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Review

Monoclonal antibodies for the structural analysis of the Na^+/H^+ antiporter NhaA from *Escherichia coli*

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Abstract

Since their advent some 25 years ago, monoclonal antibodies have developed into powerful tools for structural and functional analysis of their cognate antigens. Together with the respective antigen binding fragments, antibodies offer exclusive capacities in detection, characterization, purification and functional assays for every given ligand.

Antibody-fragment mediated crystallization represents a major advance in determining the three-dimensional structure of membrane-bound protein complexes. In this review, we focus on the methods used to generate monoclonal antibodies against the NhaA antiporter from *Escherichia coli* as a paradigm of secondary transporters. We describe examples on how antibodies are helpful in understanding structure and function relationships for this important class of integral membrane proteins.

The generated conformation-specific antibody fragments are highly valuable reagents for co-crystallization attempts and structure determination of the antiporter.

Keywords: Membrane protein; Antibody fragment; Na^+/H^+ antiporter; Crystallization

1. Introduction

Antibodies are widely used in probing the biochemical function of proteins, molecular complexes or ligand–receptor interactions. They are also becoming important tools for structural studies. Antibody Fab and Fv fragments can readily be crystallized and their structures determined at high resolution. In most cases, the common antibody folding domains are reported, but some exceptions occasionally arise when examining their structural features in greater detail. For example, uncommonly long and extended loop structures in certain complementary determining regions have been observed [1]. This fact correlates with binding modes, in which loops reach into receptor cavities, and has been explained via a mechanism of immune adaptation [2]. Also structural information for complexes of antibody Fab and Fv fragments bound to their respective antigens are becoming more and more available, with some very interesting cases of co-crystallization with flexible protein antigens or small haptens [3,4]. Moreover, diverse single antibody domains such as *Camelidae* VHH fragments generated against a number of antigens are successfully used for structural studies [5].

An intriguing possibility is represented by the use of antibody ligands for the co-crystallization of membrane proteins [6]. In 1995, the structure of a bacterial cytochrome *c* oxidase bound to an Fv fragment was presented [7]. The latter is the smallest stable domain of an antibody molecule that retains antigenic specificity and affinity amenable to its use as ‘‘crystallizing ligand’’. After this breakthrough, other significant structures of membrane protein complexes co-crystallized with the help of antibody fragments followed: the cytochrome bc_1 complex from yeast with a bound Fv fragment [8] and the high resolution structure of a potassium channel in complex with a Fab fragment [9]. In the latter case, the Fab fragment could even be exploited for structure solution. The phases were determined by molecular replacement using a known structure of an Fab fragment, as the immunoglobulin fold is highly conserved. Furthermore, the crystals of the channel–Fab fragment complex diffracted substantially better than those of the complex alone.

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The role that an antibody ligand can have in promoting the crystallization of a membrane protein is obvious: the antibody enlarges the hydrophilic surface of the antigen, thus promoting molecular interactions and facilitating nuclei formation and ordered crystal growth. Furthermore, antibodies can help in trapping a flexible protein in a fixed conformation. In the present review, we describe the generation and use of monoclonal antibodies against native epitopes of the NhaA protein, the main Na^+/H^+ antiporter from *Escherichia coli*.

2. The bacterial antiporter NhaA: functional overexpression and purification

NhaA is an important secondary pump which uses the proton gradient to actively extrude sodium and lithium ions from the bacterial cytoplasm. The protein plays an important role in adaptation of the cells at high sodium concentrations, challenging lithium toxicity and pH homeostasis (in the presence of sodium) [10]. NhaA activity has a pronounced pH dependence, changing its V_{max} by over three orders of magnitude between pH 6.5 and 8.5. This phenomenon reflects its function in vivo and it is accompanied by a drastic, fairly global conformational change which has been probed by different biochemical methods [11,12].

High resolution structural information for NhaA is not available. From a model derived from highly ordered two-dimensional crystals of the transporter [13] and from supporting biochemical evidence [14], it is clear that the protein consists of 12 putative trans-membrane segments, which are likely to be α-helices, and that it functions as a homodimer. However, identification of the precise helix arrangement and amino-acid sequence assignment will only be possible with a high resolution structure of the transporter, which can be achieved by X-ray crystallography. This will shed light on the mechanism of proton pumping, sodium and lithium specificity and pH adaptation in Gram-negative bacteria and provide further working hypotheses for understanding the basis of these phenomena in higher organisms.

