MAPPING CELL WALL POLYSACCHARIDES OF LIVING MICROBIAL CELLS USING ATOMIC FORCE MICROSCOPY

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Functionalized atomic force microscope tips were used to sense specific forces of interaction between ligand–receptor pairs and to map the positions of polysaccharides on a living microbial cell surface. Gold-coated tips were functionalized with concanavalin A using a cross-linker with a spacer arm of 15.6 Å. It was possible to measure the binding force between concanavalin A and mannan polymers on the yeast (Saccharomyces cerevisiae) cell surface. This force ranged from 75 to 200 pN. The shape of the force curve indicated that the polymers were pulled away from the cell surface for a fairly long distance that sometimes reached several hundred nanometres. The distribution of mannan on the cell surface was mapped by carrying out the force measurement in the force volume mode of atomic force microscopy (AFM). During the measurement, the maximum cantilever deflection after contact between the tip and the sample was kept constant at 10 nm using trigger mode to keep the pressing force on the sample surface as gently as possible at a force of 180 pN. This regime was used to minimize the non-specific adhesion between the tip and the cell surface. Specific molecular recognition events took place on specific areas of the cell surface that could be interpreted as reflecting a non-uniform distribution of mannan on the cell surface.

INTRODUCTION

Although atomic force microscopy (AFM) was originally introduced as a high resolution imaging device (Binnig et al., 1986), the possibility of employing it as a nanorobotic arm to sense forces of interaction between the tip and the sample in the piconewton range is receiving an ever increasing interest in many scientific disciplines. AFM was successfully used to probe the nature of fundamental forces of interactions (attractive van der Waals and attractive/repulsive electrostatic forces) between different combinations of conductors and insulators (Martin et al., 1987; Ducker et al., 1990; Moiseev et al., 1990; Butt, 1991; Wen et al., 1991; Gauthier-Manuel, 1992; Weisenhorn et al., 1992; Germann et al., 1993). In liquids, electrostatic forces between dissolved ions and other charged groups play an important role in determining the forces sensed by an AFM cantilever. An excellent review on the force measurement under fluids has been published recently by Butt et al. (1995).

Ducker and co-workers (1991) introduced a useful technique for force measurement by attaching colloidal spheres to AFM cantilevers and then measuring forces between the spheres and samples of interest. They claimed that such a technique is extremely useful because the nature of colloidal particles often determine the behavior of a great variety of materials, including paints, paper, soils, clay and, in some circumstances, cells.

In the chemical realm, Burnham and co-workers (1990) were able to distinguish two similar samples, each covered with a single chemical monolayer, the only difference between them being that one surface was terminated with CH₃ groups, the other with CF₃.
A F M was also used in the field of material science for measuring the nano-scale mechanics of thin films and clusters (H ues et al., 1993) as well as indentation and plastic deformation (Sumomogi et al., 1994).

In biology, specific intermolecular recognitions are the basis for many biochemical processes. Such recognition processes involve a multitude of weak non-covalent bonds (e.g. electrostatic, van der Waals, and/or hydrogen bonds) or hydrophobic interactions between geometrically complementary surfaces. These interactions are highly specific and can be very strong as, for example, in the molecular recognition between receptors and their ligands.

Several techniques have been developed to measure intermolecular forces. The optical trapping technique is one of them and is very sensitive to small forces, but its use has been limited to certain special samples and to measurements of less than tens of piconewtons (A shkin et al., 1987; K u and Sheetz, 1993; S voboda et al., 1993). Other techniques such as pipette suction and the surface force apparatus are also sensitive but lack spatial resolution (E van s et al., 1991; I sraelachvili, 1992).

The invention of A F M introduced a new precise technique that can probe surfaces in physiological environments with a high spatial resolution and can sense forces down to the piconewton range. Promising results were obtained from the biotin/streptavidin model systems, complementary strands of DNA, cell adhesion proteoglycans, and specific antigen/antibody interactions (F lorin et al., 1994; L ee et al., 1994a,b; M oy et al., 1994; D ammer et al., 1996; H interdorfer et al., 1996). All these measurements utilized almost the same idea of functionalizing the tip as well as the substrate with the complementary (or the same) type of molecules of interest by simple adsorption or by covalent immobilization using cross-linkers. Another novel technique for measuring the binding forces and one that foresees a technique for imaging binding sites is called sensor force microscopy. This technique was developed by H asedgrüber et al. (1995) and H interdorfer et al. (1996) in which a single biological molecule (the sensor) is attached to the A F M tip at the end of a molecular tether. The molecular tether is an elastic linker molecule a few nanometres long based on polyethylene glycol (PEG) molecules. The PEG tether gives the sensor molecule the freedom to orient itself properly to bind to its target on the surface. When a binding event takes place, the A F M detects the additional force required to break the molecular adhesion. H interdorfer et al. (1996) estimated the binding forces between human serum albumin (H S A) and its associated antibody to be in the range of 244 pN.

