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

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KEYWORDS: protein-protein interaction; protein complex modelling; acetylcholine receptor; scFv fragment; site directed mutagenesis.

ABBREVIATIONS: PDB, protein data bank; scFv, single chain Fv; nAchR, nicotinic acetylcholine receptor; CDR, complementarity determining region.

RUNNING TITLE: Biochemically driven docking simulations.

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SYNOPSIS

The structural characterisation of a complex, which a recombinant antibody fragment mimicking the acetylcholine receptor forms with α-bungarotoxin, was achieved by 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 stabilised by cation-π and hydrophobic interactions, due to tyrosilyl residues of the antibody fragment. Site-directed mutagenesis studies removing each of the latter aromatic residues, causing full inactivation of the interaction process between the antibody fragment and the neurotoxin, support the validity of the calculated structure of the complex.

INTRODUCTION

Nowadays it is more and more evident that proteins work in a concerted way [1] and, therefore, the analysis of protein-protein interactions represent a fundamental step for the understanding biological processes at 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 all the different kinds of protein-protein interactions, the ones occurring between protein ligands and their receptors assume a prominent relevance, as their delineation can be the rational basis for therapeutic interferences.

Unfortunately, structural characterisations 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, unrestrained docking simulations do not converge in general towards a limited number of structures and filters are required to reduce the possible solutions given by calculations.

Here we present the structural characterisation of the molecular complex which a recombinant single chain antibody fragment (scFv) mimicking the nicotinic receptor binding site forms with the snake neurotoxin α-bungarotoxin, α-bgt.

The anti-α-bgt scFv C12 we used in this work, was selected from a large phage library by competitive panning with the muscle acetylcholine nicotinic receptor (nAchR) [6]: this method, quicker than conventional immunisation with receptor ligands, allows 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 aminoacids in ligand binding.

The scFv C12 bind α-bgt with an affinity constant of 5.56×10^{7} 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 recognised by the scFv, mapped on α-bgt sequence by overlapping synthetic peptides, is extremely similar to the region recognised by nAchR, and peptides covering the α-bgt second loop sequence and a C-terminal region were recognised by both scFv and nAchR; 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 been already investigated in detail [6] offers the possibility to test the reliability of "biological filters" as a procedure to obtain structural results which can be verified through biological tests.

EXPERIMENTAL

Computer modelling of the antibody fragment

The three-dimensional model of the scFv C12, henceforth called C12 (for aminoacid sequence see Table 1), was built by 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.). The framework was modelled using sequence-homologous antibodies of known structure as template; CDRs from VL and CDR1 and CDR2 from VH were built using sequence-homologous known 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 minimised 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 optimised 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 and aligned along parallel axes, then the probe is translated against the target along such axis in 1.5 Å steps. For each translation the probe undergoes a full rotation around 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 (see [10] for details). All the translation/rotation pairs are then sorted according to the score obtained. The entire process is repeated after using a second set of polygons orthogonal to the first. In our case, the scFv was chosen as the target protein, and a first coarse run with rotation step of 10 degrees was carried out and approximately 5000 results were collected. The models that passed the biochemical filtering (described in Results and Discussion section) were subjected to a second run with a rotation step of 2 degrees between ±20° of the starting position in order to refine the most probable structures. The major side-chain clashes were removed by a 900 cycles minimisation in the AMBER force field [9].

Site-directed mutagenesis

For each mutation, C12 gene was used as template for the amplification of segments with primers holding each codon substitution (oligo 1 for Y32VH in Gly, oligo 2 for Y100VH in Gly, oligo 3 for Y33VL in Gly, oligo 4 for Y50VL, oligo 9 for Y32VH in Ala, oligo 10 for Y100VH in Ala, oligo 11 for Y33VL in Ala and oligo 12 for Y50VL in Ala; see Table 2 for the sequences) and a reverse primer (HisFlagFor; see Table 2) lying out 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 Y32VH, oligo 6 for Y100VH, oligo 7 for Y33VL and oligo 8 for Y50VL; these oligos are used either for Gly or Ala substitution), and an oligonucleotide (PelBback) out of the N-terminal scFv region (see Table 2 for primer sequences). For each mutated clone we had two amplified fragments which were gel purified in order to eliminate traces of initial C12 template, and then assembled by polymerase chain reaction with primers PelBback and HisFlagFor.

