Synthetic Peptides Used To Locate the α-Bungarotoxin Binding Site and Immunogenic Regions on α Subunits of the Nicotinic Acetylcholine Receptor†

Scott Ralston,‡ Virender Sarin,§ Hung Lam Thanh,‡,∥ Jean Rivier,‡ J. Lawrence Fox,§ and Jon Lindstrom*,‡

The Salk Institute for Biological Studies, San Diego, California 92138, Abbott Laboratories, Abbott Park, North Chicago, Illinois 60064, and Departement de Biologie, CEN Saclay, 91191 Gif sur Yvette Cedex, France

Received December 11, 1986

ABSTRACT: Synthetic peptides corresponding to 57% of the sequence of α subunits of acetylcholine receptors from *Torpedo californica* electric organ and extending from the NH_2 to the COOCH terminus have been synthesized. The α-bungarotoxin binding site on denatured α subunits was mapped within the sequence α185–199 by assaying binding of ^{125}I-α-bungarotoxin to slot blots of synthetic peptides. Further studies showed that residues in the sequence α190–194, especially cysteines-α192,193, were critical for binding α-bungarotoxin. Reduction and alkylation studies suggested that these cysteines must be disulfide linked for α-bungarotoxin to bind. Binding sites for serum antibodies to native receptors or α subunits were mapped by indirect immunoprecipitation of ^{125}I-peptides. Several antigenic sequences were identified, but a synthetic peptide corresponding to the main immunogenic region (which is highly conformation dependent) was not identified.

Synthetic peptides corresponding to segments of the primary sequence of the nicotinic acetylcholine receptor have been used extensively to study the structure of the receptor and potential functional sites. The ability to use such peptides resulted from determination of the primary sequence of the four receptor subunits which occur in the stoichiometry $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). The primary sequences were deduced from cDNA sequences (Noda et al., 1982, 1983a,b; Claudio et al., 1983; Devillers-Thiery et al., 1983). Analyses of these DNA sequences led to several models for the transmembrane orientation of the receptor subunits and the localization of structurally and functionally important domains, such as the main immunogenic region (MIR)[1] and the acetylcholine binding site [e.g., see Noda et al. (1982)].

Many features of these models have now been tested by immunochemical approaches using synthetic peptides. For example, the first model for the transmembrane orientation of the receptor consisted of four membrane-spanning regions (M_{1-4}) with both the NH_2 and COOH termini occurring on the extracellular surface (Noda et al., 1983b; Claudio et al., 1983; Devillers-Thiery et al., 1983). Antibodies raised against synthetic peptides corresponding to the NH_2 and COOCH termini proved this model incorrect (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Young et al., 1985). These studies determined that the COOH terminus is cytoplasmically located and, since evidence indicates that the NH_2 terminus is located extracellularly (Anderson et al., 1982), there must be an odd number of membrane-spanning regions. These data were consistent with two other models which proposed a fifth amphipathic transmembrane domain (M_5) between M_3 and M_4 (Guy, 1983; Finer-Moore & Stroud, 1984). However, Ratnam et al. (1986a) recently demonstrated that the region from α330 to the COOH terminus which includes both M_4 and M_5 occurs on the cytoplasmic surface of the membrane. These data eliminate the possibility that M_4 and M_5 exist as transmembrane domains. In addition, Criado et al. (1985a,b) used monoclonal antibodies (mAbs) against α152–167 to provide evidence for two unpredicted transmembrane domains, M_6 and M_7, on the NH_2-terminal side of M_1. The complete transmembrane orientation of the receptor subunit polypeptide chains is still unknown. The current working model based on these studies, then, is quite different from the original models based on DNA sequences alone. However, these studies with synthetic peptides would have been impossible without the prior sequence information.

Several years ago, Karlin (1969) demonstrated that a disulfide bond was within 1 nm of the acetylcholine binding site and that the affinity sulfhydryl alkylating agent [4-(*N*-maleimido)benzyl]trimethylammonium iodide (MBTA) would block receptor function and α-bungarotoxin (αBgt) binding. More recently, Gershoni et al. (1983) showed that αBgt bound to SDS-denatured α subunits and that this binding could be blocked if native receptor were labeled with MBTA prior to SDS denaturation. This led to the suggestion that the αBgt binding site on denatured α subunits was at least part of the physiologically relevant αBgt binding site on native receptor. With the demonstration that SDS-denatured α subunit would bind αBgt, it became feasible to test the hypothesis of Noda et al. (1982), Smart et al. (1984), McCormack and Atassi (1984), and Boulter et al. (1985) that the αBgt binding site is formed by the sequence between cysteine residues at positions α128 and α142.

Recent peptide mapping studies have suggested that the αBgt binding site lies between residues α153 and 241 (Wilson et al., 1985), between α152 and 313 (Neumann et al., 1986), between α179 and 207 (Dennis et al., 1986), COOH terminal

† This work was supported by grants to J.L. from the National Institutes of Health (NS11323), the Muscular Dystrophy Association, the Alexander S. Onassis Public Benefit Foundation, the U.S. Army (DAMD17-86-C-6148), and the Los Angeles and California Chapters of the Myasthenia Gravis Foundation

* Address correspondence to this author.
‡ The Salk Institute for Biological Studies.
§ Abbott Laboratories.
∥ CEN Saclay.

