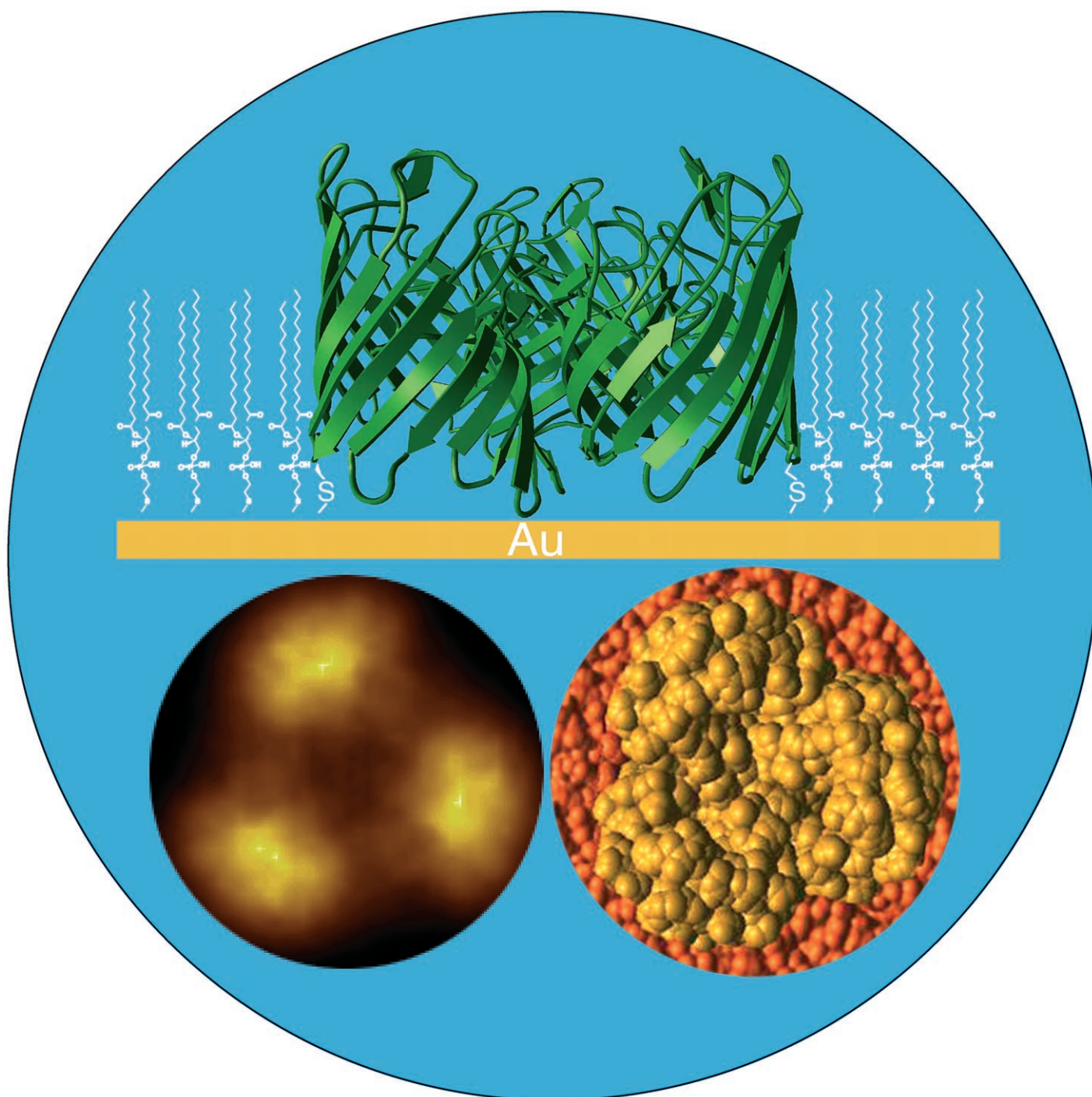


Communications



A membrane protein with a cysteine mutation, fixed in a defined orientation on a flat gold surface, can be used to obtain high-quality AFM images under aqueous conditions without 2D crystallization. Details can be found in the Communication of J. H. Lakey and co-workers on the following pages.

