The Selectivity of Visual Arrestin for Light-activated Phosphorhodopsin Is Controlled by Multiple Nonredundant Mechanisms*

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Arrestin plays an important role in quenching phototransduction via its ability to bind to the phosphorylated light-activated form of the visual receptor rhodopsin (P-Rh*). Remarkable selectivity of visual arrestin toward this functional form is determined by an elegant sequential multisite binding mechanism. Previous structure-function studies have suggested that the COOH-terminal region of arrestin (residues 356–404) is not directly involved in rhodopsin interaction, but instead plays a regulatory role. This region supports basal arrestin conformation and ensures arrestin’s transition into a high affinity rhodopsin-binding state upon an encounter with P-Rh*.

Overall, our results corroborate this hypothesis and identify three functional subregions (residues 361–368, 369–378, and 379–404) and individual amino acids involved in the control of arrestin stability and binding selectivity. Two of the most potent mutants, arrestin(1–378) and arrestin(F375A, V376A, F377A) belong to a novel class of constitutively active arrestins with high affinity for P-Rh*, dark P-Rh, and Rh* (but not dark Rh), in contrast to earlier constructed mutants arrestin(R175E) and arrestin(L2–16) with high affinity for light-activated forms only. The implications of these findings for the mechanism of arrestin-rhodopsin interaction are discussed in light of the recently determined crystal structure of arrestin.

The visual amplification cascade has long served as a model for G protein-coupled receptor signaling. Light-activated rhodopsin (Rh*) activates visual G protein transducin, which in turn stimulates cGMP phosphodiesterase. The reduction in intracellular cGMP leads to the closing of cGMP-gated sodium channels and hyperpolarization of the rod cell. Quenching of the visual signaling is initiated by rapid phosphorylation of Rh* by rhodopsin kinase, followed by highly selective binding of arrestin to P-Rh* (1). Arrestin and transducin binding sites on rhodopsin overlap (2, 3). As the result, arrestin binding precludes rhodopsin/transducin interaction and effectively shuts down signaling (1, 3).

Previously we developed an assay for assessing arrestin binding to various functional forms of rhodopsin, dark P-Rh, P-Rh*, dark Rh, and Rh* (4). Binding studies with wild type, mutant, and chimeric arrestins identified several structural and functional regions within the arrestin molecule (4–7) (Fig. 1) that have been largely confirmed by recent elucidation of the partial crystal structure of arrestin (8). The NH₂-terminal half of arrestin contains an activation-recognition site that interacts with the regions of rhodopsin that change conformation upon light activation, and a phosphorylation-recognition site that interacts with the phosphorylated COOH terminus of rhodopsin (Fig. 1) (5). These two regions serve as the primary binding sites. When arrestin encounters P-Rh*, it undergoes a conformational rearrangement (9) that results in mobilization of a secondary binding site (5) within the COOH-terminal half of the molecule (Fig. 1). According to the model of sequential multisite binding (5), this transition is triggered by simultaneous engagement of both primary sites. We hypothesized that the transition is controlled by constraining intramolecular interactions within arrestin (5–7, 10–12). Some of these constraints are disrupted as the result of arrestin binding to phosphates on phosphorhodopsin, while others are released upon arrestin binding to that part of rhodopsin, which changes conformation upon activation. When both constraints are simultaneously relieved, arrestin assumes its high affinity rhodopsin-binding conformation. Two constraining interactions have been tentatively identified, one between the basic NH₂ terminus and the acidic COOH terminus (5–7), and another between Arg-175 and its negatively charged partner(s) (10–12). The crystal structure of arrestin identifies Asp-296, Asp-303, and Asp-30 as likely partners of Arg-175 (8). Unfortunately, x-ray crystallography did not reveal any structure of the COOH terminus beyond residue 368. In this study we identify specific residues in the COOH terminus that control the selectivity of arrestin binding.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP and [3H]leucine were purchased from NEN Life Science Products. All restriction enzymes were purchased from New England Biolabs. Sepharose 2B and all other chemicals were from sources previously described (5). Rabbit reticulocyte lysate and SP6 RNA polymerase were prepared as described previously (13). 11-cis-retinal was generously supplied by Dr. R. K. Crouch.

