class at each **a** or **d** position were sequenced.

14. Substitutions were limited to Phe, Leu, Ile, Met, and Val by including an equal mixture of all four bases at the first codon position, T at the second position, and an equal mixture of G and C at the third position.
15. The number of standard deviations (Z) between the observed frequency and the expected frequency for a given amino acid at a specific position in a phenotypic class of m members is given as

$$Z = \frac{(f_a - f_b)}{\sigma}$$

where f_a is the observed frequency of the amino acid at the specified position in the given phenotypic class, f_b is the observed frequency in all sequences, and σ, the standard deviation, is calculated as

$$\sigma = \sqrt{mf_b(1 - f_b)}$$

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17. In longer coiled coils, such as those found in myosin and tropomyosin, leucine is found in the **d** position only 35% of the time (*12*).
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Identification of a Specialized Adenylyl Cyclase That May Mediate Odorant Detection

HEATHER A. BAKALYAR AND RANDALL R. REED*

The mammalian olfactory system may transduce odorant information via a G protein–mediated adenosine 3′,5′-monophosphate (cAMP) cascade. A newly discovered adenylyl cyclase, termed type III, has been cloned, and its expression was localized to olfactory neurons. The type III protein resides in the sensory neuronal cilia, which project into the nasal lumen and are accessible to airborne odorants. The enzymatic activity of the type III adenylyl cyclase appears to differ from nonsensory cyclases. The large difference seen between basal and stimulated activity for the type III enzyme could allow considerable modulation of the intracellular cAMP concentration. This property may represent one mechanism of achieving sensitivity in odorant perception.

THE VERTEBRATE OLFACTORY SYSTEM detects chemical stimuli in the environment with remarkable specificity and sensitivity. Differences in molecular structure as subtle as stereochemical configuration are discernible, and the threshold of sensitivity for some airborne odorants is in the range of parts per trillion (*1*). At least some odorants stimulate a guanosine triphosphate (GTP)–dependent increase in cAMP in the olfactory cilia, which are specialized structures that project from the apical dendrites of the olfactory sensory neurons (*2*). Adenylyl cyclase activity, which is high in olfactory tissue, is enriched in these sensory neurons (*2, 3*). The neuronal cilia also have nonspecific cation channels, which open in response to increasing cyclic nucleotide concentrations (*4*). These results, taken together, suggest that olfactory signal transduction involves an odorant-stimulated second messenger cascade that leads to sensory neuron depolarization and initiation of an action potential.

Several components of the odorant transduction pathway have evolved olfactory-specific variants. For example, the α subunit of G_{olf}, which resembles the α subunit of the stimulatory G protein, G_s, is found exclusively in olfactory neuronal cilia (*5*). A cyclic nucleotide–activated cation channel has been identified, and its mRNA has been shown to be confined to olfactory neurons (*6*). Similarly, it is possible that an olfactory-specific adenylyl cyclase exists that contributes to the high enzyme activity seen in olfactory cilia (*3, 7*). With a monoclonal antibody that recognizes the Ca^{2+}/calmodulin–sensitive cyclase in brain, an adenylyl cyclase species has been detected in olfactory cilia that is distinct in molecular size from the brain form of the enzyme (*7*).

The molecular cloning of the brain-specific type I enzyme (*8*) has allowed us to isolate cDNA clones encoding an adenylyl cyclase that may play an effector role in olfaction. A rat olfactory cDNA library was probed with an oligonucleotide based on the sequence of a tryptic fragment of purified type I adenylyl cyclase from bovine brain (*9*). By this method, we isolated a single class of cDNA clones that encoded an adenylyl cyclase (type II) (*6*) distinct from the type I enzyme. The mRNAs that encode the type I and type II enzymes were expressed in high concentrations in brain but were undetectable or present in low amounts in olfactory tissue. The olfactory cDNA library was therefore screened again at low stringency (*10*) with the coding region from the cDNAs for both the type I and type II adenylyl cyclases. A distinct class of clones was identified that weakly hybridized to both of the previously identified forms. Approximately one in every 1000 recombinant cDNA clones from the rat olfactory cDNA library represented this type III adenylyl cyclase.

