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Nuclear Magnetic Resonance (NMR) Analysis of Ligand Receptor Interactions: The Cholinergic System — A Model

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ABSTRACT: Elucidation of the molecular mechanisms that govern ligand-receptor recognition is essential to the rational design of specific pharmacological reagents. Whereas often the receptor and its binding site are the target of investigation, study of the ligand in its free and bound state can also reveal important information regarding this recognition process. Nuclear magnetic resonance (NMR) spectroscopy can be extremely useful for such studies. In this review, we discuss the attributes of NMR in the study of ligand receptor interactions. The cholinergic receptor and its binding to the neurotransmitter, acetylcholine, and cholinergic antagonists serve as a model system, illustrating the power of ligand analysis by NMR. The results discussed prove that the region of residues α 180–205 of the nicotinic acetylcholine receptor are an essential component of the cholinergic binding site and that ligand binding involves a positively charged hydrophobic motif.

KEY WORDS: nuclear magnetic resonance, ligand receptor interactions, cholinergic system, pharmacological reagents.

I. INTRODUCTION

The molecular mechanisms that govern biorecognition are the essence of cellular communication. Cell surface receptors interact specifically with ligands such as hormones, toxins, neurotransmitters, bacteria, and viruses. Thus it is obvious that a genuine, in-depth understanding of how ligands interact with their corresponding receptors would provide the means to rationally design novel receptor mediated drugs and therapeutics as well as a new spectrum of diagnostics. Therefore, much receptor-based research focuses on the ligand/receptor interaction.

Traditionally, the elucidation of the structure/function of the ligand binding site has been predominantly biochemical, encompassing methods such as affinity chromatography, Scatchard binding and kinetic analyses, protein blotting, and affinity cross-linking. With the advent of molecular genetics, cloning and mutagenesis followed by expression of receptors have become the golden standard for most receptor studies. We have even reached a new era where we have more receptors than ligands: a situation in complete contrast to the classic phar-

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macological approach for which receptors were ligand defined. For the most part, what we know about binding sites has been through the systematic analysis of the receptor and often phylogenetic comparisons of receptor genes of various beasts.

An alternative approach could be the study of the ligand proper with the intent of extrapolating from its structure to that of the corresponding domain in the receptor — essentially, studying the key with the hope of finding the lock. Two points must be considered with this approach: (a) by identifying the functional moieties of the ligand, one can assume that the binding site should contain complementary functional elements. Clearly, we would expect a negative charge of the ligand to be electrostatically charge neutralized by a positive component in the receptor. Hydrophobic regions should be complemented by opposing hydrophobic moieties and so on and so forth; (b) It may be assumed that the ligand will be substantially simpler in structure than the proteinaceous receptor. Therefore, elucidation of the three-dimensional atomic structure of the ligand could lead to the solution of the ligand binding site as well. This type of logic is based on the premise of ligand rigidity (i.e., the free ligand reflects faithfully its structure in the receptor-bound state). Unfortunately, this may not be the case and there is an increasing number of examples where it became apparent that induced conformational changes occur upon association of the ligand with its corresponding receptor. Thus, for example, antigens have been shown to both conform and induce structural changes in the Fv regions of immunoglobulins. Clearly, if one were able to study the receptor ligand complex then it would be possible to more rigorously focus on the nature of this recognition event.

The purpose of this review is to illustrate the use of NMR spectroscopy as an effective means to study ligand receptor interactions. As a case in point, we investigated the nicotinic acetylcholine receptor and its cholinergic ligands. This is not an extensive survey of all that is known about the receptor nor what has been learned about the structure of one ligand or another. It is our purpose to use the cholinergic system as a model from which the attributes of NMR will be emphasized with the hope of inspiring others to adopt this method to better understand biorecognition in their pet system.

II. STRATEGIES FOR STUDYING LIGAND STRUCTURE

The object of solving the structure of a ligand is to determine the position and orientation of its components in three-dimensional space. This can be achieved through the application of various biophysical methods, primarily, by X-ray diffraction and NMR spectroscopy. Crystallography depends on our ability to generate crystals of the ligand that defract X-rays. Once such crystals are produced, one can derive a structure of detailed atomic resolution for the ligand. Although the structure represents a ligand in an unnatural configuration — closely packed and organized — very often the crystalline structure has been found to closely represent the overall solution structure as determined by other methods (e.g., NMR). This is the case for the polypeptide cholinergic antagonists α-bungarotoxin and cobratoxin, whose solution structures determined by NMR (Kosen et al., 1988; Chin et al., 1993, respectively), are generally similar to their crystal structures (Love and Stroud, 1986; Low and Corfield, 1986, respectively). Similar comparisons can be made for *d*-tubocurarine and acetylcholine (*vide infra*).

The increasing application of NMR spectroscopy to the elucidation of the detailed structure of molecules as large as medium size proteins is due to the rapidly progressing technology in this field. Moreover, the ability to study structures in solution obviates the intrinsic objection to X-ray crystallography.

In recent years, with the development of multidimensional NMR techniques (see Wuthrich, 1986; Clore and Gronenborn, 1991), the structures of many biological determinants have been elucidated in solution. The nuclear Overhauser effect (NOE) and its two-dimensional version, NOESY, are widely applied techniques for determining the solution structures of small molecules (Wuthrich, 1986; Noggle and Schirmer, 1971; Neuhaus and Williamson, 1989).

The NOE is the change in intensity of one resonance upon irradiation of another resonance, belonging to a group situated a short distance away, so that the dipolar interaction between them affects the relaxation time. An NOE enhancement is obtained for small molecules, with tumbling correlation times, τ_c, smaller than the inverse of the resonance frequency $1/\omega_o$. For large molecules, with $\tau_c \gg 1/\omega_o$, a negative NOE is obtained. For medium size molecules, with $\tau_c \cong 1/\omega_o$, no NOE is obtained. For instance, for a field corresponding to a proton frequency of 500 MHz, $1/\omega_o = 3.18 \times 10^{-10}$ s^{-1} and molecules with τ_c close to this number have a molecular weight of 1000. For these molecules, a similar technique named ROESY (Bothner-By et al., 1984; Bax et al., 1985) can be used. In this technique, an NOE enhancement is obtained regardless of the size of the molecule. Recently, new multidimensional NOE techniques (Neuhaus and Williamson, 1989) combining the use of isotopically enriched molecules with isotope editing schemes (Wuthrich, 1989) enabled the determination of relatively large structures, including those of bound ligands (Fesik et al., 1991; Weber et al., 1991) and of protein-ligand complexes (Theriault et al., 1993).

Both X-ray and the NMR techniques, however, have had to deal with complexes that have high association constants. The main reason is that it is hard to obtain crystals adequate for X-ray studies from complexes with low association constants. Likewise, the direct NMR techniques require that the complex be the major species in the course of the time scale of the experiment. Transferred (tNOE) pulse sequences (Bothner-By and Gassend, 1972; Albrand et al., 1979; Clore and Gronenborn, 1982; Landy and Rao, 1989; Sykes and Campbell, 1993; Zheng and Post, 1993) enable the study, by NMR, of ligands bound to large proteins with relatively low association constants (0.1 m*M* to ~2 m*M*). The technique is based on the transfer of magnetization, affected by cross relaxation, from the bound ligand to the free ligand, via chemical exchange. TNOE is simple to observe because the detected species is the excess free ligand, whose resonances are narrow and relatively strong. However, tNOE is observable only when there is fast exchange between the free and bound states of the ligand. The residence time of the ligand must be smaller than its relaxation time in the bound state. Some complications due to spin diffusion in the bound state may also occur (Campbell and Sykes, 1991; Sykes and Campbell, 1993). In recent years, a growing number of studies have applied the technique to various systems (Meyer et al., 1988; Anglister et al., 1990; Milon, 1990; Ni et al., 1990; Campbell and Sykes, 1991; Cung et al., 1991; Tsikaris et al., 1993; Lippens et al., 1993).

