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Research in Microbiology 153 (2002) 395–398

Research in Microbiology

www.elsevier.com/locate/resmic

Harnessing MalE for the study of antigen/antibody recognitions

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Received 17 April 2002; accepted 11 June 2002

Abstract

The construction of hybrids between the variable fragment (Fv) of antibodies and protein MalE of *Escherichia coli* at the genetic level makes possible their preparation in a functional state, independently of any interaction with the antigen. We used such hybrids and a mutagenesis approach to study the recognition between antibody D1.3 and its antigen lysozyme, and its maturation. We subsequently transformed D1.3 into a reagentless fluorescent biosensor by knowledge-based design.

Keywords: Affinity; Amylose; Antibody; Antigen; Biosensor; Fluorescence; MalE; Maltose binding protein; Maturation; Purification; Recognition; Variable fragment

1. Introduction

This review is a summary of the talk that one of the authors (H.B.) gave on the 11th of March 2002, at the meeting in honor of Maurice Hofnung. Therefore, its introduction and conclusion are written in the first person of the singular. The remainder describes a collaborative work and is therefore in the first person of the plural.

I (H.B.) prepared my doctoral thesis in Maurice Hofnung's laboratory between 1978 and 1983. We studied the relations between structure and function for three different components of the maltose system from *Escherichia coli*, in collaboration with other laboratories. We analyzed the regulatory interval, which is located between the two divergent operons of the *malB* region [3–5]. This study has been subsequently continued by Olivier Raibaud and Evelyne Richet at the Pasteur Institute [33,34]. We analyzed the signal peptide of the maltose binding protein, MalE, in collaboration with Phil Bassford and Jon Beckwith [1,2]. Finally, we initiated an analysis of the MalE protein itself, with Pascale Duplay [20–22]. For this last analysis, we used a method of random mutagenesis by insertion of DNA linkers, which had been developed by Fred Heffron [28]. Some of our mutants are still under study at the Pasteur Institute [10].

2. The use of MalE as a protein vector

In 1984–1985, I was working on tyrosyl-tRNA synthetase with Greg Winter, at the MRC Laboratory of Molecular Biology in Cambridge [6,18]. There, David Goldenberg and Tom Creighton studied the folding of BPTI, which comprises several disulfide bonds. They were trying to export BPTI into the periplasmic space of *E. coli* and to purify it by fusion with an exported protein [26]. The difficulties that they encountered gave me the idea to use MalE as a vector to export and purify foreign proteins. I put this idea into practice on my return to France in 1986. We published a short note on this research, then submitted the corresponding manuscript to the European Journal of Biochemistry [7,8]. Simultaneously, Hiroshi Inouye, a former post-doc in Jon Beckwith's laboratory, and his collaborators (New England Biolabs) submitted a comparable work to Gene [19]. Unfortunately for us, NEB had filed a patent on the subject a few weeks before the Pasteur Institute. Nevertheless, our work provided one of the first experimental proofs showing that it is possible to export cytoplasmic proteins through a biological membrane.

We have constructed many different hybrids between foreign proteins and MalE [8,9,12–14,35]. In particular, we have shown that the construction of hybrids between the variable fragment Fv of antibodies and MalE makes possible their export into the periplasmic space, the correct formation of their essential disulfide bonds, their purification by affinity chromatography on cross-linked amylose,

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0923-2508/02/$ – see front matter
PII: S0923-2508(02)01337-2

and thus their preparation in a functional state, independently of their binding to the antigen [15–17]. We used these Fv-MalE hybrids to study the recognitions between antibodies and antigens by a mutational approach. Although these recognitions look familiar, they still pose many fundamental questions.

3. Interfaces between antibodies and antigens

As an experimental system, we used antibody mAbD1.3 and its antigen hen egg lysozyme. The crystal structure of their complex was solved by Roberto Poljak and his collaborators at the Pasteur Institute [11]. This structure defines the set of the topological contacts between the two macromolecules, that we call structural interface. It also shows that a large number of water molecules are trapped in the interface. Unfortunately, the crystal structure gives only hints about the energetic importance of these topological contacts, and about the kinetic process through which the encounter between the two molecules occurs. These data are, however, fundamental to understanding in depth the molecular recognitions. We therefore asked the following questions: Which residues of the structural interface between mAbD1.3 and lysozyme are important for the rate of association, for the rate of dissociation, and for the energy of the interaction? What is the role of the water molecules trapped in the interface?

