Chemical Scale Studies of the Phe-Pro Conserved Motif in the Cys Loop of Cys Loop Receptors

Walrati Limapichat, Henry A. Lester, and Dennis A. Dougherty

From the Divisions of *Chemistry and Chemical Engineering and ‡Biology, California Institute of Technology, Pasadena, California 91125

The functions of two conserved residues, Phe¹³⁵ and Pro¹³⁶, located at the apex of the Cys loop of the nicotinic acetylcholine receptor are investigated. Both residues were substituted with natural and unnatural amino acids, focusing on the role of aromaticity at Phe¹³⁵, backbone conformation at Pro¹³⁶, side chain polarity and volume, and the specific interaction between the aromatic side chain and the proline. NMR spectroscopy studies of model peptides containing proline and unnatural proline analogues following a Phe show a consistent increase in the population of the cis conformer relative to peptides lacking the Phe. In the receptor, a strong interaction between the Phe and Pro residues is evident, as is a strong preference for aromaticity and hydrophobicity at the Phe site. A similar influence of hydrophobicity is observed at the proline site. In addition, across a simple homologous series of proline analogues, the results reveal a correlation between receptor function and cis bias at the proline backbone. This could suggest a significant role for the cis proline conformer at this site in receptor function.

The Cys loop superfamily of neurotransmitter-gated ion channels includes the nicotinic acetylcholine receptor (nAChR), the 5-HT₃ serotonin receptor, the GABAₐ and GABAₜ receptors, and the glycine receptor (1, 2). Together, these receptors mediate both excitatory and inhibitory fast synaptic transmission throughout the central and peripheral nervous systems. They are established targets for potential treatments of Alzheimer disease, Parkinson disease, schizophrenia, attention deficit hyperactivity disorder (ADHD), addiction, and other neurological disorders.

The eponymous Cys loop, a disulfide-linked sequence Cys-Xaa₁₋₃-Cys, is located at the interface between the extracellular and transmembrane domains of the receptor, and many studies have established that the Cys loop is essential for receptor function. Not part of the agonist binding site, the Cys loop probably plays a key role in receptor gating, transmitting structural changes initiated by agonist binding to the ion channel region of the receptor (3–7).

The intervening residues of the Cys loop show considerable conservation across the family (Fig. 1). Specifically, a completely conserved Phe-Pro motif (followed by Phe or Met) lies at the apex of the Cys loop. (These are residues 135 and 136 in the α₁ subunit of the muscle-type nAChR, which is the system studied here.) Proline residues are unique among the 20 natural amino acids in several ways. Of particular interest here is the much greater tendency of prolyl peptide bonds to exist in the cis conformation (8–12). The presence of the Phe in the Phe-Pro motif makes this possibility more enticing. It is well established that an aromatic amino acid N-terminal of a proline enhances the likelihood of a cis conformation, roughly doubling the contribution of the cis peptide in the conformational equilibrium (12). Indeed, previous studies of the analogous motif in the 5-HT₃ receptor using conventional mutagenesis led to a postulation that the Pro was in a cis conformation (13).

Currently available structural information related to Cys loop receptors adds to the intrigue (Fig. 2). (Note that the acetylcholine-binding protein, arguably the most valuable structural model for the extracellular domain, does not contain a Cys loop and does not contain the Phe-Pro sequence (14).) In the medium resolution electron microscopic structure of the *To whom correspondence should be addressed: Division of Chemistry, California Institute of Technology, 1200 E. California Blvd., Pasadena, CA 91125. Tel.: 626-395-6089; Fax: 626-564-9297; E-mail: dadougherty@caltech.edu.

1 The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; SuCh, succinylcholine; Pip, piperolic acid; Aze, azetidine-2-carboxylic acid; Dhp, 3,4-dehydroproline; Mor, morpholine-3-carboxylic acid; flp, cis 4-fluoro-proline; fHlp, trans 4-fluoro-proline; 3-Me-Pro, 3-methyl-proline; 1-Me-Pro, 1-methyl-proline; Cha, cyclohexylalanine; F-Phe, 4-fluorophenylalanine; F₃-Phe, 3,4,5-trifluorophenylalanine; Me-Pro, 2-Me-Pro, 2-methyl-proline; NVP, norvaline; NVOC, O-nitroveratryloxy carbonyl; MS, mass spectrometry.

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–3.

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The feasibility of both cis and trans conformations at Pro presents the tantalizing opportunity that cis-trans isomerization of this conserved proline in the Cys loop, facilitated by the adjacent Phe, might be involved in the receptor gating mechanism. Such a cis-trans isomerization at a different proline has been shown to be essential to channel gating in the 5-HT₃ receptor (5).

In the present work, we have used a variety of tools to probe the Phe-Pro motif of the muscle-type nAChR, including unnatural amino acid mutagenesis, electrophysiology, and NMR spectroscopy of model peptides. We find evidence for a strong interaction between the two residues and an important role for the aromatic nature of the Phe. At both sites, side-chain hydrophobicity is favorable to the receptor function. In addition, some preference for cis-biased residues at the Pro site is observed, but the involvement of cis-trans isomerization or the specific role of a cis conformer, if any, remains to be firmly established.

