Tryptophan Fluorescence Reveals Conformational Changes in the Acetylcholine Binding Protein

Received for publication, August 16, 2002, and in revised form, September 12, 2002
Published, JBC Papers in Press, September 13, 2002, DOI 10.1074/jbc.C200462200

Scott B. Hansen‡, Zoran Radić‡, Todd T. Talley‡, Brian E. Molles‡, Tom Deerinck§, Igor Tsigelny‡, and Palmer Taylor¶†

From the ‡Department of Pharmacology, University of California, San Diego, La Jolla, California 92039-0636 and the §National Center for Microscopy and Imaging Research, University of California, San Diego, La Jolla, California 92039-0608

The recent characterization of an acetylcholine binding protein (AChBP) from the fresh water snail, *Lymnaea stagnalis*, shows it to be a structural homolog of the extracellular domain of the nicotinic acetylcholine receptor (nAChR). To ascertain whether the AChBP exhibits the recognition properties and functional states of the nAChR, we have expressed the protein in milligram quantities from a synthetic cDNA transfected into human kidney (HEK) cells. The protein secreted into the medium shows a pentameric rosette structure with ligand stoichiometry approximating five sites per pentamer. Surprisingly, binding of acetylcholine, selective agonists, and antagonists ranging from small alkaloids to larger peptides results in substantial quenching of the intrinsic tryptophan fluorescence. Using stopped-flow techniques, we demonstrate rapid rates of association and dissociation of agonists and slow rates for the α-neurotoxins. Since agonist binding occurs in millisecond time frames, and the α-neurotoxins may induce a distinct conformational state for the AChBP-toxin complex, the snail protein shows many of the properties expected for receptor recognition of interacting ligands. Thus, the marked tryptophan quenching not only documents the importance of aromatic residues in ligand recognition, but establishes that the AChBP will be a useful functional as well as structural surrogate of the nicotinic receptor.

Ligand-gated ion channels, of which the nicotinic acetylcholine receptor is a prototypic structure, are composed of five subunits whose α-carbon chains traverse the membrane four times (1, 2); their hydrophobicity and size preclude conventional structural studies at atomic resolution by x-ray crystallography or nuclear magnetic resonance spectrometry. Recently, an acetylcholine binding protein (AChBP) from the fresh water snail, *Lymnaea stagnalis*, has been characterized, crystallized, and its structure determined (3, 4). The crystal structure shows virtually all of the features predicted from a host of affinity labeling, site-specific mutagenesis, and subunit assembly studies conducted on the nicotinic receptor for over 2 decades (1, 2, 5). Although the isolated protein shares ligand recognition characteristics with its closest mammalian homolog, the pentameric α7 receptor (4), details on its ligand specificity, binding kinetics, and conformational changes remain unknown. These questions are critical to ascertaining whether the snail protein has the recognition properties and conformational states to serve as a functional as well as a structural surrogate of the extracellular domain of the nicotinic receptor. To this end, we have expressed the binding protein in a mammalian system from a chemically synthesized cDNA of 637 bp. The cDNA contains restriction sites at various locations to allow for substitution of encoding receptor segments into the cDNA template of the binding protein. Upon ligand binding, AChBP shows major changes in fluorescence emitted from five tryptophans on each subunit, providing an intrinsic detection system to monitor the stoichiometry and kinetics of ligand binding.

**EXPERIMENTAL PROCEDURES**

**Gene Synthesis and Protein Expression**—We synthesized seven double-stranded oligonucleotides between 80 and 126 bp reflecting codon usage in mammalian cells and containing appropriate overhangs for ligation (6). These were assembled into three ligation products that were then inserted into construction vectors and their sequence confirmed by automated sequencing. After digestion with appropriate restriction enzymes followed by band isolation, the inserts were ligated into a p3×FLAG-CMV-9 expression vector (Sigma) containing a preprotrypsin leader peptide followed by a N-terminal 3×FLAG epitope.  

The expression plasmid also contained neomycin acetyltransferase for clonal selection. Transfection of the plasmid into human embryonic kidney cells produced an epitope-attached glycoprotein secreted primarily into the medium. Selection with G418 yielded a stable cell line secreting AChBP. Ultraculture medium (Bio Whittaker) was collected at 3-day intervals from multitier flasks for several weeks. Adsorption onto a FLAG antibody column followed by elution with the 3×FLAG peptide yielded purified protein in quantities between 1 and 2.5 mg/liter.