A. Obtaining Distance Geometries by NMR

The change in the signal intensity in the NOE experiment amounts to $0.5\,\sigma/\Sigma\rho$, where σ is the cross relaxation term, $\sigma = \gamma\hbar$, and ρ is the internal relaxation (Mirau, 1988). In the two-dimensional NOE experiment (NOESY), a relaxation matrix is obtained whose diagonal and off-diagonal terms are ρ and σ, respectively. The values of the cross relaxation terms can be converted to distances by the use of Equation 1.

$$\frac{n_{ij} \cdot \sigma_{kl}}{n_{kl} \cdot \sigma_{ij}} = \left(\frac{r_{ij}}{r_{kl}}\right)^6 \qquad (1)$$

where n_{ij} and n_{kl} are the different number of protons involved in each interaction and the σ's are the calculated cross relaxation terms. By using a certain interproton distance as a "ruler," all other distances can be calculated. These distances are further processed by distance geometry algorithms that result in a calculated three-dimensional structure of the molecule being analyzed.

The distance geometry algorithm is popular because it efficiently produces structures consistent with the input distance data. The ideal case is where these distances are the result of single-nuclei interactions, but this is not the common case. Some complications occur when a single resonance represents a group of equivalent nuclei, such as the case of a methyl group or the trimethylammonium group of acetylcholine. In large molecules, where the contribution of such groups to the calculation of the overall structure is relatively small, the simple "pseudoatom" approximation is adequate. In this approximation, the nuclei of a multinuclear group are assumed to be located together and at the center of the group. For instance, the three protons of a methyl group are represented by a pseudoatom situated at the center of a triangle formed by the three of them (Tropp and Redfield, 1981). Because the pseudoatom simplification is not completely accurate, the distances calculated between methyl proton groups and other protons are further processed with wide constraints (Banks et al., 1989). Equation 1 shows that σ's and their corresponding r values are related to one another by the 6th power. Therefore, the pseudoatom approach underestimates the distance between the two groups. As an example, consider a group of two protons whose distances from a third interacting nucleus are 1 and 2 Å. Using the pseudoatom approximation, the distance between the center of the group and the third nucleus is calculated as 1.12 Å instead of the true distance of 1.5 Å. Due to this inaccuracy, we chose not to use pseudoatoms and wide constraints.

B. Application of "σ Back Calculations"

We applied a "σ back calculation" where we calculated the σ value of all the group-group interactions from all the sub interactions that are the single proton-proton distances. We iterated the structure determination and σ calculation until we obtained good correlation (10%) between the experimental and calculated σ values.

In our studies of acetylcholine distance geometry, calculations were performed with DSPACE (D. Hare Inc., version 4.0). A distance bounds matrix was created on the basis of the covalent structure of acetylcholine together with the distance constraints derived from the relaxation matrix. Methyl groups were entered as a three-hydrogen group with the known distances and angles between these atoms. The algorithm was applied such that the distance constraints

were used initially for a single pair of the hydrogen atoms i_a and j_b of the two interacting groups i and j yielding an initial geometry. From the resulting geometry, all distances from each proton in the i group to each proton in the j group were entered into Equation 1, obtaining values of σ_{cal}, and the result of summing over all the protons in the group gave the experimental σ value for the i-j interaction. In case of discrepancy, the calculated geometry was discarded and a new one was calculated and subjected to a new σ back calculation. Thus, the correct geometry must fulfill the following condition for all i-j interactions in the molecule.

$$\frac{1}{n_{ij}^*} \sum_{a,b=1}^{a,b} ia * jb * \sigma_{cal} = \sigma_{ij_{exp}} \quad (2)$$

Every calculation was done by subjecting the structure to a few cycles of minimization, annealing, and minimizing again so as to obtain a structure whose penalty function value, as defined by the DSPACE program, was minimal. The penalty function involves a combination of deviations from constraints determined by covalent distances, bond angles, as well as distance constraints determined by NOE. Typically, 20 structures for every geometrical determination were studied. The reported structures are those whose average σ value was closest to the experimental one (Equation 2).

C. Obtaining Distance Constraints by NMR

With the technological advances in NMR machines, we are able to obtain higher resolution and better sensitivity. This has allowed us to work with lower concentrations of ligands to the degree that we could work with a 1:1 complex of *d*-tubocurarine bound to a synthetic peptide. The 17-amino acid peptide that corresponds to the α182–198 sequence of the nicotinic acetylcholine receptor of *Torpedo californica* was used.

Due to $\omega\tau_c \approx 1$ in the case of this ligand-peptide complex at our field of 500 MHz, ROESY experiments were used to obtain NOE crosspeaks. Automatic processing enables performance of any number of mixing times, allowing observation of many NOE peaks that may not have been detected in a single experiment. The spectra were processed using TRIPOS' SYBYL, and peak volumes were obtained using the SYBYL automatic peak picking capability using peak refinements and defining the minimum peak width. Peaks originating from the protein were discounted on the basis of their chemical shifts.

The peak volumes from SYBYL were then transferred to matrix analysis of relaxation for discerning geometry of an aqueous structure (MARDIGRAS) (Borgias and James, 1990; Liu et al., 1995). MARDIGRAS first calculates a theoretical spectrum based on the mixing time, the chemical shift assignment, *J*-couplings, and the correlation time. This can be done independently using CORMA (Borgias and James, 1988). It then substitutes the experimental values into the complete relaxation matrix and diagonalizes it. After each iteration, the experimental values are reintroduced into the matrix and only distances based on experimental values are given at the end of the process.

MARDIGRAS solves the matrix of the whole system taking spin diffusion into account, thus allowing the use of longer mixing times in order to allow low-intensity peaks to develop. MARDIGRAS was performed on the peak volumes obtained from the experiments at each mixing time to get distance constraints. However, only those originating from crosspeaks that were present in three or more mixing times were ultimately used in the minimization proce-

dure in order to further eliminate spurious peaks. The initial model for the MARDIGRAS analysis was the X-ray structure of *d*-tubocurarine dichloride pentahydrate (Codding and James, 1973), where the proton positions were calculated. The structures were calculated using the methyl jump model, and the noise level was absolute and normalized to 0.25%. The MARDIGRAS distance-ranges were used as constraints in the SYBYL minimization procedure using a drawn and minimized structure as a starting structure. Powell minimization followed a simplex initial optimization.

III. THE CHOLINERGIC SYSTEM

A. The Nicotinic Acetylcholine Receptor

The nicotinic acetylcholine receptor is a ligand gated ion channel representative of this family of receptors that includes, for example, the glycine receptor and the $GABA_A$ receptor (for reviews regarding the acetylcholine receptor see Changeux et al., 1992; Devillers-Thiéry, et al., 1993; Unwin, 1993; Conti-Tronconi et al., 1994; Sansom, 1995). A model suggesting similarities among the ligand gated ion channel binding sites has also been published (Cockcroft et al., 1990). When the neurotransmitter acetylcholine binds to the receptor it elicits membrane depolarization of the sarcolemma that initiates muscle contraction. It is through this mechanism that the nerve communicates with the muscle. Various compounds such as *d*-tubocurarine and snake derived α-neurotoxins are competitive antagonists of acetylcholine. This system has proved to be extremely convenient for biochemical and molecular analyses due to two main reasons: (1) the receptor exists in very high concentrations in the electric organ of the ray *Torpedo* and that of the eel *Electrophorus,* and (b) the snake-derived toxins have enabled efficient purification and affinity labeling of the receptor.