**EXPERIMENTAL PROCEDURES**

**Synthesis of dCA-Amino Acids**—The preparations of amino acids coupled to the dinucleotide (dCA) have been described previously (21) with the exception of dCA-Dhp and dCA-Mor. (S)-3-morpholinocarboxylic acid HCl was purchased from Tyger Scientific Inc. (Ewing, NJ), and 3,4-dehydro-L-proline (Dhp) was from Chem-Impex International Inc. (Wood Dale, IL). The amino groups were protected as the O-nitroveratryloxycarbonyl (NVOC) group. NVOC-Cl was purchased from Aldrich. (NVOC)-3,4-Dehydroproline cyanomethyl ester and (NVOC)-morpholine cyanomethyl ester were prepared according to the representative protocol reported in Ref. 22. Products were characterized by NMR spectroscopy. The NMR spectra, both ¹H and ¹³C, are complicated because each compound shows two distinct conformations in the solution. (NVOC)-3,4-Dehydroproline cyanomethyl ester. ¹H NMR (500 MHz, CDCl₃) δ 3.95–4.03 (m, 6H), 4.34–4.43 (m, 2H), 4.69–4.87 (m, 2H), 5.20–5.21 (m, 2H), 5.43–5.67 (m, 2H), 5.76–5.83 (m, 1H), 7.01 (s, 1H), 7.71 (m, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 49.20, 49.23, 53.57, 54.20, 56.54, 56.57, 56.87, 64.66, 65.08, 65.92, 66.28, 108.34, 108.37, 110.07, 111.25, 113.96, 113.98, 123.46, 123.71, 127.44, 127.85, 130.37, 130.49, 139.88, 139.92, 148.32, 148.43, 148.53, 149.11, 149.32, 153.76, 153.81, 153.96, 161.48, 161.83. High resolution MS analysis (FAB) calcd for C₁₇H₁₈N₃O₈ m/z 392.1094, found 392.1109. (NVOC)-Morpholine cyanomethyl ester. ¹H NMR (500 MHz, CDCl₃) δ 3.24–3.48 (m, 1H), 3.51 (dt, 1H), 3.69–3.75 (m, 1H), 3.83–3.95 (m, 2H), 3.94–3.95 (m, 3H), 3.99–4.02 (m, 3H), 4.33–4.41 (m, 1H), 4.63–4.85 (m, 3H), 5.41 (dd, 1H), 5.69 (dd, 1H), 6.88–6.97 (m, 1H), 7.66–7.70 (m, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 41.22, 41.73, 49.45, 54.53, 55.05, 56.53, 56.60, 56.81, 64.91, 65.31, 66.29, 66.73, 66.99, 67.34, 108.32, 108.36, 109.91, 111.13, 113.84, 113.89, 126.93, 127.66, 139.78, 140.11, 148.32, 148.53, 153.66, 153.81, 155.14, 168.69, 168.89. High resolution MS analysis (FAB) calcd for C₁₇H₁₈N₅O₉ m/z 410.1199, found 410.1180. (NVOC)-Morpholine cyanomethyl ester. ¹H NMR (500 MHz, CDCl₃) δ 3.34–3.48 (m, 1H), 3.51 (dt, 1H), 3.69–3.75 (m, 1H), 3.83–3.95 (m, 2H), 3.94–3.95 (m, 3H), 3.99–4.02 (m, 3H), 4.33–4.41 (m, 1H), 4.63–4.85 (m, 3H), 5.41 (dd, 1H), 5.69 (dd, 1H), 6.88–6.97 (m, 1H), 7.66–7.70 (m, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 49.20, 49.23, 53.57, 54.20, 56.54, 56.57, 56.87, 64.66, 65.08, 65.92, 66.28, 108.34, 108.37, 110.07, 111.25, 113.96, 113.98, 123.46, 123.71, 127.44, 127.85, 130.37, 130.49, 139.88, 139.92, 148.32, 148.43, 148.53, 153.76, 153.81, 153.96, 161.48, 161.83. High resolution MS analysis (FAB) calcd for C₁₇H₁₈N₅O₉ m/z 410.1199, found 410.1180.
Molecular Biology—Subunits of embryonic mouse muscle nAChR were in pAMV vectors. The α subunit contains the hemagglutinin epitope tag in the M3-M4 loop. There is no significant shift in EC50 caused by the insertion of the hemagglutinin epitope tag at this location. Site-directed mutagenesis was performed using the Stratagene QuikChange protocol. For single unnatural amino acid incorporation, the site of interest was mutated to an amber stop codon. For double unnatural amino acid incorporation, the 135 site was mutated to the opal stop codon, and the 136 site was mutated to the amber stop codon. Circular cDNA was linearized with NotI or KpnI. After purification (Qiagen), linearized DNA was used as a template for run-off in vitro transcription using the T7 mMessage mMachine kit (Ambion). The resulting mRNA was purified (RNAeasy mini-kit, Qiagen) and quantified by UV-visible spectroscopy.