NhaA has been overproduced in an *E. coli* homologous system [15] and it can constitute up to 10% of the total membrane proteins when its production is induced via the *tac* promoter. NhaA is efficiently purified via Immobilized Metal Affinity Chromatography (IMAC) making use of six consecutive histidine residues (His-tag) appended at the C terminus of the protein. After a subsequent gel filtration step, NhaA becomes 99% pure as judged by SDS-PAGE. The purified dodecyl-β-D-maltoside solubilized transporter shows good monodispersity properties and is fully functional when reconstituted in liposomes of various nature and composition [16]. This protein preparation is, therefore, an excellent candidate for X-ray crystallography and other structural studies. Additionally, a wealth of information on its biochemical characteristics is available and can aid and complement the detailed knowledge of the molecular architecture of this transporter.

3. Generation of monoclonal antibodies against conformationally sensitive epitopes of NhaA: immunization and selection

For successful generation of several specific hybridoma clones, one of the first important steps is the immunization of the animals with antigen to generate a sufficient antibody titer. We immunized 10-week-old female BALB/c mice by intra-peritoneal injection of highly purified, detergent solubilized protein [100-μg protein in 50% (v/v) adjuvant (ABM2, Pan Biotech GmbH, Aidenbach, Germany) at a final volume of 250 μl]. The initial immunization was followed by four injections with protein suspension in 4-week intervals [50-μg protein in 50% (v/v) adjuvant (ABM1, same supplier) with a final volume of 250 μl]. For the final boost the latter injection was repeated on 3 consecutive days and the mouse was sacrificed the following day for removal of the spleen. Blood sera of the mice taken 2 weeks after the third immunization showed clear positive signals up to a serum dilution of 10^5 in a standard ELISA using purified NhaA as antigen [17].

It is evident that such an immunization regime in small mammals for highly homologous membrane proteins (i.e. derived from human, mouse, rat, monkey) may be less likely to produce antibodies because of self-tolerance mechanisms. There are, however, several ways that can help to stimulate immune response such as genetic immunization [18–20] or simply the use of improved lipid nanoparticle-based adjuvants [21].

A promising alternative to raising monoclonal antibodies in mice is the adaptation of phage-display antibody technology to the in vitro selection of ligands for membrane proteins. Up to now, examples for successful selection of antibodies specific for membrane proteins via phage display of nonimmune libraries are rare. We anticipate that other strategies may be worth pursuing to establish phage display-mediated screening of antibodies against these targets, namely: (1) generation of more diverse ($>10^{10}$ different elements) antibody libraries; (2) use of a modified helper phage, which allows multivalent presentation of the antibodies [22]; (3) screening for ligands after a single round of bio-panning followed by in vitro affinity maturation [23].

Selection for conformation-specific monoclonal antibodies against membrane proteins is a difficult task. For the preparation of the required pure antigen, membrane proteins have to be detergent-solubilized and they are purified as protein–detergent complexes. The type and concentration of detergent is crucial to keep the protein in its native conformation. Some detergents may prevent proteins from binding to plastic and polystyrene surfaces used as common ELISA supports. In addition, adsorption to the solid phase can cause partial denaturation of the protein [24]. Thus, an

alternative robust strategy has to be devised for proper immobilization and presentation of the "native" antigen. NhaA was immobilized on Ni^{2+}-NTA-coated ELISA plates simply following the procedure for immobilization of the transporter on the affinity matrix during IMAC purification [17]. Under these buffer conditions, the protein is fully functional, properly folded in the presence of the mild detergent dodecyl-β-D-maltopyranoside and can be subjected to the screening for antibodies. The procedure was named HIS-TAG ELISA.

Starting from more than 2000 hybridoma clones, implementation of the HIS-TAG ELISA (as opposed to a standard ELISA) allowed the isolation of six different monoclonal antibodies of the IgG type, four of which (IgG1 1F6, IgG1 2C5, IgG2a 6F9 and IgG2b 5H4; all of them containing κ-type light chains) have been further characterized. All of the antibodies obtained are conformation-sensitive and bind the detergent-solubilized antiporter as well as its membrane bound form [17]. Only one of these antibodies is positive in Western blot analysis after SDS-PAGE separation of the protein. However, this antibody (1F6) binds to a linear but conformation-specific epitope located at the N terminus of the enzyme [12]. This antibody binds NhaA in a pH-dependent fashion which mirrors the activation of the protein via pH changes and its associated conformational change as probed by trypsin digestion [11]. Such a ligand can be extremely useful for the immuno-affinity purification of the transporter from crude membrane extracts as discussed below.