[1] Abbreviations: αBgt, α-bungarotoxin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IAA, iodoacetamide; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium iodide; MIR, main immunogenic region; PBS, 10 mM sodium phosphate, pH 7.5, and 150 mM NaCl; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; mAb, monoclonal antibodies; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.

0006-2960/87/0426-3261$01.50/0

to α173 (Pedersen et a l., 1986), or between α152 and 180 (Oblas et al., 1986). Experiments with synthetic peptides suggest that the binding site is contained in residues α173–205 (Wilson et al., 1985), in residues α185–196 (Neumann et al., 1986), or in residues α127–143 (McCormack & Atassi, 1984). An apparent resolution to this discrepancy exists in the data of Kao et al. (1984), who showed that [^{3}H]MBTA bound to Cys-192 and/or -193, and from Criado et al. (1986), who showed that mAbs to α127–143 upon binding to receptor did not inhibit binding of αBgt.

Another feature of the receptor predicted by Noda et al. (1982) from the DNA sequence was the location of the MIR. The MIR is a small region on the extracellular surface of α subunits which is pathologically significant in myasthenia gravis. mAbs to the MIR bind denatured α subunits but bind native receptor with much greater affinity (Tzartos & Lindstrom, 1980; Tzartos et al., 1981, 1983; Gullick et al., 1981; Sargent et al., 1983). Half or more of the autoantibodies to receptor in sera from myasthenic patients are directed at the MIR (Tzartos et al., 1982, 1985). Noda et al. (1982) predicted that the MIR should be located at residues α161–166, α330–340, or α387–392 because these were the most hydrophilic sequences. Again, immunological work with synthetic peptides proved this prediction erroneous. Jullierat et al. (1984) and Lindstrom et al. (1984) demonstrated that MIR mAbs did not bind to α152–167, while Criado et al. (1985) showed that mAbs to α152–167 did not compete for binding to receptor with mAbs to the MIR. Ratnam et al. (1986a) then found that mAbs to the region α330–400 bound to the cytoplasmic surface of the receptor, thus eliminating this region as a candidate for the MIR. Furthermore, Ratnam et al. (1986b), in probing peptide maps of the α subunit with MIR mAbs, found that these MIR mAbs bind in the region α46–127. Barkas et al. (1986a,b), with polyclonal antibodies to synthetic peptides, MIR mAbs, and α-subunit cDNA fragments expressed in bacteria, mapped the MIR to the region α6–85, which is consistent with Ratnam et al. (1986b).

In the present work, synthetic peptides corresponding to approximately 57% of the sequence of α subunits of receptor from *Torpedo californica* and extending from the NH_2 to the COOH terminus were used to investigate functional and structural features of α subunits. The binding site for αBgt on denatured α subunits was determined. Additionally, binding sites for polyclonal antibodies to native receptors or α subunits were determined with these synthetic peptides. Several antigenic sequences which did not depend critically on the native conformation of the receptor were identified, but a synthetic peptide corresponding to the MIR was not detected.

Materials and Methods

Receptor and α-Subunit Preparation. Receptor was purified from the electric organs of *Torpedo californica* by affinity chromatography on toxin agarose (Lindstrom et al., 1981). The α subunit was purified from receptor by preparative SDS–PAGE as described (Lindstrom et al., 1979). Briefly, the preparative gel (5 mm) was lightly stained with Coomassie blue and destained. The band corresponding to the α subunit was excised and frozen. The gel containing the α subunit was sonicated in H_2O at 4 °C until the gel was dispersed. The suspension was centrifuged and filtered through a 0.22-μm filter to remove remaining gel particles. The α-subunit preparation was then dialyzed vs. H_2O and lyophilized.

Peptides and Iodination. Peptides corresponding to various segments of the α subunit were synthesized by V. Sarin, J. L. Fox, H. L. Thanh, and J. Rivier using the Merrifield method (1965) as previously described (Lindstrom et al., 1984; Criado et al., 1986; Ratnam et al., 1986a) or were purchased from Bachem (Los Angeles, CA) (Lindstrom et al., 1984; Ratnam et al., 1984). These peptides are listed in Table I. For radioiodination, several of the peptides contained an additional tyrosine at the NH_2 or COOH terminus. Peptides were labeled with ^{125}I by using chloramine-T to specific activities of 9×10^{16} to 3×10^{18} cpm/mol (Lindstrom et al., 1981; Ratnam et al., 1986a).

Preparation and Assay of Antibodies. Antisera were raised in rats against both native receptor and α subunits as previously described (Lindstrom et al., 1981). Antisera were assayed by incubation overnight with ^{125}I-αBgt-labeled receptor (1 nM) or ^{125}I-labeled α subunit in 100 μL of 10 mM sodium phosphate (pH 7.5)/100 mM NaCl/0.5% Triton X-100/10 mM NaN_3 (buffer). Immune complexes were precipitated with 100 μL of goat anti-rat immunoglobulin, diluted with 1 mL of buffer, and centrifuged. Precipitates were washed with 2 × 1 mL of buffer and counted for radioactivity, as previously described (Lindstrom et al., 1981). The binding of antibodies to 10 nM ^{125}I-peptides was assayed similarly and has been described (Ratnam et al., 1986a).