To answer these questions, we tested the functional importance of about ten topological contacts between mAbD1.3 and lysozyme. These contacts involved residues of the three CDR loops of the heavy-chain variable domain V_H, and of the three CDR loops of the light-chain variable domain V_L. We removed these contacts by mutagenesis of a Fv-MalE hybrid and then measured the consequences of the mutations on the kinetics of interaction between the hybrid and immobilized lysozyme, by Biacore. The mutations affected k_{on} weakly, by less than 3-fold, and they could affect k_{off} strongly, by more than 1000-fold. The mutations which most strongly affected k_{off} belonged to the CDR3 loop of V_H and the CDR3 loop of V_L. Some of the corresponding residues were aromatic, like Trp92 in V_L, and others were charged like Asp100 in V_H.

The crystal structure shows that residue L-Tyr32 could interact with lysozyme indirectly, by the intermediate of two water molecules, and its change into Phe should abolish these interactions. We found that the mutation of L-Tyr32 into Phe decreased the affinity by 40-fold, corresponding to 2.2 kcal/mol. Thus, such indirect bonds, mediated by water molecules, can be energetically important [23].

Fig. 1 summarizes the results: Some topological contacts were not functionnally important. The important residues were in a limited number, mainly in the CDR3 loops of V_H and V_L, at the centre of the interface with lysozyme, and could be hydrophobic or charged. Some indirect contacts, established through water molecules, also played a significant role. Thus, the structural and functional interfaces did not coincide.

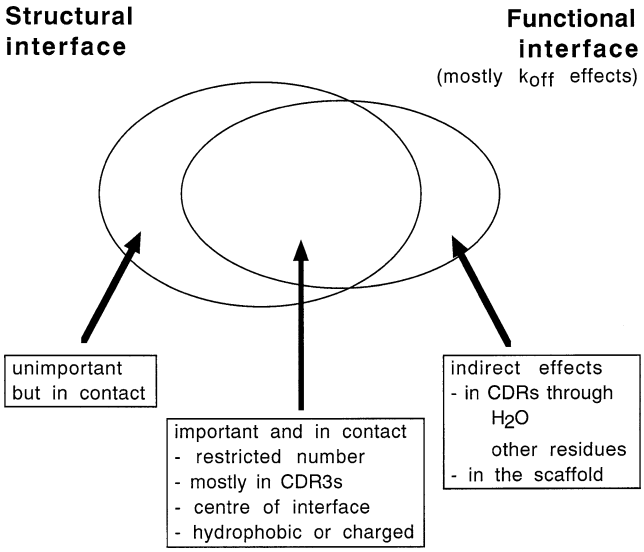

Fig. 1. Relations between the residues which belong to the structural and functional interfaces, in a complex between antibody and antigen.

4. Affinity of antibodies and presentation of their antigen to T cells

We had constructed 10 mutations or so in the Fv-MalE hybrid derived from mAbD1.3. In collaboration with P. Guermonprez and C. Leclerc, we used the mutant hybrids to study the relations between the affinity of an antibody and the presentation of its antigen to T cells. The presentation to T cells was measured by the release of the IL2 cytokine. We found that the dissociation constant between the Fv-MalE hybrids and lysozyme determined the efficiency of its capture by the B cells and presentation to the T cells, with a threshold value of 0.6 μM. Therefore, the affinity maturation in the B lymphocytes could increase their capacity to collaborate with the T cells, and thus contribute to the selection of the B cell clones having a high affinity for the antigen [27].

5. Structural mechanism of affinity maturation

Somatic mutations improve the affinity of antibodies during the secondary phase of the immune response against an antigen. A precise understanding of their mechanism of action would certainly be useful to engineer antibodies rationally. mAbD1.3 contains five somatic mutations. Only one of the mutated residues, L-Tyr50, is in contact with lysozyme in the complex. We asked the following questions: Can we reconstitute the germinal antibody from which mAbD1.3 derived? What is the affinity of the germinal antibody for lysozyme? What are the contributions of the

individual mutations for affinity? Are these contributions synergistic or additive? Can the structure explain these contributions? Can these contributions explain the selection of the mutations?

To answer these questions, we started from mAbD1.3, in a Fv-MalE format, and reverted its five somatic mutations, by mutagenesis, to reconstitute the germinal antibody. Then, we reintroduced each somatic mutation, taken individually or in combination, into this germinal context and measured its kinetic and energetic importance by Biacore.

The mutations had no effect on the association rate and induced a 60-fold improvement in the dissociation rate. Most of the affinity improvement (30-fold) was due to only one mutation, L-N50Y, which affects a residue in direct contact with lysozyme. Whether and how the other mutations were selected remains unknown [24].