Another application of A F M force measurements is to image or quantify electrical surface charges of biological materials and to measure their mechanical properties (B utt, 1992; L eng and W illiams, 1993; T ao et al., 1992; R admacher et al., 1995; I kai, 1996; M itsui et al., 1996; R ief et al., 1997).

In this study, we have tried to use the force volume mode of A F M to map the distribution of a particular species of polysaccharides on a living microbial cell surface. We carried out a direct measurement of binding force between a functionalized tip (carrying a receptor protein) and its corresponding polysaccharide ligands on a microbial cell surface, as a trial to develop an antigen-sensitive probe. Such a probe should be capable of identifying its target molecules among a heterogeneous population of molecules on the surface, thus helping us draw a map of the distribution of the molecules of interest, unlike the previous studies in which only preselected complementary molecules were used to carry out the measurement. The resulting force curves showed that binding events occurred presumably between the receptor protein on the tip and its sugar ligands on the cell surface. For this purpose, a lectin from Canavalina ensiformis namely concanavalinin (A) (conA) of molecular weight 102,300 that can bind to mannose residues of the yeast cell wall mannan was used. The protein was derivatized with long chain succinimidyl 6-[3′-(2-pyridyldithio)-propionamido]hexanoate (LC-SPDP) to introduce free sulfhydryl groups by reduction immediately before carrying out the measurement. The derivatized protein was first cross-linked to a gold-coated cantilever via Au-S bonds in a buffer solution. Yeast cells (Saccharomyces cerevisiae) were immobilized almost in a monolayer form on a glass substrate that was previously coated with conA. The results indicated that a force in the order of 75–200 pN is needed to rupture the bonds formed between a single pair of ligand-receptor molecules. An interesting process was usually observed in which the sandwiched ligand-receptor complex was extended up to 500–600 nm, or even more in some cases, before the tip was freed. This type of force curve was only obtained when we used a covalently immobilized protein to the tip. We concluded that this behaviour may demonstrate a mechanical pulling of certain cell wall components, most likely mannoprotein polymers.
MATERIALS AND METHODS

Cell culture

A haploid strain of Saccharomyces cerevisiae X2180-1A from the Yeast Genetic Stock Center, Berkeley was used in this study. They were grown in 10 ml of a medium containing 1% yeast extract (Difco), 2% polypeptone, 2% glucose at 25°C with reciprocal shaking at 150 strokes/min.

Sample preparation

Protein. conA was purchased from Sigma Co. (St Louis, MO, U.S.A.) as highly purified, essentially salt-free, lyophilized powder. It was reconstituted into a tetramer in phosphate-buffered saline (PBS) at pH 7 and was derivatized with LC-SPDP as previously described by Mitsuda et al. (1995). The derivatized protein was reduced with dithiothreitol (DTT) to make thiol groups available for the reaction with a gold-coated tip immediately before AFM measurement, as cross-linking between protein molecules was to be avoided. The excess DTT was removed by passing the solution of the thiolated protein through a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden). The average number of thiol groups that were introduced ranged from 4 to 6 as determined by spectrophotometric methods (Mitsuda et al., 1995). The reduced protein solution was concentrated by passing it through a Vivapore 10 ml concentrator from Vivascience (Lincoln, U.K.). Before using the derivatized protein, its biological activity was tested by a simple agglutination test on a slide glass. That was done by mixing two droplets of yeast cell suspension and the derivatized conA solution. Large aggregates of cells were observed under a light microscope. Moreover, the characteristic appearance of the agglutination reaction could be distinguished by the naked eye. A control experiment was done by mixing PBS with a cell suspension instead of the protein solution. All the chemicals used in this experiment were of analytical grade.

Immobilization of cells. Cells were harvested by centrifugation and washed twice using deionized water. A 15-mm clean cover glass was coated with conA by applying 20 ml of 1 mg/ml native protein solution for 15 min. The coated substrate was gently washed by holding it with a forceps and dipping and withdrawing it diagonally into a PBS buffer solution for several times. Fifty millilitres of a highly concentrated cell suspension was applied on a coated cover glass and was rotated slowly for several seconds. Cells were then allowed to agglutinate for 15 min. Finally, the sample was washed in buffer and was left to dry partially for 10 minutes. This treatment allowed the cells to be stably imaged under PBS during AFM measurements.

