Biochemical filtering of a protein–protein docking simulation identifies the structure of a complex between a recombinant antibody fragment and α -bungarotoxin

Luisa BRACCI^{*1}, Alessandro PINI^{*}, Andrea BERNINI^{*†}, Barbara LELLI^{*}, Claudia RICCI^{*}, Maria SCARSELLI^{*†}, Neri NICCOLAI^{*†} and Paolo NERI^{*}

^{*}Department of Molecular Biology, University of Siena, Via Fiorentina 1, I-53100 Siena, Italy, and [†]Biomolecular Structure Research Centre, University of Siena, Via Fiorentina 1, I-53100 Siena, Italy

The structural characterization of a complex of α -bungarotoxin with a recombinant antibody fragment that mimics the acetylcholine receptor was achieved using docking simulation procedures. To drive the computer simulation towards a limited set of solutions with biological significance, a filter, incorporating general considerations of antigen–antibody interactions, specificity of the selected antibody fragment and results from α -bungarotoxin epitope mapping, was adopted. Two similar structures were obtained for the complex, both of them stabilized by cation– π and hydrophobic interactions due to tyrosyl

residues of the antibody fragment. Site-directed mutagenesis studies, removing each of the latter aromatic residues and causing full inactivation of the interaction process between the antibody fragment and the neurotoxin, support the validity of the calculated structure of the complex.

Key words: acetylcholine receptor, protein complex modelling, protein–protein interaction, scFv fragment, site-directed mutagenesis.

INTRODUCTION

It has become increasingly evident that proteins work in a concerted way [1] and therefore the analysis of protein–protein interactions represents a fundamental step for understanding biological processes at the molecular level. Thus several interactome projects have been launched to define clusters of interacting proteins in high throughput investigations related to genomic and/or proteomic studies [2]. Among the different kinds of protein–protein interactions, those which occur between protein ligands and their receptors assume a prominent relevance, as their delineation can be the rational basis for therapeutic interference. Unfortunately, structural characterizations of protein–protein complexes available in the Protein Data Bank (PDB) [3] are not enough for a general predictive set of references in interactome projects. Computer simulations of intermolecular protein interactions could be extensively used through suitable docking algorithms [4,5]. Nonetheless, in general, unrestrained docking simulations do not converge towards a limited number of structures, and filters are required to reduce the possible solutions given by calculations.

In the present study, we present the structural characterization of the molecular complex formed between a recombinant single chain antibody fragment (scFv) mimicking the nicotinic receptor binding site and the snake neurotoxin α -bungarotoxin, α -bgt. The anti- α -bgt scFv C12 used in this work was selected from a large phage library by competitive panning with the muscle acetylcholine nicotinic receptor (nAChR) [6]; this method, which is quicker than conventional immunization with receptor ligands, allows the direct selection of receptor-competing anti-ligand

scFv. Moreover, it allows the controlled modification of antibody sequence by gene mutation in order to increase affinity or to test the relevance of single amino acids in ligand binding. The scFv C12 binds α -bgt with an affinity constant of $5.56 \times 10^7 \text{ M}^{-1}$ and seems to mimic the receptor toxin-binding site, since i) it fully competes with the receptor for toxin binding; ii) the epitope recognized by the scFv, mapped on α -bgt sequence by overlapping synthetic peptides, is extremely similar to the region recognized by nAChR, and peptides covering the α -bgt second loop sequence and a C-terminal region are recognized by both scFv and nAChR; and iii) several positively charged residues included in these regions were found to be critical for peptide recognition by both scFv and nAChR.

The fact that functional aspects of this system have already been investigated in detail [6] offers the possibility of testing the reliability of ‘biological filters’ as a method of obtaining structural results which can be verified through biological tests.