6. Reagentless fluorescent biosensors

In a further development, we transformed a single-chain derivative of FvD1.3 into a reagentless fluorescent biosensor. For such a biosensor, the formation of a complex with the antigen generates a fluorescence signal which is directly measurable, without any addition of reagents. We devised the following strategy (Fig. 2). We start from a single chain variable fragment (scFv) of a mAb. A residue of this fragment is identified by rules of design: (1) it must be in the vicinity of the antigen in the complex. (2) It must not be functionally important. (3) It must be accessible to the solvent in the structure of the free antibody. This residue is changed into a cysteine by mutagenesis. A fluorophore is then chemically coupled to the mutant cysteine in conditions that do not cleave the two essential disulfide bonds. Binding of the antigen modifies the electronic environment of the fluorophore and can be detected by a change of fluorescence.

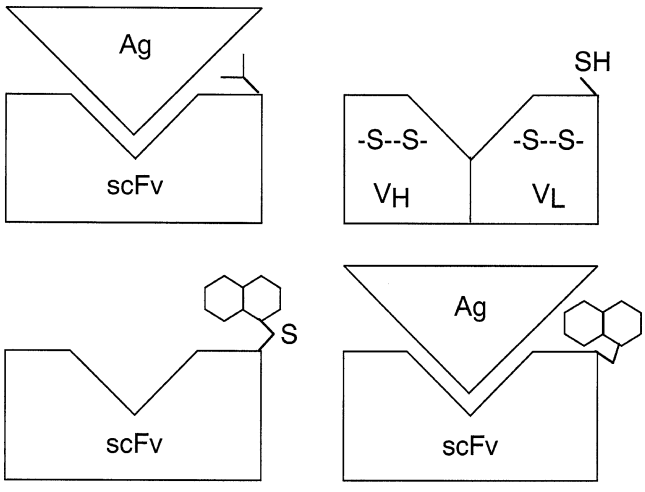

Fig. 2. Construction of a reagentless fluorescent biosensor from an antibody fragment. *Top left:* A residue, close to the paratope but non-essential for the interaction with the antigen, is identified. *Top right:* This residue is mutated into a cysteine. *Bottom left:* A fluorophore, sensitive to its microenvironment, is coupled to the reduced thiol group without altering the disulfide bonds of the antibody fragment. *Bottom right:* The binding of the antigen modifies the environment of the fluorophore and leads to a change in its fluorescence. Adapted from [32].

To demonstrate the validity of the design rules, we used mAbD1.3. We created conjugates involving 10 residues which fully satisfied the design rules, and 7 residues which failed to satisfy one of them. Six of the 10 conjugates which fully satisfied the design rules, had a relative variation of fluorescence intensity upon antigen binding which was larger than 10%, and their affinities for lysozyme remained unchanged. In contrast, only two of the seven conjugates which failed to satisfy one of the rules had such an increase of fluorescence. Note that the affinity of one of these two conjugates, constructed from L-Trp92, was decreased 1000-fold.

We studied one of the conjugates, involving residue L-Ser93, in more detail. Its fluorescence intensity varied linearly with the concentration of lysozyme, and allowed us to specifically titrate lysozyme either in a defined buffer or in a complex mixture like serum, with a detection threshold of 10 nM. The conjugate could distinguish between hen lysozyme and turkey lysozyme, a closely related protein [32].

We are now extending our rules of design to antibodies of unknown structure. This type of reagentless fluorescent biosensor could then have numerous applications in the micro- and nano-analytical sciences, in particular in the form of protein chips or at the tip of optical nano-fibers.

7. Conclusion

Thanks to the extremely fertile atmosphere that Maurice Hofnung created and to the vast array of new technologies that were made available in his laboratory, I (H.B.) was able to simultaneously undertake projects on the control of the maltose system, the export of protein MalE and the relations between the structure and functions of this protein. My thesis work thus led me naturally to the study of proteins by the methods of protein engineering and directed evolution.

The use of MalE as a vector for the production and study of proteins was very fruitful because it became apparent that MalE is uncommonly effective at promoting the stability of polypeptides to which it is fused [16,29]. Fusions with MalE enabled us to study the mechanisms of recognition between antibodies and antigens from structural, energetic, and kinetic viewpoints. In turn, our studies on antibodies provided us with the knowledge and concepts necessary to transform antibodies into reagentless fluorescent biosensors. Other groups transformed MalE into a fluorescent biosensor for maltose, and then used MalE as a scaffold to construct biosensors with designed specificities [25,30,31]. Both approaches may turn out to be general.

Acknowledgements

We thank Shamila Naïr for critical reading of the manuscript. This research was funded by grants from the Ministry of National Education, Research and Technology (programme for fundamental research in microbiology, infectious and parasitic diseases) and from the Ministry of Defense (contract 99 34 043/DSP/STTC).

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