The band of the right molecular weight (whole mutated scFv gene; mutations were confirmed by DNA sequencing) was gel purified and cloned between NcoI and NotI restriction sites of pDN268 [11] expression vector and electroporated in TG1 E.Coli cells. The pDN268 plasmid allows the expression of recombinant proteins in bacterial periplasmic space and in supernatants of cell culture. A rapid purification by Ion Metal Affinity Cromatography onto Nickel resin columns

(Qiagen, Chatsworth, CA) was performed taking advantage of this expression system. Expression and purification of scFvs were performed following protocols described in [12].

ELISA

ELISA of bacterial supernatants and purified antibodies was performed on streptavidin precoated microplates (SA plates, Boehringer Mannheim, Mannheim, Germany) coated with 10 nM biotinylated α-bgt and blocked with 3% bovine serum albumin (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 the 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 antigen binding [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 of polar type (14 Serines, 3 Threonines, 2 Glutamines), six aromatic (5 Tyrosines, 1 Phenilalanin), 10 apolar (6 Glycines, 3 Alanines, 1 Proline), and only 4 charged (2 Arginines, 1 Lysine, 1 Aspartic acid). Charged aminoacids are a few and 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 was obtained, the α-bgt solution structure determined by NMR [14] was used for the docking simulation. As described in the experimental section, such process has been carried out in two steps, as a high number of different C12/α-bgt complexes were obtained from a first docking simulation. Afterwards, these structural solutions were filtered through a set of rules, derived from experimental observations, to decrease the quantity of the candidate structures.

Biochemical criteria can be introduced for this structural selection, such as the followings: i) since the complex of an antibody with a large antigen usually involve a large number of CDRs [13] only those complexes which showed contacts between the toxin and 3 or more CDRs were considered;

ii) since CDR3s are 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, 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.

Analysis of the resulting models

The resulting two final models, henceforth called M34 and M38, are represented in Figure 1. The difference between the two that is clearly revealed by a first visual analysis is the orientation of α-bgt, that appears to be rotated by about 180 degrees along the axis perpendicular to the centre of the combining site. Beside this, a similar role in the interaction is suggested for toxin fingers I (*i.e.* residues T5-I11) and II (*i.e.* residues W28-G37), in both cases inserted at the two sides of the variable heavy domain (VH) CDR3 loop, but in swapped positions, and lying across the combining site. In more details, the M34 model presents the α-bgt loop II and loop I inserted respectively between CDR3, and CDR2 of the variable light domain (VL), and between CDR3-VH and CDR3-VL, with the α-bgt C-terminal K70-G74 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], so it could be arranged in a hardly predictable manner. As already mentioned, the other model, M38, presents the α-bgt rotated by about 180 degrees and loop I and II swapped in position respect to M34, while the toxin C-terminal segment lays on top of CDR1-VH and CDR2-VH. It is worth noting that all the 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 Y33-VL (CDR1-VL) and toxin K70, scFv Y50-VL (framework, close to CDR2-VL which starts at residue 51) and toxin R36, scFv Y32-VH (CDR1-VH) and toxin K26 (Figura 3). Furthermore, in the M34 model, toxin W28 appears to interact with two opposite tyrosines of scFv, namely Y100-VH of CDR3-VH and Y32-VH of CDR1-VH. Consequently, a double cation-π/hydrophobic interaction appears to involve scFv Y32-VH which interacts with both K26 and W28 (Figure 2). In the M38 model, hydrophobic interactions can be predicted between scFv W47-VH (framework, two residues

before CDR2-VH) and scFv Y33-VL (CDR1-VL) with respectively toxin F32 and W28. Moreover, scFv Y33-VL seems to interact also with toxin K26, while another cation-π interaction can be predicted between toxin K70 and scFv Y32-VH (CDR1-VH).

