Following single antibody binding to purple membranes in real time

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Antibody binding to surface antigens in membranes is the primary event in the specific immune defence of vertebrates. Here we used force microscopy to study the dynamics of antibody recognition of mutant purple membranes from *Halobacterium salinarum* containing a genetically appended anti-Sendai recognition epitope. Ligation of individual anti-Sendai antibodies to their antigenic epitopes was observed over time. Their increase in number within a small selected area revealed an apparent kinetic on-rate. The membrane-bound antibodies showed many different conformations that ranged from globular to V- and Y-like shapes. The maximum distance of two Fab fragments of the same antibody was observed to be ~18 nm, indicating an overall strong intrinsic flexibility of the antibody hinge region. Fab fragments of bound anti-Sendai antibodies were allocated to antigenic sites of the purple membrane, allowing the identification and localization of individual recognition epitopes on the surface of purple membranes.

Keywords: AFM; dynamic force microscopy; MACmode; epitope mapping; purple membranes

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INTRODUCTION

Binding geometry and structural changes invoked upon interaction between antibodies and membrane proteins are of considerable interest for a better understanding of the mechanisms of underlying receptor recognition important in a variety of biological processes such as cell signalling and transmembrane signal transduction (Mammen *et al*, 1998). The binding of antibodies to their corresponding antigenic receptors in cell membranes can frequently induce a dimerization or oligomerization of receptors and can consequently induce changes in the distribution of molecules at the membrane interface (Heldin, 1995). In biological signalling through oligomerization of membranous proteins, the

valency of binding can have a significant effect; that is, a bivalent binding of an antibody can differ markedly from their monovalent constituents, because multivalent interactions may achieve higher binding affinity and afford larger contact areas between the interacting molecules (Yang *et al*, 2003).

Recognition of receptors by antibodies involves molecular flexibility to varying extents inside the binding pocket (induced fit) and of the overall molecule conformation. Antibodies consist of highly flexible linkers connecting the hypervariable antigen-binding sites (Fab fragments) to the effector domain (Fc fragment), which allows adaptation to a vast number of shapes and conformations, while retaining the covalent link between the Fab and Fc domains (Saphire *et al*, 2002). The many different conformations that were recently stabilized in crystal lattices may represent a snapshot within the wide spectrum of conformers that can be attained by the antibody molecule in solution. Conformations of antibodies in physiological aqueous conditions and specifically bound to antigens on membrane surfaces, however, have not been reported before.

The binding dynamics of the antibody–antigen interaction is commonly expressed in kinetic parameters, on-rate and off-rate, usually quantified in ensemble average techniques (Yang *et al*, 2003). Owing to the high flexibility of the Fab and Fc fragments, the two Fab fragments of an antibody can bind independently of each other (Sinha & Smith-Gill, 2002). The kinetics of antibody binding to cell membrane antigens is therefore determined by the inherent binding properties of the Fab fragment–antigen interaction, the flexibility and movement of the antibody molecule, and the lateral arrangement and mobility of the antigenic binding sites (Kaufman & Jain, 1992).

The atomic force microscope (AFM) opens the possibility of studying such details of antibody binding at the single-molecule level under physiological conditions. Topographical imaging of biological samples was mainly achieved in contact-mode AFM, in which the cantilever is incessantly in touch with the sample during scanning (Mueller *et al*, 1995a). However, contact-mode imaging turned out to be less suitable for weakly attached samples, because biomolecules are often pushed away by the AFM stylus during scanning (Karrasch *et al*, 1993). We therefore used dynamic force microscopy (DFM), which has been previously used to track single-molecule processes with submolecular

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resolution (Han *et al*, 1996, 1997) and to capture dynamic features of individual molecules on the millisecond timescale (Raab *et al*, 1999; Viani *et al*, 2000). In DFM, a cantilever oscillates and touches the biological sample only intermittently at the end of its downward movement, which reduces the contact time and minimizes friction forces. Therefore, DFM is particularly suited for high-resolution imaging of soft and complex biological structures under their native environment (Kienberger *et al*, 2003, 2004).

In the following study, the individual binding of antibodies to purple membranes (PMs) was followed *in situ* using DFM. Owing to the high surface density of antigenic receptors, many different antibody conformations were observed on the membrane. The well-characterized three-dimensional structure of PMs makes this system ideally suited for showing the potential of DFM as a new immunological tool for biological membranes.