The acetylcholine receptor comprises five polypeptide chains ($\alpha_2\beta\gamma\delta$), all of which are integral membrane glycoproteins spanning the lipid bilayer four times. Affinity labeling of the receptor identified a number of residues in the α-subunit that are in close proximity to the ligand binding site. These are a cysteine pair (Cys192 and Cys193) (e.g., Kao and Karlin, 1984; Kao and Karlin, 1986; Pedersen et al., 1986; McCormick et al., 1993) and a number of aromatic residues (e.g., Tyr51, Tyr93, Tyr189, Tyr190, Trp86, Trp149, and Trp184) (e.g., Dennis et al., 1988; Galzi et al., 1990; Cohen et al., 1991; Fraenkel et al., 1991a; Galzi et al., 1991b; McCormick et al., 1993; McLane et al., 1994; Barchan et al., 1995). Recently, the γ and δ subunits have been labeled by reactive agonists, indicating that the acetylcholine binding sites are located at the interfaces of the α–γ and α–δ subunits (Czajkowski et al., 1993; Conti-Tronconi et al., 1994; Utkin et al., 1994; Czajkowski and Karlin, 1995; Fu and Sine, 1994). The α–γ and α–δ binding sites differ, as can be seen not only by their different components but also by the difference in their affinity toward agonists and antagonists: The α–γ binding site has been consistently found to have a higher affinity toward *d*-tubocurarine than the α–δ binding site (Utkin et al., 1994; O'Leary et al., 1994; Fairclough et al., 1993). Furthermore, α-conotoxins and α-neurotoxins also show different affinities for the two binding sites (Utkin et al., 1994; Kreienkamp et al., 1994; Martinez et al., 1995). The differential binding may be the result of glycosylation that selectively interferes with the binding of α-bungarotoxin (Kreienkamp et al., 1994). It has been shown that certain conserved residues in the γ and δ subunits may promote different quaternary arrange-

ments (Kreienkamp et al., 1995). Lipid-protein interactions have been shown to affect receptor function, suggesting that the lipid composition may have an effect on the secondary structure of the receptor effecting binding allosterically (Lee et al., 1994; Fernandez-Ballester et al., 1994; Blanton et al., 1994; Narayanaswami et al., 1993; Narayanaswami and McNamee, 1993; Sunshine and McNamee, 1994). The identification of a binding component of the binding site within the α-subunit has been possible through the direct means of ligand overlay of protein blots of either acetylcholine receptor-purified subunits, proteolysed subunits, bacterially expressed recombinant peptides, or synthetic peptides (Gershoni, 1983; Gershoni, 1987a; Gershoni, 1988; Neumann, 1985; McCormick et al., 1993; McLane et al., 1993b; Fulachier et al., 1994; Barchan et al., 1995). All these studies support that the region of α183–204 (residues GWKHWVYYTCCPDTPLYDITYH in *Torpedo californica*) can directly associate with various cholinergic ligands such as α-bungarotoxin and *d*-tubocurarine (see also Aronheim et al., 1988; Ohana, 1990; Ohana, 1991). Though this sequence does not contain all the components of the complete binding site, it specifically binds agonists and contains essential elements for ligand binding.

Although the structure of the acetylcholine receptor has not yet been solved, at atomic resolution the many diverse methods used to study the receptor have yielded a detailed picture of the receptor and its function. The beautiful works by Unwin provide crystallographic images at 9 Å resolution of the receptor in its closed and open states, enabling postulations as to the structural basis of channel gating (Unwin, 1993; Unwin, 1995; Sansom, 1995). Fluorescence can indicate the proximity of substructures to binding sites (e.g., Valenzuela et al., 1994). Photolabeling can show distances between different moieties within the receptor and can give us the distance between bound ligands and specific amino acids, presumably from within the active site (Machold et al., 1995; Cohen et al., 1991). It can also give evidence for the existence of distinct binding sites for different ligands, as in the case of physostigmine (Schrattenholz et al., 1993). Conformational changes in the secondary structure of the receptor upon binding ligands can be investigated using Fourier transform infrared spectroscopy (e.g., Castresana et al., 1992; Baenziger et al., 1993; Méthot et al., 1994) and Raman spectroscopy (Aslanian et al., 1993). Site-directed mutagenesis determines the degree to which binding is dependent on specific amino acids in the different subunits (see above). Taken together, a picture of the structure and function of the receptor is emerging and greater detail relating specific amino acids to the binding process is being realized (Changeux et al., 1992; Léna and Changeux, 1993; Devillers-Thiéry et al., 1993; Changeux, 1995; see also Kachalsky et al., 1995).

B. Aromatic Residues Involved in Choline Recognition

Dougherty and Staufer (1990) studied synthetic receptors for acetylcholine and showed the importance of the interaction of the quaternary ammonium group with aromatic residues. In their studies they explain the unexpected cation-aromatic interactions based on theoretical calculations and experimental findings. The quaternary ammonium group of acetylcholine interacts with aromatic residues rather than with negative charges, as in the classic electrostatic model. Dougherty had recently coined such interactions Cation-π interactions and reviewed their scope in biological systems (Dougherty, 1996). Studying another natural acetylcho-

line binding site, that of the acetylcholine esterase, has also been fruitful. It has revealed the presence of tryptophan in the active site of acetylcholine esterase by spectroscopic and chemical modification studies (Blumberg and Silman, 1978; Goeldner and Hirth, 1980). A recent affinity labeling study identified Trp84 as part of the putative acetylcholine binding site in the enzyme (Weise et al., 1990). Moreover, with the elucidation of the three-dimensional crystal structure of acetylcholine esterase (Sussman et al., 1991), additional information regarding the requirements for acetylcholine binding was obtained. First, this study confirmed the presence of Trp84 in the binding site. It has also reaffirmed and accentuated the presence of aromatic residues in the vicinity of the binding site. Sussman et al. (1991) found a substructure they coined the 'active site gorge.' This gorge, which contains the binding site, is lined with 14 aromatic residues. These form an 'aromatic guidance' structure that is the primary point of interaction of acetylcholine with the esterase.

Structural data obtained from the complex formed between acetylcholine esterase and three different ligands reveal the fact that the bound ligands are within close proximity of aromatic residues (Silman et al., 1994). In the decamethonium-acetylcholine esterase complex, the two quaternary ammonium groups are in Van der Waals contact with Trp84 and Trp279. The same phenomenon occurs for the edrophonium-acetylcholinesterase and tacrine-acetylcholinesterase complexes, where the ammonium groups lay in close proximity to Trp84 (Silman et al., 1994). Thus, the so-called 'anionic' part of the cholinergic binding site is characterized by its high content of aromatic residues rather than by negatively charged amino acids. This experimental evidence adds to the increasing data found in numerous studies (*vide infra*) that the contribution of aromatic residues is important to the binding of cholinergic ligands.

1. NMR Analysis of Acetylcholine Binding to Recombinant Peptides

An important point in favor of NMR as a tool for studying geometry is the fact that the NMR study is done in solution; thus, one can study and monitor the dynamics of the interaction (e.g., association/dissociation). Valensin et al. (1982) showed that the selective T_1 relaxation technique is an important tool for studying interactions of small ligands with macromolecules. Kushnir and Navon (1983) quantitated the T_1 relaxation technique for determining binding constants and applied it to the measurement of the binding of carbobenzoxyglycine to carboxypeptidase A. This method was applied to the measurement of the binding of various agonists to the intact acetylcholine receptor of *Torpedo californica* (Behling et al., 1988; Navon et al., 1988). Once the region of α162–210 was identified as the α-bungarotoxin binding site (Neumann et al., 1985), synthetic peptides corresponding to this area were produced and shown to bind a variety of cholinergic antagonists (e.g., 182–198) (Mulac-Jericevic and Atassi, 1986), 185–196 (Neumann et al., 1986a; Neumann et al., 1986b), 188–201 (Gotti et al., 1988), 179–191 (Radding et al., 1988), 173–204 (Wilson and Lentz, 1988), 185–200 (McCormick et al., 1993), and 181–200 (McLane et al., 1994). These experiments confirmed that ligand recognition can exist for a fragment of an isolated subunit, and the preconception that complicated tertiary and quaternary conformational constraints are a prerequisite for binding does not necessarily hold. In order to evaluate which residues are directly involved in ligand binding, various synthetic peptides were tested

and recombinant binding sites were constructed and expressed in bacteria. This allowed scrutinizing them using the arsenal of methods molecular biology affords (Barkas et al., 1987; Gershoni, 1987a). Binding sites derived from a diversity of organisms were produced and tested for their binding activity (Ohana and Gershoni, 1990; Ohana, et al., 1991; Barchan, et al., 1995).