Plasmid Construction and Site-directed Mutagenesis—Bovine visual arrestin cDNA (14) was generously supplied by Dr. T. Shinohara. Plasmid pARR-VSP was constructed as described (11). This pGEM2-based plasmid encodes bovine wild type arrestin with an “idealized” 5′-untranslated region (13) under control of SP6 promoter. Plasmid pARR-VSP was modified by polymerase chain reaction-based site-directed mutagenesis, as follows. Oligonucleotides 5′-gtc atg ggg ata ctg tct tac cag-3′ (codons 320–328) (IV) and 5′-t ggc cac ttc act gga tgt cag ctc tcc cag gag tcc tga cca gag cgg-3′ were used as forward and reverse primers, respectively, to generate an 88 bp fragment. Oligonucleotide 5′-gag gcc tcc tgg cag tct tcc tct tcc cag gag tcc tga cca gag cgg-3′ (anticodons 349–334) were used as forward and reverse primers, respectively, to generate an 88 bp fragment. Oligonucleotide 5′-aat ttt gtt ttc gaa gag tct gcc tcc gag-3′ (codons 191–198) (I) and the 88 bp fragment were used as forward and reverse primers, respectively, to generate a 473-bp fragment. Oligonucleotides 5′-aat ttt gtt ttc gaa gag tct gcc tcc gag-3′ and 5′-aat ttt gtt ttc gaa gag tct gcc tcc gag-3′ (codons 374–387) and
5′-agcatattgtggccgc-3′ (antisense modified 3′-UTR) (II) were used as forward and reverse primers, respectively, to generate a 152-bp fragment. Oligonucleotide 5′-ca tcc agt gas gtt ggc act gag gtc c-3′ (codons 343–352) (III) and the 152-bp fragment were used as forward and reverse primers, respectively, to generate a 244-bp fragment. The 473- and 244-bp fragments (which have an 18-bp overlap) were used without template to generate a 700-bp “primer-dimer,” which was subsequently reamplified using oligonucleotides I and II, purified, digested with XhoI and NotI, and subcloned into XhoI/NotI-digested pARR-VSP. In the resulting construct (pARR-SC), which was used for all further mutagenesis, several silent mutations were introduced (underlined bases in oligonucleotides) into the visual arrestin open reading frame, creating unique restriction sites SpeI (codons 323–325), BstBI (codons 377–378), NruI (codons 380–382), and eliminating sites BamHI (codons 322–324), StuI (codons 336–338), SacI (codons 341–342), and BalI (codons 347–349). All mutations were introduced by polymerase chain reaction using appropriate mutagenizing oligonucleotide and oligonucleotide II as forward and reverse primers, respectively, and the 244-bp fragment as a template. Oligonucleotide IV and resulting fragments of various lengths were then used as forward and reverse primers, respectively, with the 700-bp fragment as a template to generate 314-bp fragments with corresponding mutations. These fragments were purified, digested with NotI/StyI (mutations in positions 361–364), StyI/ NruI (369–379), or BstBI/HindIII (387–404), and subcloned into appropriately digested pARR-SC. Double mutants were constructed by exciting appropriate NruI/NotI fragment and subcloning it into NruI/ NotI digested plasmid encoding arrestin(E379Q). Cluster mutants were constructed similarly, subcloning polymerase chain reaction-generated mutant StyI/NruI or BstBI/HindIII fragments into appropriately digested pARR-SC. The sequences of polymerase chain reaction-generated portions of all constructs were confirmed by dideoxy sequencing. 

In vitro Transcription, Translation, and Evaluation of Mutants’ Stability—Plasmids were linearized with HindIII, NruI, BstBI, or StyI before in vitro transcription, to produce mRNAs encoding full-length, truncated (1–380), (1–376), or (1–367) arrestin proteins. In vitro transcription and translation were performed as described previously (5, 13). All arrestin proteins were labeled by incorporation of [3H]leucine with specific activity 20–40 Ci/mmol, resulting in the specific activities of 200–300 Ci/mmol (440–660 dpm/fmol). The translation of each of the arrestin mutants used in this study produced a single labeled protein band with the expected mobility on SDS-polyacrylamide gel electrophoresis. Two parameters were used for the assessment of mutant stability. First, protein yields in the in vitro translation were known to correlate with stability, most likely because misfolded or denatured proteins are rapidly destroyed by proteases present in rabbit reticulocyte lysate (13). Second, denatured proteins tend to aggregate and are pelleted by centrifugation at 350,000 $\times$ g for 1 h. As an estimate of a mutant’s relative stability we used its yield multiplied by the percentage of protein remaining in the supernatant after incubation for 10 min at 37 °C followed by centrifugation. This integral parameter calculated for a mutant was expressed as a percent of that for wild type arrestin.