Tip functionalization

Since the AFM used in this study did not have a combined light microscope to locate the cells, the sample was scanned by a bare gold-coated tip and a large cell surface was zoomed into. The same tip was functionalized by disassembling the liquid cell as a whole from the optical head while holding the cantilever to minimize its displacement. Care was taken not to touch the spring that holds the cantilever in its housing in the liquid cell. It was then washed by injecting PBS buffer through the inlet rubber tubing of the liquid cell, and finally functionalized by immersing it in a droplet of the SH-derivatized protein solution in a petri dish by laying the liquid cell on a support of rolled vinyl tape. After 15 min of incubation at room temperature, the unbound protein was washed away by injecting more PBS as described before. A model of the set-up configuration is shown in Figure 1a.

Atomic force microscopy

A cover glass on which yeast cells were immobilized was glued on a metal disc of a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) atomic force microscope. The liquid cell of the microscope was set up without the usual rubber o-ring. A limited volume of PBS was injected into the liquid cell so that a thin water interface is formed between the sample surface and the liquid cell. Gold-coated cantilever (OM CL-TR 400PB-1) (Olympus Co., Tokyo, Japan) having nominal spring constant 0.025 N/m were used in this study. They are manufactured by coating silicon nitride cantilevers of spring constant 0.02 N/m with a 50 nm thick gold layer under epitaxial growth conditions, according to Olympus. These coated cantilevers are useful for immobilizing biological molecules through Au-sulfhydryl bond. This bond is almost as strong as a covalent bond. To get the best possible measurement of binding forces we have measured spring constants of individual cantilevers used in this study as reported previously by Mitsui et al. (1996). This was done by pressing a macroscopic cantilever made of a piece of gold wire with known dimensions and Young’s modulus. The deflection...
of the macroscopic cantilever was compared with that of the AFM cantilevers to calculate the spring constants of the latter from the theoretical value of the former. The average spring constant of the cantilevers we used was found to be $0.018 \text{ N/m} \pm 30\%$ of the nominal value. This value was used to calculate the binding force between concanavalin A and mannose residue. Spring constants of

Fig. 1. (a) Model of experimental set-up configuration. (b) A speculative model explaining the stretching phenomenon of mannan polymer.
functionalized tips did not show any significant change.

Force volume measurements were performed at 1 μm Z scan range as gently as possible at 0.5 Hz to secure enough contact time for a ligand and receptor pair to recognize each other. In the force volume mode, force curves were recorded while a square area of the sample was raster scanned under the tip. The maximum upward cantilever deflection was kept at 10 nm using the relative trigger mode to minimize the contact area between the tip and the cell surface. The scanned area was 3 μm × 3 μm and the number of collected data was 16 × 16 for each scan.

RESULTS AND DISCUSSION

A typical AFM force plot is shown in Figure 3(a). The horizontal axis represents the Z position of the tip relative to the sample while the vertical axis represents the cantilever deflection that can be expressed in terms of the applied force on the sample by Hooke’s law. On the other hand, the force volume mode has a force mapping capability in which a force plot is taken at each point along a sample surface. For detailed anatomy of the force curve and detailed explanation of force mapping, refer to Digital Instruments support note No. 228, Rev. A, entitled force imaging.

Figure 2 shows a deflection mode image of the yeast cells (Gad and Ikai, 1995) under PBS buffer forming almost a monolayer. There was a possibility that the tip may be lowered to an intercellular space between the closely packed cells. To avoid this possibility, the sample was imaged before functionalizing the tip and after carrying out the measurement with the functionalized tip to make sure that tip was hitting a cell surface. We could also distinguish the cell surface in the low resolution height image of the force volume mode. There has always been an unavoidable shift of about 1 μm on setting up the liquid cell after tip functionalization but it was found that in most cases the tip normally encountered a cell surface thanks to the large cell surface area that exceeded 9 μm².

A control experiment was carried out by measuring the adhesion force between a bare gold coated tip and yeast cell surface. There was only a weak adhesion between the tip and the sample. It is difficult to speculate the origin of such weak adhesions because the measurement was carried out on a heterogeneous surface that contained...
several classes of macromolecules. Two typical force curves from the control experiment explained above are shown in Figure 3(b) and (c). In Figure 3(b) the plot was recorded using the trigger mode to limit the tip deflection to 10 nm, whereas the plot in Figure 3(c) was recorded without switching on the trigger mode. It is clear that both the approaching and retracting traces of Figure 3(c) are similar to each other indicating that there is no adhesion between the tip and the cell surface, despite the high loading force in this case. A slight curvature can be noticed in the contact area between the tip and the sample; such a curvature is typical for a force curve measured on a soft surface. The
separation distance between the two positively deflected lines shows that the cell has been indented by the tip, which insured that the tip was contacting a cell surface and not the glass substrate.

