Atomic Force Microscope: The Crystallographer's Best Friend?

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The pioneering days of biological scanning probe microscopy were filled with high hopes that this new tool would revolutionize structural biology. I doubt that many protein crystallographers feared unemployment in the face of the new tools. It soon became clear that scanning probe microscopes suffer from limited resolution, sample damage, and an inability to probe internal structure. The strength of the new methods lies in monitoring processes at the nm scale. This is demonstrated beautifully in the work of Yip and Ward (1996; published in this issue).

These investigators describe a careful atomic force microscopy (AFM) investigation of the growth of insulin crystals. By exploiting evaporation to bring about supersaturation in situ in the microscope liquid cell, the layer-by-layer growth of the crystal was studied in real time. In addition to monitoring the quality of the crystal surface (out to the micrometer scale), these investigators studied the role of defects, measured the growth rate, and used the measured-step radius to estimate the step-free energy. Molecular resolution images permitted confirmation of the space group, direct measurement of the lattice parameters, and identification of polymorphs. This paper constitutes a striking proof-of-principle for the use of in situ AFM in the study of protein crystal growth. Because the growth of large, high quality crystals remains the main impediment to x-ray crystal structure determination, it appears that the AFM will play a role in high-resolution structure determination after all.

The in situ study of dissolution and growth was initiated some years ago in the Hansma laboratory (Gratz et al., 1991; Giles et al., 1995) and carried on by others cited in Yip and Ward's paper. However, this pioneering work used inorganic crystals (or samples with a high mineral content), which are much more robust and easier to handle than most protein crystals.

It is worth recalling that some protein structures have been imaged at rather high resolution by AFM, notably by the groups of Shao (Shao and Yang, 1995) and Engel (Schabert and Engel, 1994). To date, much of this work has been carried out using contact-mode AFM in solution, working on self-assembled two-dimensional films of the protein. In the case of the insulin crystal, Yip and Ward found that the normal contact mode, even operated in solution, was too destructive to permit imaging of the crystal surface. They used the so-called tapping mode, in which the cantilever is oscillated at with an amplitude of some nm and the approach to the surface is detected as a fall in this amplitude as the cantilever interacts transiently with the surface. This has the advantages that the average contact force is smaller, and that the tip delivers an impulse predominately normal to the surface, a direction in which these samples are generally stronger.

Despite the quality of the present work, it is clear that there is much room for improvement. To begin with, the work is tricky, as anyone who has tried AFM in a fluid cell will testify. More importantly, the probe obstructs the free-diffusion of molecules onto the surface and this must play some role in modifying growth as studied in situ. Yip and Ward have been careful to test for this possibility, and it does not appear to be a problem in the present work. However, our (unpublished) in situ studies of electrochemical corrosion and deposition show that mass-transport is often modified by the presence of the probe. Finally, tapping mode AFM, as presently practiced in solution, leaves a lot to be desired. When the probe is acoustically driven, coupling through the fluid medium to resonances of the sample cell leads to spurious responses that make the microscope hard to operate and characterize (Florin et al., 1993). And whereas tapping offers a great improvement on contact mode, it still involves rather violent encounters between the probe and sample. If, for example, a 10% change in amplitude is detected using a free-oscillation amplitude of 10 nm and a cantilever of 0.1 N/m spring constant, elementary mechanics leads to the result that $\sim 10^{-18}$ J is deposited into the sample at each strike of the probe. This is several hundred times thermal energy. Yip and Ward have done beautiful work with relatively primitive tools. It gives a hint of what may come in the future as better methods and microscopes are developed.

REFERENCES

Florin, E. L., M. Radmacher, B. Fleck, and H. E. Gaub. 1993. Atomic force microscope with magnetic force modulation. *Rev. Sci. Instrum.* 65:639–643.

Giles, R., S. Manne, D. E. Morse, G. D. Stucky, and P. K. Hansma. 1995. Inorganic overgrowth of aragonite on molluscan nacre examined by atomic force microscopy. *Biol. Bull.* 188:8–15.

Gratz, A. J., S. Manne, and P. K. Hansma. 1991. Atomic force microscopy of atomic-scale ledges and etch pits formed during dissolution of quartz. *Science.* 251:1343–1346.

Schabert, F. A., and A. Engel. 1994. Reproducible Acquisition of E. Coli Porin Surface Topographs by Atomic Force Microscopy. *Biophys. J.* 67:2394–2403.

Shao, Z., and J. Yang. 1995. Progress in high resolution atomic force microscopy in biology. *Q. Rev. Biophys.* 28:195–251.

Yip, C. M., and Ward, M. D. 1996. Atomic force microscopy of insulin single crystals: direct visualization of molecules and crystal growth. Biophys. J. 71:1071–1078.

