PDGF A or PDGF B to stimulate DNA synthesis in a variety of fibroblasts in culture (*20, 21*). We and others have observed that PDGF B is a more potent mitogen than PDGF A for NIH 3T3 cells (*21*). Yet, both PDGF receptors can effectively couple with mitogenic signaling pathways (*8*). Thus, the greater transforming potency of those PDGF chimeras capable of triggering β as well as α PDGF receptors likely reflects increased levels of receptor activation. The ability to further localize amino acid residues specifically required for β PDGF receptor binding or activation, or both, should aid in efforts to develop antagonists of β PDGF receptor function.

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Design and Synthesis of a Peptide Having Chymotrypsin-Like Esterase Activity

KARL W. HAHN, WIESLAW A. KLIS, JOHN M. STEWART*

A peptide having enzyme-like catalytic activity has been designed and synthesized. Computer modeling was used to design a bundle of four short parallel amphipathic helical peptides bearing the serine protease catalytic site residues serine, histidine, and aspartic acid at the amino end of the bundle in the same spatial arrangement as in chymotrypsin (ChTr). The necessary "oxyanion hole" and substrate binding pocket for acetyltyrosine ethyl ester, a classical ChTr substrate, were included in the design. The four chains were linked covalently at their carboxyl ends. The peptide has affinity for ChTr ester substrates similar to that of ChTr and hydrolyzes them at rates ~0.01 that of ChTr; total turnovers >100 have been observed. The peptide is inhibited by ChTr specific inhibitors and is inactive toward benzoyl arginine ethyl ester, a trypsin substrate. The peptide is inactivated by heating above 60°C, but recovers full catalytic activity upon cooling and lyophilization from acetic acid.

THE DESIGN AND SYNTHESIS OF MOLecules having catalytic activity and substrate specificity resembling that of natural enzymes has long been a goal of chemists (*1*). Some nonprotein organic molecules such as cyclodextrins and paracyclophanes show some enzyme-like characteristics. Recently monoclonal antibodies that catalyze specific reactions have been evoked by immunization with molecules that resemble sterically the transition state intermediate of the substrate being transformed (*2*). Although progress has been made in design and synthesis of peptides that assume a desired conformation in solution (*3–5*), the step of adding an enzyme-like constellation of catalytic amino acid residues to these synthetic molecules has not been previously accomplished. We report the design and synthesis of a 73-residue peptide "chymohelizyme-1" (CHZ-1) that shows characteristics resembling those of ChTr.

Department of Biochemistry, University of Colorado Medical School, Denver, CO 80262.

*To whom correspondence should be addressed.

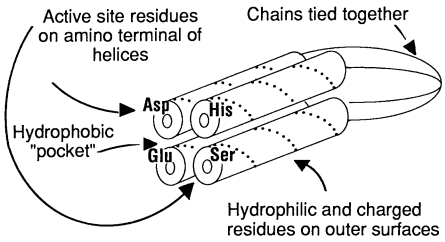

Fig. 1. Generic structure of a "chymohelizyme."

The mammalian serine proteases, of which ChTr is a much-studied example, possess a "catalytic triad" of amino acid residues so positioned as to confer hydrolase activity on the protein. In ChTr (which is synthesized as a single chain of 245 amino acid residues), these are His^{57}, Asp^{102}, and Ser^{195}. The His and Asp residues confer special reactivity on the hydroxyl group of Ser^{195}; the oxygen of this hydroxyl group attacks the electropositive carbonyl carbon of the substrate. A portion of the protein backbone chain (at Gly^{193} and Ser^{195}) is so positioned as to form strong hydrogen bonds to the tetrahedral oxyanion intermediate of the carbonyl group of the peptide or ester bond being broken; this binding site constitutes the so-called "oxyanion hole" of the enzyme (*6, 7*). The bacterial protease subtilisin possesses the same three catalytic amino acid residues positioned in space in a totally analogous way, but the 274-residue chain of this protein shows no homology to that of the mammalian serine proteases, suggesting that very different structures can effectively hold the active site residues in position for catalysis.