DOI: 10.1002/anie.200504506

An Approach To Prepare Membrane Proteins for Single-Molecule Imaging**

David A. Cisneros, Daniel J. Muller, Sofian M. Daud, and Jeremy H. Lakey*

Membrane proteins are under represented in the database of high-resolution structures obtained from X-ray crystallographic and NMR spectroscopic methods.^[1] Furthermore, they often form large and sometimes transient supramolecular complexes. Alternative approaches such as cryo-electron microscopy (EM) and Atomic force microscopy (AFM) are essential tools that are able to provide complementary information on the structure–function relationship of membrane proteins embedded in the lipid membrane. Two approaches increase the resolution of these methods, 2D crystallization and single-particle reconstruction. In EM, crystals provide electron diffraction data which can increase resolution, whereas single-particle averaging can be used for proteins > 200 kDa. High-resolution AFM topographs can reveal structural details of single native membrane proteins but, as a prerequisite, the proteins must be adsorbed to atomically flat mica and densely packed in a membrane to restrict their lateral mobility.^[2] Although averaging of single particles shows their common structures, selected examples can be used to characterize structural flexibility, variability, and conformational changes.^[3] Many examples of AFM analysis of self-assembled monolayers on gold have been published,^[4] and thiolipids have been developed to create membrane mimetic surfaces on gold.^[5–7] However, these have not yet been combined as a means to image membrane proteins.

[*] Prof. J. H. Lakey
 Institute for Cell and Molecular Biosciences
 University of Newcastle upon Tyne
 Newcastle upon Tyne, NE2 4HH (UK)
 Fax: (+44) 191-222-8865
 E-mail: j.h.lakey@ncl.ac.uk

D. A. Cisneros, Prof. D. J. Muller
 Biotechnologisches Zentrum
 Technische Universität Dresden
 Tatzberg 49, 01307 Dresden (Germany)

S. M. Daud
 Institute for Nanoscale Science and Technology
 University of Newcastle
 Newcastle upon Tyne, NE2 4HH (UK)

[**] This work was supported by the BBSRC (ISIS Travel Grant, and Research Development Fellowship & Project Grant E19051 to J.H.L.), the DFG (GK864 to D.A.C.), the European Union, the BMBF, and the state of Saxony (D.J.M.). We thank Helen Ridley for excellent technical assistance and Neil Keegan for helpful discussions.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

Herein we demonstrate a covalent assembly approach to membrane protein imaging that avoids crystallization. Atomically flat gold, engineered proteins, and chemically modified lipids are combined to rapidly assemble immobile and fully oriented samples (Figure 1). The resulting tapping-mode AFM topographs of single membrane proteins, set against a