**Rodopsin Preparations**—Urea-treated rod outer segment membranes were prepared, phosphorylated with rhodopsin kinase, and re-generated with 11-cis-retinal as described elsewhere (12). The stoichiometry of phosphorylation for the rhodopsin preparations used in these studies was 1.2–1.6 mol of phosphate/mol of rhodopsin.

**Arrestin Binding to Rhodopsin**—In vitro translated tritiated arrestins (100 fmol) were incubated in 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl$_2$, 1.5 mM dithiothreitol, 50 mM potassium acetate with 7.5 pmol of the various functional forms of rhodopsin in a final volume of 50 µl for 5 min at 37 °C either in the dark or in room light (5). The samples were immediately cooled on ice and loaded under dim red light onto 2 ml of Sepharose 2B columns equilibrated with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA. Bound arrestin eluted with the rod outer segments in the void volume (between 0.5 and 1.1 ml). Nonspecific binding determined in the presence of 0.3 µg of liposomes (10% of the total binding and <0.5% of the arrestin present in the assay) was subtracted.

**RESULTS**

The removal of 37 COOH-terminal amino acids (Figs. 1 and 2) substantially reduces the stability of arrestin and its selectivity toward P-Rh$^*$ (4, 5). To localize more precisely the residues involved we utilized restriction sites NruI and BstBI to produce truncated mRNAs encoding arrestin(1–380) and (1–376). These mutants were expressed by in vitro translation and compared with full-length arrestin and arrestin(1–367) (Fig. 2). Arrestin(1–380) demonstrates a 20% decrease in P-Rh$^*$ binding and a 4-fold increase in binding to dark P-Rh and Rh$^*$. The binding of arrestin(1–376) to both dark P-Rh and Rh$^*$ is twice that of arrestin(1–380), while its binding to P-Rh$^*$ is virtually the same. The deletion of 9 additional residues in (1–367) slightly reduces the binding without further change in selectivity (Fig. 2). The absolute binding of any mutant reflects both its affinity for a particular form of rhodopsin and its stability under assay conditions, whereas its relative binding to different functional forms (selectivity profile) is not affected by its stability. To interpret the effects of mutations on absolute binding levels, we used an independent assessment of stability. We found that truncations (1–380), (1–376), and (1–367) reduce stability to 17, 14, and 10% of that for full-length arrestin. Thus, it appears that multiple residues within the COOH-terminal domain are critical for arrestin stability and selectivity.

Several lines of evidence suggest functional importance of negatively charged residues in the COOH terminus of visual arrestin (5, 6). Therefore we first introduced Glu → Gln and Asp → Asn mutations, and compared these mutants with wild type arrestin and a phosphorylation-independent mutant arrestin(R175E) (10, 11) (Fig. 3). Point mutants clearly fall into three distinct groups. Mutations in the subregion I (361–368) (E361Q, D362N, D364N, and E368Q) result in a moderate general decrease of arrestin binding. Thus, the subregion 361–368 appears to be more involved in supporting arrestin stability than in selectivity control. The most potent of these mutations (E368Q) reduces the stability by 35%.

Mutations in the subregion II (372–378) (D372N, E373Q, and E378Q) reduce arrestin binding to P-Rh$^*$ and moderately increase its binding to both dark forms of rhodopsin (Fig. 3). Reversal of all three negative charges (arr-3R = D372R,
E373R, and E378R) results in less than a 2-fold increase in dark P-Rh binding, as well as a 3-fold increase in binding to Rh* (Fig. 4), without affecting P-Rh* and dark Rh interaction. Neither of these mutations affects arrestin stability.