Affinity Labeling of Receptor with MBTA and Blotting onto Nitrocellulose. Receptor was affinity labeled with MBTA as described by Walker et al. (1984). Receptor (2 μM) in 10 mM Tris, pH 8.0, containing 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, and 1 mM EDTA was reduced with 1 mM dithiothreitol (DTT) for 30 min. MBTA (10 mM) was added, and after 10 min, the reaction was terminated by rapid chromatography on Sephadex G-25. For some experiments, part of the MBTA-labeled receptor was then denatured with 1% SDS.

Aliquots of receptor, MBTA-labeled receptor, SDS-denatured receptor, and SDS-denatured MBTA-labeled receptor were applied to a nitrocellulose membrane in a slot-blot apparatus (Schleicher & Schuell, Keene, NH). The remaining reactive membrane sites were quenched with 2% BSA in PBS + NaN_3 from 1 to 14 h, and the membrane was rinsed with five changes of PBS + NaN_3. ^{125}I-αBgt was applied overnight followed by washing with several changes of buffer over 1 h. Blots were autoradiographed on preflashed Kodak XAR5 film. The membranes were then cut so that the cpm bound to each spot could be quantitated.

Binding ^{125}I-αBgt to Synthetic α-Subunit Peptides. A slot-blot apparatus was used to apply synthetic peptides to a Biodyne immunoaffinity membrane (Pall, East Hills, NY) in 16 mM borate, pH 9.0, and 15 mM NaCl. Remaining reactive membrane sites were quenched overnight with 5% Carnation dried milk and 50 mM Tris, pH 7.5, with 0.01% anti-foam A (quench buffer), and the membrane was rinsed with five changes of buffer. ^{125}I-αBgt (5 nM) was applied overnight in the quench buffer containing 0.5% Triton X-100. The membrane was washed with buffer and autoradiographed on preflashed XAR5 film.

Reduction and Alkylation of α172–205. Aliquots of 100 μL containing 1×10^{-6} M α172–205 were reduced with 1 mM dithiothreitol (DTT) for 30 min at 23 ± 2 °C. Iodoacetamide (IAA) was then added to a final concentration of 3 mM and incubated for 30 min at 23 ± 2 °C in the dark. The samples were then diluted to a final concentration of 1×10^{-7} M α172–205 and applied to a Biodyne immunoaffinity membrane for analysis of αBgt binding. In some experiments, the peptide was not reduced prior to the addition of IAA.

Results and Discussion

Binding of ^{125}I-αBgt to Native and MBTA-Labeled Receptor. Figure 1 shows the results obtained by exhaustively

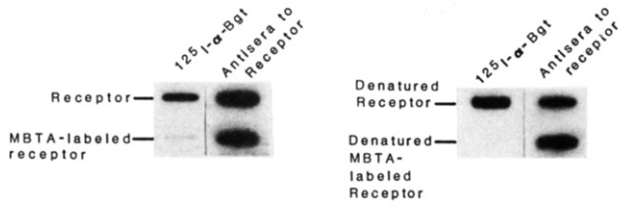

FIGURE 1: Inhibition of αBgt binding to acetylcholine receptor by affinity labeling with MBTA. Aliquots (50 μL) containing 4×10^{-12} mol of receptor were applied to a nitrocellulose membrane via a slot-blot apparatus. Remaining reactive sites were quenched with 2% BSA in PBS + NaN_3 and rinsed with five changes of PBS + NaN_3. ^{125}I-αBgt (2×10^{-9} M) was applied overnight followed by washing with 0.5% Triton X-100 in PBS + NaN_3. Blots were autoradiographed on flashed XAR5 film using an intensifer screen for 1 h with native receptor and 10 h with denatured receptor. The MBTA-labeled receptor was prepared as described under Materials and Methods. SDS (1%) was used to denature the receptor.

labeling the receptor with MBTA and performing ^{125}I-αBgt binding experiments. Native receptor without MBTA bound ^{125}I-αBgt (44 800 cpm), but MBTA-labeled receptor bound only 10% as much ^{125}I-αBgt (4300 cpm), even though comparable amounts of receptor were loaded onto the nitrocellulose membrane. This maximum of 90% inhibition of toxin binding by MBTA agrees precisely with that observed by Walker et al. (1984), who also affinity labeled in the presence of a high concentration of reducing agent (1 mM DDT). SDS-denatured receptor lacking MBTA was able to bind some ^{125}I-αBgt (4600 cpm), but MBTA-labeled receptor denatured with SDS bound no ^{125}I-αBgt. These results confirm those of Gershoni et al. (1983), showing that this ^{125}I-αBgt binding site on denatured α subunits is near the site for binding small cholinergic ligands on native receptor.