The novel HIS-TAG ELISA is a powerful method, which combines the sensitivity, specificity and high signal/noise ratio of a common immunosorbent assay with recombinant protein chemistry. It can easily be adapted to all recombinant proteins, which are purified with the help of a histidine tag.

Alternative methods for the presentation of membrane proteins in their native conformation have been reported, such as the selection with lipid reconstituted samples [25], native enriched membrane fractions or complete cells [26]. However, the HIS-TAG ELISA procedure is to our knowledge the first example of selection of antibodies against conformational epitopes using a detergent-solubilized membrane protein, i.e. the most common state for growth of type II three-dimensional crystals.

4. Production of antibody fragments in the Fab and Fv format

For the purpose of antibody fragment-mediated crystallization of NhaA the recombinant Fv fragments 2C5 and 5H4 were obtained by cloning the genes of the variable domains of the respective monoclonal antibodies [27]. They were inserted in the di-cistronic operon of the plasmid pASK68 allowing periplasmic expression in *E. coli* under the control of the inducible *lac* promoter [28]. A streptavidin binding peptide (Strep-tag) is appended at the C terminus of the heavy chain for purification via streptavidin affinity chromatography, while a myc-tag (recognized by the monoclonal antibody 9E10) is present at the C terminus of the VL domain and is used for detection of the antibody fragment in a Western blot or ELISA [29].

It can be argued that *E. coli* will not be the most suitable expression host for antibodies binding to, and inhibiting, a transporter that naturally resides in the cytoplasmic membrane of the host. Even though most antibodies did have an inhibitory effect on the activity of NhaA [17], the transporter is vital only at certain growth conditions, e.g. high sodium or lithium concentration or at alkaline pH in the presence of sodium, and expression of the antibody fragments did not interfere with the normal growth of cells.

In bacterial expression systems, Fv and Fab fragments are normally produced in the periplasmic space, where oxidizing conditions lead to the formation of the required disulfide bonds essential for proper folding and activity of the antibodies. Protein yields vary for the different antibody fragments, with maximum yields of 1.5 mg protein per liter of bacterial culture for stable Fv fragments. Fab fragments are expressed at an even lower yield (0.1–0.4 mg/l of culture) most likely due to the higher complexity of their disulfide bond patterns and the larger mass. Their production creates a bottleneck for providing sufficient material for co-crystallization trials.

While Fv fragments cannot be produced via protease digestion of an entire IgG molecule, Fab fragments are also commonly made using papain or pepsin cleavage of the antibody in its hinge region. This procedure is costly, often inefficient (highest yield of pure Fab fragment is rarely better than 25–30% of the starting protein sample) and involves several purification steps: after monoclonal antibody production, preferentially under serum-free conditions to avoid undesirable contamination with bovine IgG, Fab fragments were purified by, for example, protein A depletion of the split constant domain of the antibody, followed by size-exclusion or ion exchange chromatography to isolate pure Fab fragment suitable for crystallization attempts.

Our strategy was to produce Fab fragments as chimeric proteins with the CH1 and CL constant domains of another antibody (the anti-lysozyme D1.3 monoclonal antibody) by subcloning the VH and VL domains into the vector pASK85-D1.3. This plasmid provides an histidine-tag at the C terminus of the heavy chain for purification via immobilized metal affinity chromatography. It is designed for periplasmic expression in *E. coli* under the control of the tetracycline repressor/promoter system [29]. We observed a clear correlation of the expression properties between Fv and Fab fragments of the same antibody: 2C5 antibody fragments are produced at good levels with 1.5 and 0.5 mg/l in Fv and Fab format, respectively. Antibody fragments of 5H4 were produced at much lower yields (0.1 and 0.03 mg/l for Fv and Fab fragments, respectively). Exchange of charged and bulky residues at the N terminus of the variable

domain of the light chain site improved the folding and solubility properties of the latter antibody [27].

An alternative strategy to scale up antibody fragment production is the expression in an oxidizing bacterial cytoplasm using the *E. coli* strain FA113. This leads to high yields of pure, functional Fab fragment suitable for crystallization studies [31]. In addition, scFv fragments derived from phage display libraries have recently been successfully expressed in a similar strain, which co-expresses molecular chaperones [32]. The production of Fv fragments in the FA113 strain is currently being tested.

These systems provide noticeable higher yields if compared to the expression of antibodies in the periplasm of bacteria.

5. Immuno-affinity purification of NhaA with the antibody 1F6

The monoclonal antibody 1F6 detects a pH-dependent conformational change, which involves the N terminus of NhaA in a response to activation of the antiporter [12]. Such an antibody specificity can be successfully exploited for the immuno-affinity purification of the antigen under native conditions.