RESULTS AND DISCUSSION

PM patches with diameters of 1–5 μm were isolated from the membrane of *Halobacterium salinarum* and adsorbed on mica (Mueller *et al*, 1995a). Imaging of PMs in aqueous solutions using AFM revealed a hexagonal arrangement of trimeric bacteriorhodopsin (BR) molecules embedded in a lipid bilayer (Mueller *et al*, 1995a; Moeller *et al*, 1999). BR is a light-driven proton pump, with its photoactive retinal embedded in seven (A–G) tightly packed α -helices (Henderson & Unwin, 1975; Oesterhelt

et al, 2000) that can undergo photochemically (Roussio *et al*, 1997; Moeller *et al*, 2000) or mechanically induced conformational changes (Mueller *et al*, 1995b). In contrast to wild-type PM used in previous studies, our PMs (Sendai PM) had a 13-amino-acid-long Sendai epitope genetically fused in the cytoplasmic E–F loop of BR (Fig 1A). The latter peptide sequence represents a specific high-affinity binding target for monoclonal anti-Sendai antibodies (Einberger *et al*, 1990). The specificity of anti-Sendai antibody binding to Sendai PM is shown in the topography image of Fig 1B. After incubation of the Sendai PM with antibodies, single protein molecules were clearly visible on the flat membrane surface (Fig 1B, left panel). In contrast, no antibodies were detected on wild-type PMs even after prolonged times of antibody incubation (Fig 1B, right panel). Additionally, enzyme immuno-adsorbent assays (EIAs) yielded specific binding of anti-Sendai antibodies to Sendai PM and no binding to native PM (data not shown).

To study the kinetics of binding of single antibodies to BR, a dilute concentration of anti-Sendai antibodies was injected into the fluid cell of the AFM that contained a high surface density of strongly adsorbed Sendai PM patches, during imaging. Before antibody injection, the membrane patches appeared as flat structures in large-scale images (Fig 1C, at 0 min). Continual binding of single antibodies was clearly resolved by observing additional structures on top of the membrane surface (arrows in Fig 1C, 48 and 72 min). Their number steadily increased over time

Fig 1 | Kinetics of anti-Sendai antibody binding to Sendai PM. (A) Schematic representation of a Sendai BR molecule. In contrast to a wild-type BR molecule, Sendai BR has a 13-amino-acid-long Sendai epitope genetically fused in the cytoplasmic E–F loop. (B) Specificity of the anti-Sendai antibody–antigen interaction. The specificity of the antibody–antigen interaction is shown on AFM topography images. The left image shows anti-Sendai antibodies (visible as bright dots) covering Sendai PM after 20 min of incubation. No anti-Sendai antibodies were detected on wild-type PM even after 2 h incubation time (right image). Scan size was 500 nm on both images. Scale bar, 150 nm. (C) Successive AFM topography images of Sendai PM at (0 min) and after (48 and 72 min) antibody incubation. Upon binding to PM, they appear as bright structures (see arrows) 2–3 nm in height (see height false colour bar, 0–8 nm). Scan size was 300 nm on all three images. Scale bar, 100 nm. (D) Diagram depicting the increase in the number of bound antibodies (n_{ABBR}) in the image frame of (C) with incubation time. An initial lag phase (from 0 to 40 min) is followed by a linear increase in the number of membrane-bound antibodies (from 48 to 88 min).

within the selected scan area (from 0 to 2 to 8 in a period of 72 min, as shown in Fig 1C). Following antibody binding for 90 min revealed an initial lag phase arising from mass transport limitation (Nygren, 1994), succeeded by a linear increase in the number of membrane-bound antibodies (Fig 1D). The increase in the number of antibodies bound to the membrane (n_{AbBR}) was described by a simple Langmuir interaction model assuming a pseudo-first-order reaction according to $\text{Ab} + \text{BR} \rightarrow \text{AbBR}$, resulting in $d n_{\text{AbBR}}/dt = k_{\text{on}} C_{\text{Ab}} C_{\text{BR}}$, with C_{Ab} being the free antibody concentration in solution and C_{BR} the effective density of BR at the PM surface. Fitting the number increase of bound antibodies to the aforementioned model yielded an apparent on-rate constant of $k_{\text{on}} = 2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, which falls in the range of known antibody–antigen interactions determined by bulk and AFM measurements (Hinterdorfer *et al*, 1996).