Mapping the α-Bgt Binding Site with Synthetic Peptides. Recently, peptide mapping evidence has been presented which suggests that the αBgt binding site is located between α153 and 241 (Wilson et al., 1985), between α152 and 313 (Neumann et al., 1986), between α179 and 207 (Dennis et al., 1986), COOH terminal to α173 (Pedersen et al., 1986), or between α152 and 180 (Oblas et al., 1986). Studies with synthethic peptides suggest that the binding site is contained in residues α173–204 (Wilson et al., 1985), residues 185–196 (Neumann et al., 1986), or residues α127–143 (McCormack & Atassi, 1984). We addressed this problem with synthetic peptides α127–143, α172–189, α194–212, α172–205, and α185–199. Figure 2 shows the results obtained when 2.5 pmol of each peptide was coupled to a Biodyne membrane via a slot-blot apparatus, except for α185–199 where 70 pmol was applied. αBgt bound to the α subunit, as expected, and to the peptide α172–205, confirming the work of Wilson et al. (1985), and also to the smaller peptide α185–199, which is consistent with Neumann et al. (1986). No binding occurred to the other peptides, α127–143, α172–189, or α194–212. In addition, the ^{125}I-αBgt binding to α172–205 could be blocked by the addition of 0.2 μM cold αBgt (Figure 2). The IC_{50} for cold αBgt of 0.5 μM obtained with α172–205 (data not shown) was consistent with Wilson et al. (1985). A 100-fold higher concentration of cold αBgt (20 μM) was required to block ^{125}I-αBgt binding to α185–199. However, to detect ^{125}I-αBgt binding to α185–199, it was necessary to apply 28 times as much α185–199 to the Biodyne membrane. The affinity for αBgt of this short peptide appears to be the same as the affinity of α172–205 for αBgt, as shown by the observation that binding curves for both ^{125}I-peptides to αBgt on Biodyne membranes were superimposable (data not shown). Thus, when α185–199 is bound to a Biodyne membrane, amino acid residues must often be involved which would otherwise interact

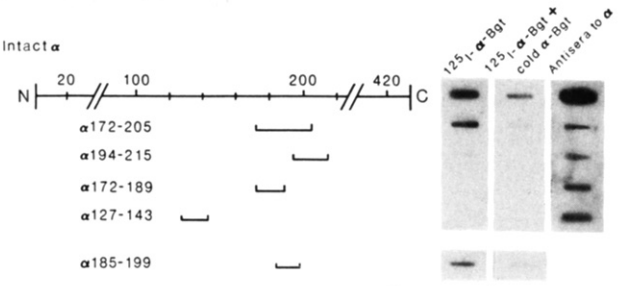

FIGURE 2: Binding of ^{125}I-αBgt to synthetic peptides from α subunits. Aliquots (25 μL) of 1×10^{-7} M synthetic peptide in 16 mM borate buffer, pH 9.0, were applied to a Biodyne immunoaffinity membrane via a slot-blot apparatus. Remaining reactive sites were quenched overnight with 5% Carnation dried milk in Tris buffer (see Materials and Methods) and washed with five changes of 0.5% Triton X-100 in PBS + NaN_3. ^{125}I-αBgt (5×10^{-9} M) was applied in the 10^{-7} Carnation milk quench buffer plus or minus 2×10^{-7} M unlabeled αBgt. Washing and autoradiography were as described in Figure 1, except that exposure was for 14 h. Antiserum to α subunits followed by ^{125}I anti-antibody was used to demonstrate that comparable amounts of all peptides were bound to the membrane. A 25-μL aliquot of 2.8×10^{-6} M α185–199 was required to bind αBgt as shown here. In addition, 2×10^{-5} M unlabeled αBgt was required to block the binding of ^{125}I-Bgt to α185–199.

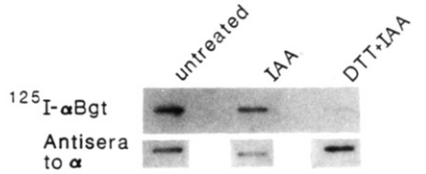

FIGURE 3: Binding of ^{125}I-αBgt to reduced and alkylated α172–205. Aliquots (25 μL) of 1×10^{-7} M α172–205, which were untreated or had been treated with IAA, or reduced with DTT and then reacted with IAA, were applied to a Biodyne immunoaffinity membrane and processed as described in Figure 2.

with ^{125}I-αBgt. Antisera to α subunits were used to confirm that these peptides bound to the membrane. Antisera, however, failed to react with α185–199, but the αBgt binding confirmed that this peptide bound to the Biodyne membrane.

Since αBgt did not bind to peptides α172–189 or α194–212, but did bind to the peptides α172–205 and α185–199, amino acids in the sequence α190–193 of the α subunit appear to be critical for binding of αBgt. In addition, α172–205 treated with IAA continued to bind ^{125}I-αBgt. However, if α172–205 was first reduced with dithiothreitol and treated with IAA, it no longer bound ^{125}I-αBgt (Figure 3). This indicates that Cys residues α192 and 193 must be disulfide cross-linked for αBgt binding to occur. These data are consistent with those of Kao and Karlin (1986), which demonstrated that, in native receptor, Cys-192 and Cys-193 are disulfide cross-linked, and those of Criado et al. (1986), who also suggested that the toxin binding site disulfide occurs between Cys-192 and Cys-193. We conclude that the peptide α172-205 and the smaller fragment α185–199 contain the αBgt binding site of the SDS-denatured α subunit which can be blocked with MBTA prior to SDS denaturation. However, αBgt exhibits much greater affinity for the native receptor and no doubt requires the native conformation of this part of the amino acid sequence of α subunits; it may also require interaction with the native conformation of other parts of the sequence of α.