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Shuffling Protons in Bacteriorhodopsin: Long-Distance Coupling Between the pK_as of Two Carboxylic Groups

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How are protons transported across membranes? This question can be more specifically asked of the light-driven proton pump, bacteriorhodop-

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sin, than of other more complex pumps. Until recently, the proton translocation pathway in this small protein with seven transmembrane helices was thought to be basically understood. After photoisomerization of the retinal chromophore, a proton is transferred from the retinal Schiff base to D85 in the extracellular region, followed by reprotonation of the Schiff base from D96 in the cytoplasmic region. These internal events are linked to proton release to the extracellular surface and to proton uptake to reprotonate D96 from the cytoplasmic surface. This simple mechanism became complicated, however, by the observation that the proton at the extracellular surface is not released directly from D85. Where this proton, named residue X, originates and what triggers the release of the proton when D85 becomes protonated have been unanswered questions. In this issue of the *Biophysical Journal*, Govindjee et al. (1996) present the last in a series of articles in which they provide the answer to these questions in a novel mechanism for the proton release in which the pK_as of D85 and the proton release group (a glutamate) are coupled.

A seemingly unrelated, and possibly obscure, observation was made earlier by the same group (Balashov et al. 1995): D85 in the unphotolyzed protein titrated with two pK_as. Titration of D85 is simple enough to do because protonation of D85 shifts the absorption maximum of the chromophore toward the red, but the higher of the dissociation constants had never been noticed before because the amplitude of the transition is very small. It was the correlation of the rate of thermal isomeric equilibration of the retinal ("dark adaptation") with the protonation state of D85, demonstrated with the aid of mutants of R82 where the pK_a of D85 is greatly altered, that made observation of the biphasic titration curve possible. The first important insight by these workers was that the explanation for the complex titration behavior lay in the interaction of D85 with an unidentified group, X′ (labeled to make a distinction from the previously termed "X"). Thus, in their proposed scheme, which accounted quantitatively for the results, D85 acquires a lower pK_a when X′ becomes protonated, and conversely X′ acquires a lower pK_a when D85 becomes protonated.

The second important insight (Balashov et al. 1995, 1996) was the realization that such a relationship of X′ to D85 is just what one would expect for the proton release group X. According to the scheme, X would have a high pK_a (and therefore be protonated under physiological conditions) before photoexcitation when D85 is anionic, but its pK_a would drop once D85 became protonated (and therefore X would dissociate and release its proton). What, then, is X′, and is it in fact the same residue as X?

Previously, all that was known about X was that its apparent pK_a drops during the photocycle from ~9 to ~6. Below pH 6, proton release in the photocycle is delayed until the last step, presumably because X will not dissociate at pH below its pK_a. Under these conditions proton uptake at the cytoplasmic surface occurs before release at the extracellular surface, i.e., there is net proton uptake rather than release in the photocycle. R82, located between D85 and the extracellular surface, seemed a good candidate to a number of investigators even though arginine residues seldom have such low proton affinities. A tyrosine located nearby, Y57, was another guess, or possibly water liganded to either or both of these groups. Mutations in either R82 or Y57 abolished, or at least greatly diminished, the early proton release, and the net proton release was replaced by net proton uptake in the photocycle. Removal of the positive charge of R82 by mutation greatly increased the pK_a of D85 (and presumably vice versa).

Last year, however, a third residue was demonstrated to be the proton release group. The photocycle of E204Q, but not E204D, lacked early proton release like the R82 and Y57 mutants, but more importantly the easily dissected negative C=O stretch band of the carboxylic group of D204 indicated that residue 204 dissociates after protonation of D85 and must therefore be X (Brown et al. 1995). Subsequently, the prediction that X′ is identical to X was proved correct by showing that the higher of the two pK_as of D85 is missing in titrations of the E204Q mutant (Richter et al., 1996). The paper by Govindjee et al. (1996) now provides another piece of the puzzle by distinguishing the roles of R82 and E204 in the proton release. They found that at elevated pH proton release becomes normal in R82Q, but not in E204Q. This removes any doubts that E204, rather than R82, is the proton release group. The earlier reports on R82 mutants can be now understood: the influence of R82 decreases the pK_a of E204 to be in the range for proton release at neutral pH in the photocycle. In the R82Q mutant the pK_a is above this range. As expected from this, the pK_a of E204 in the R82K mutant is intermediate between the wild-type R82Q (Govindjee et al. 1996).

The intriguing question that remains is exactly how the protonation states of D85 and E204 can influence one another. These residues are ~10 Å apart, with D85 near the center of the protein and E204 closer to the extracellular surface, while R82 is located between them. Molecular dynamics calculations predicted that a chain of hydrogen-bonded water connects these three residues. Although this possibility would be consistent with charge transfer along hydrogen bonds that would accomplish the observed pK_a shifts, uncovering the molecular mechanism for long-distance coupling in this structure remains a challenge for the future.

REFERENCES

Balashov, S. P., R. Govindjee, E. S. Imasheva, S. Misra, T. G. Ebrey, Y. Feng, R. K. Crouch, and D. R. Menick. 1995. The two pK_a's of aspartate-85 and control of thermal isomerization and proton release in the arginine-82 to lysine mutant of bacteriorhodopsin. *Biochemistry*. 34:8820–8834.