By a comparison of the main features of the two complexes, summarised in Table 3, 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

Selection and characterisation of C12 has been previously described [6], whilst production and purification of C12 were performed according to procedures reported elsewhere [12].

For the reasons above outlined, M34 was preferred to M38 as a reference model for rational design of mutants. From M34 model, Tyr32VH, Tyr100VH, Tyr33VL, Tyr 50VL appear to be critical for binding α-bgt (Figure 2). In order to confirm the reliability of the proposed filtered docking simulation, all these Tyr were systematically mutated in Gly and in Ala. These substitutions were performed by site-directed mutagenesis using polymerase chain reaction and designed primers (Table 2). Mutated scFv genes were cloned in the expression vector pDN268, described elsewhere [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 in Gly or in Ala) caused the full drop of antibody activity (not shown), confirming their fundamental role in the complex stability. The same culture supernatants were also assayed in BIACORE on SA sensor chip coated with biotin-α-bgt (not shown). The presence of scFvs in culture supernatants was checked by a further ELISA test where wells were coated with anti-flag antibody and scFvs revealed by an anti-His-tag antibody. Results from this test allowed to verify that the absence of α-bgt-binding activity was not due to lacking or lower scFv expression in mutants culture supernatants.

CONCLUSIONS

With the aim to construct a model system for the rational design of interaction surfaces we propose the computational complex of a α-bgt interacting scFv. For the structure reconstruction of the complex we propose molecular docking in conjunction with binding data from toxin epitope mapping, in order to achieve a simulation driven by case-specific experimental criteria. Indeed, the compact structure of the two proposed α-bgt/scFv complexes (Figure 2) by construction fully

accounts 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 deducted on the basis of both models. In particular, in M34 model cation-π interactions can be predicted between i) C12 Tyr33, located within the CDR1-VL and toxin K70; ii) C12 Y50-VL and toxin R36 and iii) C12 Y32, located within the CDR1-VH and α-bgt K26 (see Figure 2). Furthermore, in the same model, W28 of α-bgt appears to interact at the same time with two opposite tyrosines of C12, namely Y100 of CDR3-VH and Y32 of CDR1-VH. Consequently, C12 Y32, simultaneously involved in cation-π and hydrophobic interactions with both K26 and W28, seem to play a central role in stabilising the complex formation.

In M38, hydrophobic interactions can be predicted between C12 W47, located in proximity of CDR2-VH, and C12 Y33, CDR1-VL, respectively with α-bgt F32 and W28. Moreover, C12 Y33-VL seems to interact also with K26 of the toxin, while another cation-π interaction is predictable between α-bgt K70 and C12 Y32 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, indeed, the molecular basis of neurotoxin binding by nicotinic receptors, as aromatic and hydrophobic residues located in 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 K26 has been proposed to interact with receptor Y190 through a cation-π interaction [20, 22].

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

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Table 1.

Amino acid sequence of C12 scFv

Primary sequence of the anti-α-bgt scFv C12 in 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].

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	Y	A	T	S	W
V	R	Q	A[40]	P	G	K	G	L	E	W	V
S	A[50]	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	Y[100]	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	Y	L	A	W	Y	Q	Q	K[40]	P	G	Q
A	P	R	L	L	I	Y[50]	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							

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Table 2.

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 ggc 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 ggc cct3'-
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 ctc ctc atc ggt ggt gca-3'
12	5’-gct ccc agg ctc ctc atc gcc ggt gca-3’
HisFlagFor	5'-gtg ctt gtc gtc gtc gtc ctt gta-3'

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Table 3.

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.

