight effect on the rate of modification (data not shown). The rate of modification of all three sulfhydryl groups is also very senstive to the presence of a denaturing agent. In the presence of 0.5 M urea, the rate of modification increases by a factor of 2.5, and at 1 M urea it increases by a factor of 6 (data not shown). In 0.5 M urea, the reactivities of all three sulfhydryl groups of arrestin are indistinguishable. Increases in urea concentration up to 5 M continue to lead to an increase in the rate of sulfhydryl modification, presumably as a result of the loss of structural integrity of arrestin [see also Kotake et al. (1991)].

Because of reports that Ca^{2+} and ATP bind to arrestin (Glitscher & Rüppel, 1989; Huppertz et al., 1990), we evaluated the effect of these and other potential ligands on sulfhydryl modification. Addition of $CaCl_2$, $MgCl_2$, inorganic phosphate, MgATP, or MgGTP had little effect on the rate of modification of the cysteine residues under conditions in which all groups react at similar rates (Table I). Heparin and inositol hexakisphosphate, which have been shown to induce conformational changes in arrestin (Palczewski et al., 1991a,c), block modification of one sulfhydryl group of arrestin but increase the reactivity of the other two.

Our results are in agreement with and extend the observations of Pogozheva et al. (1989).

Circular Dichroism. The CD spectrum of arrestin shown in Figure 3 (an average of eight measurements) has a particularly low intensity. Typically, proteins have an intensity in the range of 2–8 L/(cm·mol·amide) at the 210–220-nm minimum (Johnson, 1990). The range for the arrestin spectrum is also shown as the highest and lowest measurements. Although this range is larger than usually observed, and appears quite high on a relative basis, it is not particularly high on an absolute basis. The primary variability was due to instability in the protein that made each sample solution a little different, but this difference is magnified in the figure because of the exceedingly low CD measurement of arrestin. Similar

Table I: Pseudo-First-Order Rate Constants for the Modification of Arrestin by NbS_2[a]

	$[-SH]_1$		$[-SH]_{2,3}$	
	$k_{obs} \times 10^3$ (s^{-1})	ΔA_1	$k_{obs} \times 10^3$ (s^{-1})	$\Delta A_{2,3}$
control	0.43	0.181	0.43	0.362
Salt				
1.00 M KF	3.39	0.211	0.03	0.388
0.85 M KF	2.57	0.210	0.06	0.358
0.70 M KF	2.21	0.171	0.11	0.358
0.50 M KF	2.17	0.182	0.28	0.342
0.30 M KF	1.71	0.181	0.33	0.352
0.20 M KF	1.00	0.155	0.40	0.367
Inhibitors of the Arrestin–Rhodopsin Interaction				
200 μg/mL heparin	blocked		0.62	0.355
100 μg/mL heparin	blocked		0.64	0.355
50 μg/mL heparin	blocked		0.60	0.361
200 μM $InsP_6$[b]	blocked		1.71	0.350
75 μM $InsP_6$[b]	blocked		1.19	0.392
25 μM $InsP_6$[b]	blocked		1.01	0.402
Potential Ligands				
1 mM $CaCl_2$	0.42	0.185	0.42	0.370
10 mM $MgCl_2$	0.42	0.183	0.42	0.366
10 mM P_i	0.43	0.181	0.43	0.362
100 μM ATP/1 mM $MgCl_2$	0.44	0.185	0.44	0.370
100 μM GTP/1 mM $MgCl_2$	0.45	0.185	0.45	0.370

[a] The modification was performed at 30 °C in 20 mM Hepes buffer, pH 7.5, containing 100 mM KCl. The concentration of arrestin was 13 μM, and that of NbS_2 was 3.17 mM. The reaction was monitored at 412 nm as described in Materials and Methods. [b] Inositol hexakisphosphate.

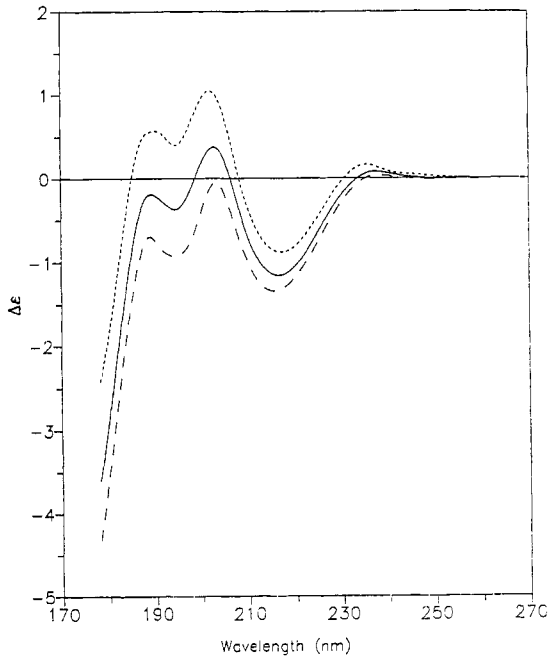

FIGURE 3: Circular dichroism of arrestin: average (solid line), highest recorded scan (dotted line), and lowest recorded scan (dashed line). The average spectrum is derived from eight independent measurements.