Substrate specificity of enzymes is determined by the arrangement of groups in the enzyme that bind structural features characteristic of the substrate molecule. Protein chains are hydrolyzed by ChTr at the carboxyl group of the aromatic amino acids Phe, Tyr, and Trp. Aromatic side chains of these amino acids are bound in a hydrophobic "pocket" adjacent to the catalytic triad; this pocket is composed of amino acid hydrophobic side chains and is approximately perpendicular to the plane of the catalytic triad residues. Esters and amides of aromatic amino acids having blocked amino groups are hydrolyzed by ChTr. Acetyltyrosine ethyl ester (ATEE), benzyloxycarbonyltyrosine *p*-nitrophenyl ester (ZTONP), and benzoyltyrosine ethyl ester (BTEE) are convenient ChTr substrates.

Given the recent great increase in understanding of the features in peptides that

promote formation of α helices (*8, 9*) and recent improvements in solid-phase peptide synthesis (*10*), it seemed reasonable to attempt the design and synthesis of a bundle of short amphipathic α-helical peptides that would hold a serine protease catalytic triad of amino acid residues in the necessary conformation to provide catalysis and also provide a hydrophobic pocket to bind the aromatic side chain of ChTr substrate esters. The essential features of this design are shown in Fig. 1. The three residues of the catalytic triad are situated at the amino terminus of separate peptide chains; the fourth chain, terminating in a Glu residue, stabilizes the entire structure and provides backbone hydrogen bonds to constitute the "oxyanion hole." Carboxyl ends of the four helices are linked covalently through the side chain amino groups of Orn and Lys residues. We promoted parallel association of the helices by linking the four chains covalently in this way, designing amphipathic helices having distinct hydrophilic and hydrophobic sides (which should adhere by hydrophobic interaction), and manipulating the helix-induced dipole by use of appropriate amino acids in the sequence (*8, 9*), even though helical regions in native proteins and in designed long synthetic peptides tend to associate in antiparallel bundles (*3*).

Computer molecular graphics (Tripos MENDYL software on a Silicon Graphics IRIS computer) was used to display the three-dimensional (3-D) structure of ChTr from the Brookhaven Protein Data Bank (*11*). The protein was discarded, leaving the catalytic triad in proper 3-D space. A supporting framework of amphipathic helical peptides was designed to hold these residues in the correct position. The ChTr substrate ATEE was used throughout the design to assure that proper spatial relations for substrate binding were incorporated. "Leucine zipper" concepts (*12*) were used to promote association of the large hydrophobic amino acid side chains near the carboxyl ends of the peptides; amino acids having smaller side chains were used near the amino end to provide the substrate binding pocket. External Lys and Glu residues were positioned so that their side chains could provide ionic bonds between turns of the helices (*9*) as well as between helices.

Energy minimization algorithms were used repeatedly for fine-tuning the design (*13*). The Orn and Lys residues at the carboxyl terminus provided side chain functional groups at the correct distances to form covalent bonds to hold the chains in the desired conformation. Side and end stereo views of the design are shown in Fig. 2, and the amino acid sequence is given in Fig. 3. For convenience in identification, amino acid residues of the catalytic triad are assigned sequence numbers in CHZ-1 identical to those of the same active site residues in ChTr, although CHZ-1 has no overall sequence homology with ChTr. The fourth chain, which begins with acetyl glutamic acid (Ac-Glu, no. 1) and provides the oxyanion hole, is numbered arbitrarily. The four chains contain 17, 19, 22, and 15 residues, respectively. Helix formation is promoted by acetylation of the amino termini of the chains and amidation of the single carboxyl terminus. Except for the catalytic elements, the structure of CHZ-1 bears no relation whatever to the protein sequence or overall conformation of ChTr. CHZ-1 is designed to be nearly fully helical, whereas ChTr contains ~5% helix in its structure (*11*). With our design significant enzyme activity can be generated only if the entire peptide assumes the designed 3-D structure.