Location of the amino acids involved in binding acetylcholine may best be achieved with a photoaffinity labeling reagent capable of reacting with many amino acids. The primary site of reaction of such a label has been reported to be within the peptide α197–207 (Dennis et al., 1986), which is consistent with the αBgt binding results. Small amounts of reaction were

Table I: Synthetic Peptides of the α Subunit of the Acetylcholine Receptor

peptide	sequence
[Tyr-11]α1–11	SEHETRLVANY
[Tyr-60]α44–60	DEVNQIVETNVRLRQQY
[Tyr-83]α66–83	RWNPADYGGIKKIRLPSY
[Gly-89,Tyr-90]α73–90	GGIKKIRLPSDDVWLPGY
α78–93	IRLPSDDVWLPDLVLY
[Tyr-104]α89–104	DLVLYNNADGDFAIVY
[Tyr-100]α100–116	YAIVHMTKLLLDYTGKI
α112–127	YTGKIMWTPPAIFKSY
	S–S
α127–143	YCEIIVTHFPFDQQNCT
[Tyr-170]α159–170	SPESDRPDLSTY
α172–189	ESGEWVMKDYRGWKHWVY
α185–199	KHWVYYTCCPDTPYL
α172–205	ESGEWVMKDYRGWKHWVYYTCCP-DTPYLDITYHF
α194–212	PDTPYLDITYHFIMQRIPL
α261–277	VELIPSTSSAVPLIGKY
[Tyr-347]α330–347	KRASKEKQENKIFADDIY
[Tyr-365]α349–365	SDISGKQVTGEVIFQTY
[Tyr-379]α360–379	VIFQTPLIKNPDVKSAIEGY
[Tyr-386]α371–386	DVKSAIEGVKYIAEHY
[Tyr-409]α389–409	DEESSNAAEEWKYVAMVIDHY
[Tyr-427]α427–437	YGRLIELSQEG

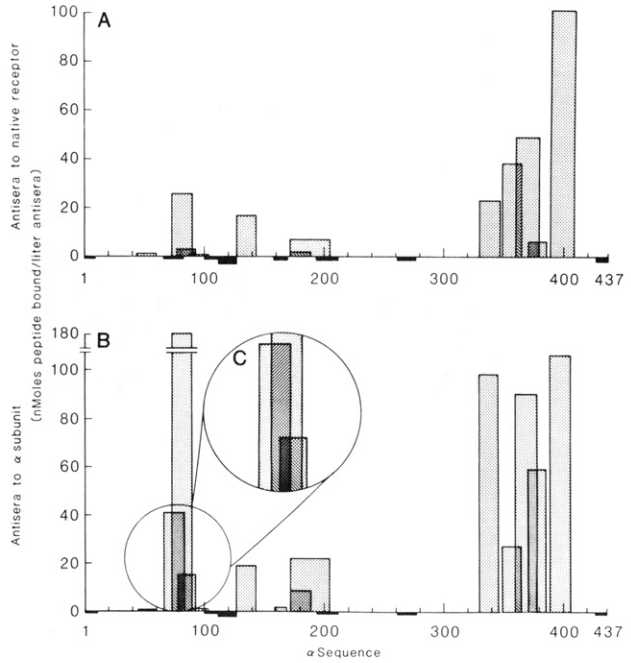

FIGURE 4: Reaction of synthetic peptides with antisera to native receptor and α subunits. Synthetic peptides corresponding to segments of the α subunit sequence, indicated by the bars, were labeled with ^{125}I as described under Materials and Methods. Antisera (5–10 μL) were assayed by reacting with ^{125}I-peptide (10 nM) in 100 μL of 10 mM sodium phosphate buffer (pH 7.5), 100 μM NaCl, 0.5% Triton X-100, and 10 mM NaN_3. The immune complexes were precipitated with 100 μL of goat anti-rat immunoglobulin. Solid black bars below the base line indicate that antisera did not react with these peptides. Panel A shows the reaction of antisera to native receptor with the synthetic peptides. Panel B shows the reaction of antisera to SDS-denatured α subunits. The insert C shows a magnified view of the overlap which occurred with antisera to α subunits vs. [Tyr-83]α66–83, [Gly-89,Tyr-90]α73–90, and α78–93.

also noted in two other unidentified peptides. The acetylcholine binding site is clearly close to α192–193 in the native receptor, and some amino acids in this part of the sequence may contribute to the binding site, but amino acids from distant parts of the sequence of α subunits may also be involved.

Reaction of Synthetic α Peptides with Antisera to Native Receptor and α Subunits. Synthetic peptides corresponding to approximately 57% of the sequence of the α subunit were synthesized (Table I). These peptides were labeled with ^{125}I and tested for reaction with antisera to native receptor and α subunits (Figure 4A,B). Some of these data concerning the peptides near the C-terminus was previously reported (Ratnam et al., 1986b) and is included here to provide a comprehensive view of the antigenicity of the denatured α subunit.