Balashov, S. P., E. S. Imasheva, R. Govindjee, and T. G. Ebrey. 1996. Titration of aspartate-85 in bacteriorhodopsin: what it says about chromophore isomerization and proton release. *Biophys. J.* 70:473–481.

Brown, L. S., J. Sasaki, H. Kandori, A. Maeda, R. Needleman, and J. K. Lanyi. 1995. Glu-

tamic acid 204 is the terminal proton release group at the extracellular surface of bacteriorhodopsin. *J. Biol. Chem.* 270:27122–27126.

Govindjee, R., S. Misra, S. P. Balashov, T. G. Ebrey, R. K. Crouch, and D. R. Menick. 1996. Arginine-82 regulates the pK_a of the group responsible for the light-driven proton release in bacteriorhodopsin. *Biophys. J.* 71: 1011–1023.

Richter, H-T., L. S. Brown, R. Needleman, and J. K. Lanyi. 1996. A linkage of the pK_a's of asp-85 and glu-204 froms part of the reprotonation switch of bacteriorhodopsin. *Biochemistry.* 35:4054–4062.

Water Chains in Lipid Bilayers

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Several reports have appeared recently that present molecular dynamics simulations of water in channels. (Sagnella and Voth, 1996; Pomes and Roux, 1996; Breed et al., 1996) In this issue of the *Biophysical Journal*, Marrink et al. extend such simulations to the stability of hydrogen-bonded water chains in pure lipid bilayers. The properties of water molecules in lipid bilayers might seem to be so fundamental that the basic facts would have been established decades ago. And yet, nearly 30 years after Cass and Finkelstein (1967) proposed that water partitions into bilayers and diffuses as individual molecules, it is still possible to open a recent journal and find evidence that water can move through porelike discontinuities (Jansen and Blume, 1995). The truth is probably somewhere in between, with most of the water present as individual molecules and a small fraction involved in collective behavior within transient defects in the bilayer.

Marrink et al. use molecular dynamics simulations to estimate the stability of hydrogen-bonded water strands in the bilayer. Such structures are of interest because of earlier proposals that water strands in rare transient defects could account for the high permeability of bilayers to protons (see Marrink et al. (1996)for pertinent references). It is generally considered that the low permeability of bilayers to ions like potassium is attributable to a very high electrostatic energy barrier, in which Born energy is the major component. However, protons do not depend on diffusion alone to move through aqueous media. Instead a proton can hop along chains of hydrogen-bonded water molecules. If such chains are present in lipid bilayers, protons could bypass the electrostatic energy barriers by a wirelike conductance.

This brings us to the question addressed by Marrink: Do such chains have sufficiently long lifetimes to provide a permeation pathway? Because the proposed transient hydrogen-bonded water chains are much too rare to observe as spontaneous events, the simulation was necessarily performed by establishing a water chain within the bilayer and then observing its stability over time. Chains typically last for 2–5 ps, just long enough to permit a single-proton transfer event. By extrapolating from the lifetimes and energy required to produce water chains, it was estimated that ~100 chains form per second in a 0.2-mm diameter liposome. This might seem sufficient to account for the proton permeation anomaly, but there is a problem. If one uses reasonable assumptions for the rate at which protons might be delivered to a given chain at 10^{-7} M H+ (pH 7), the estimated flux falls short by eight orders of magnitude. A similar problem was encountered by Benz and McLaughlin (1983) when attempting to account for the rate at which protonophores carried protons across bilayers. In both cases, it is possible that buffers play a role. Instead of assuming that the rate of proton delivery is a direct function of the pH, it may be that much higher numbers of protons can be delivered as buffer ions interact with the site.

The molecular dynamics simulations of water in channels and in bilayers are intrinsically interesting, allowing us to "see" rare events that are not readily detected by direct measurements. Such simulations will likely spur a new round of experimentation that will advance our understanding of how water and ions interact with bilayers and channels.

REFERENCES

Benz, R. and S. McLaughlin. 1983. The molecular mechanism of action of the proton ionophore FCCP. *Biophys. J.* 41:381–398.

Breed, J., R. Sankararamakrishnan, I. D. Kerr, and M. S. P. Sansom. 1996. Molecular dynamics simulations of water within models of ion channels. *Biophys. J.* 70:1643–1661.

Cass, A., and A. Finkelstein 1967. Water permeability of thin lipid membranes. *J. Gen. Physiol.* 50:1765–1779.

Jansen, M., and A. Blume. 1995. A comparative study of diffusive and osmotic water permeation across bilayers composed of phospholipids with different head groups and fatty acyl chains. *Biophys. J.* 68:997–1008.

Marrink, S. J., F. Jahnig, and H. J. C. Berendsen. 1996. Proton transport across transient single-file water pores in a lipid membrane studied by molecular dynamics simulations. *Biophys. J.* 71:632–647.

Pomes, R., and B. Roux. 1996. Structure and dynamics of a proton wire: a theoretical study of H^+ translocation along a single file water chain in the gramicidin channel. *Biophys. J.* In press.

Sagnella, D. E., and G. A. Voth. 1996. Structure and dynamics of hydronium in the ion channel gramicidin A. *Biophys. J.* 70:2043–2051.

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