EXPERIMENTAL

Computer modelling of the antibody fragment

The three-dimensional model of the scFv C12 (for amino acid sequence, see Figure 1) was built using the on-line modelling service WAM (<http://antibody.bath.ac.uk>) [7], which uses an updated version of the algorithm first implemented in AbM (Oxford Molecular Ltd., Oxford, U.K.). The framework was modelled using sequence-homologous antibodies of known structure as template: complementarity determining regions (CDRs) from antibody light (VL) variable chains and CDR1 and CDR2 from antibody heavy (VH) variable chains were built using

Abbreviations used: α -bgt, α -bungarotoxin; CDR, complementarity determining region; nAChR, nicotinic acetylcholine receptor; PDB, Protein Data Bank; scFv, single chain antibody fragment.

¹ To whom correspondence should be addressed (e-mail braccil@unisi.it).

E ¹	V	Q	L	V	E	S	G	G	G ¹⁰	L	V
Q	P	G	G	S	L	R	L ²⁰	S	C	A	A
S	G	F	T	F	S ³⁰	S	<u>M</u>	A	T	S	W
V	R	Q	A ⁴⁰	P	G	K	G	L	E	W	V
S	<u>A⁵⁰</u>	I	S	G	S	G	G	S	T	Y	Y ⁶⁰
A	D	S	V	K	G	R	F	T	I ⁷⁰	S	R
D	N	S	K	N	T	L	Y ⁸⁰	L	Q	M	N
S	L	R	A	E	D ⁹⁰	T	A	V	Y	Y	C
A	K	A	<u>M¹⁰⁰</u>	S	T	S	F	D	Y	W	G
Q	G ¹¹⁰	T	L	V	T	V	S	S	G	G	G
G	S	G	G	G	G	S	G	G	E	G	S
G	G	G	G	S	E ¹	I	V	L	T	Q	S
P	G	T ¹⁰	L	S	L	S	P	G	E	K	A
T ²⁰	L	S	C	R	A	S	Q	S	V	S ³⁰	S
S	<u>M</u>	L	A	W	Y	Q	Q	K ⁴⁰	P	G	Q
A	P	R	L	L	I	<u>Y⁵⁰</u>	G	A	S	S	R
A	T	G	I	P ⁶⁰	D	R	F	S	G	S	G
S	G	T ⁷⁰	D	F	T	L	T	I	S	R	L
E ⁸⁰	P	E	D	F	A	V	Y	Y	C	Q ⁹⁰	Q
T	G	Q	P	P	F	T	F	G ¹⁰⁰	Q	G	T
K	V	K	I	K							

Figure 1 Amino acid sequence of C12 scFv

Primary sequence of the anti- α -bgt scFv C12 in the configuration N-terminal–VH–linker–VL–C-terminal. Single amino acid codes are used according to standard IUPAC nomenclature. Amino acids in the CDRs are underlined. Amino acids in the flexible linker are in italic. Tyrosines mutually substituted with glycines and alanines, in order to confirm the reliability of model M34, are highlighted. The VH chain derives from DP47 human germ-line gene and the VL from DPK22 human germ-line gene [6].

known sequence-homologous loops of the same canonical class; the non-canonical CDR3-VH was modelled using a modified CAMAL method [8], which is based on a combined database/conformational search approach. The resulting structure was minimized with a 900 cycles run with the AMBER software suite [9] and the linker chain between the two domains was manually inserted, since there is no available structure in PDB databases for this moiety. The obtained substructure was optimized by a simulated annealing dynamic with AMBER. Although its conformation is not accurate, this linker was considered in the final C12 model, since its high hydrophobicity could be a relevant factor in the formation of large overlapping complexes.

Docking simulation

The docking procedure was carried out with ESCHER software [10], whose algorithm is based on a rigid body approach using polygons. The structures of a target and a probe protein are first simplified in stacks of polygons separated by 1.5 Å (1 Å = 0.1 nm) and aligned along parallel axes, then the probe is translated against the target along such axes in 1.5 Å steps. For each translation, the probe undergoes a full rotation around