A whole membrane patch of Sendai PM textured with a moderate surface density of specifically bound antibodies appearing singly distributed is shown in Fig 2A. Details of their substructures became visible by zooming into smaller areas (Fig 2B). Many different morphologies could be distinguished that were combined into three major classes (Fig 2C). A globular-like shape of the antibody (I) was obtained when the antibody bound monovalently or when the two Fab fragments bound close together, obscuring structural details of the molecule. In contrast, V-shaped antibodies with various apex angles (II, III) were formed with the two Fab fragments bound distant from each other and the Fc portion of the antibody freely rotating, whereas additional adsorption or oppression of the Fc part resulted in a Y-shaped antibody feature (IV). Owing to the high surface density of antigenic sites in the membrane and the strong intrinsic conformational flexibility of the two Fab fragments when screening for binding sites, we observed that at least two-thirds of the antibodies bound bivalently (corresponding to either V- or Y-shaped morphology). Additionally, antibodies unspecifically adsorbed on mica were occasionally observed (Fig 2A), but with less coverage than on the membranes.

The size of the Fab and Fc fragments observed on the topographical images agrees well with the known three-dimensional morphology and size of antibodies (lower-right sketch in Fig 2C; Silverton *et al*, 1977). The distance between single Fab epitopes within one antibody varied from a few nanometres in nearly globular appearance to about 18 nm in V-shaped or spread-out morphologies. These results are again consistent with the recent findings from crystallographic studies that revealed the Fab fragments of an antibody to be highly flexible with a maximum distance of 15–20 nm between the Fab epitopes (Saphire *et al*, 2002). Although recent studies using carbon nanotube AFM tips (Cheung *et al*, 2000) and non-contact AFM (San-Paulo & Garcia, 2000) revealed Fab fragments of dried (Cheung *et al*, 2000; San-Paulo & Garcia, 2000) or ice-bound (Han *et al*, 1995) IgG antibodies, conformations of antibodies specifically bound to antigens embedded in membranes have not been detected so far (Mueller *et al*, 1996). Here, morphological features of antibodies specifically attached to membrane-embedded antigens in their physiological environment were resolved for the first time.

In high-magnification images, the hexagonal pattern of the trimeric BR of PM and the detailed arrangement of the two Fab fragments of a bound antibody were simultaneously resolved (Fig 3). The topographical image (Fig 3A) shows the hexagonal lattice structure of the membrane and an antibody with its two specifically bound Fab fragments appearing dispersed (white box in Fig 3A). In this particular conformation, the Fab fragments are bound distant from each other at an angle of almost 180° , whereas the Fc portion of the antibody is freely rotating and therefore not observable (class II/III of Fig 2C). The trimeric arrangement of the BR molecules within the hexagonal lattice appeared clearer in the magnified image (Fig 3B); a lateral resolution of 1.5 nm was determined by Fourier analysis. In drawing contour lines of the two hidden BR trimers (dotted lines), distinct BR molecules (marked in black) that are bound to their respective Fab fragment were identified (Fig 3B). As evident from the image, the two occupied BR molecules are each owned by

Fig 2 | Substructures of antibodies specifically bound to Sendai PM. (A) Large membrane patches of Sendai PM covered with antibodies. The white box indicates the region that was enlarged for (B). Antibodies appear as small dots 10–20 nm in diameter and 2–3 nm in height. Scan size was 1 μm . Scale bar, 200 nm. (B) Structural details of the membrane-attached antibodies obtained in smaller scan areas. Several different arrangements of Fab fragments can be easily discerned. Scan size was 150 nm. Scale bar, 40 nm. (C) Classes of antibody conformations. Antibody shapes were divided into three major classes, corresponding to their Fab and Fc arrangement: (i) globular, (ii) and (iii) V-shaped, and (iv) Y-shaped. The lower-right sketch shows the three-dimensional morphology and size of an antibody (Silverton *et al*, 1977). Image size was 35 nm in all four images. Scale bar, 20 nm.

Fig 3 | Localization of antibody-binding epitopes on Sendai PM. (A) Topographical image of the cytoplasmic side of Sendai PM to which an antibody is bound. The hexagonal lattice structure of the membrane can be discerned over the whole scan size in this Fourier-filtered image. The white box indicates the region for subsequent analysis. Image size was 40 nm. Scale bar, 10 nm. (B) The hexagonal lattice structure of BR trimers and the arrangement of the two Fab fragments of the antibody are clearly visible at higher magnification. The dotted contour lines depict BR trimers covered by the antibody. The BR molecules that are engaged by the two Fab fragments of the antibody are marked in black. Image size was 20 nm. Scale bar, 4 nm. (C) Three-dimensional presentation of (B) showing the heights of the structures. The BR trimers (indicated with dotted lines) protrude 0.8 nm out of the lipid bilayer, whereas the two Fab fragments (see labelling) of the antibody are 2–3 nm in height.

a different trimer. A three-dimensional presentation of the same image shows that the BR structures protrude 0.8 nm out of the lipid bilayer and the antibody with its two Fab fragments appears 2–3 nm in height (Fig 3C).