We obtained the nucleotide and deduced amino acid sequences of the cDNA that encoded type III adenylyl cyclase (Fig. 1) (*11*). A potential initiation codon at position −173 is followed by stop codons in three reading frames. The methionine codon at nucleotide +1 is contained within a canonical eukaryotic translation initiation sequence (*12*) and is followed by an open reading frame that encodes 1144 amino acids.

Type III adenylyl cyclase appears to be topographically similar to the 1134–amino acid type I enzyme (*8*). A comparison of the hydropathy profiles for both proteins (Fig. 2A) revealed that each protein has two extremely hydrophobic regions: one near the NH_2-terminus and the second between amino acid residues 600 and 850. The hydrophobic regions of the type III protein each contain six potential membrane-spanning segments in a pattern analogous to that predicted for the bovine brain type I cyclase, suggesting a similar orientation in the membrane. The type I enzyme, which is glycosylated, contains a consensus sequence for N-linked carbohydrate addition between membrane-spanning regions 9 and 10 on the putative extracellular face of the molecule (*8*). The type III protein also has a potential N-glycosylation site between transmembrane regions 9 and 10, and biochemical evidence confirmed that this protein is a substrate for N-linked glycosylation (Fig. 3B). Treatment of olfactory cilia with peptide:N-glycosidase F (PNGaseF) altered the mobility of the protein, from that corre-

Department of Molecular Biology and Genetics, The Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

*To whom correspondence should be addressed.

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   1 MTEDQGFSDP EYSAEYSAEY SVSLPSDPDR GVGRTHEISV  RNSGSCLCLP RFMRLTFVPE SLENLYQTYF KRQRHETLLV   80
  81 LVVFAALFDC YVVVMCAVVF SSDKLAPLMV AGVGLVLDII  LFVLCKKGLL PDRVSRKVVP YLLWLLITAQ IFSYLGLNFS  160
 161 RAHAASDTVG WQAFFVFSFF ITLPLSLSPI VIISVVSCVV  HTLVLGVTVA QQQQDELEGM QLLREILANV FLYLCAIIVG  240
 241 IMSYYMADRK HRKAFLEARQ SLEVKMNLEE QSQQQENLML  SILPKHVADE MLKDMKKDES QKDQQQFNTM YMYRHENVSI  320
 321 LFADIVGFTQ LSSACSAQEL VKLLNELFAR FDKLAAKYHQ  LRIKILGDCY YCICGLPDYR EDHAVCSILM GLAMVEAISY  400
 401 VREKTKTGVD MRVGVHTGTV LGGVLGQKRW QYDVWSTDVT  VANKMEAGGI PGRVHISQST MDCLKGEFDV EPGDGGSRCD  480
 481 YLDEKGIETY LIIASKPEVK KTAQNGLNGS ALPNGAPASK  PSSPALIETK EPNGSAHASG STSEEAEEQE AQADNPSFPN  560
 561 PRRRLRLQDL ADRVVDASED EHELNQLLNE ALLERESAQV  VKKRNTFLLT MRFMDPEMET RYSVEKEKQS GAAFSCSCVV  640
 641 LFCTAMVEIL IDPWLMTNYV TFVVGEVLLL ILTICSMAAI  FPRAFPKKLV AFSSWIDRTR WARNTWAMLA IFILVMANVV  720
 721 DMLSCLQYYM GPYNVTTGIE LDGGCMENPK YYNYVAVLSL  IATIMLVQVS HMVKLTLMLL VTGAVTAINL YAWCPVFDEY  800
 801 DHKRFQEKDS PMVALEKMQV LSTPGLNGTD SRLPLVPSKY  SMTVMMFVMM LSFYYFSRHV EKLARTLFLW KIEVHDQKER  880
 881 VYEMRRWNEA LVTNMLPEHV ARHFLGSKKR DEELYSQSYD  EIGVMFASLP NFADFYTEES INNGGIECLR FLNEIISDFD  960
 961 SLLDNPKFRV ITKIKTIGST YMAASGVTPD VNTNGFTSSS  KEEKSDKERW QHLADLADFA LAMKDTLTNI NNQSFNNFML 1040
1041 RIGMNKGGVL AGVIGARKPH YDIWGNTVNV ASRMESTGVM  GNIQVVEETQ VILREYGFRF VRRGPIFVKG KGELLTFFLK 1120
1121 GRDRPAAFPN GSSVTLPHQV VDNP                                                            1144
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Fig. 1. The amino acid sequence of type III adenylyl cyclase (128,852 daltons) predicted from clone pROCS6+10, which includes 366 nucleotides 5′ to the presumed initiating ATG codon and 742 nucleotides 3′ to the TGA stop codon at position 3432. The GenBank accession number for the nucleotide sequence is M55075. A potential site for N-glycosylation (*13*) between predicted transmembrane spans 9 and 10 is at amino acid residue 734. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