Table 1 Primer sequences used in site-directed mutagenesis

Oligo name	Sequence
pelBback	5'-agc cgc tgg att gtt att ac-3'
5	5'-gct aaa ggt gaa tcc aga ggc tgc-3'
1	5'-gga ttc acc ttt agc agc gcc gcc acg-3'
9	5'-gga ttc acc ttt agc agc gcc gcc acg-3'
6	5'-ttt cgc aca gta ata tac ggc cgt-3'
2	5'-tat tac tgt gcg aaa gcg ggt agt acg-3'
10	5'-tat tac tgt gcg aaa gcg gcc agt acg-3'
7	5'-gct gct aac act ctg act gcc cct-3'
3	5'-cag agt gtt agc agc agc ggt tta gcc-3'
11	5'-cag agt gtt agc agc agc gcc tta gcc-3'
8	5'-gag gag cct ggg agc ctg gcc agg-3'
4	5'-gct ccc agg ctg ctg atc ggt ggt gca-3'
12	5'-gct ccc agg ctg ctg atc gcc ggt gca-3'
HisFlagFor	5'-gtg ctt gtc gtc gtc gtc ctt gta-3'

the axis perpendicular to polygons with a configurable step. For each step, the shape and charge complementarity of adjacent polygon edges from target and probe is evaluated and a score given according to a particular scoring function [10]. All the translation/rotation pairs are then sorted according to the score obtained. The entire process is repeated using a second set of polygons orthogonal to the first. In the present study, the scFv was chosen as the target protein, and a first coarse run with a rotation step of 10 degrees was carried out and approx. 5000 results were collected. The models that passed the biochemical filtering (described in the Results and Discussion section) were subjected to a second run with a rotation step of 2 degrees between $\pm 20^\circ$ of the starting position in order to refine the most probable structures. The major side-chain clashes were removed by a 900 cycles minimization in the AMBER force field [9].

Site-directed mutagenesis

For each mutation, the C12 gene was used as template for the amplification of segments with appropriate primers for each codon substitution [oligo 1 for Tyr32VH into Gly (i.e. oligo 1 is the primer used to mutate a tyrosine residue at position 32 in the VH region of the scFv fragment into a glycine), oligo 2 for Tyr100VH into Gly, oligo 3 for Tyr33VL into Gly, oligo 4 for Tyr50VL into Gly, oligo 9 for Tyr32VH into Ala, oligo 10 for Tyr100VH into Ala, oligo 11 for Tyr33VL into Ala and oligo 12 for Tyr50VL into Ala; see Table 1 for the sequences] and a reverse primer (HisFlagFor; see Table 1) for the C-terminal scFv region. A parallel amplification was carried out with primers overlapping the 5' end of the first forward primers (oligo 5 for Tyr32VH, oligo 6 for Tyr100VH, oligo 7 for Tyr33VL and oligo 8 for Tyr50VL; these oligos were used either for Gly or Ala substitution), and an oligonucleotide (PelBback) out of the N-terminal scFv region (see Table 1 for primer sequences). For each mutated clone, two amplified fragments were gel purified in order to eliminate traces of initial C12 template, and then assembled by PCR with primers PelBback and HisFlagFor.

The band of the correct molecular mass (whole mutated scFv gene; mutations were confirmed by DNA sequencing) was gel purified and cloned between *Nco*I and *Not*I restriction sites of pDN268 expression vector [11] and electroporated in TG1 *Escherichia coli* cells. The pDN268 plasmid allowed the expression of recombinant proteins in the bacterial periplasmic space and the cell culture supernatant. A rapid purification using ion metal affinity chromatography on to nickel resin columns (Qiagen, Chatsworth, CA, U.S.A.) was performed taking ad-

vantage of this expression system. Expression and purification of scFvs were performed following protocols described previously [12].

ELISA

ELISA of bacterial supernatants and purified antibodies was performed on streptavidin pre-coated microplates (SA plates, Boehringer Mannheim, Mannheim, Germany) coated with 10 nM biotinylated α -bgt and blocked with 3 % BSA. The anti-FLAG M2 monoclonal antibody (Kodak, Milan, Italy) followed by a peroxidase-conjugated anti-mouse IgG monoclonal antibody (Sigma Aldrich, Milan, Italy) was used to detect binding.