The question whether any of these sites specifically recognizes the cholinergic agonist could not be addressed by standard biochemical analyses. This is due to the fact that Scatchard analysis and pharmacological competition assays demand efficient separation of bound ligands from free ones within a time frame shorter than the half-life of the site/ligand complex, a condition that could not be satisfied due to the low affinity agonists show for the synthetic or recombinant peptides. Here, selective T_1 relaxation has proved to be an ideal method. The principle for this assay is that one monitors the behavior of a specific moiety of the ligand in the presence or absence of the candidate binding site. If the association/dissociation is very rapid (rapid chemical exchange) and the duration of ligand occupancy of a site is markedly less than the relaxation time, then even relatively few binding sites can affect the overall relaxation rates of a concentrated solution of the ligand. Thus, the acetyl methyl group of acetylcholine could be shown to relax (Figure 1b demonstrates a general picture of the effect) at a faster rate in the presence of recombinant binding sites, and no such effect was measured for the negative controls (Fraenkel et al., 1990).

By determining the K_D's of acetylcholine, nicotine, *d*-tubocurarine, and gallamine to the various genetically engineered sequences derived from the acetylcholine receptor of a few species (See Figure 2 for their sequences), we were able to demonstrate that the α183–204 sequence contains the binding site for acetylcholine and other cholinergic ligands (Fraenkel et al., 1990; Fraenkel et al., 1991).

To further substantiate the selectivity of these effects, one could demonstrate that the accelerated rate of relaxation was quantitatively lost at ever increasing concentrations of α-bungarotoxin. This titration of the binding sites was used to measure both the number of binding sites in a given sample (see Figure 3a) and their affinity for such ligands as acetylcholine, nicotine, and gallamine.

2. How Snakes Escape the Effect of Their Own Toxin

Snakes have been shown to be resistant to cobratoxin because their receptor cannot bind this antagonist even at concentrations up to 10^{-5} *M* (Burden et al., 1975). Mongooses and hedgehogs, which feed on snakes, do not bind α-bungarotoxin, although they bind acetylcholine (Kachalsky et al., 1993; Kachalsky et al., 1995; Barchan et al., 1995). What is the molecular basis for this resistance?

Once the sequence of the area of cobra acetylcholine receptor α183–204 was determined (Neumann et al., 1989) the recombinant binding site system (Gershoni, 1987b; Aronheim et al., 1988; Ohana et al., 1990) was applied to the site of cobra (Ohana et al., 1991). Indeed it was found that the site did not bind α-bungarotoxin even at concentrations of 10^{-2} *M*. Furthermore, systematic site-directed mutagenesis proved the particular importance of position 189, which undergoes a transition from tyrosine to asparagine when going from *Torpedo californica* to snake. It was necessary, however, to evaluate what effect all of the six amino acids that differ between the snake and *Torpedo* had on acetylcholine binding. For this, selective T_1 relaxation NMR was

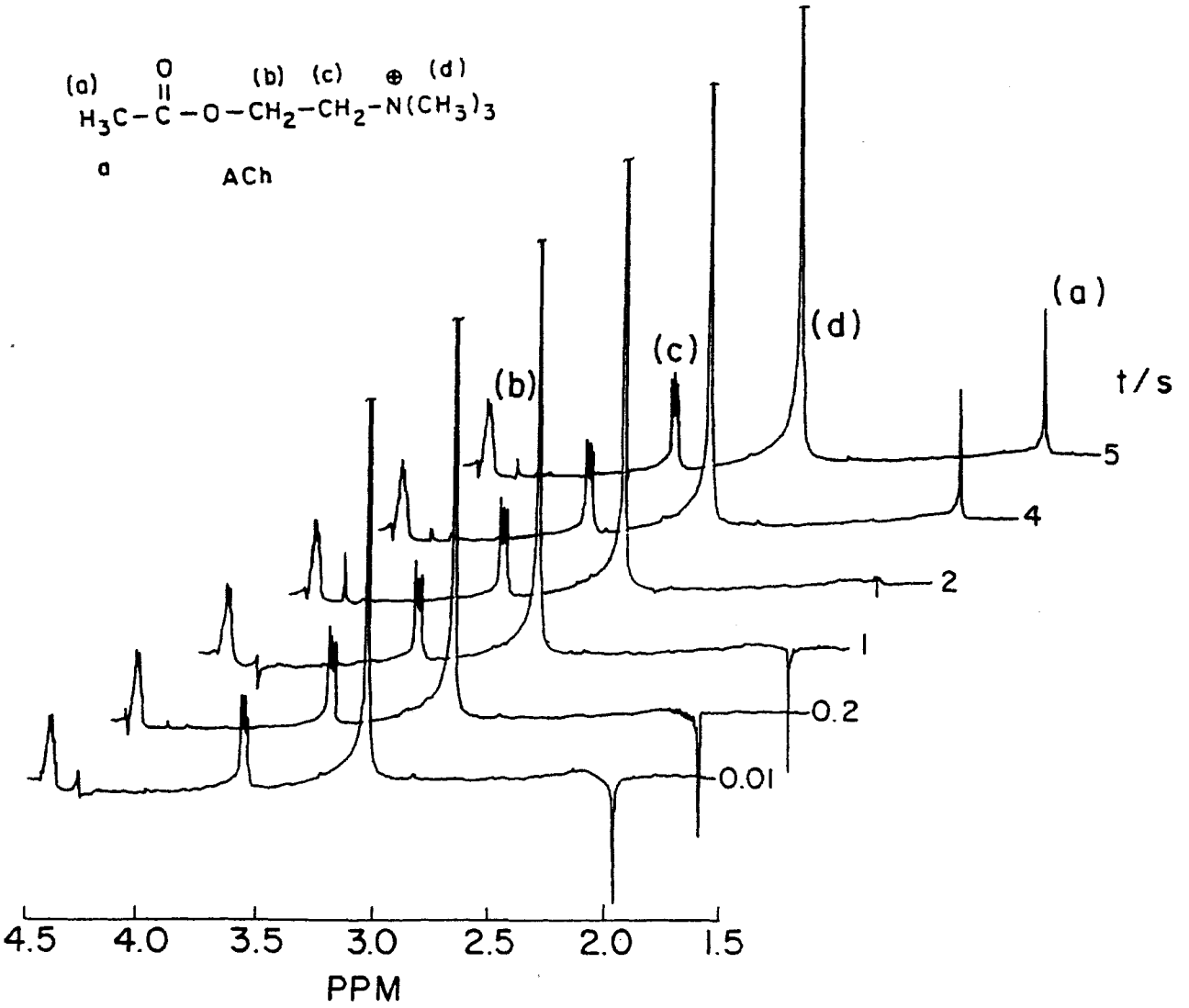

FIGURE 1. (A) Structure of acetylcholine. (B) 1D spectra of acetylcholine demonstrating the selective T_1 measurement of the acetylmethyl group.

Torpedo	G-W-K-H-W-V-Y-Y-T-C-C-P-D-T-P-Y-L-D-I-T-Y-H
Chicken	G-W-K-H-W-V-Y-Y-**A**-C-C-P-D-T-P-Y-L-D-I-T-Y-H
Xenopus	G-W-K-H-W-V-Y-Y-T-C-C-P-D-**K**-P-Y-L-D-I-T-Y-H
Mouse	G-W-K-H-W-V-**F**-Y-**S**-C-C-P-**T**-T-P-Y-L-D-I-T-Y-H
Calf	G-W-K-H-W-V-**F**-Y-**A**-C-C-P-**S**-T-P-Y-L-D-I-T-Y-H
Human	G-W-K-H-**S**-V-**T**-Y-**S**-C-C-P-D-T-P-Y-L-D-I-T-Y-H
Snake	G-**F**-**W**-H-**S**-V-**N**-Y-**S**-C-C-**L**-D-T-P-Y-L-D-I-T-Y-H

FIGURE 2. Amino acid sequences for 183–204 domains on the α-subunit of the acetylcholine receptor for selected species. The differences from *Torpedo californica* are emphasized.

found to be indispensable. The snake binding site did affect the relaxation rate of acetylcholine; however, titration with α-bungarotoxin was obviously impossible. Therefore, selectivity was demonstrated by titrating the sites with *d*-tubocurarine (see Figure 3b). These experiments illustrate well the utility of this NMR strategy that enabled the explanation of the selective binding activity of the snake receptor.