Microinjection—Stage V-VI Xenopus laevis oocytes were employed. For wild-type receptor and receptors containing conventional mutations, quantified mRNAs of all subunits were mixed in a ratio of $\alpha/\beta/\gamma/\delta = 2:1:1:1$ by mass. If an unnatural amino acid was to be incorporated into the α subunit, the mRNA stoichiometry was $\alpha/\beta/\gamma/\delta = 10:1:1:1$ by mass. Total amount of injected mRNA was 0.5–5 ng/cell for the wild type, 5–50 ng/cell for conventional mutations, and 25–125 ng/cell for suppression mutations. More mRNA was used in the double mutation experiments and with some mutations that gave abnormally low expression level. Equal volumes of the mRNA mixture and unprotected tRNA-amino acid were mixed prior to injection. Approximately 15 ng of tRNA/cell was used in the single suppression experiments, and 50 ng was used in the double suppression experiments. Each oocyte was injected with 50 nl of RNA solution, and cells were incubated for 18–72 h at 18 °C in culture medium (ND96+ with 5% horse serum). In the case of low expressing mutant receptors, a second injection was required. As a negative control for all suppression experiments, 76-nucleotide tRNA (dCA ligated to 74-nucleotide tRNA) was co-injected with mRNA in the same manner as fully charged tRNA.

Electrophysiology—Acetylcholine chloride and succinylcholine dihydrate were purchased from Sigma. Drug dilutions were prepared from 1 m stock solutions in the calcium-free ND96 buffer.

Ion channel function in oocytes was assayed by current recording in two-electrode voltage clamp mode using the OpusXpress 6000A (Axon Instruments). For dose-response experiments, 1 ml of each drug solution was applied to the cells, and between 12 and 16 concentrations of drug were used. Oocytes were clamped at $-60$ mV. Cells were perfused in calcium-free ND96 solution at flow rates of 1 ml/min before agonist application, 4 ml/min during agonist application, and 3 ml/min during wash. Drug application was 15 s in duration. Data were sampled at 125 Hz and filtered at 50 Hz.

Data Analysis—All dose-response data were obtained from at least five cells and at least two batches of oocytes. Data were normalized ($I_{\text{max}} = 1$) and averaged. EC50 and Hill coefficient ($n_H$) were determined by fitting averaged, normalized dose-response relations to the Hill equation. Dose responses of individual oocytes were also examined and used to determine outliers. Individual dose-response data with $n_H > 2$ or $n_H < 1$ were discarded.

The coupling parameter ($\Theta$) between any two mutations at residues 135 and 136 was calculated from Equation 1,

$$\Theta = \frac{(EC50(\text{double mutation}) \times EC50(\text{wild type}))}{(EC50(135 \text{ mutation}) \times EC50(136 \text{ mutation}))} \quad (\text{Eq. 1})$$

Side chain logP values were obtained using the ChemDraw program (CambridgeSoft Corp.).

Synthesis of Fmoc-protected Amino Acid—Fmoc-CI was purchased from Fluka. (S)-3-Morpholinocarboxylic acid HCl was purchased from Tsger Scientific Inc. (Ewing, NJ), (2S,3S)-3-methylpyrrolidine-2-carboxylic acid (3-Me-Pro) was from Acros Organics USA (Morris Plains, NJ), α-methyl-l-proline (2-Me-Pro) was from Fluka, and Dhp was from Chem-Impex International Inc. (Wood Dale, IL). The amino acids were coupled to the Fmoc protecting group using the following protocol.

1-Amino acid (0.06 mmol) was dissolved in water (2 ml), resulting in a solution with pH $\sim 9$. To this solution was added Fmoc-CI (1.5 eq) in dioxane (2 ml) at room temperature. Diisopropylethylamine was added dropwise while the reaction mixture was added Fmoc-Cl (1.5 eq) in dioxane (2 ml) at room temperature. The aqueous layer was acidified with 6N HCl to pH 2, resulting in a solution with pH $\sim 1$ (solution became cloudy) and extracted with ether (5 ml) three times. The combined organic layers were dried over Na2SO4, and the solvent was removed under reduced pressure. Crude product was dried under vacuum overnight and was used in the next step (solid-phase peptide synthesis) without further purification. N-Fmoc-2-methyl-proline. $^1$H NMR (500 MHz, CDCl3) $\delta$1.26–1.62 (m, 3H), 1.75–1.98, (m, 3H), 2.15–2.42 (m, 1H), 2.51–2.63 (m, 3H), 2.62–2.72 (m, 3H), 3.51–3.63 (m, 2H), 3.85–3.97 (m, 1H), 4.12–4.28 (m, 1H), 4.33–4.46 (m, 2H), 7.27–7.41 (m, 4H), 7.53–7.62 (m, 2H), 7.70–7.77 (m, 2H).