RESULTS AND DISCUSSION

C12 three-dimensional model

In the absence of experimental structural data, a model for C12, a single chain antibody fragment which mimics the acetylcholine receptor site for neurotoxin binding [6], was built as described in the Experimental section. As expected for large antigens [13], the combining site of the antibody fragment appears as a planar surface. From the analysis of the side chain composition of the combining site exposed surface, a marked dual polar/hydrophobic nature is apparent. Indeed, of the 57 residues constituting the six CDRs, 38 show a surface-exposed side chain, being 19 polar residues (14 serines, 3 threonines, 2 glutamines), 6 aromatic (5 tyrosines, 1 phenylalanine), 10 apolar (6 glycines, 3 alanines, 1 proline), and only 4 charged (2 arginines, 1 lysine, 1 aspartic acid), located in positions near the edge of the combining site surface.

Filtering criteria for the docking simulation of C12 with α -bgt

Once the three-dimensional model of the scFvC12 had been obtained, the α -bgt solution structure determined by NMR [14] was used for the docking simulation. As described in the Experimental section, this process was carried out in two steps, as a high number of different C12/ α -bgt complexes were obtained from a first docking simulation. These structural solutions were then filtered through a set of rules derived from experimental observations, to decrease the number of candidate structures.

The following biochemical criteria were introduced for this structural selection: i) since the complex of an antibody with a large antigen usually involves a large number of CDRs [13], only those complexes which showed contacts between the toxin and three or more CDRs were considered; ii) since CDR3s were the only variable CDRs in the phage library used to select C12 [15,16], only complexes involving at least one CDR3 were selected; iii) since results from epitope mapping obtained by overlapping synthetic peptides [6] indicated the critical role of toxin residues Arg36, Lys70, Arg25 and Lys26 for the interaction with C12, only complexes where Arg36, Lys70 and one from the couple Arg25/Lys26 were in contact with the antibody were taken into account. Models which passed through the biochemical filters were clustered in groups whenever they differed less than 5 Å in translation and less than 20 degrees in rotation. Only two main families, populated by 20 and 16 complexes each, were obtained (for a total of 36 models accepted by the filter), and on the most representative structure of each group, a second docking fine run was carried out. At this step, the best solutions of each run were chosen and then carefully visually analysed.

Figure 2 Models of the resulting scFv C12/ α -bgt complexes

The scFv C12/ α -bgt complexes M34 (top) and M38 (bottom) as resulted from docking study represented as spacefill: the C12 light chain is shown in blue with CDR1, CDR2 and CDR3 coloured in purple, cyan and blue respectively, while the heavy chain is shown in brown, with CDR1, CDR2 and CDR3 coloured in orange, yellow and red respectively. The α -bgt is represented in ivory as backbone only and loops are numbered with roman numerals.

Analysis of the resulting models

The two final resulting models, M34 and M38, are represented in Figure 2. The difference between the two models, which is clearly revealed by a first visual analysis, is the orientation of α -bgt, which appears to be rotated by approx. 180 degrees along the axis perpendicular to the centre of the combining site. In addition, a similar role in the interaction is suggested for toxin fingers I (i.e. residues Thr5–Ile11) and II (i.e. residues Trp28–Gly37), which, in both cases, are inserted at the two sides of the VH CDR3 loop, but in inverted positions, and lying across the combining site. In more detail, the M34 model presents the α -bgt loop II and loop I inserted between CDR3 and CDR2 of the variable light domain (VL), and between CDR3-VH and CDR3-VL respectively, with the α -bgt C-terminal Lys70–Gly74 sequence inserted between CDR1-VL and CDR2-VL. It should be noted that the C-terminal portion of α -bgt shows disordered conformations in both X-ray and NMR structures [14,17,18], and so it could be arranged in a barely predictable manner. As already mentioned, the alternative model, M38, presents the α -bgt rotated by approx.