The entire 73-residue peptide was assem-

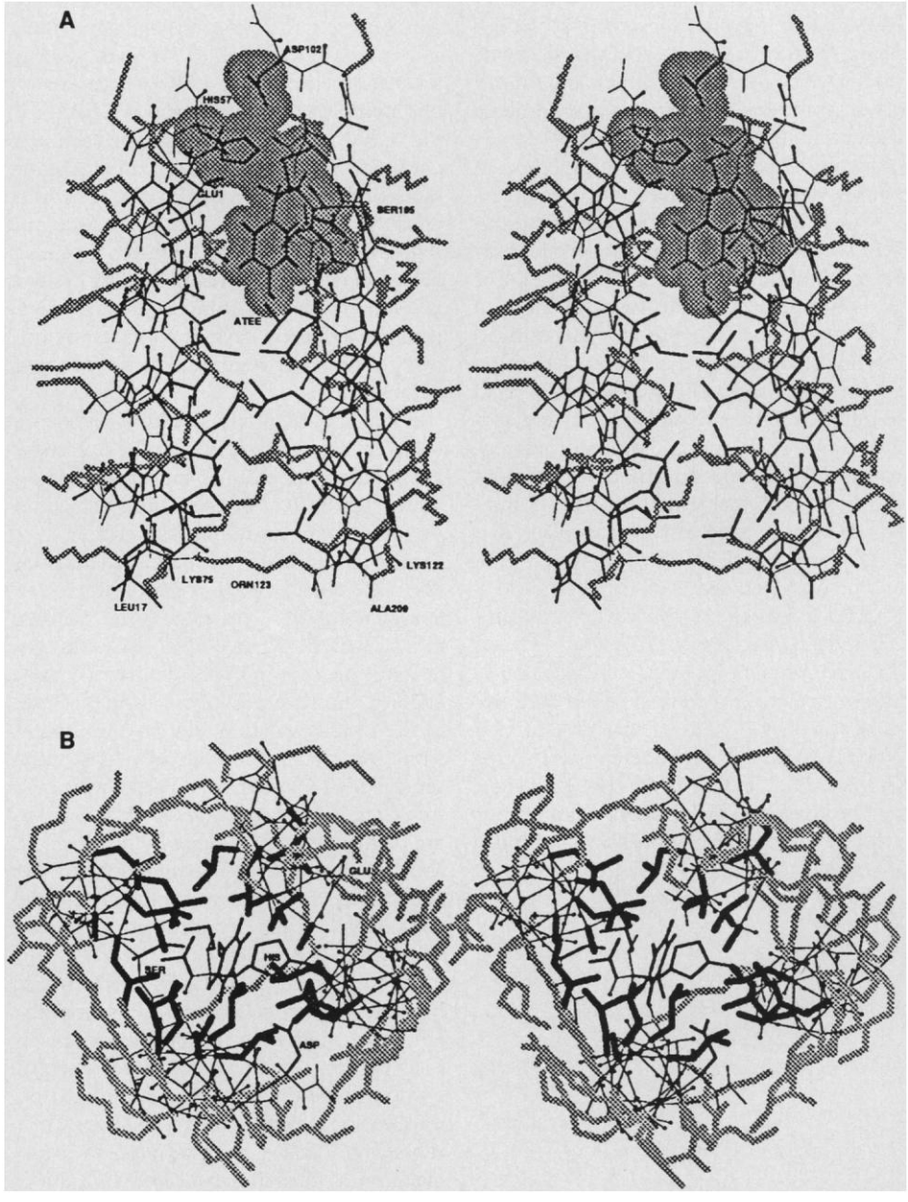

Fig. 2. Relaxed stereo drawings of CHZ-1 structure. The substrate acetyltyrosine ethyl ester is docked in the active site. Internal amino acid hydrophobic side chains are dark and heavy; external polar side chains are broad, light, and stippled. Helix backbone chains are in narrow lines. Hydrogen atoms are indicated on α-carbons, backbone nitrogens, and the substrate; backbone hydrogens are indicated as small knobs to assist in following the peptide chains. (**A**) Side view. The amino-terminal active site is at the top; the carboxyl-terminal linking structure is at the bottom. The Glu^{1} and Ser^{195} chains are in the foreground. Van der Waals shells were added to the substrate and to the catalytic triad side chains. (**B**) End view. The amino-terminal catalytic site is in the foreground. The aromatic ring of the substrate is just to the left of the imidazole of His^{57}.

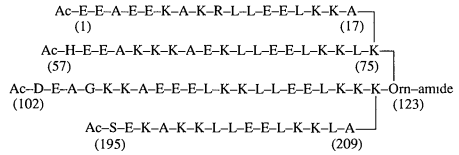

Fig. 3. Amino acid sequence of CHZ-1. Branches are formed by acylation of the Orn and Lys side-chain amino groups by the carboxyl-terminal residues of the subsidiary peptide chains. Abbreviations for the amino acid residues are: A, Ala; D, Asp; E, Glu; G, Gly; H, His; K, Lys; L, Leu; R, Arg; and S, Ser.

bled in one operation by stepwise solid-phase synthesis on methylbenzhydrylamine (MBHA) polystyrene resin in a Beckman 990B synthesizer (*14*). Standard solid-phase chemistry (*15*) modified by use of the recently developed chaotropic salt method to improve coupling reactions (*10*) was used.