Neutralization of negative charges in subregion III (379–404) (E379Q, D387N, E390Q, E393Q, E394Q, D397N, E399Q, D403N, and E404Q) increases arrestin binding to all functional forms of rhodopsin, especially to dark P-Rh and Rh* (Fig. 3). The most potent mutations in this group are E379Q, D387N, and E393Q. The effect of each mutation on Rh* and dark P-Rh binding is rather moderate (less than 2-fold), unlike the dramatic 10–15-fold increase in Rh* binding due to R175E mutation (Fig. 5). On the other hand, elimination of 7 (out of 9) negative charges within this 24-residue subregion stimulates binding. Conceivably these residues function in concert, interacting with multiple positively charged partners. Alternatively, some of them might be involved in the interaction with Arg-175 (the lack of information on three-dimensional structure of arrestin COOH terminus (8) does not allow us to exclude this possibility). If the first scenario is correct, we expected that: 1) double mutations would have a slightly stronger effect than either single mutation alone, and 2) the effect of charge reversal would not be substantially greater than charge neutralization. If these residues interact with Arg-175, double mutations and/or charge reversal may result in a dramatic change in arrestin selectivity. To test these possibilities, we constructed four double mutants, each combining E379Q with another stimulatory mutation (D387N, E394Q, or D397N) and seven mutants with charge reversal (E379R, D387R, E390R, E393R, E394R, D397R, E399R, and D403R) (Fig. 4). The second mutation in each case enhances the effect of E379Q, especially on Rh* binding, but neither combination causes a change comparable to that of R175E. The effects of charge reversal are essentially the same as those of neutralization. Apparently, negatively charged residues in this subregion do not interact with Arg-175. Subregion 379–404 likely interacts with multiple positive charges elsewhere. To further test this hypothesis, we constructed additional mutants: arrestin(1–378), in which the whole subregion 379–404 is deleted; arr-4R (D387R, E390R, E393R, and E394R); arr-5R (E379R, D387R, E390R, E393R, and E394R) with multiple charge reversals; arr-4R(1–394); and arr-5R(1–394), in which charge reversals are combined with the truncation of the remainder of the subregion III. Arrestin(1–378) demonstrates an increased binding
Inhibition is indicative of the involvement of the hydrophobic dopsin (5). A reduced sensitivity of the binding to high salt the secondary site for the interaction with these forms of rho-
activation-recognition, respectively), and/or the mobilization of the corresponding primary binding site (phosphorylation-
and arrestin(1–378) are similar (roughly 1:3:2).

To simplify the interpretation of the data, we assume that mutations, which result in less than 20% change in protein stability accompanied by a dramatic 10–20-fold increase in dark P-Rh and/or Rh* binding, primarily disrupt selectivity control. More profound decreases in stability suggest that the mutated element supports structural integrity of the molecule, while concomitant changes in binding selectivity may be either the direct result of the mutation or a secondary effect of a “loose” conformation.

Arrestin Stability—Elements involved in supporting arrestin stability appear to be distributed throughout COOH-terminal region. These include at least 9 negatively charged residues (4 in subregion I and 5 in subregion III), and residues 395–404. The effects of point mutations are weak, whereas multiple mutations produce substantial changes. Thus, groups of these residues function in concert, and an individual mutation has a profound effect only when it is “the last straw.” Similarly, the deletion of residues 395–404 in the context of arr-R4 has virtually no effect on binding selectivity, whereas the effect of the same deletion in arr-R5 is more significant (Fig. 5). Interestingly, dark P-Rh:P-Rh*:Rh* binding ratios of arr-R4 (1–394) are similar (roughly 1.3:2).

In addition to negative charges, other COOH-terminal elements are well conserved among the members of arrestin family (Fig. 1), including a cluster of three bulky hydrophobic residues FVF (375–377), the hydrophobic Phe-380, and the positively charged Arg-382. Properties of a mutant with triple alanine substitution arr-3A (F375A,V376A,F377A) were found to be similar to those of the arrestin(1–378); the binding to dark P-Rh, dark Rh, and Rh* was increased 22-, 9-, and 10-fold, respectively, and the binding to P-Rh* was substantially increased (Fig. 5). The effects of individual mutations in this cluster are similar, although less dramatic, suggesting that these residues act in concert. The effects of F380A and R382N mutations resemble those of F375A and F377A (Fig. 5). Neither these residues act in concert. The effects of F380A and R382N mutations, which result in less than 20% change in protein stability accompanied by a dramatic 10–20-fold increase in dark P-Rh and/or Rh* binding, primarily disrupt selectivity control. More profound decreases in stability suggest that the mutated element supports structural integrity of the molecule, while concomitant changes in binding selectivity may be either the direct result of the mutation or a secondary effect of a “loose” conformation.