**Fluorescence Assays**—Fluorescence measurements were performed on a Jobin Yvon-SpeX Fluoromax 2 fluorometer (Instruments S. A., Inc., Edison, NJ). AChBP was excited at 280 nm and emission intensity monitored for 0.1-s intervals at unitary wavelengths between 337 and 345 nm.

**Stopped-flow Kinetics**—Stopped-flow measurements were obtained using an Applied Photophysics SX.16MV (Leatherhead, UK) stopped-flow spectrophotometer. Excitation was at 280 nm, and a cut-off filter at 305 nm was used to collect the fluorescence signal. Measurements of binding of the dansyl choline analogues employed 280 nm excitation and measured the enhanced fluorescence using a 420-nm cut-off filter. Rates of association and dissociation were estimated from the slope and ordinate intercept of plots of overall rate of fluorescence change versus ligand concentration. Dissociation rates were also estimated in several cases by reacting the preformed complex with a large excess of galan...
lamine in the stopped-flow instrument and observing the time course of the increase in fluorescence.

RESULTS AND DISCUSSION

Purification of AChBP secreted into the medium produced a single band on SDS gels migrating at \(-35 \text{kDa}\) (Fig. 1a). Treatment with PNGase to remove N-linked oligosaccharides enhances the migration rate considerably. N-terminal sequencing and matrix-assisted laser desorption ionization mass spectrometry after deglycosylation yielded a sequence and mass consistent with cleavage of the leader peptide. Negative staining electron microscopy showed typical rosette structures expected of a pentameric subunit assembly (Fig. 1, b and c). Hydrodynamic analysis revealed a Stokes radius of 57 Å from gel filtration and a sedimentation coefficient of 4.9 S from sucrose density gradients; values were also consistent with pentamer formation.

Stoichiometry of ligand binding was estimated from AChBP tryptophan quenching through titration by high affinity ligands (Fig. 2). In separate preparations, this yielded values of 4.7–5.6 mol/mol of pentamer or 0.94 to 1.1 mol/mol of 26,551-dalton subunit based on quantitative amino acid analysis. These data show with epibatidine as a high affinity ligand \(-50\%\) quenching of the intrinsic tryptophan fluorescence, a typical quenching value for most of the quaternary and tertiary structures expected of a pentameric subunit assembly (Fig. 1, b and c). Hydrodynamic analysis revealed a Stokes radius of 57 Å from gel filtration and a sedimentation coefficient of 4.9 S from sucrose density gradients; values were also consistent with pentamer formation.

FIG. 1. Characterization of AChBP. a, SDS-PAGE electrophoresis showing apparent molecular weight of untreated and PNGase F-treated AChBP (1 µg each lane). Electron micrographs of b, Torpedo californica nAChR in isolated membrane vesicles prepared by density gradient centrifugation (27) and c, AChBP by negative staining with 2% uranyl acetate.

FIG. 2. Equilibrium titration of AChBP with \(\alpha\)-bungarotoxin and epibatidine. AChBP at 300 nM was titrated in a 4 \(\times\) 4-mm cuvette with incremental quantities of the peptide antagonist and the alkaloid agonist. Since quenching by \(\alpha\)-bungarotoxin is only 15–20% of the unliganded receptor fluorescence, a receptor-gallamine complex was formed by addition of 2 µM gallamine to enhance fluorescence prior to the \(\alpha\)-bungarotoxin addition. The contribution of the fluorescence from the added \(\alpha\)-bungarotoxin was subtracted from the titration curve. The single tryptophan in \(\alpha\)-bungarotoxin has less than 2% of the emission intensity at 355 nm of the tryptophans in the receptor-gallamine complex, so only a small correction is necessary. Fluorescence was recorded in a SPEX Fluoromax2 spectrophotometer at 25 °C. Protein content was determined by quantitative amino acid analysis with a subunit molecular weight of 26,551. Fluorescence excitation was at 280 nm; emission maxima were measured over the range of 337–343 nm.
all fluorescence quenching. The slower \( \alpha \)-neurotoxin kinetics of association, which also has been well documented in nicotinic receptors from several species (24–26), and the linked unimolecular step seen here are suggestive of the \( \alpha \)-bungarotoxin locking the AChBP into a distinctive conformational state. The single tryptophan in \( \alpha \)-bungarotoxin has \( \sim 2\% \) of the fluorescence intensity of the five tryptophans in each AChBP subunit.