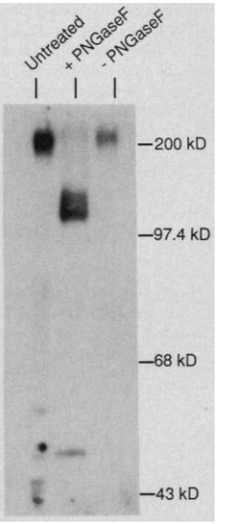


Fig. 3. Determination of apparent molecular size of native type III adenylyl cyclase. Samples (30 μg) of olfactory ciliary protein were solubilized in 1% SDS and 1% B-mercaptoethanol, and boiled for 2 min. Samples were incubated with and without 1.5 μl of PNGaseF (*23*) for 18 hours at 37°C in 5% NP40, 45 mM glycine (pH 9.3), 9 mM EDTA, 0.6% SDS, 0.6% β-mercaptoethanol, 10 μM phenylmethylsulfonyl fluoride (PMSF), and 100 μM leupeptin in a 30-μl volume. SDS was then added to 6%. After addition of gel-loading buffer (*24*), a portion of each sample (15 μg of protein) was subjected to SDS–polyacrylamide gel electrophoresis (8% acrylamide gel). The separated proteins were subsequently transferred to Immobilon (Millipore). The untreated lane contained 15 μg of olfactory ciliary protein (*25*). Type III protein was detected with purified anti–HAB-1 (*16*, *26*) diluted 1:100 and visualized with ^{125}I-labeled protein A. The positions of molecular mass standards are indicated.

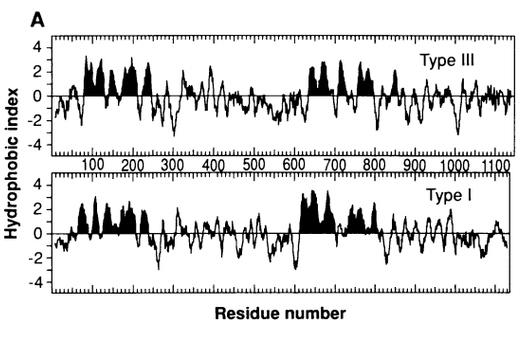
sponding to an apparent molecular size of 200 kd (*7*) to a mobility consistent with the calculated molecular size of 129 kd. Two additional potential sites for glycosylation are present on the predicted extracellular face of the type III protein (*13*).

A dot matrix alignment of the type III and type I enzymes revealed that the region of greatest similarity between the two proteins is within the putative intracellular domains (Fig. 2B). These regions show great similarity to a region of the atrial naturetic factor receptor responsible for guanosine 3′-5′-monophosphate (cGMP) formation (*8*). These domains in the mammalian adenylyl cyclases also share modest similarity with the catalytic region of yeast adenylyl cyclase, a protein whose functional domains have been characterized by genetic and biochemical analyses (*8*, *14*). The regions with membrane-spanning potential are the least well conserved between the two mammalian adenylyl cyclases, perhaps suggesting a structural rather than a sequence-specific function for these hydrophobic domains.