DISCUSSION

The COOH-terminal region of visual arrestin plays an important role in supporting basal arrestin conformation and ensuring its strict selectivity toward P-Rh*. However, a mutation that relieves any conformational constraint in the arrestin molecule can be reasonably expected to affect both stability and selectivity. The association of increased conformational flexibility with constitutive activity of a protein appears to be a general phenomenon. A recent study (31) has demonstrated that a constitutively active mutant of β2-adrenergic receptor is less stable than wild type protein. This constitutively active mutant was also found to undergo the conformational change upon activation by an agonist more readily, i.e., higher percentage of receptor appears to be in an “active” state under the same conditions. This finding parallels the substantially higher binding of constitutively active arrestins to P-Rh* determined in our experiments (Fig. 5).

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residues appear to work together, conceivably via zipper-like interactions with clusters of positively charged residues. Mutagenesis studies (5, 6) suggest that interaction with the arrestin NH$_2$ terminus is most likely involved. Although the recently published arrestin crystal structure (8) does not extend beyond residue 368, the positioning of the beginning of the COOH-terminal region and of the NH$_2$-terminal region (β-strands A and B in Granzin et al. (8)) supports this hypothesis.

**Binding Selectivity—**Point mutations in the arrestin COOH terminus do not dramatically reduce selectivity (Figs. 3 and 4). Thus, structural elements encompassing several residues appear to be involved in selectivity control. The most profound changes in arrestin selectivity are brought about by alanine substitution of VFV cluster (375–377), and by the deletion of the 379–404 region. Binding characteristics of arr-3A and arr(1–378) are similar, although not identical (Figs. 5 and 6). The proximity of these two elements suggests that they may interact with a common partner which has both a hydrophobic cluster and a series of positive charges. Alternatively, these elements may participate in two independent constraining interactions. Our data suggest that the disruption of either interaction is sufficient to promote high binding to dark P-Rh and Rh*, apparently via the mobilization of a secondary hydrophobic binding site (Fig. 6). Superimposition of arrestin crystal structure and cytoplasmic surface of rhodopsin (8) leaves a substantial part of the latter open for putative interaction with the secondary binding site of arrestin. The arrestin molecule consists of two domains connected by an extended hinge region (residues 180–192), with both domains having domelike concave surfaces (8). It is tempting to speculate that the secondary binding site is localized on such a surface of COOH-terminal domain. Then the conformational rearrangement of the arrestin molecule upon P-Rh* binding (9) and the mobilization of the secondary binding site (5) can both be identified with the movement of COOH-terminal domain toward exposed elements of rhodopsin.

**Functional and Structural Elements in Arrestin COOH Terminus—**Subregion I (361–368) appears to support the stability of arrestin. Glu-368 is the most important residue in subregion I (Fig. 3). The significantly lower stability of arr(1–355) compared with that of arr(1–367) (5) suggests that this stabilizing element likely extends further upstream. Subregions II and III are primarily involved in selectivity control (Fig. 5). Mutations in either subregion yield proteins with high binding to P-Rh*, dark P-Rh, and Rh*. These two subregions may either cooperate or function as two independent regulatory elements; however, both parts are necessary to ensure arrestin selectivity toward P-Rh*. Two lines of evidence suggest that subregion 379–404 is a separate structural element. First, limited proteolysis of purified recombinant arrestin with V8 endopeptidase removes this subregion (as determined by electrospray mass spectrometry). The remaining truncated arrestin effectively competes with 3H-labeled arrestin(1R75E) for both P-Rh* and Rh*; i.e. it behaves exactly as does 3H-labeled arr(1–378). Second, arr(1–378) is as stable as wild type protein, whereas the stability of both arr(1–376) and arr(1–380) is at least 5 times lower. Complete structural domains of proteins are often fairly stable, whereas parts of the domains, as well as proteins with partially deleted structural domains are not. The most logical interpretation of such a profound effect on stability upon removal or addition of just 2 amino acids appears to be that both mutations yield an incomplete structural element.