Figure 3 Close view of the scFv C12/ α -bgt interface in the M34 and M38 models

The scFv C12/ α -bgt complex M34 (top) and M38 (bottom) as resulting from docking studies and represented as ribbons: the C12 light chain is shown in blue, the heavy chain in red and the toxin in green. The amino acids involved in key interactions are also shown.

180 degrees and loop I and II inverted in position with respect to M34, where the C-terminal segment of the toxin lies on top of CDR1-VH and CDR2-VH. It is noteworthy that all six CDRs are in contact with the toxin in both models.

A number of possible interactions between scFv and toxin residues can be predicted in both models. In particular, in the M34 model, cation- π interactions can be predicted between scFv Tyr33-VL (CDR1-VL) and toxin Lys70, scFv Tyr50-VL (framework, close to CDR2-VL which starts at residue 51), and toxin Arg36, scFv Tyr32-VH (CDR1-VH) and toxin Lys26 (Figure 3). Furthermore, in the M34 model, toxin Trp28 appears to interact with two opposite tyrosines of scFv, namely Tyr100-VH of CDR3-VH and Tyr32-VH of CDR1-VH. Consequently, a double cation- π /hydrophobic interaction appears to involve scFv Tyr32-VH which interacts with both Lys26 and Trp28 (Figure 3). In the M38 model, hydrophobic interactions can be predicted between scFv Trp47-VH (which occurs in the framework region, two residues before CDR2-VH) and scFv Tyr33-VL (CDR1-VL) with toxin Phe32 and Trp28 respectively. Moreover, scFv Tyr33-VL also seems to interact with toxin Lys26, while another cation- π interaction can be predicted between toxin Lys70 and

Table 2 Main features of M34 and M38 models

Complex buried surface area and energy decomposition (as calculated in the AMBER force field) of M34 and M38 models. 1 kcal $\equiv$ 4.184 kJ.

Model	Buried surface area ($\text{\AA}^2$)	H-bonds energy (kcal/mol)	Electrostatic energy (kcal/mol)	Van der Waals energy (kcal/mol)
M34	2844	-7.1	-50.9	-144.3
M38	2196	-3.7	-29.8	-97.9

scFv Tyr32-VH (CDR1-VH). By comparing the main features of the two complexes, summarized in Table 2, it is apparent that M34 has more favourable energy contributions and, at the same time, a larger interaction surface.

Site-directed mutagenesis studies in relation to the proposed complex structure

The selection and characterization of C12 have been described previously [6], and the production and purification of C12 were performed according to procedures reported in [12].

For the reasons outlined above, M34 was the preferred reference model for rational design of mutants. From the M34 model, Tyr32VH, Tyr100VH, Tyr33VL and Tyr 50VL appear to be critical for binding α -bgt (Figure 3). In order to confirm the reliability of the proposed filtered docking simulation, all these tyrosine residues were systematically mutated into Gly and Ala. These substitutions were performed by site-directed mutagenesis using PCR and the appropriate primers (Table 1). Mutated scFv genes were cloned into the expression vector pDN268, described previously [11], and new clones were analysed by DNA sequencing and then tested by ELISA and BIAcore. ELISA tests of culture supernatants on α -bgt-coated wells showed that mutations of each of these tyrosines (either substituted with Gly or Ala) caused a total loss of antibody activity (results not shown), confirming their fundamental role in the complex stability. The same culture supernatants were also assayed in BIAcore on streptavidin sensor chip coated with biotin- α -bgt (results not shown). The presence of scFvs in culture supernatants was checked by a further ELISA test in which wells were coated with anti-FLAG antibody and scFv binding was revealed by an anti-His-tag antibody. Results from this test verified that the absence of α -bgt-binding activity was not due to the reduction, or lack, of scFv expression in mutant culture supernatants.