The tissue distribution of the type III adenylyl cyclase mRNA was examined by Northern analysis (Fig. 4A). RNA isolated from seven tissues, including two epithelial tissues (lung and intestine), was hybridized to the coding region from the type III cDNA; the results indicated that expression of the type III mRNA was limited to olfactory epithelium. To assess the distribution of type III mRNA among the cell types within the olfactory epithelium, we used a neuronal depletion technique. The sensory neurons of the olfactory epithelium degenerate 6 to 8 days after the removal of their target tissue, the olfactory bulb (*15*). Type III adenylyl cyclase mRNA disappeared concomitant with the loss of sensory neurons after bulbectomy (Fig. 4B).

The localization of the type III protein within olfactory sensory neurons was examined by immunohistochemical analysis (Fig. 5). Only cilia, the sensory apparatus of the olfactory neurons, were stained with an antibody to a peptide sequence specific to the type III adenylyl cyclase (*16*). At the nasal septum of a unilaterally bulbectomized rat, where normal and neuron-depleted epithelium can be seen in the same field, no staining was detected on the denervated side. Electron microscopic examination of normal rat olfactory epilthelia demonstrated that immunoreactivity was confined to ciliary structures (*17*). This localization is nearly identical with that seen for G_{olf} and suggests that these two components may interact at this site to mediate olfaction.

The biochemical properties of type III adenylyl cyclase were examined in a heterologous system. The cDNA encoding type III adenylyl cyclase was expressed under the control of the cytomegalovirus promoter (*18*) in a human kidney cell line (293 cells) that has low endogenous cyclase activity. Cell homogenates from stably transformed clonal lines were assayed for adenylyl cyclase activity. Production of cAMP was measured under basal conditions; in the presence of forskolin, a direct activator of adenylyl cyclase; and in the presence of AlF_4^-, which indirectly modulates adenylyl cyclase



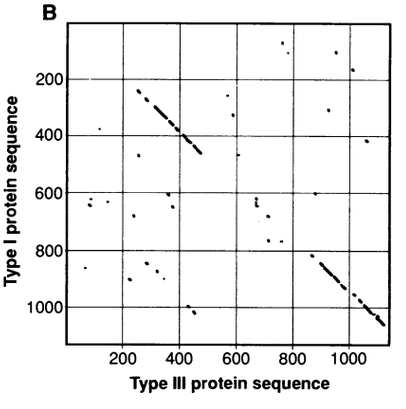


Fig. 2. (**A**) Hydropathy profiles of type III olfactory adenylyl cyclase and type I brain adenylyl cyclase. Shaded areas indicate putative membrane-spanning regions. The Strider 1.1 DNA analysis software was used to calculate the hydrophobic indices by the method of Kyte and Doolittle (*22*), averaging over a window size of nine amino acid residues. (**B**) Dot matrix comparison of type III adenylyl cyclase with type I adenylyl cyclase. Regions of high amino acid similarity between the two enzymes were located by the MacVector software (IBI) and the pam250 scoring matrix. Window size for the comparison was eight amino acids, and the stringency of match identities was set at 60%.

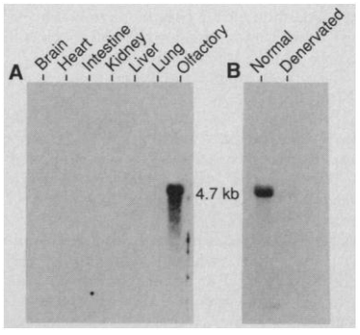


Fig. 4. Northern analysis of the tissue distribution type III adenylyl cyclase mRNA. (**A**) Total RNA (12.5 μg) isolated from seven rat tissues (*27, 28*). (**B**) Total RNA (10 μg) derived from normal rat olfactory epithelium or neuron-depleted epithelium (*5*). Previous hybridization of both blots with ^{32}P-labeled tubulin cDNA suggested each lane contained similar amounts of total RNA. Hybridization of the filter in (B) with ^{32}P-labeled cDNA for olfactory marker protein, a mature olfactory neuron marker, confirmed the loss of olfactory sensory neurons in the neuron-depleted olfactory epithelium. The autoradiograms shown were exposed for 14 hours at −80°C.