Following HF cleavage, small by-products were removed from the product by repeated ultrafiltration of a solution in 4 M guanidine hydrochloride over an Amicon YM5 membrane. The product was purified by chromatography on Sephadex LH-60 in 60% ethanol and G-50 in 25% acetic acid solution. It gave a single peak on reversed-phase high-performance liquid chromatography (HPLC) and an amino acid analysis consistent with the desired structure. Circular dichroism spectroscopy showed that CHZ-1 has ~65% helix in pure water, 75% in 0.05 M NaCl, and 85% in 95% ethanol.

CHZ-1 hydrolyzes the ChTr substrates ZTONP, BTEE, and ATEE. Hydrolysis of ZTONP is facile, since this is an activated *p*-nitrophenyl ester; the initial rate of hydrolysis observed is ~2.5% of that of ChTr for ZTONP, corrected for spontaneous hydrolysis. Specific ZTONP hydrolysis is roughly proportional to CHZ-1 concentration, but is complicated by strong product inhibition.

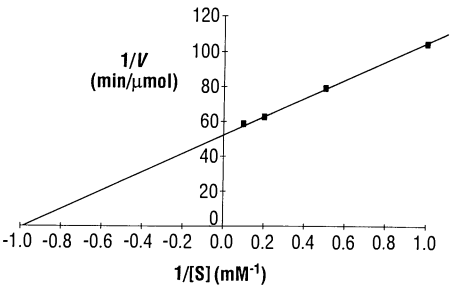

Fig. 4. Lineweaver-Burk plot of hydrolysis of ATEE by CHZ-1 at 24°C, pH 8.2, in 0.05 M NaCl solution: Enzyme concentration [E] = 0.729 μM; maximum velocity V_{max} = 18.9 ± 0.17 nmol/min; Michaelis constant K_m = 1.01 ± 0.04 mM, k_{cat} = 0.042 ± 0.0045 s^{-1}; k_{cat}/K_m = 0.041 mM^{-1} s^{-1}. Corresponding values for ChTr hydrolysis of ATEE are 0.7 mM, 190 s^{-1}, and 270 mM^{-1} s^{-1}.

Evidently the *p*-nitrophenol or the benzyloxycarbonyl group can bind in the substrate binding pocket. Hydrolysis of all three substrates is pH-dependent, and shows a maximum rate near pH 8.5, similar to ChTr. BTEE and ATEE are stable at pH 8.5; spontaneous hydrolysis is not a complicating factor with these substrates. Hydrolysis of ATEE shows saturation kinetics appropriate for an enzyme-catalyzed reaction. A Lineweaver-Burk plot of ATEE hydrolysis is given in Fig. 4. The affinity of CHZ-1 for ATEE, as shown by the Michaelis constant K_m, is very near that of chymotrypsin, whereas the rate of catalysis k_{cat} is ~0.03% of that of ChTr for ATEE (*17*); this represents an acceleration of ~10^5 over spontaneous hydrolysis. Hydrolysis was continued for >100 turnovers; the product formed coeluted from HPLC with an authentic sample of acetyltyrosine. The rate of hydrolysis increases ~30% on going from water to 0.05 M NaCl, consistent with the increased helix content in NaCl solution. The catalyst is stable in aqueous solution at room temperature for many days; it contains no aromatic amino acid residues that would cause autohydrolysis.

Initial studies of temperature dependence of hydrolysis of BTEE by CHZ-1 show approximate doubling of the rate of hydrolysis for each 10°C temperature rise, with a maximum rate at 60°C; this temperature dependence indicates an energy of activation of ~12 kcal/mol, which is reasonable for an enzyme-catalyzed reaction. Heating of CHZ-1 solution above 60°C causes loss of activity; the solution remains inactive upon cooling. However, following lyophilization of the CHZ-1 solution and relyophilization from acetic acid, the thermally inactivated sample of CHZ-1 regains full catalytic activity. Thus, in contrast to most enzymes that are permanently inactivated by heating, the short chains of CHZ-1 can refold in the appropriate environment to regenerate the secondary and tertiary structure necessary for enzyme-like activity.