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Data Analysis—All dose-response data were obtained from at least five cells and at least two batches of oocytes. Data were normalized ($I_{\text{max}} = 1$) and averaged. EC50 and Hill coefficient ($n_H$) were determined by fitting averaged, normalized dose-response relations to the Hill equation. Dose responses of individual oocytes were also examined and used to determine outliers. Individual dose-response data with $n_H > 2$ or $n_H < 1$ were discarded.

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Solid-phase Peptide Synthesis—All peptides were synthesized by solid-phase methods from Fmoc-protected amino acids using HBTU (Fluka) as a coupling reagent. Fmoc-L-proline was purchased from Sigma, Fmoc-L-pipercolic acid was from Peptech Corp. (Burlington, MA), Fmoc-L-azetidine-2-carboxylic acid was from Fluka, Fmoc-cis-2-fluoro-L-proline was from AnaSpec Inc. (San Jose, CA), N-Fmoc-glycine was from Aldrich, and Fmoc-L-phenylalanine was from Sigma. All chemicals were used as purchased without purification.

PAL resin (Sigma; estimated 0.4–0.8 mmol/g loading, 1% cross-linked with divinylbenzene, 100–200 mesh) was used to afford C-terminal primary amides. For conventional amino acids, couplings were performed with 3 eq of Fmoc amino acid, 2 eq of HBTU, and 4 eq of diisopropylethylamine. For unnatural amino acids, couplings were performed with 3 eq of Fmoc amino acid, 2 eq of HBTU, and 6 eq of diisopropylethylamine. For unnatural amino acids, couplings were performed with 2 eq of HBTU as the first trial, and no further attempt has been made to obtain the product.

NMR Spectroscopy of Model Peptides—The peptide samples were dissolved in 5 mM phosphate buffer with 25 mM NaCl in 90% H2O, 10% D2O at pH 5. Samples for NMR experiments were between 2 and 5 mM. NMR spectra were acquired on a Varian 600-MHz spectrometer, and the temperature was set to 298 K. The water signal was suppressed by presaturation. Sequential assignments were achieved using gradient-selected correlated spectroscopy (gCOSY) and total correlation spectroscopy (TOCSY) experiments. Spectra were all internally referenced to 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (~200 μM final concentration) at 0.0 ppm. The fraction of cis conformer was determined by integrating well resolved peaks in the one-dimensional 1H NMR spectra after base-line correction. NMR data were processed using the MestReNova software version 5.1.0 (Mestrelab Research S.L.).

RESULTS

Mutational Studies at Pro136—A previous study of the muscle-type nAChR in HEK293 cells showed that P136G mutations in the β and γ subunits prevented receptor assembly, whereas analogous mutations in the α or δ subunits prevented trafficking of receptors to the cell surface (26). Similarly, in previous studies of the analogous proline in the homopentameric 5-HT3 receptor, the P136A mutant revealed no surface expression in HEK293 cells (13). In the more permissive Xenopus oocyte expression system, the muscle-type nAChR containing the αP136A mutation produces <10% of the current levels seen for wild type. Surprisingly, this mutant receptor has an AC0 EC50 value similar to that of the wild type. As discussed below, this result can be interpreted in several different ways; we therefore anticipated that the more subtle mutations enabled by unnatural amino acid mutagenesis would provide a more revealing analysis of the role of this residue.

Several unnatural analogues of proline (Fig. 3) (5) were incorporated into the receptor using the in vivo nonsense suppression method. These unnatural proline analogues have varying ring size, side chain substitution, and intrinsic preferences for the cis conformer when probed in model systems (Table 1). The wild-type rescue experiment (i.e. incorporating Pro by nonsense suppression) displays the full phenotype of the wild-type receptor, including AC0 EC50 value, Hill coefficient, and current traces. This indicates that the nonsense suppression methodology is viable at the 136 site. Interestingly, Pro analogues at position 136 are generally gain-of-function (lower EC50), the sole exception being 2-Me-Pro, which gives essentially wild-type behavior. Similar to the Ala mutation mentioned above, the current levels from experiments involving 2-Me-Pro are <10% of those seen in comparable experiments with other mutations. Despite the relative subtlety of the mutations, the gain-of-function effects display the full phenotype of the wild-type receptor, including AC0 EC50 value, Hill coefficient, and current traces. This indicates that the nonsense suppression method is viable at the 136 site. Interestingly, Pro analogues at position 136 are generally gain-of-function (lower EC50), the sole exception being 2-Me-Pro, which gives essentially wild-type behavior. Similar to the Ala mutation mentioned above, the current levels from experiments involving 2-Me-Pro are <10% of those seen in comparable experiments with other mutations. Despite the relative subtlety of the mutations, the gain-of-function effects could be substantial, as seen with Pip and 3-Me-Pro, which show 13- and 22-fold decreases in EC50, respectively, relative to wild type.

Correlation between the cis-trans energy gap and the energy of channel activation could be expected if the receptor gating...
mechanism involves cis-trans isomerization of Pro. However, no simple correlation is found (Table 1). For example, although both Pip and Aze show a stronger inherent cis preference than Pro and a lower EC$_{50}$, 3-Me-Pro shows a conformational bias very similar to that of Pro but a greatly diminished EC$_{50}$. Before analyzing these results in greater detail, however, we must consider the role of Phe.