IV. GEOMETRY OF LIGANDS

The pioneering hypothesis of Beers and Reich suggested a geometry for acetylcholine and some of its agonists and antagonists when bound, as well as the nature of the amino acids involved in its binding (Beers and Reich, 1970). They based their analysis on the common structural feature among the measured X-ray structures of several ligands. These analyses gave rise to a model of the required geometry of a cholinergic ligand. Consequently, the steric and electronic requirements from the binding site, based on complementarity, were concluded. Thus, all the ligands analyzed fit into a structure that had a positive charge at a distance of 5.9 Å from a hydrogen-bound acceptor oxygen.

A. Geometry of Ligands in the Free State

The central role of acetylcholine as a neurotransmitter provided much incentive for the elucidation of its three-dimensional structure. Acetylcholine is regarded as a substituted ethane with an acetoxy group on one side and a trimethyl ammonium group on the other. Figure 1 shows the acetylcholine molecule. Behling et al. (1988a) reported an acetylcholine structure in solution, determined by the two-dimensional (2D) NOE technique. Calculated interproton distances were found to be similar to those in the crystalline chloride salt as determined by X-ray crystallography. However, applying our σ back calculation to the σ values reported for free acetylcholine in solution (Behling et al., 1988a) gave rise to a somewhat different geometry (Fraenkel et al., 1994), as shown in Figure 5a.

1. d-Tubocurarine

This alkaloid (for its structure and 1D spectrum, see Figure 4) achieved its fame from its use in South America where it is applied to poison darts to paralyze prey. Its mechanism of action is by strongly inhibiting the nicotinic acetylcholine receptor. The

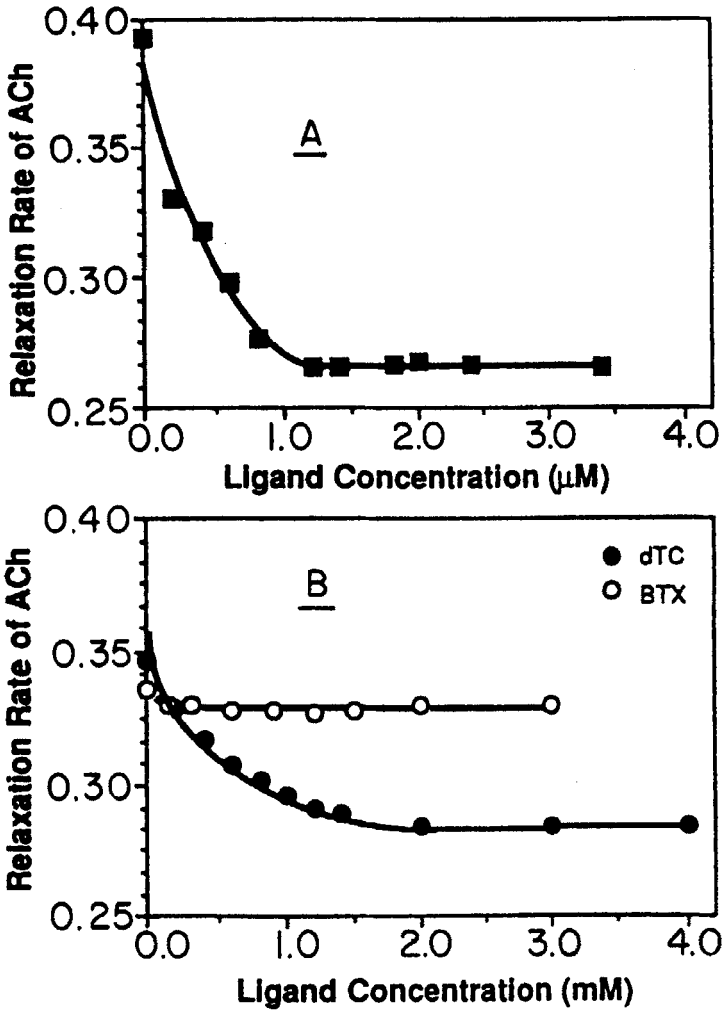

FIGURE 3. Titration curves of acetylcholine bound to *Torpedo californica* (upper graph) and to snake (lower graph) cholinergic binding sites. The T_1 NMR relaxation rate of acetylcholine was measured as described in Fraenkel et al. (1990). As can be seen, α-bungarotoxin was able to effectively displace acetylcholine from the *Torpedo* sequence (top) and had absolutely no effect on that of the snake (bottom, 0). (Note the difference in the scale for the ligand concentrations). On the other hand, *d*-tubocurarine (dTC) was able to titrate the acetylcholine in the snake (0), illustrating that this site is resistant to α-bungarotoxin although sensitive to both dTC and acetylcholine.

geometry of both the dichloride and dibromide salts of *d*-tubocurarine have been determined by X-ray crystallography. Apparently, the *d*-tubocurarine molecule can assume more than one conformation because these two crystalline structures are very different from one another. Whereas the structure of the dibromide salt is extended with an N-N distance of 10.7 Å (Reynolds et al., 1976), the dichloride salt structure is somewhat folded with an N-N distance of 9.0 Å (Codding and James,

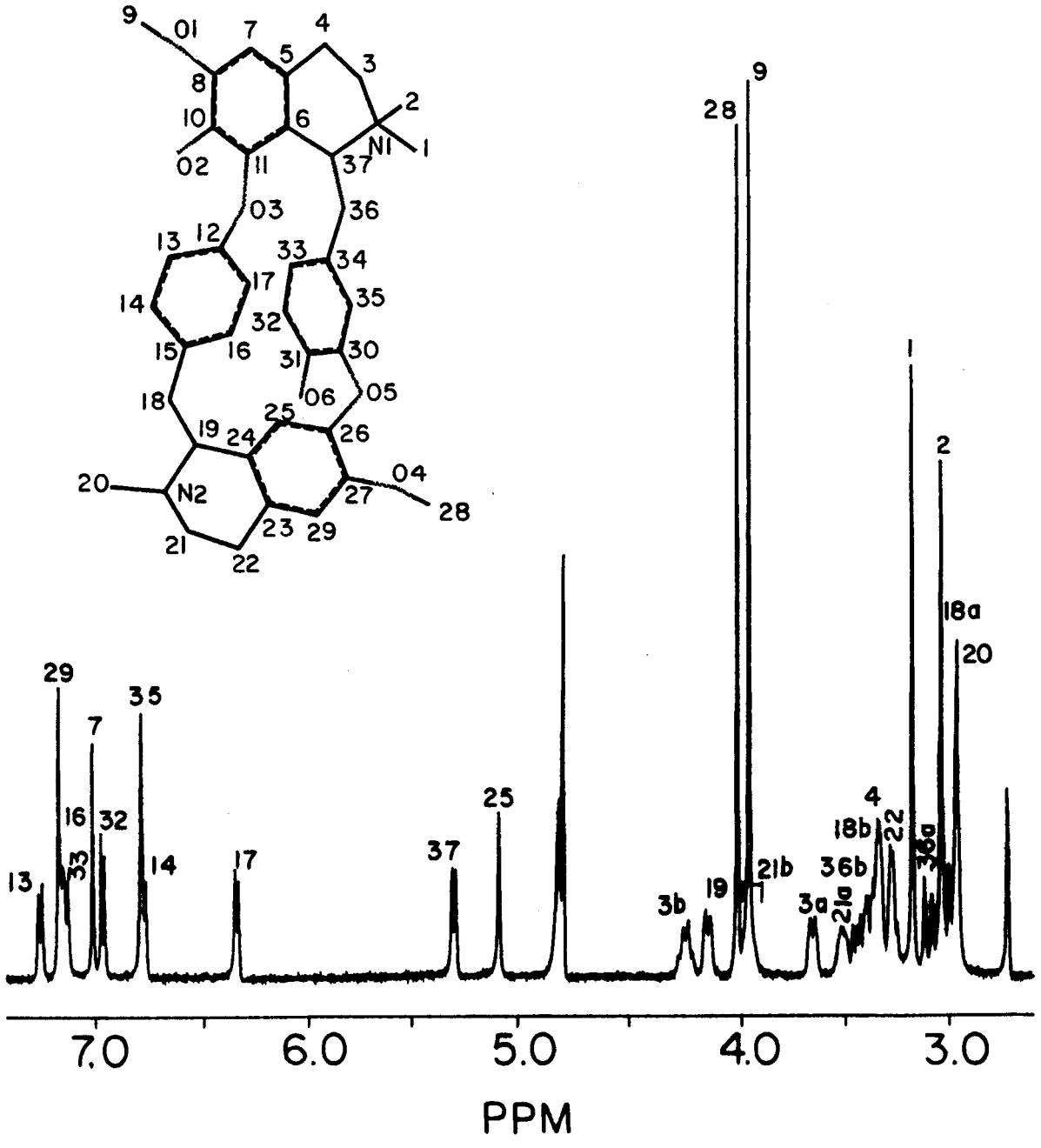

FIGURE 4. *d*-Tubocurarine structure and 1D NMR spectrum.