**Modified Model of Arrestin/Rhodopsin Interaction—**Mutagenesis studies yielded at least four arrestin mutants with minimally perturbed stability and dramatically reduced selectivity: arr-3A, arr(1–378), arr(1R75E), and arr(1–378)). The mutants fall into two distinct categories: 1) those exhibiting high binding to P-Rh*, dark P-Rh, and Rh* (arr-3A, arr(1–378)), and 2) those exhibiting high binding to only the light-activated forms of rhodopsin (arr(1R75E), and arr(1–378)). The model of sequential multisite binding predicts that there should be two types of constraining intramolecular interactions in arrestin that are disrupted upon arrestin binding to either phosphorylated or light-activated rhodopsin. Corresponding mutations yield proteins capable of phosphorylation-independent binding (mutants of the second group) or activation-independent binding (to P-Rh* and dark P-Rh only), respectively. A recent crystallographic study (8) identified 3 residues that interact with the phosphorylation-sensitive trigger Arg-175 (Asp-82 and Glu-148–Arg-171) on the putative rhodopsin-binding surface of the NH$_2$-terminal domain that may be involved in activation-recognition. However, in its simple form (5, 10) the model does not provide an adequate mechanistic explanation of the binding characteristics of the first group of mutants. In order to accommodate these data, we propose the following refinement of the model. The first tier of constraining interactions involves trigger residues that are elements of the primary binding sites (e.g. Arg-175). These constraints are relieved when primary sites interact with corresponding parts of P-Rh*. We hypothesize that these events disrupt the second tier of constraining interactions, and this disruption brings about arrestin transition into high affinity rhodopsin-binding state. Mutations that directly disrupt these putative interactions of the second tier should override both activation- and phosphorylation-dependence of arrestin binding, thereby yielding proteins with high binding to P-Rh*, dark P-Rh, and Rh* (e.g. the first group of mutants). Conceivably, several residues in 379–404 region (Glu-379, Phe-380, Arg-382, Glu-390, Glu-393, Glu-394), as well as VFV cluster (375–377) in 369–378 region participate in the constraining interactions of the second tier. These two groups of interactions do not appear to be redundant, and both are necessary for arrestin selectivity. Interestingly, even the most promiscuous mutants such as arr(1–378) and arr-3A do not bind well to dark Rh, suggesting that in order to maintain its rhodopsin-binding state arrestin must be properly oriented via a strong engagement of at least one primary binding site.

Visual arrestin has a strong potential for high affinity binding to unphosphorylated Rh*. It is possible that its evolution-
ary predecessor quenched signaling by unphosphorylated rhodopsin, as may be the case in the photoreceptors of invertebrates (15, 16), where arrestins tend to have shorter COOH termini (17–23). A two-step quenching mechanism (phosphorylation followed by arrestin binding) may be a later refinement. A splice variant of arrestin with a truncated COOH terminus is also expressed in vertebrate rods (p44) (24). This variant is localized primarily in rod outer segments (25), it binds avidly to both P-Rh* and Rh* (24), and quenches photoper- response (both in vitro (24) and in truncated rods (26)) even more effectively than does full-length arrestin. It was suggested that p44 may be relatively more important than arrestin in quenching (26). However, numerous in vivo experiments do not support this notion (27–29). Photoreceptors in transgenic mice expressing rhodopsin with a COOH-terminal truncation that eliminates rhodopsin kinase phosphorylation sites show grossly abnormal prolonged responses to stimulation by a single photon (27), whereas rhodopsin truncation per se does not prevent tight binding of truncated arrestins (5, 6). The deficit of functional rhodopsin kinase has been recently identified as the cause of several cases of Oguchi disease, a form of stationary night blindness (28) with symptoms strikingly similar to those of several cases of Oguchi disease, a form of stationary night blindness (28). Its actual role in photoreceptor cells remains to be elucidated.

The failure of x-ray crystallography to resolve the structure of COOH-terminal region of arrestin (8) suggests that this part of the molecule may exist in several alternative conformations, which is consistent with its involvement in an elaborate mechanism of selectivity control.

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