Mutational Studies at Phe — Previous single channel studies have shown that the F135A mutation in the nAChR alters the gating mechanism, leading to two uncoupled open states that produce independent gating reactions from the diliganded closed state. In our studies of the nAChR, we found that the F135A mutation nearly obliterates receptor function; only very weak ACh-induced currents are observed despite normal sur-

The F135Cha mutant receptor is functional, aromaticity at position 135 is not an absolute requirement for the receptor to function. To further explore the possible role of Phe$^{135}$ in receptor gating, wild-type and mutant receptors were probed with the partial agonist succinylcholine (SuCh). Compared with ACh, SuCh produces only 14% of the maximal current under saturating drug concentrations in the wild-type receptor (Table 2). This indicates that upon receptor activation by SuCh, the channel open-closed equilibria are shifted toward the open state, but to a much lesser extent relative to ACh activation. If a mutation produces a gain-of-function effect as a result of enhanced receptor gating, one could expect the mutation to improve the efficacy of a partial agonist like SuCh. The EC$_{50}$ trend of SuCh (Table 2) parallels that of ACh, implying that the mutants respond to both drugs in the same way. As anticipated, all of the Phe analogues that show a lowered EC$_{50}$ do increase the relative efficacy of SuCh with respect to ACh. This suggests that mutations at position 135 primarily affect receptor gating. Note that the non-aromatic analogue Cha shows essentially wild-type EC$_{50}$ for both ACh and SuCh and that this mutation has no strong effect on the relative efficacy.

Interaction between Phe$^{135}$ and Pro$^{136}$ — There is considerable evidence supporting a specific interaction in a Phe-Pro sequence that stabilizes the cis form of the Pro. This could possibly involve a polar-π interaction in which polarized C–H bonds (C$^6$–H$^6$+) on the proline interact favorably with the negative electrostatic potential on the face of the Phe side chain stacked on the Pro (9, 11). We investigated the possibility of a Phe-Pro interaction in this system by testing double mutant receptors in which Phe$^{135}$ was substituted with the non-aromatic Cha and Pro$^{136}$ was substituted with either Pip or 3-Me-Pro, the two mutations that cause the largest EC$_{50}$ shifts. These experiments required consecutive incorporation of two different unnatural amino acids, an unprecedented experiment for receptors expressed in a living cell that was made possible by recent advances in tRNA design (24, 25).

We probing the F135$^{135}$ site with an extensive series of Phe analogues. Again, the wild type rescue experiment displays the full characteristics of the wild-type receptor. The F135$^{135}$ site is sensitive to even very subtle mutations, as shown in Table 2. Similar to what is observed with Pro$^{136}$, Phe analogues consistently produce gain-of-function mutants. ACh sensitivity increases with the volume and number of hydrophobic substituents on the aromatic ring. For example, Me-Phe has a lower EC$_{50}$ than F-Phe, and Me$_2$-Phe has a lower EC$_{50}$ than Me-Phe. Surprisingly, cyclohexylalanine (Cha), which is similar to Phe in size and shape but is not aromatic (28), produces functional receptors with a small perturbation; EC$_{50}$ is near the wild-type value. Given that the F135Cha mutant receptor is functional, aromaticity at position 135 is not an absolute requirement for the receptor to function.

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The resulting current signals (1–4 μA) were quite sufficient for quantitative analysis.

The F135Cha mutation substantially diminishes the large effects of the mutations at Pro$^{136}$. As shown in Fig. 4, the 13- and 22-fold drops in EC$_{50}$ for Pip and 3-Me-Pro, respectively,
seen in a wild-type Phe background fall to \(-2.5\) fold in the presence of F135Cha. A standard evaluation of double mutants employs a mutant cycle analysis, which has been used successfully with \(\text{EC}_{50}\) values for Cys loop receptors in several instances (29–32). For the interaction of F135Cha with P136Pip and P136(3-Me-Pro), we find coupling parameters (\(\Omega\)) of 5 and 10, respectively, which correspond to coupling energies \((RT\ln(\% \text{cis}))/\% \text{cis}(\text{Pro analogue})/\% \text{cis}(\text{Pro})\). \(\Delta\Delta G\) values were calculated from electrophysiology results of mutant receptors containing the corresponding amino acid at \(\alpha_{135}\).

\[
\Delta\Delta G = -RT\ln(\% \text{cis} (\text{Pro analogue})/\% \text{cis} (\text{Pro}))
\]

Table 3 shows the percentage cis values and \(\Delta\Delta G\) values for each unnatural amino acid at \(\alpha_{135}\). Note that for the Gly-Phe-Pro-Gly peptide, the reported values in model peptides lacking the aromatic residue are based on known chemical shifts of the Gly-Phe-Pro-Gly peptide reported in Ref. 33. Using a similar solution NMR technique, it should be possible to determine \(\Delta G\) values for our series of unnatural analogues of proline following a Phe residue in model peptides.