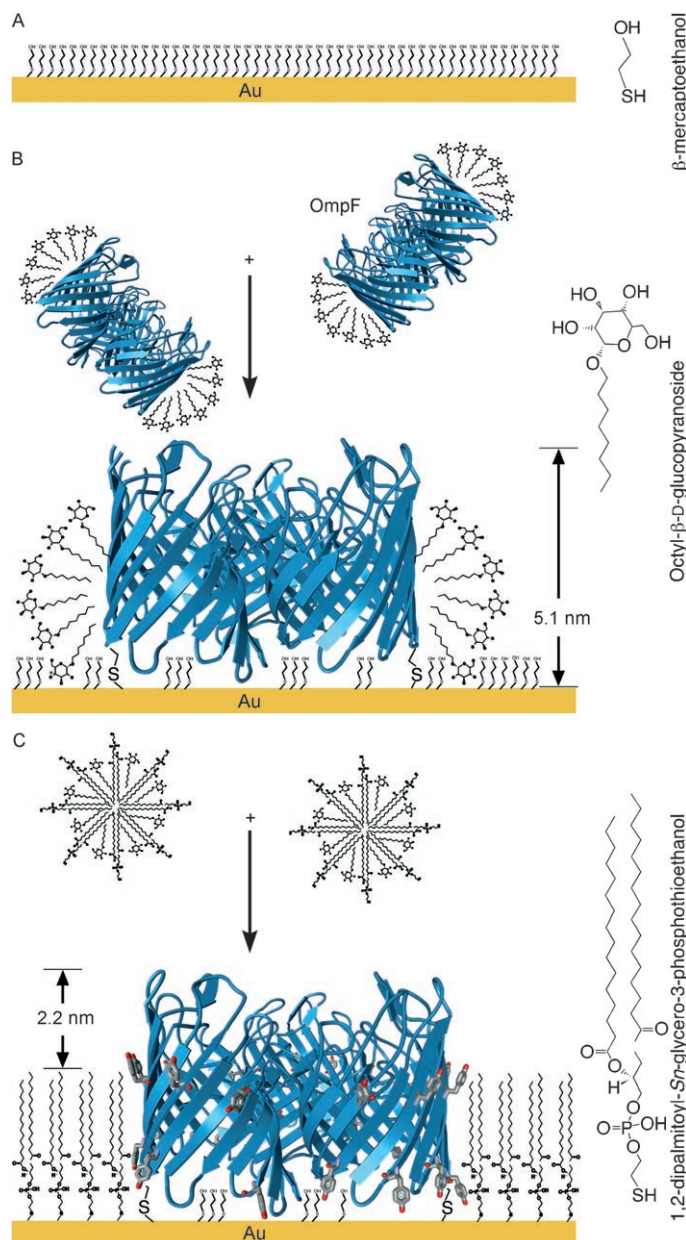


Figure 1. The attachment of OmpF porin trimers to a gold surface. A) The gold surface is passivated by β -mercaptoethanol. B) OmpF trimers in OG micelles attach to gold surfaces through cysteine mutation E183C. C) Mixed OG/thiolipid micelles are added to assemble a thiolipid monolayer. Subsequent washing with OG-containing, and later detergent-free, buffer solution removes free lipid and detergent leaving a hydrophobic half bilayer. Owing to the relatively thin cross section of outer-membrane proteins, the hydrophobic belt of the OmpF protein (indicated by the aromatic residues) is effectively stabilized by the 3-nm thick lipid monolayer. All compounds shown in the scheme are to scale.

hydrophobic background, are used to create averaged structures with a resolution approaching that of 2D crystals.

The major outer-membrane porin OmpF, from *E. coli*, is a well-characterized member of a large and widespread group of β -barrel proteins.^[8,9] We have previously shown that OmpF can be covalently assembled on gold surfaces if a single cysteine residue is inserted into the intracellular face of this protein trimer.^[7] The assembly process consists of passivating the gold surface with a short chain thiol, adding OmpF–Cys in detergent solution and then completing the remaining surface with a layer of thiolipid in which the headgroup contains a thiol that is able to chemisorb to the gold surface. FTIR, surface plasmon resonance (SPR), electrochemistry,^[7] neutron reflection (NR),^[10] and CD^[11] have shown that surface pretreatment (Figure 1 A) and thiolipid (Figure 1 C) assembly are critical in maintaining protein structure.

Template stripped gold (TS-Au)^[12] covered with a thiolipid monolayer provided a control background (Figure 2 A). This half bilayer of immobilized phospholipid probably acts like hydrophobic self-assembled monolayers (SAM) of methyl terminated alkane thiols.^[13] Recent studies show that interaction forces of the hydrophilic Si₃N₄ tips with hydro-

phobic SAM layers are very low,^[14] thus enabling the acquisition of clear AFM topographs.

OmpF–Cys on gold without thiolipid remains stable in detergent-free buffer solution (buffer B) possibly owing to the remaining tightly bound surfactant.^[15] Under these conditions, (Figure 2 B) we observed poorly defined OmpF trimers with, nevertheless, the expected height of 5.5 ± 1.3 nm ($n = 18$) corresponding to that of their 3D structure (Figure 3 D–E).^[16] However, after incubation with thiolipid, the topographs were greatly improved (Figure 2 C) and revealed highly reproducible individual trimers protruding by 2.2 ± 0.4 nm ($n = 17$). The height difference of ≈ 3.3 nm confirmed that the trimers were embedded by the thiolipid monolayer (Figure 2 C and Figure 3 A–C). From our measurements, we do not know whether a screening layer of tightly bound detergent may have resisted the washing with detergent-free buffer solutions. Such remaining detergents may shield hydrophobic surfaces that would otherwise be exposed to water. It has been shown, however, that detergents like octylglucopyranoside (OG) dissociate from similar hydrophobic surfaces after being diluted below their critical micellar concentration.^[17]