1973). For both crystalline forms the authors note the presence of a somewhat hydrophobic face. In the case of the dibromide salt, as well as for the methylated derivative of *d*-tubocurarine (Sobell et al., 1972) the hydrophilic face consists of the six oxygen atoms on the convex face of the molecule.

An NMR study was performed for *d*-tubocurarine dissolved in dimethyl sulfoxide (DMSO). The spectrum indicated that the molecule is rigid up to a temperature of 90°C, where the para substituted benzene ring rotates fast enough so that the ABCD spectrum becomes a AA′BB′ spectrum (i.e.,

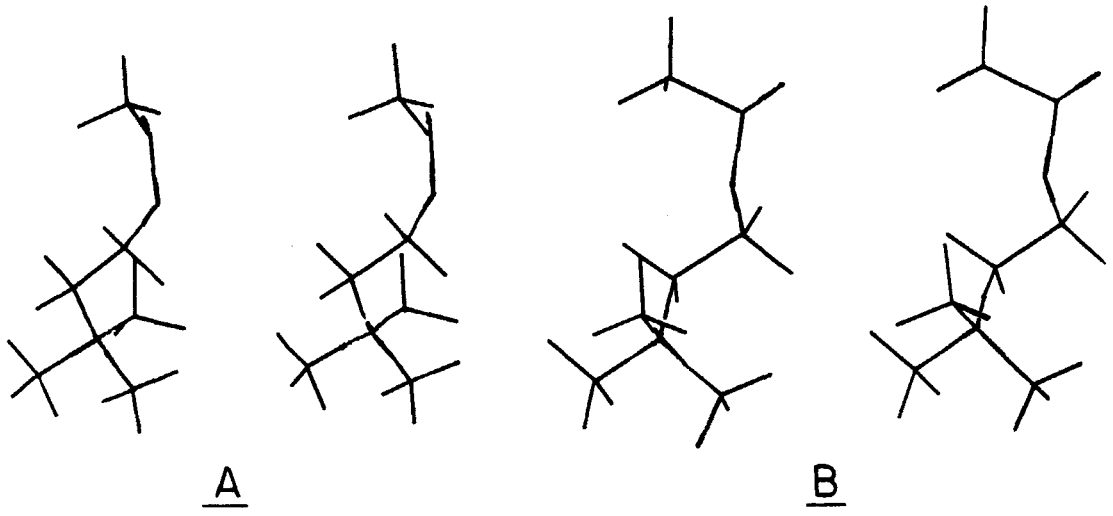

FIGURE 5. Stereoview of acetylcholine geometry, in solution, as calculated by the distance geometry algorithm DSPACE. (A) Free acetylcholine (constraints taken from Behling et al., 1988). (B) Acetylcholine bound to the recombinant α184–200 sequence of *Torpedo californica*. The two geometries are the result of our "σ back calculation" iteration.

the two pairs on the two sides of the benzene ring become chemically equivalent).

2. Semi-Rigid Ligands

The elucidation of the structure of a ligand in solution would be most useful toward understanding receptor structure if both the bound and free ligand conformations were identical — in other words, if the ligand were rigid. The following section deals with the study of semi-rigid ligands. Spivak et al. (1989) synthesized a series of semi-rigid compounds and tested their binding to the acetylcholine receptor. Assuming the validity of the Beers and Reich model, they added other factors to account for their observations of varying potencies of their ligands. They deduced the bioactive geometry of their semi-rigid acetylcholine analogs by initial computer modeling, seeking the lowest energy conformers, followed by fitting these structures to the Beers and Reich model using a 5.9 ± 0.5 Å distance between the site of coulombic interaction (N^+) and the site of hydrogen bonding (C=O) (Waters et al., 1988). However, they did not give any data based on structural techniques such as X-ray or NMR to support their proposed structures.

a. Anatoxin-a

(+)-Anatoxin-a, an alkaloid isolated from blue-green algae (Figure 7), is a very potent acetylcholine agonist. Stereospecificity of the ligand-receptor interaction is demonstrated by the fact that the natural (+) form has a much higher potency than the (–) form (Swanson et al., 1986). Initially, the molecule seemed sufficiently rigid, thus serving as a good model for cholinergic ligands. Its conformation has been determined both in the solid by X-ray and in solution by NMR, based on *J-J* coupling and one-dimensional NOE (Koskinen and Rapoport, 1985). The crystalline and the solution structures showed a strong resemblance, particularly in the ring conformation. However,

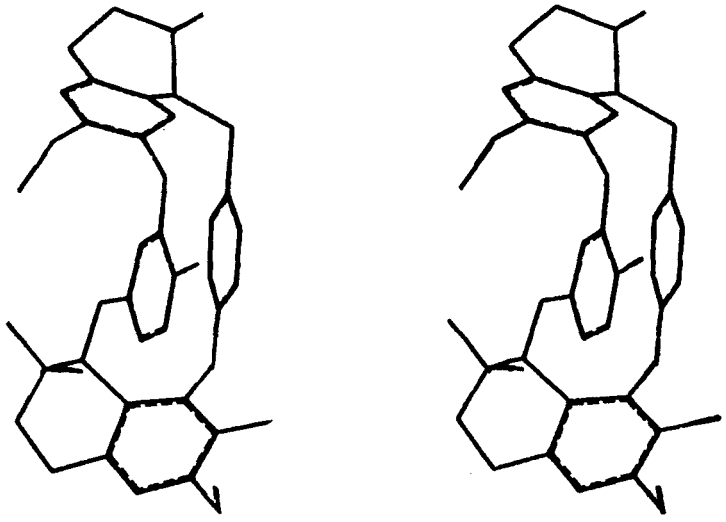

FIGURE 6. Stereoview of *d*-tubocurarine in solution bound to the 182–198 peptide of the α-subunit of the *Torpedo californica*. The structure was calculated from a ROESY spectrum using MARDIGRAS and then SYBYL's distance geometry algorithm.

there are small but rather significant differences between the two structures. These changes are in the relative stereo orientation of the carbonyl relative to the amine groups. The enone system appears to be in an s-*trans* conformation in the crystalline structure, whereas in solution both the s-*trans* and the s-*cis* conformations coexist with the latter prevailing. These two conformations differ in their N-O distance (6.04 vs. 4.48 Å). The small energy difference between the two conformations, as demonstrated by their coexistence in solution, may allow either of them to be stabilized by binding to the acetylcholine receptor.

b. Lophotoxins

This is a family of neurotoxins (Figure 8) isolated from corals that bind covalently and irreversibly to the acetylcho-

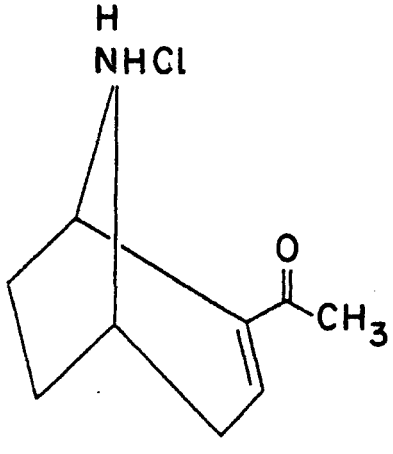

FIGURE 7. (+)-Anatoxin-A.