Model peptides Gly-Phe-\(X_{\text{Pro}}\)-Gly, where \(X_{\text{Pro}}\) represents Pro, Pip, Aze, Flp, Mor, 3-Me-Pro, 2-Me-Pro, and 3-Me-Pro, were synthesized via standard solid-phase peptide synthesis methods. These peptides were then subjected to solution NMR experiments similar to those in Ref. 33. Protons were assigned by two-dimensional gCOSY and/or TOCSY experiments. The proportion of each of the two conformers in solution was measured by integration of a corresponding, well-resolved peak after base-line correction. (Representative sample spectra are shown in supplemental Fig. 2). Conformational assignments were based on known chemical shifts of the Gly-Phe-Pip-Gly peptide reported in Ref. 33.

The results from the solution NMR experiments (Table 3) show that the cis preferences are indeed higher than the reported values in model peptides lacking the aromatic residue (Table 1). Note that for the Gly-Phe-(2-Me-Pro)-Gly peptide, the cis form was not observed. The model peptide containing Mor has a very high cis propensity; nearly 50% of the peptide is in the cis form. Moreover, one of the protons attached to the C atom of the Mor ring displays a large upfield shift in the cis peptide compared with that of the trans peptide (supplemental Table 1), as has also been reported with the structurally similar Pip (33). In the Pip-containing peptide, the chemical shifts of C protons are 1.72 and 2.15 ppm in the trans conformation and 0.35 and 1.90 ppm in the cis conformation. Likewise, for the Mor-containing peptide, the chemical shifts change from

### Table 2

| \(\alpha\text{Phe}^{135}\) | ACh | SuCh |
|---|---|---|
| | \(\text{EC}_{50}\) | Hill constant | \(n\) | \(\text{EC}_{50}\) | Hill constant | \(n\) | Efficacy\(^a\) |
| Phe | 23 \(\mu\text{M}\) | \(1.5 \pm 0.02\) | 35 | 59 | 1.3 \(\pm 0.03\) | 13 | 0.14 \(\pm 0.01\) |
| Phe\(^b\) | 23 \(\mu\text{M}\) | \(1.5 \pm 0.03\) | 8 | 32 | 1.4 \(\pm 0.04\) | 9 | 0.54 \(\pm 0.02\) |
| F-Phe | 2.6 \(\mu\text{M}\) | \(1.6 \pm 0.02\) | 7 | 8.1 | 1.6 \(\pm 0.05\) | 8 | 0.86 \(\pm 0.02\) |
| F\(_1\)-Phe | 1.0 \(\mu\text{M}\) | \(1.5 \pm 0.05\) | 15 | 11 | 1.5 \(\pm 0.04\) | 11 | 0.82 \(\pm 0.02\) |
| Me\(_1\)-Phe | 1.0 \(\mu\text{M}\) | \(1.6 \pm 0.04\) | 12 | 1.6 | 1.5 \(\pm 0.07\) | 7 | 0.93 \(\pm 0.04\) |
| Me\(_2\)-Phe | 0.22 \(\mu\text{M}\) | \(1.6 \pm 0.07\) | 9 | 60 | 1.6 \(\pm 0.03\) | 9 | 0.10 \(\pm 0.01\) |

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\(\text{ACh EC}_{50}\) results from single and double mutation experiments at residues 135 and 136 in comparison with the wild-type value. For F135Cha/P136Pip, \(\text{EC}_{50} = 6.5 \pm 0.2 \mu\text{M}\), Hill constant = 1.7 \(\pm 0.07\), and \(n = 9\). For F135Cha/P135(3-Me-Pro), \(\text{EC}_{50} = 6.9 \pm 0.2 \mu\text{M}\), Hill constant = 1.5 \(\pm 0.06\), and \(n = 12\).

\(\Delta\Delta G\) values were calculated from electrophysiology results of mutant receptors containing the corresponding amino acid at \(\alpha_{136}\).

### Table 3

| \(X_{\text{Pro}}\) | Percentage cis | \(\Delta\Delta G\text{(c-t)}\) | \(\Delta\Delta G\text{(EC}_{50}\) |
|---|---|---|---|
| Pro | 17 | 0 | 0 |
| Pip | 39 | 0.65 | 1.5 |
| Aze | 30 | 0.42 | 0.81 |
| Flp | 32 | 0.49 | 0.38 |
| 3-Me-Pro | 12 | -0.24 | 1.8 |
| 2-Me-Pro | 0 | -0.056 |
| Mor | 48 | 0.87 | 0.57 |

\(\Delta\Delta G\text{(c-t)}\) calculated from the percentage of cis results of solution NMR experiments for each amino acid and the \(\Delta\Delta G\text{(EC}_{50}\) calculated from electrophysiology results of mutant receptors containing the corresponding amino acid at \(\alpha_{135}\).
3.74 and 4.37 ppm in the trans conformation to 2.10 and 3.74 ppm in the cis conformation. Most importantly, the Phe residue can alter the trends in cis-trans preferences, as shown for the simple homologous series Aze, Pro, Pip (Table 1 compared with Table 3).