Figure 4 shows a typical force volume data frame. The upper left image represents a height image for the area of interest, whereas the upper right image represents a map of the elastic properties of the same area, it is also known as the force volume image. It can be identified as a map of the cantilever deflection at each point on the sample surface at a constant Z position of the sample (Laney et al., 1997). This image will not be considered in the present study. The lower right plot represents the window where force curves of each data point in the height image are displayed. The plot in this figure shows 60 superimposed curves. It can be noticed that a long distance was frequently travelled by the tip with a continued downward reflection suggesting that parts of the sample were pulled away from the cell surface. It is possible that several strands of mannoprotein polymer were being pulled out by the receptor ligand bonds.

Unfolding of conA does not seem to be a major reason because conA is a relatively low molecular weight tetrameric protein of 102,300 that would never be extended to such a long distance when stretched. Furthermore, the shape of the negative cantilever deflection in a representative curve like the one shown in Figure 5 suggests that mannan polymers may be stuffed as random coils on the cell surface. This point will be considered more carefully in a future study. Figure 1(b) shows a speculative model for such a phenomenon. Experiments with the surface force apparatus (Helm et al., 1991) and with pipette suction techniques (Evans et al., 1991) have revealed that lipids can be pulled out of a membrane by means of receptor–ligand bonds.

The force curves collected in this study resemble the ones already reported by Florin et al. (1994) and Dammer et al. (1995) in having multiple jump-off steps. When these authors analysed their data, they only considered the final jump offs claiming that the other steps were a non linear convolution of multiple unbinding processes. We have, however, considered two types of pull off points.
for each force curve. The first is the maximum deflection and the second is the final pull off point. Data collected from the maximum deflections were used to construct a map for the sugar distribution on the cell surface while the last pull off point was used to estimate the binding force between a single pair of conA and mannose residue as described before by Florin et al. (1994) and Dammer et al. (1995). Since it is still difficult to hang a single small molecule like conA from the tip, we tried to limit the number of the active protein molecules by injecting 0.25% mannose solution into the liquid cell to block most of the available active sites hoping to obtain single jump-off events. Figure 6(a) represents adhesion force frequencies between a conA functionalized tip and yeast cell wall mannan before injecting mannose, whereas, Figure 6(b) shows the adhesion frequency change at the same area after injecting mannose. The binding force was estimated to be in the order of 75–200 pN.

Eight surface graphs constructed from the maximum force values measured at each point of a force map for an area of 9 μm² are shown in Figure 7. A pair of two adjacent graphs represent subsequent scans of the same area in different experiments. The distribution of force in the two subsequent frames suggests that molecular recognition events can be reproduced to a large extent, indicating that conA functionalized tips are actually mapping the distribution of mannan on the cell surface. After several force map recordings, force profiles may change due to the following possibilities. The first possibility is the inactivation of proteins on the tip due to the mechanical stress to which they were exposed during force measurement cycles. The second possibility was a piezo scanner shift in the scan area or thirdly cell movement. Since mannan polymers may be floating as random coils on the cell surface, their movement may contribute to an increase in the frequency of the measured force within a small local area of several nanometres. This is due to their repeated interactions with conA on the tip as a result of their motion.

In conclusion, a functionalized AFM tip can be used to map the distribution of a molecular species on chemically heterogeneous surfaces like living cell surface. From our data we found that mannan is not uniformly distributed on some of the studied areas. Autoradiography and gold markers have been used by other workers to study
the location of mannan on the yeast cell. Farkas et al. (1974), and Horisberger and Vanlanthen (1977) showed that mannan was uniformly distributed on the outer layer of the cell wall. Such a contradiction may be due to the difference in resolution between the above-mentioned techniques and that of AFM.

The specific binding force between conA and mannose residues on the surface of a living yeast cell was measured directly by the AFM and estimated to be in the range of 75–200 pN. Although this technique has obvious applications in other fields, there are desirable technical improvements that need to be dealt with in the future to achieve more accurate measurements. The most important of which is developing reliable methods to immobilize a single functional molecule to the AFM tip. In the future, we think that data of kinetic importance can be obtained by studying the time dependence of specific molecular recognition events at increasing scan speeds. This will also require a precise technique to immobilize the molecules in a particular orientation at the tip surface to expose their active sites. Furthermore, it will be a necessity to compare numbers obtained from the energetics of the reaction and to study the effect of external parameters such as temperature and pH.

To the least of our knowledge, this is the first study to employ AFM force mapping for investigating molecular distribution on a living cell surface.

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