Table II: Analysis of Arrestin CD Spectra for Secondary Structure[a]

scan	H	A	P	A + P	T	O	total
high	0.03 ● 0.01	0.36 ± 0.02	0.08 ● 0.01	0.44 ± 0.03	0.12 ± 0.01	0.37 ± 0.01	0.96
low	0.02 ± 0.01	0.28 ± 0.02	0.11 ± 0.03	0.39 ± 0.01	0.21 ± 0.01	0.43 ± 0.01	1.04
av	0.03 ± 0.01	0.31 ± 0.01	0.10 ± 0.01	0.41 ± 0.02	0.18 ± 0.01	0.39 ± 0.01	1.01

[a] The CD spectrum was analyzed for five secondary structures: α-helix (H), antiparallel β-strands (A), parallel β-strands (P), β-turns (T), and other structures (O) as described in Materials and Methods. The average spectrum is derived from eight independent measurements.

instability of arrestin also was reported by Kotake et al. (1991), employing tryptophan fluorescence spectroscopy.

The CD measurement of arrestin in 0.01 M potassium phosphate buffer (pH 7.5) with 0.1 M potassium fluoride added, with 0.5 M potassium fluoride added, or with 400 μg/mL heparin added, all gave CD spectra within the range shown in the spectra in Figure 3. Heparin does have a CD spectrum of its own, but at 400 μg/mL it has no measurable CD over the wavelength range studied. Therefore, within the limits of our measurements, neither heparin nor salt induced a change in the CD spectrum. Analysis of the average CD scan for secondary structure and analysis of the highest and lowest scans are given in Table II. Except for β-turn, the variability in the CD measurement had little effect on the analysis for secondary structure. Arrestin shows virtually no α-helical structure, 41 ± 3% β-structure, 18 ± 6% β-turns, and 39 ± 4% other structure.

Discussion

Modification of sulfhydryl groups is a sensitive method that can be used to monitor the degree of denaturation of arrestin and the binding of ligands. The kinetics of sulfhydryl group modification are sensitive to KF or KCl concentration, suggesting that salt may interfere with ion pairing within arrestin, as has been proposed (Palczewski et al., 1991a,b). Using this and other methods (Palczewski & Hargrave, 1990), we were unable to confirm the earlier reports of Ca^{2+}, MgATP, or MgGTP binding to arrestin (Glitscher & Rüppel, 1989; Huppertz et al., 1990). We found no changes in arrestin conformation upon exposure to Ca^{2+}, MgATP, or MgGTP. Although it is plausible that the hypothetical binding of Ca^{2+}, MgATP, or MgGTP to arrestin does not provoke conformational change, lack of evidence for binding of these ligands to the protein has been strengthened by complementary analytical methods (Palczewski & Hargrave, 1990; Palczewski et al., 1991a). Our previous finding using equilibrium dialysis, gel filtration, fluorescence, and absorption spectroscopy supports the conclusion that they are not ligands. Our results may differ from those of Glitscher and Rüppel (1989) and Huppertz et al. (1990) because of the methods these authors use for the determination of ATP and Ca^{2+} binding to arrestin. Alternatively, if the arrestin prepared by these authors was contaminated by ROS membranes, artifactual binding of ATP (Sitaramayya & Hakki, 1990) and Ca^{2+} would be observed. Heparin and inositol hexakisphosphate, on the other hand, are proven inhibitors of the interaction between arrestin and photolyzed phosphorylated rhodopsin (Palczewski et al., 1991a,c). Heparin and inositol hexakisphosphate block one of the sulfhydryl groups of arrestin by inducing conformational changes, so that the sulfhydryl group is not exposed.

More than one model may explain our kinetic studies of the modification of arrestin by NbS_2. In the first model, arrestin exists in two or more conformational states. The reactivity of one of the sulfhydryl groups is equivalent in all states, but the two additional sulfhydryl groups have different reactivities in the different conformations. The transition among the conformations is the slowest step in the modification, and the modification of these groups, therefore, is independent of the reagent concentration. In the second model, modification of the first cysteine causes a conformational change in arrestin, which then exposes the addition two cysteine residues. This arrangement is the slowest step in the modification, making the modification independent of the reagent concentration. The flexibility of the arrestin conformation is confirmed by an increase in the rate of modification in the presence of as little as 0.5 M urea.

Shinohara et al. (1987) have measured the CD of arrestin to 195 nm, and we confirm their finding that there is a negative band typical of all-β proteins at about 215 nm. Their negative band is blue-shifted compared to ours, presumably because they used a long analog time constant to collect their data, rather than modern digital methods. Also, the intensity of their band is twice the magnitude of ours. We believe our intensity to be correct because (1) our measured $\epsilon(190)$ is in the expected range and (2) our predicted secondary structure sums to 101% even before doing variable selection.

Our CD measurements detected no significant change in secondary structure of arrestin upon the addition of either 400 μg/mL heparin or 0.5 M potassium fluoride. Thus the changes in arrestin that protect the sulfhydryl group from modification must be rather minor, and they may be due to changes in the conformation of side chains rather than the backbone, which gives rise to the CD signal.

We have characterized the conformation of arrestin by sulfhydryl reactivity with NbS_2. Presumably, arrestin assumes two major conformations: a free conformation and a bound conformation (Palczewski et al., 1991a). The bound conformation is induced by phosphorylated and photolyzed rhodopsin or by heparin. Transition between these conformations shows high Arrhenius activation (Schleicher et al., 1989). Lack of change in the secondary structure of arrestin in the presence of salt and heparin, as monitored by CD, suggests that the conformational changes of arrestin between the free and bound conformations are due to minor rearrangement of polypeptide side chains rather than to overall changes in the composition of secondary structural elements.

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