The improvement of the sample background after thiolipid assembly may be due to two effects, the removal of poorly attached OmpF–Cys after competition with thiolipid, and the reduction of possible lateral displacements by a rigid thiolipid layer. Although the protein solution used contains non-aggregated individual trimers (gel filtration chromatography,^[9] data not shown), we observed small groups (25% of 393 trimers) of closely contacting trimers arranged as in 2D crystals. This possibly implies a cooperative assembly of OmpF trimers at the surface (Figure 2 C, white circles). The SPR, FTIR,^[7] and NR^[10] data were collected on evaporated and therefore rougher gold surfaces. Comparable topographs from these samples are presented in the Supporting Information.

Topographs of single OmpF trimers assembled on TS-Au were averaged (Figure 3 F–G) by using the same approach as used previously on 2D crystals (Figure 3 H, I). The resulting topographs clearly resolve the three individual protrusions of the extracellular OmpF trimer surface.^[16] The majority (>90%) of structures observed in our topographs were trimeric. All of these structures showed the characteristic structural appearance of the extracellular surface of the OmpF porin trimer as identified in previous work.^[18] Therefore, these data show that the cysteine mutation ensures 100% orientation of the proteins in the final sample (as in Figure 1). Additionally, the exposed hydrophobic face of the thiolipid monolayer provided a smooth noninteracting background for AFM imaging. Although the method is immediately applicable to many outer-membrane proteins, it could be extended to the study of other types. In particular, by avoiding crystallization, the tiny quantities required make the study of hard-to-purify complexes more attractive. The limiting step would be to purify the membrane protein and keep it stable in detergent solution. Here, the proteins are fixed close to the surface but thiolipids with hydrophilic spacers could be used to stabilize the proteins with water-soluble domains.^[5] Finally, the engineering scaffold properties of the β -barrel proteins suggests that these oriented layers are

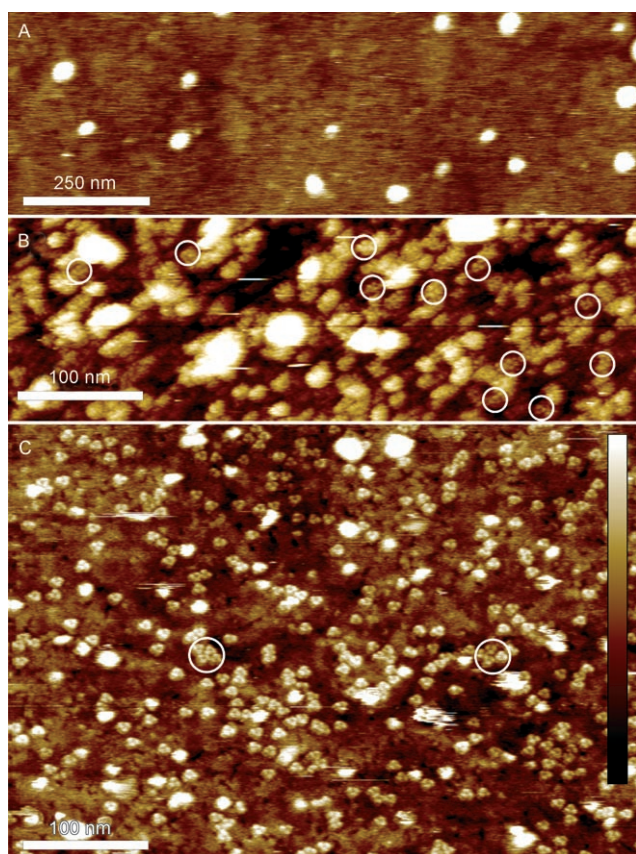


Figure 2. Assembly of porin on flat TS-Au surfaces. A) Control surface of thiolipid assembled on TS-Au surface without OmpF. B) OmpF assembled on TS-Au. Individual trimers are circled. C) Sample as in (B) followed by overnight thiolipid assembly. OmpF trimers are clearly resolved and areas of apparent intermolecular organization are circled. The vertical height scale given in (C) corresponds to 10 nm and is the same for all topographs.