CONCLUSIONS

With the aim of constructing a model system for the rational design of interaction surfaces, we studied the computational complex of an α -bgt-interacting scFv. For the structure reconstruction of the complex, we examined molecular docking in conjunction with binding data from toxin epitope mapping, in order to achieve a simulation driven by case-specific experimental criteria. It was found that the compact structure of the two α -bgt/scFv complexes (Figure 3) proposed by construction did indeed fully account for the previously reported binding data [11]. It is worth noting that both complexes obtained by docking simulation of C12 with α -bgt share the same pattern of interaction, with α -bgt fingers I and II lying on the combining site across the CDR3-VH loop. A large number of possible interactions between residues of C12 and α -bgt can be deduced on the basis of both models. In particular, in the M34 model, cation- π interactions can be predicted between i) C12 Tyr33, located

within the CDR1-VL and toxin Lys70; ii) C12 Tyr50-VL and toxin Arg36; and iii) C12 Tyr32, located within the CDR1-VH and α -bgt Lys26 (see Figure 3). Furthermore, in the same model, Trp28 of α -bgt appears to interact at the same time with two opposite tyrosines of C12, namely Tyr100 of CDR3-VH and Tyr32 of CDR1-VH. Consequently, C12 Tyr32, simultaneously involved in cation- π and hydrophobic interactions with both Lys26 and Trp28, seems to play a central role in stabilizing the complex formation.

In M38, hydrophobic interactions can be predicted between C12 Trp47, located in CDR2-VH, and C12 Tyr33, located in CDR1-VL, with α -bgt Phe32 and Trp28 respectively. Moreover, C12 Tyr33-VL also seems to interact with Lys26 of the toxin, while another cation- π interaction is predictable between α -bgt Lys70 and C12 Tyr32 of CDR1-VH. The resulting groove-type of interaction is consistent with other experimentally derived structures of antibody-antigen complexes [13], which supports the reliability of the calculated models. Thus both models can provide useful information about the molecular basis of toxin-receptor interaction, as they point out the presence of hydrophobic and cation- π interactions involving tyrosine residues of the antibody with aromatic and cationic residues of α -bgt. This kind of interaction could reproduce the molecular basis of neurotoxin binding by nicotinic receptors, as aromatic and hydrophobic residues located in the receptor binding site have been described as critical for neurotoxin binding [19,20]. Moreover, cation- π interactions are most probably involved in agonist [21] and neurotoxin binding to nicotinic receptors [20]. Interestingly, the snake neurotoxin invariant residue Lys26 has been proposed to interact with receptor Tyr190 through a cation- π interaction [20,22].

These results support the hypothesis that it is possible to obtain reliable structures of interacting protein systems from docking calculations, provided that the computer simulation can be driven by experimental biological data. The biochemically restrained computational procedure may yield information that is particularly useful for rational design of ligands with enhanced affinity and for defining, *in silico*, steric aspects of protein-protein interactions.

The authors thank Silvia Scali, Serena Lorenzini, and Camilla Bacci for their technical support. This work was supported by grants from the Italian "Ministero dell'Università e della Ricerca Scientifica e Tecnologica" (MURST-PRIN 2000) and from the University of Siena.