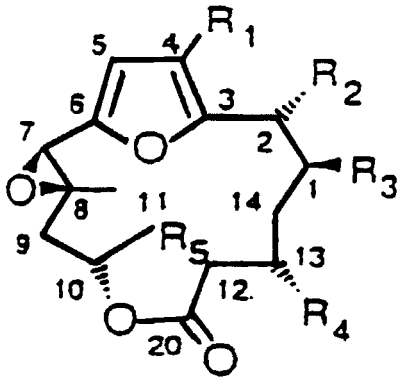

FIGURE 8. General structure of naturally occurring Lophotoxin.

line binding site in the acetylcholine receptor thereby preventing acetylcholine binding (Culver et al., 1985). Testing a series of lophotoxin analogs gave rise to the conclusion that the interacting determinants in the lophotoxin are the electron-deficient C8 that mimics the nitrogen in the classic cholinergic ligands, and the lactone carbonyl C20 for the hydrogen bonding moiety according to the Beers and Reich model (Abramson et al., 1991). X-ray crystallography revealed a structure similar to the minimum energy conformation of the toxin obtained by molecular modeling. Because the toxin ring seems rigid, it was assumed that this is also the solution conformation. As these (predicted) two points of contact (C8 and C20) are connected exactly as in acetylcholine, namely by a $-CH_2-CH-O-C=O$ sequence, the distance between them was of particular interest. The distance between C8 and C20 was found to be 4.9 Å, and the dihedral angle showed a gauche conformation (Abramson et al., 1991). It should be added, however, that although the gauche conformation of the particular sub-lophotoxin structure correlates with the gauche acetylcholine structure, Behling et al. (1988) have shown this is true only for free acetylcholine in solution. In the bound state, acetylcholine adopts a different geometry, and the dihedral angle corresponds to a *trans* conformation. The distance of 4.9 Å, which is 1 Å short of the distance proposed by the Beers and Reich model, does correlate, however, with the bound conformation of acetylcholine (Fraenkel et al., 1994).

B. Geometry of Bound Ligands — Changes in Conformation

Recent studies have demonstrated that, upon binding to their target protein, ligands can undergo profound conformational changes (Fesik et al., 1991; Weber et al., 1991; Rini et al., 1992). Even ligands that are presumed to be rigid or "semi-rigid" are still prone to undergo significant conformational changes. Unless the prerequisite of rigidity is fully maintained, the premise that the structure in the crystalline form will reflect the complementary interface of the binding site in solution (Beers and Reich, 1970) may not be correct. It should be noted that even small differences in the position of key functional groups can have important mechanistic implications. Thus, the geometry of a free ligand either in solution or in a crystal may not necessarily be applicable to the study of the receptor binding

site. The biologically active conformation is that of the bound ligand. Pictorially the binding site may be regarded as a template that forces the ligand to adopt a certain geometry. It is therefore relevant to look at the effect that the binding can have on the ligand's geometry (mutually induced conformational rearrangements are also possible).

1. Bound Acetylcholine

Behling et al. (1988) carried out a study of acetylcholine bound to the purified and solubilized acetylcholine receptor obtained from the electric organ of *Torpedo californica* using the 2D-NOE technique followed by geometrical computations. The nonspecific effect of acetylcholine binding to the membrane was subtracted by using an identical solution containing added α-bungarotoxin as a reference. This specifically displaced the acetylcholine from its binding sites. Because a high concentration of acetylcholine was present in this study, the receptor was in its desensitized state. The result of their study was that bound acetylcholine is no longer an elongated molecule but rather a bent molecule with the acetyl methyl group residing at a distance of 3.3 Å from the trimethylammonium group, in sharp contrast to the values of 5.5 and 5.3 Å present for the free ligand in the crystal and in solution, respectively. The authors made no reference to the N-O distance or whether the bound acetylcholine geometry satisfies the Beers and Reich model. A CPK model built with their reported interproton distances showed a N-O distance of 6.6 Å. This distance contradicts the Beers and Reich model that serves as a base for defining pharmacophores of the acetylcholine receptor (Spivak et al., 1989). The geometry calculation performed by Behling et al. was based on the pseudoatom approximation for the methyl and the trimethylammonium groups. Repeating the calculation using the σ back calculation (Section I) yielded an N-O distance of 5.0 ± 0.1 Å (Fraenkel et al., 1994).

Following our previous finding that the α184–200 sequence of *Torpedo californica* contains the cholinergic binding site (Fraenkel et al., 1990), the next step was to determine the geometry of acetylcholine bound to the α184–200 sequence and learn whether it resembles that of acetylcholine bound to the intact receptor. Applying the transferred 2D-NOE technique followed by solving the relaxation matrix, we obtained interproton distances that are practically the same as those of acetylcholine bound to the intact receptor (Behling et al., 1988). These distance constraints were used to obtain three-dimensional geometry with the distance geometry algorithm DSPACE (Hare Research, Inc.), and the σ back calculation iteration was applied. The resulting geometry, shown in Figure 5b, is somewhat different than that of Behling et al. (1988) but fits the Beers and Reich model in that the N-O distances, both in the bound and free ligand as calculated using the σ back calculation iteration, were found to be practically the same 5.0 ± 0.1 Å.

In the past, conclusions regarding the geometry of bound acetylcholine were not well accepted because they seemed to contradict the so-called pharmacophoric points of contact of acetylcholine, namely the positive nitrogen and the carbonyl oxygen. The bent geometry seemed to alter the distance between these two points of contact to one different from that predicted. Our calculations on the free and bound acetylcholine show that the N-O distance is unchanged despite the conformational change observed upon binding. Thus, to the extent that the X-ray structure of crystalline acetylcholine reflects a biologically active conformation, our results indicate that apparently there

TABLE 1
Interproton Distances in Angstroms between Aminomethyls and Methoxymethyls in *d*-tubocurarine Bound to the α182–198 (T17) Peptide

	Distances in angstroms					
	O1	O2	O3	O4	O5	O6
N1	6.3	6.0	3.9	6.4	6.2	8.1
N2	11.9	9.8	8.0	6.4	5.9	8.5

TABLE 2
Distances in Angstroms between Oxygens and Nitrogens in *d*-tubocurarine Bound to the α182–198 (T17) Peptide

Aminomethyl protons	Methoxymethyl protons	T17 distance (A)
C1	C9	7.6
C1	C28	4.8
C2	C9	6.9
C2	C28	6.9
C20	C9	7.1
C20	C28	8.4

exists no contradiction with respect to the N-O distance. The pharmacologists focused on the N-O distance, whereas NMR has revealed the bent geometry so as to create a hydrophobic domain with similar N-O distance.

2. Bound d-Tubocurarine

As mentioned above, acetylcholine bound to the α184–200 sequence shows a similar conformation to that bound to the intact receptor. Thus, the α184–200 sequence was further used to obtain the bound conformation of *d*-tubocurarine. The structure and assignment of *d*-tubocurarine are given in Figure 4. The configuration of bound *d*-tubocurarine is a bent molecule with an inner cleft, as shown in Figure 6. The interior is hydrophobic, with the outer six oxygen atoms forming a hydrophilic shell. The conformation shown in Figure 6 shows the same main features as those given in Fraenkel et al., 1991b for *d*-tubocurarine bound to the recombinant peptide. However, due to an error in assignment this structure is currently being revised. The correct N-O distances are shown in Table 1. Though *d*-tubocurarine has no carbonyl oxygens, both nitrogens have N-O distances that fulfill the Beers and Reich model (see Table 1; N1-O2 6.0 Å and N2-O5 5.9 Å). The interproton distances between the methoxy groups of C9 and C28, and the amino methyls C1, C2, and C20 are shown in Table 2. These distances can be compared with the analogous distance in bound acetylcholine, which is 3.3 Å. Note that the shortest dis-

tance between a methoxy methyl and an amino methyl in *d*-tubocurarine is between the C1 amino methyl and the C28 methoxy methyl and is 4.8 Å. The conformation of *d*-tubocurarine bound to the 15-mer α184–198 peptide was also determined. *d*-Tubocurarine bound to the 17-mer α182–198 peptide is more bent than when bound to the 15-mer, suggesting that the two amino acids α182 and α183 may play a role in forcing the *d*-tubocurarine to fold. The *d*-tubocurarine contains what seems to be an essential component of the bound acetylcholine: a positive charge hidden by methyl groups. Such bulky methyl groups prohibit close contact between a negative charge and the buried positive charge. An aromatic moiety is most suited for complementing such a structure: a charged hydrophobic domain.