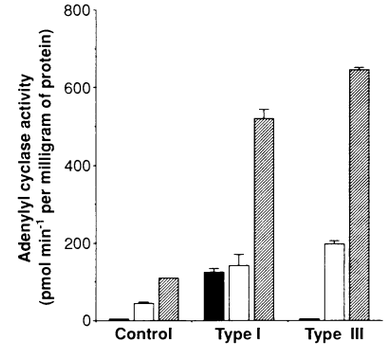


Fig. 6. Adenylyl cyclase activity of clonal 293 cell lines (*29, 30*) carrying parental vector pCIS (*18*), type III adenylyl cyclase cDNA in pCIS, or type I adenylyl cyclase cDNA in pCMV5 (*8*). Cell homogenates were prepared in 50 mM tris-HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 1 μM PMSF, leupeptin (50 U/ml), and egg-white trypsin inhibitor (50 U/ml). Adenylyl cyclase assays (100 μl) were performed in duplicate and contained 5 mM $MgCl_2$, 20 mM creatine phosphate, 1 mM cAMP, creatine phosphokinase (100 U/ml), 15 mM tris-HCl (pH 8.0), 1 mM dithiothreitol, deoxyribonuclease I (15 μg/ml), 1 mM adenosine triphosphate (ATP), and 3 μCi of [α-^{32}P]ATP. Homogenate protein, determined with an assay kit (Bio-Rad), was between 100 to 200 μg per reaction. The following substances were included as indicated: 100 μM forskolin (striped bars), AlF_4^- (20 μM $AlCl_3$ + 10 mM NaF) (open bars), or no additions (solid bars). After 45 min at 31°C, the reactions were stopped with 200 μl of 2% SDS, and cAMP formation was assessed (*31*). Assays of transiently transfected 293 cells demonstrated similar results (type I basal activity equals 19.4 ± 1.3 pmol min^{-1} per milligram of protein, type I forskolin activity equals 252.8 ± 53.0 pmol min^{-1} per milligram of protein; type III basal activity equals 2.9 ± 1.0 pmol min^{-1} per milligram of protein; type III forskolin activity equals 317.9 ± 38.0 pmol min^{-1} per milligram of protein; pCIS basal activity equals 2.4 ± 0.2 pmol min^{-1} per milligram of protein; pCIS forskolin activity equals 169.6 ± 17.0 pmol min^{-1} per milligram of protein), although the forskolin-stimulated activities of type III and type I cDNA–transfected lines was only two- to threefold that of control cells, compared to the five- to sixfold enhancement observed on the stable lines.

through G protein activation. In the presence of forskolin (100 μM) (*19*), adenylyl cyclase activity of cell lines that expressed the type I and type III enzymes was five to six times that of control cells (Fig. 6). In addition, the increase in adenylyl cyclase activity in extracts of type III cDNA–transfected cells in response to AlF_4^- was greater than that seen in control cell homogenates. These results indicate that the endogenous G_s protein present in the 293 cell line can activate type III adenylyl cyclase. Previous experiments have shown that G_{olf}, which shares 88% amino acid identity with G_s, is able to interact with the adenylyl cyclase present in the S49 *cyc*$^-$ *mouse cell line (5, 20)*. Olfactory cilia preparations have high adenylyl cyclase activity, apparently resulting from activated G proteins present in the absence of exogenous odorant (*2*). The detailed characterization of the interaction between type III cyclase and G_{olf} will require the reconstitution of purified components.