The simultaneous measurement of the hexagonal pattern of the trimeric BR and the two Fab fragments of a bound antibody therefore allowed localization of the binding epitope of the respective Fab fragments attached to the PM with a resolution of 1.5 nm under physiological conditions. The resolution obtained is in close proximity to other published DFM studies of PMs (Moeller *et al*, 1999). Contact-mode AFM revealed a somewhat better resolution (0.5 nm; Mueller *et al*, 1995a), but was not able to achieve morphological details of membrane-bound antibodies (Mueller *et al*, 1996).

As time-dependent processes can be followed at high lateral resolution, DFM can be used as a new immunological tool on biological membranes, revealing details impossible to resolve by conventional techniques. Further applications of this technique to other biological systems may enable a broad range of single-molecule structure-binding studies of transmembrane receptors and ion channels in membranes and cells.

METHODS

Construction and expression of Sendai BR. The derivative bacteriorhodopsin with the additional nucleotide sequence coding for the C-terminal epitope of the Sendai virus L-protein (DGLSGDIEPYDSS) in the EF loop of BR was obtained by PCR mutagenesis. A PCR fragment was generated with the primers *bop-a-down* (5'-TGGATCTGGCTAGCGCTCGGTACGG) and *bop-sen-ef-up* (5'-GCAATTCCCCTTACGCAGTACTTTGAACGTGGATGC GACCTCGCTCGAGTCGTACGGCTCGATGTCGCCAGGGAGC CGTCCGAGGTGAACCCG), recombined into the wild-type bacteriorhodopsin (*bop*) of the halobacterial expression vector pFG6 (Gropp *et al*, 1995), transformed in *H. salinarum* L-33 and cultured as described previously (Oesterhelt & Stoekenius, 1974).

AFM imaging. PMs were adsorbed on freshly cleaved mica for 20 min in a buffer solution (300 mM KCl, 20 mM Tris, pH 8.8) and

subsequently washed with the same buffer at pH 7.6. The AFM images were acquired in buffer with a magnetically driven DFM (Molecular Imaging, Phoenix, AZ) using magnetically coated MacLevers™ (Molecular Imaging, Phoenix, AZ) at a 5 nm oscillation amplitude. The cantilevers had a spring constant of 100 pN/nm, as calibrated in solution using the equipartition theorem (Butt & Jaschke, 1995). Images were taken with the lowest possible amplitude reduction at a line scan rate of 1.3 Hz. The topographical image in Fig 3A was Fourier-filtered using IMAGIC (Image Science Software GmbH, Berlin, Germany), whereas Fig 3B was averaged over 18 unit cells using Matlab Verison 4.2c.1 (MathWorks Inc., Natick, MA). To follow the ligation of individual antibodies to Sendai PM over time, anti-Sendai antibodies were injected into the fluid cell of the AFM at a final concentration of 3 µg/ml during imaging.

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REFERENCES

- Butt HJ, Jaschke M (1995) Thermal noise in atomic force microscopy. *Nanotechnology* **6**: 1–7
- Cheung CL, Hafner JH, Lieber CM (2000) Carbon nanotube atomic force microscopy tips: direct growth by chemical vapor deposition and application to high-resolution imaging. *Proc Natl Acad Sci USA* **97**: 3809–3813
- Eimerberger H, Mertz R, Hofschneider PH, Neubert WJ (1990) Purification, renaturation, and reconstituted protein kinase activity of the Sendai virus large (L) protein: L protein phosphorylates the NP and P proteins *in vitro*. *J Virol* **64**: 4274–4280
- Gropp F, Gropp R, Betlach MC (1995) Effects of upstream deletions on light- and oxygen-regulated bacterio-opsin gene expression in *Halobacterium halobium*. *Mol Microbiol* **16**: 357–364
- Han W, Mou J, Sheng J, Yang J, Shao Z (1995) Cryo atomic force microscopy: a new approach for biological imaging at high resolution. *Biochemistry* **34**: 8215–8220
- Han W, Lindsay SM, Jing J (1996) A magnetically driven oscillating probe microscope for operation in liquid. *Appl Phys Lett* **69**: 4111–4113