The phenomenon of two structurally different ligands, acetylcholine and *d*-tubocurarine, displaying the same general substructure as a result of binding to the same receptor was also found for two haptens that bind to antiphenylphosphocholine antibodies. These two haptens differ in their NMR-determined solution structure, but when bound, they share common structural features. This result is consistent with the idea that the binding site provides a molecular template for the bound conformation of the ligand (Bruderer et al., 1992).

The change in configuration of a ligand after binding must be taken into consideration, especially when using the structure of the ligand to glean information on the binding site. Sine (1993) used the crystallographic distance between the two nitrogens of *d*-tubocurarine to gauge the distance between the α subunit and the non-α subunit that make up the ligand-binding pocket. The internitrogen distances we found in the *d*-tubocurarine-peptide complexes were on the scale of 2 Å smaller, and could be even smaller when bound to the intact receptor.

V. POSITIVELY CHARGED HYDROPHOBIC DOMAINS

Figure 2 shows the amino acid content of the α183–204 region from different species. A number of experimental and theoretical approaches have been used to find the amino acids within the putative binding site. Comparing the different K_D values of α-bungarotoxin binding (Ohana and Gershoni, 1990) or of other cholinergic ligands binding (Fraenkel et al., 1991b) to some of these slightly different sequences did not give a clear picture regarding the importance of any particular amino acid because the difference in K_D's are not that large and the effect might result from the overall change in sequence. A more fruitful approach was measuring K_D's of point mutated sequences either synthetically (McLane et al., 1991; McCormick et al., 1993; McLane et al., 1994) or genetically engineered peptides (Ohana and Gershoni, 1990; Ohana et al., 1991; Kachalsky et al., 1995; Barchan et al., 1995). These studies point to the importance of the aromatic residues. Affinity labeling (Dennis et al., 1988; Galzi et al., 1990; Cohen et al., 1991) has also revealed the importance of aromatic residues. *d*-Tubocurarine binding to the receptor is reduced if Tyr93 or Tyr190 in the α subunit are substituted by phenylalanine. Substitution of Trp55 in the γ subunit also brings about a severe reduction in binding, whereas substitution of Trp57 in the δ subunit does not effect binding (O'Leary et al., 1994). Furthermore, substitution of Tyr198 in the α subunit by phenylalanine leads to increased affinity toward *d*-tubocurarine, possibly due to an additional interaction of the phenylalanine with one of the aromatic rings of the *d*-tubocurarine (Filatov et al., 1993). A covalent bond formed between the coral derived neurotoxin, lophotoxin, and tyrosine-190 from the α-subunit of the ace-

tylcholine receptor is additional evidence for the importance of the aromatic residues (Abramson et al., 1989). Low and Corfield (1986) analyzed the X-ray structure of *Erabotoxin-b*, a 62-amino acid polypeptide. Their detailed analysis gave rise to the identification of a 'trp-cleft,' leading them to conclude that there exists a tryptophan in the receptor binding site that could complement the cleft found in the structure. Additional support for the importance of aromatic amino acids in acetylcholine binding comes from acetylcholine esterase. Determination of the X-ray structure of the esterase revealed that the binding site contains numerous aromatic residues and only a small number of negative charges (Sussman et al., 1991). The interaction of neuronal acetylcholine receptors with κ-neurotoxins has also been shown to involve aromatic residues (McLane et al., 1993).

Thus, all recent experimental findings point to the importance of the aromatic residues in the active site of the acetylcholine receptor in binding of cholinergic ligands. These residues do not carry any charge and therefore do not seem to comply with the Beers and Reich model. It was therefore initially predicted that the lone pair electrons on the oxygen and nitrogen of the tyrosine and tryptophan, respectively, would neutralize the positive charge common to most of the cholinergic ligands (Dennis et al., 1988). Only later it became apparent that the bulk of the aromatic ring is the moiety that might be interacting with the positive charge (Galzi et al., 1990). Changeux and co-workers proposed the three-loop model that showed that three aromatic amino acids, Tyr92, Trp148, and Try187, play a significant role in binding by getting a sort of three-fingered hold on agonists (Galzi et al., 1990a; Galzi et al., 1991b; Changeux et al., 1992; Changeux, 1995). This hypothesis is reinforced by the experimental and theoretical findings showing that the charge of tetraalkyl ammonium ion can be accommodated efficiently in a pore composed of tyrosines and tryptophans (Dougherty and Stauffer, 1990). Dougherty and Stauffer synthesized a cyclic molecule composed of tryptophans and tyrosines that was found to serve as an efficient acetylcholine "receptor." Furthermore, they found that when the *p*-(*tert*-butyl) *N,N,N*-trimethyl anilinium ion binds to their "receptor," it is the ammonium group rather than the *tert*-butyl group that is bound in the cavity. This occurs despite the fact that the ionic ammonium group must be better solvated by water than the neutral *tert* butyl, and binding must involve considerable desolvation. Their synthetic receptor bound quaternary ammonium compounds better than RNH_3^+ structures, like the native receptor, but unlike other synthetic receptors that are based on charge-charge interactions. Therefore, they proposed that the aromatic moiety can accommodate both the positive charge through interactions with the aromatic π electrons, whereas the bulk can interact with the hydrophobic environment created by the alkyl groups. Charge-charge interactions, predicted by Beers and Reich, were not ruled out but they do not seem to be of first priority in the process of recognition of cholinergic ligands.

We applied NMR to the study of this problem. By performing a transferred 2D-NOE experiment on acetylcholine bound to the α184–200 of *Torpedo californica* and to the human α183–204 sequences, we detected an interaction between trp-184 and bound acetylcholine (Fraenkel et al., 1991a). Further, the absence of trp-184 from the *Torpedo* α186–198 sequence might explain the reduced affinity of this sequence toward acetylcholine (Fraenkel et al., 1991b). Apparently, within the binding site, acetylcholine interacts with different amino acids than those required for α-bungarotoxin binding although interactions with an aromatic amino

acid in the binding process is retained. One can expect different ligands, in particular the neurotransmitter and its antagonists, to bind to different amino acids within the same binding site. This is certainly the case in the cholinergic binding site of the snake that has lost affinity for α-bungarotoxin yet continues to bind both *d*-tubocurarine and acetylcholine (Ohana et al., 1991). In fact, this might even be one of the ways that the receptor differentiates between agonists and antagonists that bind to a common domain but display different effects there. The NMR data, however, reveal additional information regarding the distance between the bound acetylcholine and the trp-184. We found that the acetyl methyl and the amino methyls approach each other in bound acetylcholine and both interact with trp-184 (Fraenkel et al., 1991a). To obtain a better understanding of the important structural features of the active form of the ligands (i.e., when bound) we determined their bound geometry. The assumption is that based on complementarity, the bound conformation will reveal important features required by the ligand that are present in the occupied binding site. Indeed the bound conformations of acetylcholine and *d*-tubocurarine demonstrate a unique phenomenon in that the positive charge is buried inside a cloud of methyls and is part of a positively charged hydrophobic domain. This seems to complement nicely the dual role attributed to the aromatic residues. The hydrophobic domain could reside close to the aromatic ring, and as a result of the close contact the aromatic π electron system may efficiently neutralize the positive charge buried inside the hydrophobic domain. The role of the aromatic residues is further supported from the geometry of the bound ligand, justifying our initial theory that studying the bound ligand can provide information regarding the binding site.

In the course of our studies we have applied NMR spectroscopy to further our understanding of cholinergic ligand recognition. NMR has contributed to two aspects of this issue: (a) it has been possible to demonstrate that α183–204 not only binds antagonists but also the neurotransmitter acetylcholine and nicotine, and (b) we show a functional motif in the ligand, namely, a positively charged hydrophobic domain, which becomes apparent through the elucidation of the three-dimensional structure of bound acetylcholine and *d*-tubocurarine.

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