The type III and type I cDNA–transfected cell lines showed similar responses to forskolin and AlF_4^-. We expected that the increased amounts of adenylyl cyclase present in these cell lines, demonstrated by forskolin activation, would also be reflected in an increased basal activity. Whereas an increased basal activity was observed in homogenates from cells expressing the type I enzyme (125.6 ± 8.8 pmol min^{-1} per milligram of protein), the basal adenylyl cyclase activity of the type III enzyme–expressing cell line (4.7 ± 0.1 pmol min^{-1} per milligram of protein) was similar to that seen for control cells (4.0 ± 0.3 pmol min^{-1} per milligram of protein) (*21*). Expression of the nonsensory type II adenylyl cyclase in this system also led to markedly increased basal activity (*6*).

The large difference seen between basal and stimulated activity for the type III enzyme could allow considerable modulation of the intracellular cAMP concentration. In the absence of stimulatory ligand, low amounts of cAMP could be maintained even in an environment where the adenylyl cyclase protein is highly concentrated. On activation of adenylyl cyclase, the combination of an abundance of enzyme and the large surface-to-volume ratio in cilia would allow a rapid increase in cAMP concentration. The dynamic modulation of cAMP concentration afforded by the enzymatic properties of the type III cyclase could contribute to the sensitivity in odorant detection that is characteristic of this sensory system.

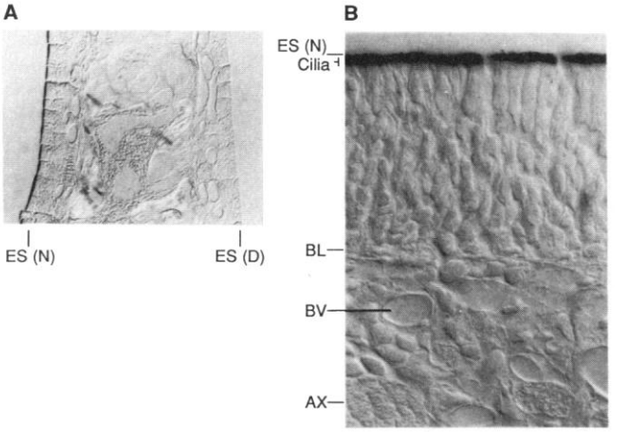


Fig. 5. Subcellular localization of type III adenylyl cyclase. Olfactory tissue from a unilaterally bulbectomized rat was embedded in paraffin, and immunocytochemistry was performed with the use of Vectastain ABC rabbit IgG (immunoglobulin G) kit (Vector Labs). Dewaxed sections were blocked with 2% normal goat serum in phosphate-buffered saline (PBS), and incubated at room temperature with affinity-purified antisera to HAB-1 (anti–HAB-1) (*16, 26*) diluted 1:100 with PBS. Secondary antibody was used at 1:200 dilution and visualized with diaminobenzidine and ABC–horseradish peroxidase. Prior incubation of the antisera with an excess of HAB-1 peptide eliminated staining. (**A**) Photograph taken at an optical magnification of 200× of anti–HAB-1 staining through the nasal septum of a unilaterally bulbectomized rat. (**B**) Photograph taken at an optical magnification of 1000× of normal olfactory epithelium. ES, epithelial surface; N, neuron-containing normal epithelium; D, neuron-depleted epithelium. The cilia in this preparation collapsed during the embedding process. BL, basal lamina; BV, blood vessel; AX, axon bundle.