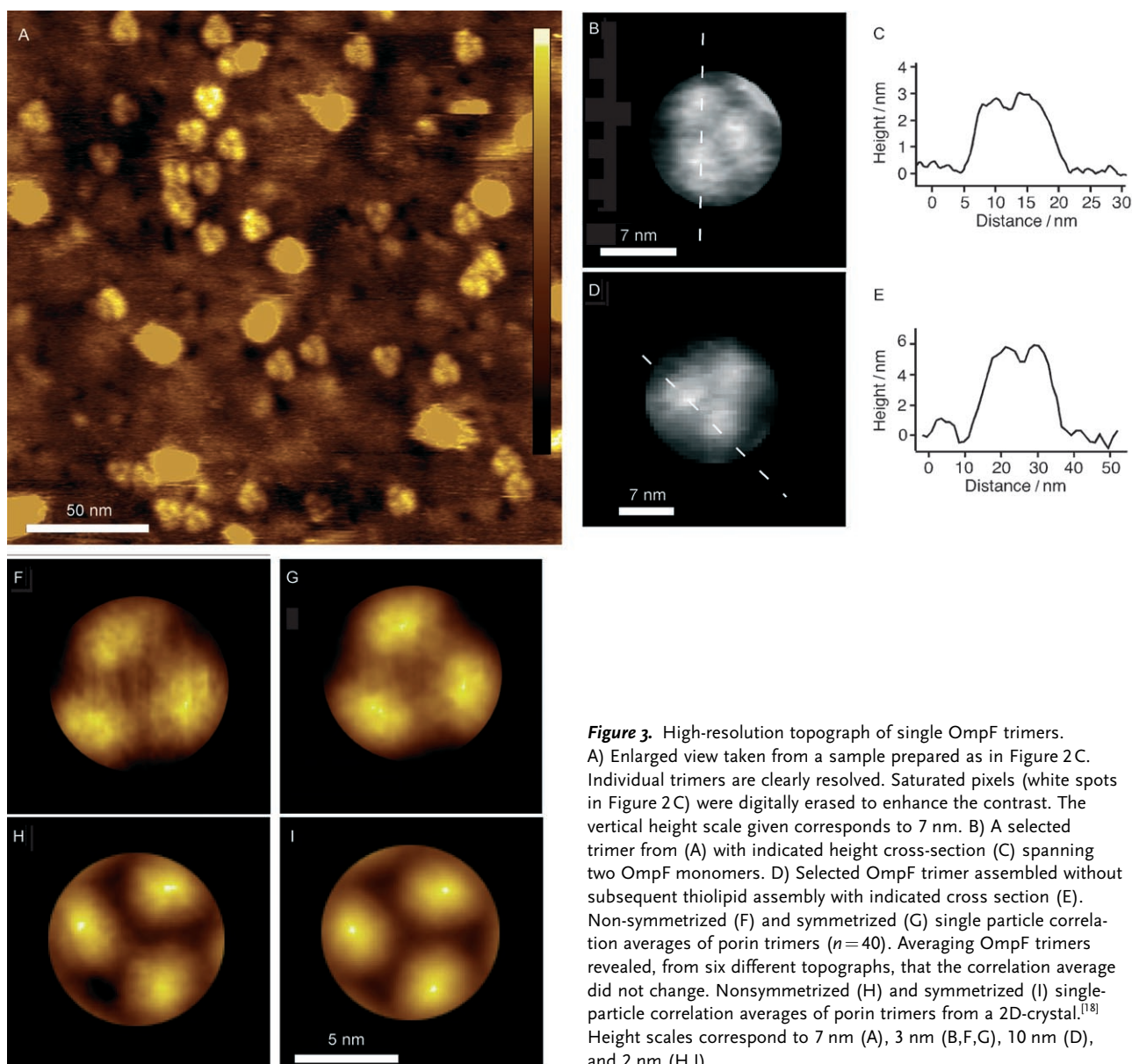


Figure 3. High-resolution topograph of single OmpF trimers.