There are two regions on the α subunit which account for most of its antigenicity in the denatured state. The first one is located between residues 330 and 400 and is also highly antigenic in native receptor (Ratnam et al., 1986a,b). The region α330–410 accounts for approximately 60% of the antigenicity of denatured α subunits as indicated by the titer of α-subunit antisera with synthetic peptides from this region and by reaction with several mAbs in our library (Ratnam et al., 1986b). Binding of antisera against native receptor to synthetic peptides in the region α330–410, as well as the fact that mAbs directed against α subunits and mapped to this region also bind native receptor with high affinity, suggests that this sequence forms an extended flexible region largely exposed on the cytoplasmic surface of the receptor. Recent data from Tzartos and Kordossi (1987) agree with the hypothesis that the cytoplasmic region from α330 to the COOH terminus occurs as an extended, flexible region. They reached this conclusion from antibody competition experiments on native receptor, finding that anti-α-subunit mAbs 187, 142, and 155, which bind respectively to the segments α339–346, α352–360, and α371–378, could bind simultaneously to the receptor.

The region α330–410 contains the putative membrane-spanning regions M_4 and M_5 (Noda et al., 1982; Guy, 1983; Finer-Moore & Stroud, 1984). However, two types of studies suggest that all of the region from residue 330 to the COOH terminus is located on the cytoplasmic side of the membrane (Ratnam et al., 1986a,b). One study investigated binding and competition experiments with mAbs to this region on native receptor in closed and permeabilized membrane vesicles. The second study utilized immunoelectron microscopy with the colloidal gold technique (Wray & Sealock, 1984; La Rochelle et al., 1985), using mAbs and native receptor in lysed vesicles. Both types of experiments demonstrated that the region from residue 330 to the COOH terminus of the α subunit is located on the cytoplasmic side of the membrane and, therefore, that M_4 and M_5 do not form membrane-spanning domains. The cytoplasmic location and failure to react with mAbs to the MIR also eliminated this region as a candidate for the MIR, even though the region α330 to the COOH terminus is highly immunogenic. Souroujon et al. (1986) also found that this region is highly immunogenic in α subunits; however, their study did not address whether this region is cytoplasmic or extracellular.

The second region of high antigenicity was determined in the present work to lie between α73 and 93 (Figure 4B) and accounts for approximately 33% of the antigenicity of denatured α subunits. However, the experiments with synthetic peptides in this region and antisera to native receptor suggest that this region is only moderately antigenic in native receptor (Figure 4A) in terms of sequential or continuous epitopes. These studies with synthetic peptides corresponding to 75% of the region on α subunits from the NH_2 terminus to residue α212 suggest that this region of α is folded with a high degree of tertiary structure in the native receptor. The segment of denatured α subunit, α73–93, which is immunogenic must be slightly exposed in the native receptor or may simply occur in another conformation in the native receptor. Alternatively, the antisera to native receptor reactive with [Gly-89,Tyr-

90]α73–90 and α78–93 may simply result from the presence of a small amount of denatured receptor in the preparation used to produce the antisera or denaturation during antigen processing.

The three peptides [Tyr-83]α66–83, [Gly-89,Tyr-90]α73–90, and α78–93 titered with antiserum to native receptor (Figure 4A) illustrate one of the problems encountered with using small synthetic peptides to address functional and structural features of a large polypeptide which contains much secondary and tertiary structure. Antisera to native receptor did not bind [Tyr-83]α66–83, but with [Gly-89,Tyr-90]α73–90, there was a moderate degree of binding, as indicated by a titer of 26 nM. This would suggest that the immunogenic amino acids on this region of α subunits were contained approximately in the six residues α83–88. One would then assume that the peptide α78–93 would have a titer at least equal to [Gly-89,Tyr-90]α73–90, since α78–93 contains the same six residues in α83–88. However, this was not the case. We found that antisera to native receptor bound less well to α78–93. One explanation for this anomaly is that the two small peptides [Gly-89,Tyr-90]α73–90 and α78–93 could have very different conformations as a result of their different sequences, even though they share residues α83–88. Another possible explanation is that antibodies bound to the NH_2 terminus of α73–90 and were made to peptide fragments generated in the immunized rats. Examples are known of antisera to overlapping synthetic α-subunit peptides which do not cross-react because the antibodies are specific for the termini of the peptides (Dryberg & Oldstone, 1986). A similar situation occurred with these peptides and antisera to α subunits but to a different degree (Figure 4B,C). Again, there was greater reactivity with [Gly-89,Tyr-90]α73–90 than with [Tyr-83]α66–83 and α78–93 even though there is a considerable degree of sequence overlap between these peptides.

The MIR was previously mapped to lie between α46 and 127 (Ratnam et al., 1986b). We, therefore, hoped to use synthetic peptides from this region to precisely locate the MIR. However, none of the synthetic peptides from this region appeared to react specifically in RIAs with any of the MIR mAbs known to bind to SDS-denatured α subunit. In another attempt to identify an MIR peptide, all of the peptides from α46–143 (including α52–70 which we did not investigate by RIA) were applied to a nitrocellulose membrane, diazophenolthiol paper, and a Biodyne membrane via a slot blot. After being quenched, the membranes were probed with antisera to native receptor and α subunits, as well as several MIR mAbs. None of the MIR mAbs bound to the peptides (data not shown), and binding of antisera to the peptides reflected the data shown in Figure 4. Ratnam et al. (1986b) localized the MIR to α46–127 using large proteolytic fragments of α and differential binding of mAbs. Our present inability to locate the MIR with small synthetic peptides was perhaps due to the small peptides' inability to assume a conformation that is retained in SDS-denatured α subunits or large fragments of α subunits. These data would suggest that in the native receptor the region from α46–212 of α subunits is tightly folded and packed so that the antigenic determinants forming the MIR are discontinuous. Indeed, Barlow et al. (1986) recently suggested that all native protein antigenic determinants are discontinuous to some extent. This situation appears to exist with the NH_2-terminal, extracellular portion of α subunits in native receptors; however, as mentioned above, the cytoplasmically located COOH-terminal region consists of determinants which must be somewhat continuous since a number of mAbs bind with high affinity both to synthetic peptides in the region α330–408 and to native receptor.