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10. Low stringency wash conditions: twice for 20 min in 2× SSC (standard saline citrate) at 55°C.
11. The complete cDNA of the rat type III adenylyl cyclase was obtained by ligation of two clones. Clone pROSC10-4 (nucleotides −366 to +3183) and clone pROSC6-4 (nucleotides 465 to 4167) were fused at an overlapping Bst EII site at nucleotide 3038 to generate the complete pROSC6+10 construct.
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27. Samples were treated with glyoxal and separated on a 1% agarose gel as described (*28*). The fractionated material was transferred to nitrocellulose and prehybridized at 42°C in 50% formamide, 6× SSPE (saline, sodium phosphate, EDTA), 5× Denhardt's solution, 0.1% SDS, and single-stranded DNA (100 μg/ml). The Eco RI fragment representing the full-length type III cDNA was labeled with ^{32}P and hybridized to the immobilized RNA samples for 48 hours at 42°C. Filters were washed once for 15 min in 1× SSC and 0.1% SDS at 25°C, and then twice for 20 min in 1× SSC and 0.1% SDS at 65°C.
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Isotope-Edited NMR of Cyclosporin A Bound to Cyclophilin: Evidence for a *Trans* 9,10 Amide Bond

S. W. FESIK,* R. T. GAMPE, JR., T. F. HOLZMAN, D. A. EGAN, R. EDALJI, J. R. LULY, R. SIMMER, R. HELFRICH, V. KISHORE, D. H. RICH

The binding of a ^{13}C-labeled cyclosporin A (CsA) analog to cyclophilin (peptidyl prolyl isomerase) was examined by means of isotope-edited nuclear magnetic resonance (NMR) techniques. A *trans* 9,10 peptide bond was adopted when CsA was bound to cyclophilin, in contrast to the *cis* 9,10 peptide bond found in the crystalline and solution conformations of CsA. Furthermore, nuclear Overhauser effects (NOEs) were observed between the ζ_3 and ϵ_3 protons of the methylleucine (MeLeu) residue at position 9 of CsA and tryptophan^{121} (Trp^{121}) and phenylalanine (Phe) protons of cyclophilin, suggesting that the MeLeu^9 residue of CsA interacts with cyclophilin. These results illustrate the power of isotope-edited NMR techniques for rapidly providing useful information about the conformations and active site environment of inhibitors bound to their target enzymes.

CYCLOSPORIN A (CsA) IS A CYCLIC undecapeptide (Fig. 1) that is widely used as an immunosuppressive agent in organ transplantation (*1*). Although its precise mechanism of action is unknown, the immunosuppressive activity of CsA may be linked to its affinity for the protein cyclophilin (molecular mass ~17.8 kD), because the binding to cyclophilin appears to correlate with the relative immunosuppressive activities of CsA analogs (*2–4*). Recently, cyclophilin was shown (*5, 6*) to have the same amino acid sequence as peptidyl prolyl isomerase, an enzyme that catalyzes the *cis-trans* isomerization of Xaa-proline peptide bonds (where Xaa is any amino acid). Cyclosporin A was found to be a potent inhibitor of this enzyme, suggesting that some of the biological effects of CsA (*7*) may be mediated by blocking the enzymatic activity of cyclophilin (*8, 9*).

In order to aid in the design of CsA analogs with reduced toxicity and potentially greater clinical utility, structural information on the binding of CsA to cyclophilin is desired. To date, however, only the conformation of CsA by itself has been determined in the crystalline state by x-ray crystallography and in apolar solvents by NMR spectroscopy (*10*). In three different crystal forms of CsA involving different intermolecular contacts and in two different solvent systems (*10, 11*), CsA was found to adopt an antiparallel β-pleated sheet consisting of residues 11 → 7 with a type-II′ β-turn at residues 2 → 5. The remaining residues (8 to 10) form a loop defined by a *cis* peptide bond between MeLeu residues 9 and 10. The only differences in the crystal and solution conformations are in the orientation of the 1-MeBmt [(4*R*)-*N*-methyl-4-butenyl-4-

S. W. Fesik, R. T. Gampe, Jr., T. F. Holzman, D. A. Egan, R. Edalji, J. R. Luly, R. Simmer, R. Helfrich, Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, IL 60064.
V. Kishore and D. H. Rich, School of Pharmacy and Department of Chemistry, University of Wisconsin–Madison, Madison, WI 53706.

*To whom correspondence should be addressed.