The fluorescence change in the slow step could emerge from enhanced fluorescence of the single tryptophan of the toxin in the complex or a slight enhancement of the AChBP tryptophans after formation of the intial complex. Irrespective of the tryptophans contributing to the signal differences, the slower unimolecular isomerization points to differing positions of the tryptophans in the initial complex and the final equilibrium state.

The *tris*-quaternary antagonist, gallamine, when associated with the receptor, results in an enhancement of the tryptophan fluorescence, suggesting that the stabilization of this ligand may differ from the other agonists and antagonists studied. The three triethylammonioethyl groups that emanate from the pyrogallol ring probably preclude full insertion of the ring into the aromatic pocket. Rather stabilization involving the quaternary ammonium ligands and anionic moieties at the subunit interface account for the different binding orientation of gallamine, resulting in fluorescence enhancement (Fig. 4). Since gallamine binding appears mutually exclusive with the other agonists and antagonists listed in Table I, reaction of the various ligand-AChBP complexes with gallamine by stopped-flow provided a valuable means of confirming the dissociation rates

![Image](317x298 to 561x737)

**Fig. 4. Docking of gallamine to the AChBP using DOT (28).** A, crystal structure of the subunit interface (3) showing the \( \alpha \) carbon chain and the space filling residues for two subunits. The exposed portion of the docked gallamine is shown in green, tryptophan side chains in yellow, and selected anionic side chains in red. B, expanded view of the \( \alpha \) carbon chain with the tryptophan side chains in yellow, tyrosine in blue, and anionic residues in red. Note the positioning of the triethylammonio moieties near the anionic side chains (Glu-110, Glu-149, Glu-163, Glu-190; Asp-108) at the binding site. The pyrogallol ring is sandwiched between the vicinal cystines at 187 and 188 and isoleucine 112 side chain on the neighboring subunit. The position of gallamine docked by computation may be contrasted with HEPES found in the crystal structure (3).

**Table I**

AChBP ligand binding kinetics

| Ligand                  | \( k_1 \) \( \times 10^6 \ s^{-1} \) | \( k_{-1} \) \( s^{-1} \) | \( K_d \) \( \mu M \) |
|-------------------------|---------------------------------|--------------------------|-----------------|
| Decamethonium           | 3.2 ± 0.17                      | 120 ± 17                 | 380              |
| Dansyl-C6-choline\(^a\) | 2.1                             | 2.9                      | 14               |
| Gallamine               | 2.5 ± 0.14                      | 36 ± 6.5                 | 140              |
| d-Tubocurarine          | 2.0                             | 30                       | 150              |
| (+)-Epibatidine         | 1.7 ± 0.26                      | 0.027 ± 0.037            | 0.16             |
| (-)-Nicotine            | 1.5                             | 5.7                      | 38               |
| Dansyl-C2-choline\(^b\) | 1.3                             | 7.6                      | 58               |
| Acetylcholine           | 1.1 ± 0.12                      | 120 ± 16                 | 1000             |
| Waglerin-1              | 0.048                           | 31                       | 6500             |
| \( \alpha \)-Cobratoxin | 0.033                           | 0.011                    | 3.2              |
| \( \alpha \)-Bungarotoxin| 0.0097 ± 0.0017                 | 0.0003 ± 0.0003          | 1.8              |

\(^a\) 5-Dimethylaminonaphthybenzamidoethyltrimethylammonium.  
\(^b\) 5-Dimethylaminonaphthybenzamidoethoxyltrimethylammonium.
of the various antagonists (Table I), as well as measuring the stoichiometry of ligand binding for ligands that quench to a lesser extent than epibatidine (Fig. 2).

Quite apart from establishing ligand specificity for the AChBP, intrinsic tryptophan fluorescence quenching affords a universal means of directly following ligand binding to the AChBP without the necessity of developing competition studies with radioactive or fluorescent ligands. Substitution of sequences unique to particular receptor subtypes may allow one to examine selectivity of various AChBP-receptor chimeric sequences fashioned after the neuronal or muscle subtypes of the many subtypes of nicotinic receptor. Physical measurements in solution should enable one to correlate conformation with kinetic parameters of ligand recognition, and add another dimension to investigating specificity of this unique binding protein in relation to the larger family of receptor-related offspring.