Model	Buried surface area (A^2)	H-bonds energy (Kcal/mole)	Electrostatic energy (Kcal/mole)	Van der Waals energy (Kcal/mole)
M34	2844	-7.1	-50.9	-144.3
M38	2196	-3.7	-29.8	-97.9

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Caption to figure 1:

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 L chain is shown in blue with CDR1, CDR2 and CDR3 coloured in purple, cyan and blue, respectively, while the H chain is shown in brown, with CDR1, CDR2 and CDR3 coloured in orange, yellow and red, respectively. The α-bungarotoxin is represented in ivory as backbone only. α-bgt loop numbering is reported as roman numbers.

Caption to figure 2:

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 resulted from docking study represented as ribbons: the C12 L chain is shown in blue, the H chain in red and the toxin in green. The aminoacids involved in key interactions are also shown.

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Figure 1

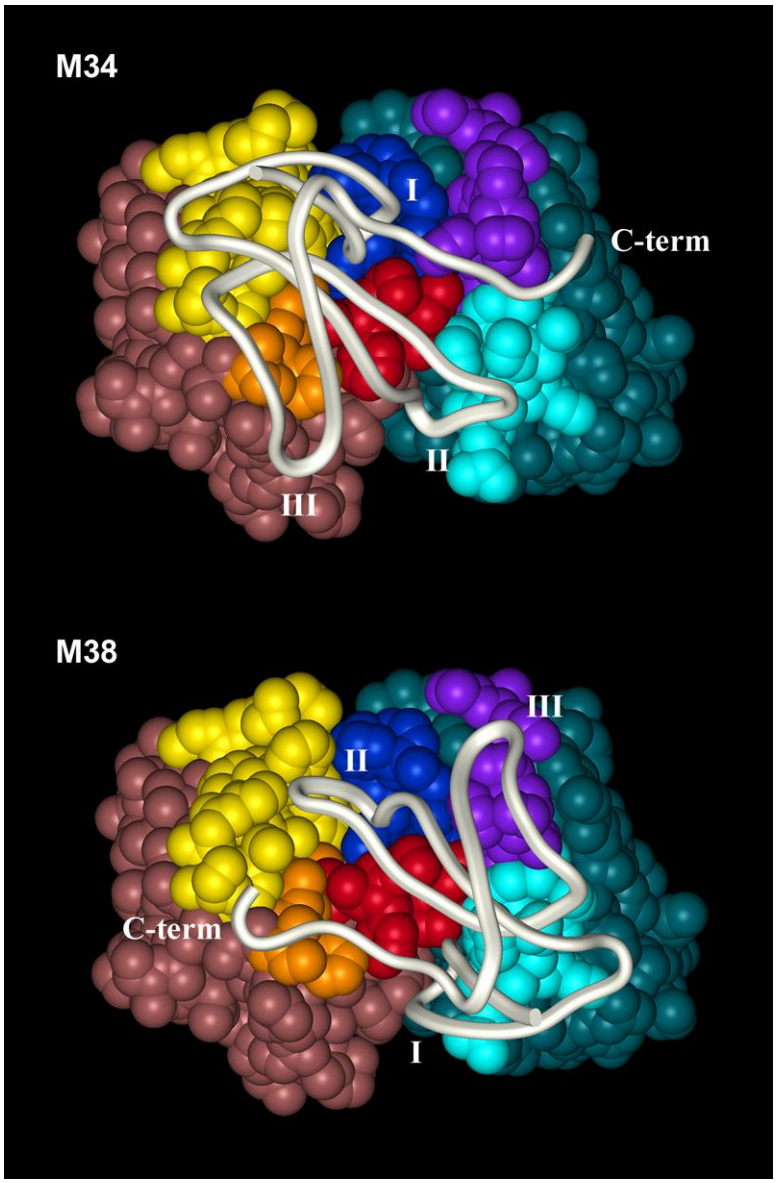

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Figure 2