A) Enlarged view taken from a sample prepared as in Figure 2C. Individual trimers are clearly resolved. Saturated pixels (white spots in Figure 2C) were digitally erased to enhance the contrast. The vertical height scale given corresponds to 7 nm. B) A selected trimer from (A) with indicated height cross-section (C) spanning two OmpF monomers. D) Selected OmpF trimer assembled without subsequent thiolipid assembly with indicated cross section (E). Non-symmetrized (F) and symmetrized (G) single particle correlation averages of porin trimers ($n=40$). Averaging OmpF trimers revealed, from six different topographs, that the correlation average did not change. Nonsymmetrized (H) and symmetrized (I) single-particle correlation averages of porin trimers from a 2D-crystal.^[18] Height scales correspond to 7 nm (A), 3 nm (B,F,G), 10 nm (D), and 2 nm (H,I).

capable of contributing to the development of precise nano-scale biointerfaces.^[7,19]

Experimental Section

OmpF-Cys was purified as described previously^[7] except that buffer solution A contained NaCl (300 mM), Tris-HCl (50 mM; pH 7.5), and OG (1%; Melford, UK). TS-Au surfaces^[12] were treated with β -mercaptoethanol (10 mM) for 20 min before washing and incubation with protein (0.25 mg mL⁻¹ in buffer solution A plus tris(2-carboxyethyl)phosphine hydrochloride (1 mM)). Protein incubations were for a minimum of 1 h. After this, the samples were washed with buffer solution A before incubation with buffer solution A containing thiolipid (1,2-Dipalmitoyl-sn-Glycero-3-Phosphothioethanol; 0.5 mg mL⁻¹; Avanti Polar Lipids, USA) for at least 1 h at 45°C. To remove loosely bound proteins, thiolipids, and detergent the sample was repeatedly (3x) washed with buffer solution A followed by buffer solution B (NaCl (150 mM), Tris-HCl (50 mM); pH 7.8). The AFM (MultiMode, DI-Vecco, CA) was operated in buffer solution B by

using a fluid cell. The piezoelectric scanner had a scan range of $\approx 120 \times 120 \mu\text{m}^2$. Oxide sharpened Si₃N₄ cantilevers had a nominal force constant of 0.08 N m⁻¹ (OMCL TR400PS, Olympus Ltd, Tokyo). Imaging was performed in tapping mode with a drive frequency close to the resonance frequency of the cantilevers (9.5 kHz) immersed in buffer solution B. The drive amplitude of the cantilever was set to a root-mean-square (RMS) value of 18–30 nm. All samples were imaged in buffer solution at room temperature (23°C). Single particles extracted from the topograph (trace and retrace) shown in Figure 3A were aligned and correlation averaged by using the software Semper. The final average was threefold symmetrized. This procedure was identical for the topographs from 2D crystals.^[18]

Received: December 19, 2005

Revised: March 10, 2006

Published online: April 24, 2006

Keywords: lipids · membrane proteins · porin OmpF · protein structures · scanning force microscopy