**DISCUSSION**

Cys loop neurotransmitter-gated ion channels are remarkable molecular machines. In response to the binding of a small molecule ligand, these large proteins undergo a global conformational change, opening a selective ion channel and thereby converting a chemical event (i.e. ligand binding) to an electrical signal. The precise mechanism of this process is a central issue in molecular neurobiology. Recently, chemical scale studies have provided valuable insights into the structure and function of these receptors, yet significant challenges still remain.

Here we have evaluated the highly conserved and structurally intriguing Phe$_{135}$-Pro$_{136}$ motif of the prototypic Cys loop receptor, the nAChR. Proline is well appreciated to display novel conformational behaviors compared with all other natural amino acids. Additionally, it has been proposed that prolines might play a key role in the conformational changes that are essential to the function of many types of receptors (34). Several lines of evidence establish that local amino acids flanking proline can influence proline conformational preferences (9, 11, 12, 35). In particular, an aromatic residue preceding the proline is found to enhance the fraction of the cis isomer for peptides in solution (12). As shown in Fig. 2, Pro$_{136}$ can exist in both cis and trans conformations, and the two crystal structures with a cis peptide bond (2QC1 and 3EAM) show stacking of the Phe-Pro side chains. Given the complete conservation of the Phe-Pro motif and the available structural data, it seemed reasonable to speculate that the cis conformer of Pro$_{136}$ could be involved in receptor function.

Our primary measure of receptor function is EC$_{50}$, the effective concentration of agonist required to achieve half-maximal response. Agonist binding to a receptor induces step-by-step conformational changes that lead to opening of the ion channel; therefore, EC$_{50}$ is a value that reflects the composite effect of the agonist binding affinity and the sequential gating events. The Phe-Pro motif is remote from the agonist binding site, and the Cys loop is firmly established to play an essential role in gating (36). In addition, we find that a number of mutations at residue 135 greatly increase the efficacy of the partial agonist SuCh, supporting the notion that this residue participates in the gating mechanism. As such, we interpret changes in EC$_{50}$ to reflect primarily, if not exclusively, changes in receptor gating.

The involvement of the Phe-Pro motif in gating is further supported by a previous single channel study on the F135A mutation, which indicated that the gating mechanism is modified as a result of this mutation (27). The new mechanism appears to be much less efficient at coupling agonist binding to channel opening, consistent with our macroscopic observations of greatly reduced current for this mutant.

Conventional mutations at Pro$_{136}$ also have strong effects on the receptor. When expressed in HEK293 cells, both Gly mutants in the nAChR subunits and an Ala mutant in the related 5-HT$_3$ receptor (13) gave receptors that were substantially impaired in the ability to assemble and/or traffic to the surface. In the Xenopus oocyte system, we find that the P136A mutant gave $<10\%$ of the current levels seen from wild type, again suggesting a disruption of assembly and/or trafficking or a disruption of gating.

Similarly, in an earlier study of Pro$_{308}$ in the M2-M3 loop of the 5-HT$_3$ receptor, in which a compelling correlation between cis propensity of incorporated proline analogues and receptor function was demonstrated, structural disruption by conventional mutagenesis produced ambiguous results (5). In that study, Ala, Cys, Gly, Lys, Val, and Gln conventional mutants gave nonfunctional receptors. More recently, studies of an orthologous 5-HT$_3$ receptor showed that His and Trp mutants did give functional receptors (37). We note that aromatic amino acids, such as His and Trp, are more than twice as likely to be in a cis conformation as other non-proline natural amino acids (38). Again, the implications of the conventional mutagenesis results are open to debate.

Using conventional mutagenesis to probe the role of the cis conformation of a highly conserved proline is, in our view, unlikely to produce compelling results. Such studies frequently assume that simply seeing a functional receptor with a non-proline natural amino acid incorporated rules out a role for the cis conformer. However, previous studies have demonstrated that in some cases, when a cis proline is mutated to an alanine, the main-chain cis bond is preserved, presumably because the three-dimensional structure favors the cis conformation (11). In such cases, the Pro to Ala mutation often reduces the stability of the protein, which could manifest as lower expression levels, as we see with the P136A mutant. In addition, as with Pro, the presence of an aromatic amino acid (such as Phe) N-terminal to an aliphatic residue (such as Ala) doubles the probability of a cis conformation (38). Alternatively, replacement of a proline with another natural amino acid could produce functional receptors via a different gating path that has become more energetically accessible, parallel to what is seen with the F135A mutation (27).

When studying such a structurally distinctive motif as Phe-Pro, the benefits of unnatural amino acid mutagenesis are amplified. The subtle perturbations allow one to maintain the essential motif while probing its intrinsic features. We have used unnatural amino acids to probe several aspects of the Phe-Pro motif, including the importance of Phe aromaticity, the roles of side chain hydrophobicity and volume, and the possibility of cis-trans isomerization at the proline backbone.