REFERENCES
1. Karlin, A. (2002) Nat. Rev. Neurosci. 3, 102–114
2. Corringer, P. J., Le Novère, N., and Changeux, J. P. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 431–458
3. Brejc, K., van Dijk, W. J., Klaassen, R. V., van der Oost, J., Smit, A. B., and Sixma, T. K. (2001) Nature 411, 269–276
4. Smit, A. B., Syed, N. I., Schaap, D., van Minnen, J., Klumperman, J., Kals, K. S., Lodder, H., van der Schors, R. C., van Elk, R., Sorgedrager, B., Brejc, K., Sixma, T. K., and Geraerts, W. P. (2001) Nature 411, 261–268
5. Taylor, P., Osaka, H., Molles, B., Keller, S. H., and Malany, S. (2000) in Handbook of Experimental Pharmacology (Clementi, F., Fornasari, D., and Gotti, C., eds) Vol. 144, pp. 79–100, Springer-Verlag, Berlin
6. Itakura, K., Hirose, T., Crea, R., Riggs, A. D., Heyneker, H. L., Bolivar, F., and Boyer, H. W. (1977) Science 198, 1056–1063
7. Lackiewicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2 Ed., pp. 185–210, Kluwer Academic/Plenum Publishers, New York
8. Lockless, S. W., and Ranganathan, R. (1999) Science 286, 295–299
9. Radiz, Z., and Taylor, P. (2001) J. Biol. Chem. 276, 4622–4633
10. Sussman, J. L., Harel, M., Frolov, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991) Science 253, 872–879
11. Galzi, J. L., Bertrand, D., Devillers-Thiery, A., Revah, F., Bertrand, S., and Changeux, J. P. (1999) FEBS Lett. 294, 198–202
12. Xie, Y., and Cohen, J. B. (2001) J. Biol. Chem. 276, 2417–2426
13. Beebe, D. L., Brandt, G. S., Zhong, W., Zacharias, N. M., Lester, H. A., and Dougherty, D. A. (2002) Biochemistry 41, 10262–10269
14. Zheng, W., Gallivan, J. P., Zhang, Y., Li, L., Lester, H. A., and Dougherty, D. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12088–12093
15. Colquhoun, D., and Sakmann, B. (1985) J. Physiol. (Lond.) 369, 501–557
16. Figl, A., Labarca, C., Davidson, N., Lester, H. A., and Cohen, B. N. (1996) J. Gen. Physiol. 107, 369–379
17. Bouzat, C., Barrantes, F., and Sine, S. (2000) J. Gen. Physiol. 115, 663–672
18. Prince, R. J., and Sine, S. M. (1998) Biophys. J. 75, 1817–1827
19. Salamone, F. N., Zhou, M., and Auerbach, A. (1999) J. Physiol. (Lond.) 516, 315–330
20. Grossman, C., and Auerbach, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14102–14107
21. Wang, H. L., Auerbach, A., Bren, N., Ohno, K., Engel, A. G., and Sine, S. M. (1997) J. Gen. Physiol. 109, 757–766
22. Unwin, N., Miyaizawa, A., Li, J., and Fujiyoshi, Y. (2002) J. Mol. Biol. 319, 1165–1176
23. Wenninger, M., and D¨uger, J. P. (2001) Mol. Pharmacol. 60, 790–796
24. Weiland, G. K., and Schmidt, J. (1980) J. Biol. Chem. 255, 7648–7656
25. Brookes, J. P., and Hall, Z. W. (1975) Biochemistry 14, 2092–2099
26. Wang, G. K., and Schmidt, J. (1980) J. Biol. Chem. 255, 11156–11162
27. Planagan, S. D., Barondes, S. H., and Taylor, P. (1976) J. Biol. Chem. 251, 855–865
28. Mandell, J. G., Roberts, V. A., Pique, M. E., Klotovyi, V., Mitchell, J. C., Nelson, E., Tsigelny, I., and Ten Eyck, L. F. (2001) Protein Eng. 14, 105–113