Barkas et al. (1987) have produced several long fusion proteins containing different portions of the mouse α subunit. With these methods, they have mapped the αBgt binding site to residues α160–216, which is consistent with Wilson et al. (1985), Neuman et al. (1986), and the present data. In addition, they report that fusion proteins containing residues α6–85 bind MIR mAbs and, furthermore, that two MIR epitopes are contained within this sequence based on differential mAb binding to one fusion protein containing residues α6–37 and a second fusion protein containing residues α37–216. Their conclusion that the MIR is located between residues α6 and 85 is consistent with that of Ratnam et al. (1986b), who concluded that the MIR is located between residues α46 and 127. However, the binding of these MIR mAbs to the fusion proteins is both weak (K_D approximately micromolar) and inefficient (only a tiny fraction of the peptide binds mAb at all) and could, therefore, pose problems in making absolute conclusions as to the peptide regions of α subunits which form the MIR.

The peptide α172–205 was modestly immunogenic with antisera to α subunits (Figure 1, right panel) and less so with antisera to native receptor (Figure 1, left panel). There was only a slight reaction of antisera with the smaller fragment, α172–189, and no reaction with α194–212. Attempts to produce antisera against α172–205 which would bind to native receptor have thus far proven unsuccessful. This further emphasizes that this part of the native receptor is to a high degree conformationally restrained. Antibody binding sites in such regions are likely to be formed by surface structures of novel shapes formed by several amino acids from distant parts of the sequence, as illustrated by recent X-ray crystallographic studies of complexes of Fab with lysozyme (Amit et al., 1986).

Another problem we have encountered in our efforts to use immunological techniques with synthetic peptides to map structural and functional regions of the receptor has been that mAbs raised against synthetic peptides tend not to bind well to the native receptor (Ratnam & Lindstrom, 1984; Criado et al., 1985a,b, 1986; S. Ralston et al., unpublished results). Antibodies raised against 12 different peptides failed to react well with native receptor. These data support the suggestion of Barlow et al. (1986) that most antigenic determinants on native proteins are discontinuous and our hypothesis that particularly the NH_2-terminal portion of the receptor is tightly folded and packed so that mAbs to this region require the native conformation of the receptor.

In summary, we have used synthetic peptides corresponding to segments of α subunits from the acetylcholine receptor to map the αBgt binding site and immunogenic regions. The data presented here demonstrate that the αBgt binding site on SDS-denatured α subunits is at least part of the physiologically functional αBgt site on native receptor and near to the site for binding of acetylcholine to the native receptor. This binding site is contained within the residues α185–199, and a disulfide bond between cysteines-α192 and -193 is critically important.

We report the identification of a highly immunogenic region on denatured α subunits within residues α73–93. This region is much less immunogenic in native receptor. Experiments with the overlapping peptides [Tyr-83]α66–83, [Gly-89,Tyr-90]α73–90, and α78–83 illustrate that factors other than sequence, such as conformation, may be critical in immunogenically mapping large proteins with small synthetic peptides. Factors such as these are perhaps involved in our inability to detect a synthetic peptide reactive with mAbs to the MIR, even

though we covered the entire region α46–127 to which Ratnam et al. (1986b) mapped the MIR. The MIR is highly conformation dependent and most likely includes amino acids from noncontiguous parts of the sequence. The MIR is defined by competitive binding of mAbs (Tzartos et al., 1981). A mAb binding site occludes a protein surface about 20 × 30 Å involving 16 or 17 amino acids (Amit et al., 1986). Clearly, several competing antibodies could bind to partially overlapping areas, yet recognize different amino acids and in no case exhibit high affinity for a short α-subunit sequence. Another approach will therefore be required to determine the peptide(s) which form(s) the MIR.

ACKNOWLEDGMENTS

We thank John Cooper for performing the iodinations and Maya Spies for typing the manuscript.

REFERENCES

Amit, A., Mariuzza, R., Phillips, S., & Poljak, R. (1986) *Science (Washington, D.C.) 233*, 747–753.

Anderson, D. J., Walter, P., & Blobel, G. (1982) *J. Cell Biol. 93*, 501–506.

Barkas, T., Gabriel, J. M., Juillerat, M. A., Kokla, A., & Tzartos, S. J. (1986) *FEBS Lett. 198*, 237–241.

Barkas, T., Roth, B., Gabriel, J. M., Tzartos, S. J., Juillerat, M., Alliod, C., & Ballivet, M. (1987) *Ann. N.Y. Acad. Sci.* (in press).

Barlow, D. J., Edwards, M. S., & Thornton, J. M. (1986) *Nature (London) 332*, 747–748.

Boulter, J., Luyten, W., Evans, K., Mason, P., Ballinet, M., Goldman, D., Stengilen, S., Martin, G., Heinemann, S., & Patrick, J. (1985) *J. Neurosci. 5*, 2545–2552.