- Han W, Lindsay SM, Dlakic M, Harrington RE (1997) Kinked DNA. *Nature* **386**: 563
- Heldin C (1995) Dimerization of cell surface receptors in signal transduction. *Cell* **80**: 213–223
- Henderson R, Unwin PNT (1975) Three-dimensional model of the purple membrane obtained by electron microscopy. *Nature* **257**: 28–32
- Hinterdorfer P, Baumgartner W, Gruber HJ, Schilcher K, Schindler H (1996) Detection and localization of individual antibody–antigen recognition events by atomic force microscopy. *Proc Natl Acad Sci USA* **93**: 3477–3481
- Karrasch S, Dolder M, Schabert F, Ramsden J, Engel A (1993) Covalent binding of biological samples to solid supports for scanning probe microscopy in buffer solution. *Biophys J* **65**: 2437–2446
- Kaufman EN, Jain RK (1992) Effect of bivalent interaction upon apparent antibody affinity: experimental confirmation of theory using fluorescence photobleaching and implications for antibody binding assays. *Cancer Res* **52**: 4157–4167
- Kienberger F et al (2003) Dynamic force microscopy imaging of native membranes. *Ultramicroscopy* **97**: 229–237
- Kienberger F, Zhu R, Moser R, Blaas D, Hinterdorfer P (2004) Monitoring RNA release from human rhinovirus by dynamic force microscopy. *J Virol* **78**: 3203–3209
- Mammen M, Choi S-K, Whitesides GM (1998) Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew Chem Int Ed* **37**: 2754–2794
- Moeller C, Allen M, Elings V, Engel A, Mueller DJ (1999) Tapping-mode atomic force microscopy produces faithful high-resolution images of protein surfaces. *Biophys J* **77**: 1150–1158
- Moeller C, Bueldt G, Dencher N, Engel A, Mueller DJ (2000) Reversible loss of crystallinity on photobleaching purple membrane in the presence of hydroxylamine. *J Mol Biol* **301**: 869–879
- Mueller DJ, Schabert FA, Bueldt G, Engel A (1995a) Imaging purple membranes in aqueous solutions at sub-nanometer resolution by atomic force microscopy. *Biophys J* **68**: 1681–1686
- Mueller DJ, Bueldt G, Engel A (1995b) Force-induced conformational change of bacteriorhodopsin. *J Mol Biol* **249**: 239–243
- Mueller DJ, Schoenenberger CA, Bueldt G, Engel A (1996) Immuno-atomic force microscopy of purple membrane. *Biophys J* **70**: 1796–1802
- Nygren H (1994) Kinetics of antibody binding to surface-immobilized antigen. Analysis of data and an empirical model. *Biophys Chem* **52**: 45–50
- Oesterhelt D, Stoerkenius W (1974) Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol* **31**: 667–678
- Oesterhelt F, Oesterhelt D, Pfeiffer M, Engel A, Gaub HE, Muller DJ (2000) Unfolding pathways of individual bacteriorhodopsins. *Science* **288**: 143–146
- Raab A, Han W, Badt D, Smith-Gill SJ, Lindsay SM, Schindler H, Hinterdorfer P (1999) Antibody recognition imaging by force microscopy. *Nat Biotechnol* **17**: 901–905
- Rouso I, Khachatryan E, Gat Y, Brodsky I, Ottolenghi M, Sheves M, Lewis A (1997) Microsecond atomic force sensing of protein conformational dynamics: implications for the primary light-induced events in bacteriorhodopsin. *Proc Natl Acad Sci USA* **94**: 7937–7941
- San-Paulo A, Garcia R (2000) High-resolution imaging of antibodies by tapping-mode atomic force microscopy: attractive and repulsive tip–sample interaction regimes. *Biophys J* **78**: 1599–1605
- Saphire E et al (2002) Contrasting IgG structures reveal extreme asymmetry and flexibility. *J Mol Biol* **319**: 9–18
- Sinha N, Smith-Gill SJ (2002) Protein structure to function via dynamics. *Protein Peptide Lett* **9**: 367–377
- Silverton EW, Navia MA, Davies D (1977) Three-dimensional structure of an intact human immunoglobulin. *Proc Natl Acad Sci USA* **74**: 5140–5144
- Viani MB et al (2000) Probing protein–protein interactions in real time. *Nat Struct Biol* **7**: 644–647
- Yang T, Baryshnikova OK, Mao H, Holden MA, Cremer PS (2003) Investigations of bivalent antibody binding on fluid-supported phospholipid membranes: the effect of hapten density. *J Am Chem Soc* **125**: 4779–4784