As anticipated, CHZ-1 does not hydrolyze the trypsin substrate benzoylarginine ethyl ester, since the substrate binding pocket does not contain a negatively charged group to bind the basic arginine side chain; the catalytic activity is thus specific for ChTr substrates. CHZ-1 is inactivated by treatment with phenylmethylsulfonyl fluoride, an irreversible inhibitor of serine proteases especially active against ChTr. It is inhibited reversibly by indole and *p*-cresol, which are reversible inhibitors of ChTr. Hydrolysis of BTEE by CHZ-1 is inhibited ~60% by indole at a concentration equal to that of BTEE. CHZ-1 shows some spontaneous dimerization; dimers have decreased, but significant, catalytic activity.

These results indicate that the present state of knowledge of protein and peptide folding, of computer molecular graphics, and of peptide synthesis is adequate to allow design and synthesis of peptides having catalytic activity resembling that of native enzymes. Because peptides like CHZ-1 can be synthesized readily by solid-phase peptide synthesis, modifications of the structure to generate enzymes having other substrate specificities can be readily accomplished; we have designed peptides having anticipated activities like trypsin and acetylcholine esterase. Modifications to CHZ-1 can probably be made to increase the stability of the catalytic site, to increase overall helix content, and to provide for specific covalent attachment to insoluble supports. Although design of enzymes that require extended substrate-binding sites is likely to be more difficult, design and synthesis of a variety of enzymes for processing of small substrates is clearly within the scope of present technology (*18*).

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13. The standard TRIPOS force field (parameter set TRIPOS 5.05) was used in the default configuration with the PROTEIN dictionary, conjugate gradient minimization, and no electrostatics. Simplex techniques were applied as needed and aggregates were used to concentrate on various portions of the molecule. Bad contacts were analyzed with the BUMP-CHECK option in the SEARCH module at standard van der Waals radii.
14. Four different categories of blocking groups were required. Stable side chain blocking was provided by standard benzyl-related groups; these were removed simultaneously with cleavage of the peptide from the resin by HF. Boc groups were used for most α-amino temporary protection, and Fmoc (*15*) and Npys (*16*) groups were used for selective protection of Lys and Orn functions. Npys groups were also used for synthesis of the entire Asp chain. Npys-Lys(Boc) was coupled to Orn(Fmoc)-MBHA resin. The Boc group was deprotected and Boc-Ala coupled. The Npys group was removed and the Ac-Asp chain assembled (Npys chemistry). The Boc group was removed and the Ac-Ser chain was assembled (Boc chemistry). The Fmoc group was removed and Boc-Lys(Npys) was coupled to the Orn residue. The

Npys group was removed, and Npys-Ala was coupled. The Boc group was removed and the Ac-His chain was assembled (Boc chemistry). The Npys group was removed and the Ac-Glu chain was assembled (Boc chemistry). Every coupling reaction was monitored for completeness and repeated if necessary. Ac, acetyl; Boc, *tert*-butyloxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; Npys, 3-nitro-2-pyridylsulfenyl.

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β-Arrestin: A Protein That Regulates β-Adrenergic Receptor Function

MARTIN J. LOHSE, JEFFREY L. BENOVIC,* JUAN CODINA, MARC G. CARON, ROBERT J. LEFKOWITZ

Homologous or agonist-specific desensitization of β-adrenergic receptors is thought to be mediated by a specific kinase, the β-adrenergic receptor kinase (βARK). However, recent data suggest that a cofactor is required for this kinase to inhibit receptor function. The complementary DNA for such a cofactor was cloned and found to encode a 418–amino acid protein homologous to the retinal protein arrestin. The protein, termed β-arrestin, was expressed and partially purified. It inhibited the signaling function of βARK-phosphorylated β-adrenergic receptors by more than 75 percent, but not that of rhodopsin. It is proposed that β-arrestin in concert with βARK effects homologous desensitization of β-adrenergic receptors.

HOMOLOGOUS DESENSITIZATION is a widespread process that causes specific dampening of cellular responses to stimuli such as hormones, neurotransmitters, or sensory signals (*1*). It is defined by a loss of responsiveness of receptors that have been continuously or repeatedly stimulated, while responses of other receptors remain intact. The β-adrenergic receptor–G_s–adenylyl cyclase system has been studied as a model system for this phenomenon (*1*). Homologous desensitization of β receptors has been shown to be associated with phosphorylation of the receptors, which appears to be catalyzed by a specific kinase, called β-adrenergic receptor kinase (βARK) (*2*). This kinase phosphorylates only agonist-occupied receptors, which may explain the receptor specificity of homologous desensitization. βARK-mediated phosphorylation has been shown for several G protein–coupled receptors. Several lines of evidence indicate that βARK participates in homologous desensitization of β-adrenergic receptors (*3*). The recent cloning of the cDNA encoding βARK suggests that there are several isoforms of this enzyme whose specificities have not yet been determined (*4*).