Immuno-affinity columns have been applied to purify a number of soluble proteins to homogeneity. An example is the use of column matrices with immobilized antibodies to isolate the HIV-1 envelope glycoprotein gp120 from cell culture supernatants [33]. However, the reusability of these columns, as well as the adaptation for membrane protein purification, is often limited by the fact that very low pH (2.8) or very high pH (10.5) conditions are required to disrupt the binding of the antibody; this treatment may irreversibly denature the antigen and/or rapidly deteriorate the binding capacity of the immobilized antibody. For pH-sensitive membrane proteins, this is usually not an option. With linear epitopes, an alternative but expensive way is the elution of the protein by competition with a molar excess of the peptide matching the epitope.

In the case of the anti-NhaA 1F6 antibody, a limited pH gradient is applied using conditions, in which NhaA is active and does not denature (pH range 4.5 to 8): this allows specific elution of all bound transporter molecules from the affinity matrix [12]. After immuno-affinity purification, NhaA retains its full activity and the capacity to recycle through the same pH-dependent conformational change. For immuno-affinity purification of the transporter, a matrix based on recombinant protein A was used to properly orient the antibody molecule before cross-linking with the bifunctional chemical disuccinimidyl (DSS) substrate. Because the recombinant protein A has a very low unspecific binding activity and the cross-linker DSS is stable at low pH values, such a column can be reused at least 10 times. This fact renders the overall procedure cost-effective and reproducible.

6. Crystallization attempts of NhaA–antibody fragment complexes

The availability of suitable amounts of highly purified NhaA is the basis for three-dimensional crystallization. 3D crystals of the antiporter have been reported, but diffraction quality is poor [10,30]. Disorder in the crystals may be caused by the antiporter existing in various conformations. In addition, NhaA exhibits only small hydrophilic domains, which are essential for stable crystal contacts. Thus, enlarging the hydrophilic surface and stabilizing defined conformations by binding antibody fragments is a promising approach to obtain well-ordered crystals. It has been shown that the addition of stabilizing ligands increases the chance of getting crystals suitable for diffraction analysis [34].

Antibody fragments successfully used for co-crystallization of membrane proteins were derived from monoclonal antibodies obtained by classical hybridoma technology [7–9]. They recognize native, nonlinear epitopes of their respective antigen. Stoichiometric antibody–antigen complexes purified by size-exclusion chromatography were used for crystallization, indicating the high affinity of the antibodies. However, we do not have a comprehensive overview of binding constants for all antibodies, which have been co-crystallized with their cognate antigen. Therefore, we cannot specify yet which affinity threshold is required to successfully obtain crystals of antibody complexes, nor can we rule out the possibility that lower affinities may result in reduced chances of crystallization. All NhaA-specific antibodies and their respective fragments discussed here possess affinities in the nanomolar range as measured in a Biacore analysis [30] (Table 1). However, their different k_{on} and k_{off} kinetic rates could be important variables to be taken into account when setting up crystallization trials; for example, the Fv fragment 2C5 shows a rapid dissociation rate compared to the antibody 5H4. The fragments form stable complexes with the antiporter, which can be purified by

Table 1
Kinetic and binding constants for the NhaA-antibody interactions

	k_{on} (1/M s)	k_{off} (1/s)	K_D (M)
Mab 2C5	7.0×10^4	2.0×10^{-3}	3.4×10^{-8}
Fab 2C5	9.0×10^5	2.0×10^{-3}	2.2×10^{-9}
Fv 2C5	3.3×10^5	2.0×10^{-3}	5.0×10^{-9}
Mab 5H4	2.6×10^4	1.2×10^{-4}	2.0×10^{-9}
Fab 5H4	7.0×10^4	1.2×10^{-4}	4.0×10^{-9}
Fv 5H4	5.0×10^4	5.5×10^{-3}	1.0×10^{-7}
Mab 6F9	1.7×10^4	4.6×10^{-4}	2.8×10^{-8}

NhaA was immobilized onto a Biacore™ CM5 (carboxy-methylated dextran hydrogel) chip, the antibody solutions were applied and their interactions with the antigen measured quantitatively. NhaA binds covalently to the chip via its free amino-groups which form a stable hydroxy-succinimide ester bond after activation of the surface. No binding was detectable in the case of the antibody 1F6 which binds the linear peptide HLHRFFSS located at the very N terminus of the antiporter. It is conceivable that this epitope is disturbed by the coupling procedure of NhaA to the chip.

size-exclusion chromatography. Extensive crystallization trials of NhaA in complex with either Fv or Fab fragments are in progress.

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