Several intriguing observations emerge from the unnatural amino acid mutagenesis studies. Considering Pro$_{136}$, subtle mutations produce noticeable changes in EC$_{50}$. For example, simply adding a methyl group (3-Me-Pro) can lower EC$_{50}$ 22-fold, and adding a single CH$_2$ group to the ring (Pip) can lower EC$_{50}$ 13-fold. Mutations are generally gain-of-function; EC$_{50}$ decreases. The only residue that is not gain-of-function but instead gives nearly wild-type EC$_{50}$ is 2-Me-Pro. Similar to Ala, 2-Me-Pro also produces much smaller whole cell currents.

As with the proline, subtle mutations of Phe$_{135}$ can produce substantial changes in EC$_{50}$; a 100-fold shift arises from just the addition of two methyl groups fairly remote from the protein backbone (Me$_2$-Phe). Paralleling the proline results, all of the
unnatural amino acid mutants are gain-of-function. Moreover, an interesting trend is evident; Fig. 5 shows a plot of log(EC50) for the receptor versus the side chain logP, a measure of its hydrophobicity. Although the cyclohexyl compound (Cha) is clearly an outlier, a significant correlation is seen among the aromatic side chains. These results indicate that hydrophobicity is an important determinant at position 135, with an increase in hydrophobicity making the channel easier to open. This is consistent with a molecular dynamics simulation of the α7 nAChR that places Phe135 in a hydrophobic pocket in an open state (39). In addition, the logP analysis (Fig. 5) highlights the role of aromaticity at residue 135 because Cha has essentially the same hydrophobicity as both Me-Phe and F3-Phe but a much higher EC50. As such, the F135Cha mutant, having being more hydrophobic than the wild-type Phe but lacking the aromaticity, appears to have a nearly wild-type ACh EC50. From these data, we conclude that both hydrophobicity and aromaticity at position 135 are important in receptor function.

The results of our double mutant studies have confirmed an important interaction between residues 135 and 136; the large effects caused by mutation at Phe135 are attenuated when Phe135 is simultaneously mutated to the non-aromatic Cha (Fig. 4). Mutant cycle analysis shows significant coupling energies between residues 135 and 136.

We noted above the intriguing possibility that cis-trans isomerization at Pro136 is involved in receptor gating. In the present work, we did not see a simple correlation between EC50 and logP for mutations at Phe135. Note that the Cha point was not included in the linear fit.

Concerning the more dramatic proline mutations, a simple percentage cis correlation is not evident. It is clear from the Phe135 mutational studies that receptor function is highly sensitive to side chain polarity at the 135 site (Fig. 5), with increased side chain hydrophobicity lowering EC50. It seems reasonable to expect a similar effect at the adjacent Pro136 because Phe and Pro interact, as shown by the mutant cycle analysis. Indeed, our results suggest a preference for side chain hydrophobicity at the Pro136 site as well. Mor is structurally very similar to Pip, but it does not fit into the Aze-Pro-Pip correlation. We propose that EC50 for Mor is anomalously high because of the increased polarity relative to Pip. Similarly, flp has a significantly higher
percentage cis than Pro but only a modest decrease in EC$_{50}$, apparently due to the increased polarity of the fluorine substituent. In fact, a second linear correlation can be seen in Fig. 6 involving the Pro-flp-Mor series, although the structural variation across this series is less consistent than in the Aze-Pro-Pip trio. 3-Me-Pro shows a smaller percentage cis than Pro but the lowest EC$_{50}$ among the amino acids at the 136 sites. Interestingly, adding a single CH$_3$ group to Pro$_{136}$ has the same effect on EC$_{50}$ as adding a single CH$_3$ group to Phe$_{135}$ (3-Me-Pro and Me-Phe show the same EC$_{50}$). Inspection of simple molecular models leads to an observation that the two CH$_3$ groups could point into nearly the same region of the receptor when the proline is in the cis form. Perhaps each CH$_3$ fits into a hydrophobic pocket, stabilizing the open state of the receptor and lowering EC$_{50}$.

As shown Fig. 6, in the most conservative structural series (Pro, Pip, and Aze), we do find a trend that is suggestive of cis-trans isomerization at Pro$_{136}$. Importantly, this trend is seen only when the perturbing effect of the Phe residue is included, justifying the consideration of the Phe-Pro unit as a single motif. Residues that involve more complex changes do not fit the correlation, but generally the deviation is consistent with the notion that increasing side chain hydrophobicity lowers EC$_{50}$. From our data, we propose that both cis propensity and side-chain hydrophobicity at Pro$_{136}$ simultaneously are determinants of nAChR function. Moreover, the possibility of cis-trans isomerization at Pro$_{136}$ being involved in gating cannot be ruled out.

**Summary**—The subtle mutations enabled by unnatural amino acid mutagenesis have allowed a detailed study of the Phe-Pro motif in the Cys loop of a Cys loop receptor. Mutant cycle analysis reveals a strong interaction between the two residues and a strong preference for an aromatic residue at position 135. In addition, a clear trend is evident whereby increasing hydrophobicity at either Phe$_{135}$ or Pro$_{136}$ lowers EC$_{50}$. Although the analysis of residue Pro$_{136}$ is complex, the data provide evidence supporting a role for the cis conformer in receptor function.

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