-
- [1] S. H. White, *Protein Sci.* **2004**, *13*, 1948.
- [2] D. J. Müller, H. Janovjak, T. Lehto, L. Kuerschner, K. Anderson, *Prog. Biophys. Mol. Biol.* **2002**, *79*, 1.
- [3] A. Engel, D. J. Müller, *Nat. Struct. Biol.* **2000**, *7*, 715; S. Scheuring, D. J. Müller, H. Stahlberg, H. A. Engel, A. Engel, *Eur. Biophys. J.* **2002**, *31*, 172.
- [4] G. J. Leggett, *Anal. Chim. Acta* **2003**, *479*, 17; A. Tinazli, J. Tang, R. Valiokas, S. Picuric, S. Lata, J. Piehler, B. Liedberg, R. Tampe, *Chemistry* **2005**, *11*, 5249; J. C. Love, L. A. Estroff, J. K. Kriebel, R. G. Nuzzo, G. M. Whitesides, *Chem. Rev.* **2005**, *105*, 1103; P. Wagner, F. Zaugg, P. Kernén, M. Hegner, G. Semenza, *J. Vac. Sci. Technol. B* **1996**, *14*, 1466; P. Wagner, P. Kernén, M. Hegner, E. Ungewickell, G. Semenza, *FEBS Lett.* **1994**, *356*, 267.
- [5] H. Lang, C. Duschl, H. Vogel, *Langmuir* **1994**, *10*, 197.
- [6] S. Terrettaz, T. Stora, C. Duschl, H. Vogel, *Langmuir* **1993**, *9*, 1361; T. Stora, J. H. Lakey, H. Vogel, *Angew. Chem.* **1999**, *111*, 402; *Angew. Chem. Int. Ed.* **1999**, *38*, 389; R. Naumann, S. M. Schiller, F. Giess, B. Grohe, K. B. Hartman, I. Karcher, I. Koper, J. Lubben, K. Vasilev, W. Knoll, *Langmuir* **2003**, *19*, 5435; K. Tamada, M. Hara, H. Sasabe, W. Knoll, *Langmuir* **1997**, *13*, 1558.
- [7] S. Terrettaz, W.-P. Ulrich, H. Vogel, Q. Hong, L. G. Dover, J. H. Lakey, *Protein Sci.* **2002**, *11*, 1917.
- [8] M. P. Bos, J. Tommassen, *Curr. Opin. Microbiol.* **2004**, *7*, 610; A. Bateman, L. Coin, R. Durbin, R. D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, M. Marshall, S. Moxon, E. L. L. Sonnhammer, D. J. Studholme, C. Yeats, S. R. Eddy, *Nucleic Acids Res.* **2004**, *32*, D138.
- [9] V. Visudtiphole, M. B. Thomas, D. A. Chalton, J. H. Lakey, *Biochem. J.* **2005**, *392*, 375.
- [10] S. A. Holt, J. H. Lakey, S. M. Daud, N. Keegan, *Aust. J. Chem.* **2005**, *58*, 674.
- [11] N. Keegan, N. G. Wright, J. H. Lakey, *Angew. Chem.* **2005**, *117*, 4879; *Angew. Chem. Int. Ed.* **2005**, *44*, 4801.
- [12] M. Hegner, P. Wagner, G. Semenza, *Surf. Sci.* **1993**, *291*, 39; P. Wagner, M. Hegner, H. J. Guntherodt, G. Semenza, *Langmuir* **1995**, *11*, 3867.
- [13] C. D. Bain, G. M. Whitesides, *Science* **1988**, *240*, 62.
- [14] G. Yang, N. A. Amro, Z. B. Starkewolfe, G. Y. Liu, *Langmuir* **2004**, *20*, 3995; S. Subramanian, S. Sampath, *Pramana* **2005**, *65*, 753.
- [15] R. K. Thomas, *Annu. Rev. Phys. Chem.* **2004**, *55*, 391; J. L. Popot, E. A. Berry, D. Charvolin, C. Creuzenet, C. Ebel, D. M. Engelmann, M. Flotenmeyer, F. Giusti, Y. Gohon, P. Herve, Q. Hong, J. H. Lakey, K. Leonard, H. A. Shuman, P. Timmins, D. E. Warschawski, F. Zito, M. Zoonens, B. Pucci, C. Tribet, *Cell. Mol. Life Sci.* **2003**, *60*, 1559.
- [16] S. W. Cowan, T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Paupit, J. N. Jansonius, J. P. Rosenbush, *Nature* **1992**, *358*, 727.
- [17] G. B. Sigal, M. Mrksich, G. M. Whitesides, *Langmuir* **1997**, *13*, 2749.
- [18] D. J. Müller, A. Engel, *J. Mol. Biol.* **1999**, *285*, 1347.
- [19] H. Lang, *Int. J. Med. Microbiol.* **2000**, *290*, 579.
-