REFERENCES

- Pawson, T. (1995) Protein modules and signalling networks. *Nature (London)* **373**, 573–580
- Pawson, T., Raina, M. and Nash, P. (2002) Interaction domains: from simple binding events to complex cellular behavior. *FEBS Lett.* **513**, 2–10
- Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. and Bourne, P. E. (2000) The Protein Data Bank. *Nucleic Acids Res.* **28**, 235–242
- Smith, G. R. and Sternberg, M. J. (2002) Prediction of protein-protein interactions by docking methods. *Curr. Opin. Struct. Biol.* **12**, 28–35
- Halperin, I., Ma, B., Wolfson, H. and Nussinov, R. (2002) Principles of docking: An overview of search algorithms and a guide to scoring functions. *Proteins* **47**, 409–443
- Bracci, L., Pini, A., Lozzi, L., Lelli, B., Battistin, P., Spreafico, A., Bernini, A., Niccolai, N. and Neri, P. (2001) Mimicking the nicotinic receptor binding site by a single chain Fv selected by competitive panning from a synthetic phage library. *J. Neurochem.* **78**, 24–31
- Whitelegg, N. R. J. and Rees, A. R. (2000) WAM: an improved algorithm for modelling antibodies on the WEB. *Protein Eng.* **13**, 819–824
- Martin, A. C. R., Cheetham, J. C. and Rees, A. R. (1989) Modelling antibody hypervariable loops: a combined algorithm. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9268–9272
- Pearlman, D. A., Case, D. A., Caldwell, J. W., Ross, W. S., Cheatham, T. E. III, Ferguson, D. M., Seibel, G. L., Chandra Singh, U., Weiner, P. K. and Kollman, P. A. (1995) AMBER 4.1, University of California, San Francisco
- Ausiello, G., Cesareni, G. and Helmer Citterich, M. (1997) ESCHER: a new docking procedure applied to the reconstruction of protein tertiary structure. *Proteins* **28**, 556–567
- Neri, D., Petrucci, H., Winter, G., Light, Y., Marais, R., Britton, K. E. and Creighton, A. M. (1996) Radioactive labeling of recombinant antibody fragments by phosphorylation using human casein kinase II and [γ - 32 P]-ATP. *Nature Biotechnol.* **14**, 485–490
- Pini, A., Spreafico, A., Botti, R., Neri, D. and Neri, P. (1997) Hierarchical affinity maturation of a phage library derived antibody for the selective removal of cytomegalovirus from plasma. *J. Immunol. Methods* **206**, 171–182
- Webster, D. M., Henry, A. H. and Rees, A. R. (1994) Antibody-antigen interactions. *Curr. Opin. Struct. Biol.* **4**, 123–129
- Scarselli, M., Spiga, O., Ciutti, A., Bracci, L., Lelli, B., Lozzi, L., Calamandrei, D., Bernini, A., Di Maro, D., Klein, S. and Niccolai, N. (2002) NMR structure of α -bungarotoxin free and bound to a mimotope of the nicotinic acetylcholine receptor. *Biochemistry* **41**, 1457–1463
- Pini, A., Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P. and Neri, D. (1998) Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J. Biol. Chem.* **273**, 21769–21776
- Viti, F., Nilsson, F., Demartis, S., Huber, A. and Neri, D. (2000) Design and use of phage display libraries for the selection of antibodies and enzymes. *Methods Enzymol.* **326**, 480–505
- Zeng, H., Moise, L., Grant, M. A. and Hawrot, E. (2001) The solution structure of the complex formed between α -bungarotoxin and an 18-mer cognate peptide derived from the α 1 subunit of the nicotinic acetylcholine receptor from *Torpedo californica*. *J. Biol. Chem.* **276**, 22930–22940
- Love, R. A. and Stroud, R. M. (1986) The crystal structure of α -bungarotoxin at 2.5 Å resolution: relation to solution structure and binding to acetylcholine receptor. *Protein Eng.* **1**, 37–46
- Spura, A., Russin, T. S., Freedman, N. D., Grant, M., McLaughlin, J. T. and Hawrot, E. (1999) Probing the agonist domain of the nicotinic acetylcholine receptor by cysteine scanning mutagenesis reveals residues in proximity to the α -bungarotoxin binding site. *Biochemistry* **38**, 4912–4921
- Ackermann, E. J., Ang, E. T., Kanter, J. R., Tsigelny, I. and Taylor, P. (1998) Identification of pairwise interactions in the α -neurotoxin-nicotinic acetylcholine receptor complex through double mutant cycles. *J. Biol. Chem.* **273**, 10958–10964
- Zhong, W., Gallivan, J. P., Zhang, Y., Li, L., Lester, H. A. and Dougherty, D. A. (1998) From *ab initio* quantum mechanics to molecular neurobiology: a cation- π binding site in the nicotinic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 12088–12093
- Ackermann, E. J. and Taylor, P. (1997) Non-identity of the α -neurotoxin binding sites on the nicotinic acetylcholine receptor revealed by modification in α -neurotoxin and receptor structures. *Biochemistry* **36**, 12836–12844

Received 2 September 2002/2 January 2003; accepted 9 January 2003

Published as BJ Immediate Publication 9 January 2003, DOI 10.1042/BJ20021369