The phosphorylation of purified reconstituted β_2 receptors by crude βARK preparations causes significant impairment of their capacity to activate G_s (*5*), the guanine nucleotide binding protein that serves as a signal transducer for β receptors. However, this inhibitory effect is almost completely lost when βARK is purified, although the pure preparations retain the phosphorylating activity (*5*). These data suggest that a cofactor (or more) is necessary to effect βARK-mediated inhibition of receptor function, and that this cofactor is lost during the purification of βARK.

The existence of such cofactors is further suggested by analogies with the light-activated rhodopsin-phosphodiesterase system in the retina. Phosphorylation of light-activated rhodopsin by rhodopsin kinase requires the binding of an additional protein, termed arrestin, to inhibit rhodopsin function (*6*). This analogy is supported by the observation that arrestin enhances the inhibitory effects of βARK-mediated phosphorylation on β-receptor function, as assessed in a reconstituted system with the pure components (*5*). However, large amounts of arrestin are required for a relatively modest inhibition, indicating that arrestin itself is not the protein that serves to inhibit β-receptor function. These observations suggested that there might be one (or more) arrestin-like protein that inactivates βARK-phosphorylated β receptors, and possibly other G protein–coupled receptors.

The search for arrestin-like cDNA's was performed with a 1262-bp probe comprising the entire coding sequence of arrestin (*7*). Screening of a bovine brain library revealed positive clones at a frequency of 1 in 10,000 at relatively high stringency (0.5× SSC at 65°C), eight of which were isolated and sequenced. All contained the full-length or part of the same open reading frame (ORF) of

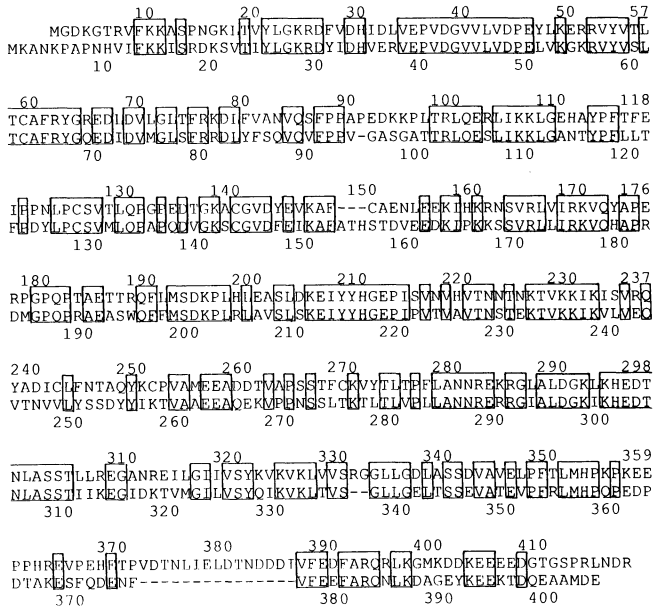

Fig. 1. Deduced amino acid sequence of β-arrestin (top sequence) and arrestin (bottom sequence). Gaps were introduced to obtain maximum similarity. Boxes surround identical residues. The sequence of arrestin was taken from Shinohara *et al.* (*7*). A randomly primed, size-selected (2 to 4.4 kb) bovine brain library in λZAP with 300,000 independent clones (*20*) was screened with a 1262-bp Hpa I–Nhe I fragment of bovine arrestin cDNA (*7*, *20*) containing the entire coding sequence of arrestin. Eight positive clones were isolated, sequenced in both directions (dideoxynucleotide method), and found to contain all or parts of the same 1254-bp open reading frame. The 3′ ends of the clones varied from 0.6 to 2.5 kb, but none contained a poly(A) tail. The complete nucleotide sequence is deposited in GenBank (accession number M33601) or is available from the authors.

M. J. Lohse, J. L. Benovic, M. G. Caron, R. J. Lefkowitz, Howard Hughes Medical Institute, Departments of Medicine, Biochemistry and Cell Biology, Durham, NC 27710.
J. Codina, Department of Cell Biology, Baylor College, Houston, TX.

*Present address: Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA 19140.