Claudio, T., Ballivet, M., Patrick, J., & Heinemann, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 111–115.

Criado, M., Hochschwender, S., Sarin, V., Fox, J., & Lindstrom, J. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2004–2008.

Criado, M., Sarin, V., Fox, J. L., & Lindstrom, J. (1985b) *Biochem. Biophys. Res. Commun. 128*, 864–871.

Criado, M., Sarin, V., Fox, J. L., & Lindstrom, J. (1986) *Biochemistry 25*, 2839–2845.

Dennis, M., Giraudaut, J., Kotzba-Hibert, F., Goeldner, M., Hirth, C., Chang, J.-Y., & Changeux, J.-P. (1986) *FEBS Lett. 207*, 243–249.

Devillers-Thiery, A., Giraudat, J., Bentaboulet, M., & Changeux, J.-P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2067–2071.

Dryberg, T., & Oldstone, M. (1986) *J. Exp. Med. 164*, 1344–1349.

Gershoni, J. M., Hawrot, E., & Lentz, T. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4973–4977.

Gullick, W., Tzartos, S., & Lindstrom, J. (1981) *Biochemistry 20*, 2173–2180.

Guy, R. (1983) *Biophys. J. 45*, 249–261.

Juillerat, M. A., Barkas, T., & Tzartos, S. (1984) *FEBS Lett. 168*, 143–148.

Kao, P., Dwork, A. J., Kaldany, R. J., Silver, M. L., Wideman, J., Stein, S., & Karlin, A. (1984) *J. Biol. Chem. 259*, 11662–11665.

Karlin, A. (1969) *J. Gen. Physiol. 54*, 245s–264s.

La Rochelle, W. J., Wray, B. E., Sealock, R., & Froehner, S. C. (1985) *J. Cell Biol. 100*, 684–691.

Lindstrom, J., Merlie, J., & Yogeeswaran, A. (1979) *Biochemistry 18*, 4465–4470.

Lindstrom, J., Einarson, B., & Tzartos, S. (1981) *Methods Enzymol. 74*, 432–460.

Lindstrom, J., Criado, M., Hochschwender, S., Fox, J. L., & Sarin, V. (1984) *Nature (London) 311*, 573–575.

McCormack, D. J., & Atassi, M. Z. (1984) *Biochem. J. 224*, 995–1000.

Merrifield, R. B. (1965) *Science (Washington, D.C.) 150*, 178–185.

Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., & Fuchs, S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3008–3011.

Noda, M., Takahashi, H., Tanabe, T., Toyosota, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature (London) 299*, 793–797.

Noda, M., Takahashi, H., Tanabe, T., Toyosota, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1983a) *Nature (London) 301*, 251–255.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983b) *Nature (London) 302*, 528–532.

Oblas, B., Singer, R. H., & Boyd, N. D. (1986) *Mol. Pharmacol. 29*, 649–656.

Pederson, S. E., Dreyer, E. B., & Cohen, J. B. (1986) *J. Biol. Chem. 261*, 13735–13743.

Raftery, M., Hunkapillar, M., Strader, C., & Hood, L. (1980) *Science (Washington, D.C.) 208*, 1454–1457.

Ratnam, M., & Lindstrom, J. (1984) *Biochem. Biophys. Res. Commun. 122*, 1225–1233.

Ratnam, M., Le Nguyen, D., Rivier, J., Sargent, P. B., & Lindstrom, J. (1986a) *Biochemistry 25*, 2633–2643.

Ratnam, M., Sargent, P. B., Sarin, V., Fox, J. L., Le Nguyen, D., Rivier, J., Criado, M., & Lindstrom, J. (1986b) *Biochemistry 25*, 2621–2623.

Reynolds, J., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.

Sargent, P., Hedges, B., Tsavalar, L., Clemmons, L., Tzartos, S., & Lindstrom, J. (1983) *J. Cell Biol. 98*, 609–618.

Smart, L., Meyers, H.-W., Hilginfeld, R., Saenger, W., & Maelicke, A. (1984) *FEBS Lett. 178*, 64–68.

Souroujon, M., Neumann, D., Pizzighella, S., Sofran, A., & Fuchs, S. (1986) *Biochem. Biophys. Res. Commun. 135*, 82–89.

Tzartos, S. J., & Lindstrom, J. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755–759.

Tzartos, S., & Kordossi, A. A. (1987) *NATO ASI Ser., Ser. H 3*, 35–47.

Tzartos, S. J., Seybold, M., & Lindstrom, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 188–192.

Tzartos, S., Langeburg, L., Hochschwender, S., & Lindstrom, J. (1983) *FEBS Lett. 158*, 116–118.

Tzartos, S., Sophianos, D., & Efthimcadis, A. (1985) *J. Immunol. 134*, 2343–2349.

Walker, J. W., Richardson, C. A., & McNamee, M. G. (1984) *Biochemistry 23*, 2329–2338.

Wilson, P. T., Lentz, T. L., & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8790–8794.

Wray, B. E., & Sealock, R. (1984) *J. Histochem. Cytochem. 31*, 1117–1120.

Young, E. F., Ralston, E., Blake, J., Ramachandran, J., Hall, Z. W., & Stroud, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 626–630.