Na,K-ATPase in crystalline form investigated by scanning force microscopy

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Na,K-ATPase has been isolated in purified membrane fragments from kidney tissue and crystallized by phospholipase treatment to obtain two-dimensional, membrane-bound protein crystals. Scanning force microscopy has been used to identify and analyze the topography of the membrane fragments. Specific patterns in accordance with electron microscopic images have been found. In biological material under physiological conditions the scanning force is a crucial parameter for the resulting image at high resolution.

1. Introduction

Since its invention by Binnig et al. [1], the scanning force microscope (SFM) has been developed to become a powerful instrument in surface science. In contrast to its precursor, the scanning tunneling microscope (STM), the SFM takes images not only from conducting, but also from nonconducting surfaces. In principle, sample preparation for SFM is very easy; samples can be imaged in vacuum, air or liquids and in a wide range of temperatures from 4 K to room temperature [2]. If the forces applied to the samples are small enough, scanning force microscopy can be regarded as a nondestructive imaging technique. The SFM is today the only instrument which can picture biological samples with nm resolution under physiological conditions. Therefore it seems to be obvious that SFM will evolve into a very important tool also for biological research. In fact, images of Langmuir–Blodgett films [3], DNA [3], bacteriorhodopsin [4], blood cells and bacteria [5], and even the polymerization of fibrin [6] have been taken with the SFM. Unfortunately these already impressive results lack up to now the

reproducibility and resolution of results taken with SFM or STM of flat, crystalline surfaces. The reduction and better control of imaging forces represents the main challenge for SFM on biological samples. Pushing the resolution of SFM down to atomic scale at high scan rates will not be easy, but we think that it will open a new frontier: direct observation of nature “working” at the biochemical level would then be possible.

We have chosen to image Na,K-ATPase by SFM because it is a well investigated membrane-bound transport protein. Na,K-ATPase is easy to prepare, and has extensively been studied by electron microscopy and other independent biochemical techniques. For a review, see ref. [7]. Na,K-ATPase can be crystallized and shows a well defined structure at nm resolution. With an appropriate resolution geometrical differences in its conformations should be distinguishable.

The Na,K-ATPase is located in the cytoplasmic membrane of animal cells and carries out transport of sodium and potassium ions against their electrochemical gradients at the expense of free energy of ATP hydrolysis. During the pump cycle 3 Na⁺ are transported out of the cell and 2

K^+ into the cytoplasm per hydrolyzed ATP. Two main conformations have been established, which are characterized by different properties [7]: E_1 with high affinity binding sites for Na^+ at the cytoplasmic interface and E_2 with high affinity binding sites for K^+ at the extracellular interface. Further differences of the main conformations are the tryptic digestion patterns and different two-dimensional crystals induced in membrane fragments. Typical dimensions of Na,K-ATPase dimers determined by electron-microscopic techniques from vanadate-induced crystals are: total length perpendicular to the membrane approx. 10 nm, cytoplasmic and extracellular protrusion approx. 4 and 2 nm, respectively [8]. From top view a unit cell with a cross section of $8\text{ nm} \times 12\text{ nm}$ has been found [9]. In fig. 1 an electron microscopic image of membrane fragments is shown, containing Na,K-ATPase crystallized by a phospholipase A_2 treatment [10]. The lattice constant of $13 \times 5\text{ nm}^2$ proposes an upper limit on the size of the dimer.

2. Sample preparation

Na,K-ATPase was isolated and purified from the outer medulla of rabbit kidney [11]. The remaining membrane preparation had typically an enzymatic activity of $2000\text{ }\mu\text{mol P}_i$ per mg protein per hour. Extraction of lipids by dialysis from membrane fragments [10] in the presence of $3.4\text{ }\mu\text{g/ml}$ phospholipase A_2 for 48 h initializes crystallization of the Na,K-ATPase as shown in fig. 1. After the period of incubation with phospholipase A_2 the enzyme was spun down by centrifugation and resuspended in a phospholipase-free buffer and kept at 0°C . The residual activity of the crystallized enzyme was determined to be $20\text{ }\mu\text{mol P}_i$ per mg protein per hour. To avoid bacterial growth in the solution containing the membrane fragments, which otherwise was observed, azide was added to the buffer. To identify the adhesion of membrane fragments to the substrate by an independent method, some of the enzyme preparations have been labelled with 5-iodoacetamidofluorescein (5-IAF) as described by Stürmer et al. [12].

Fig.1. Electron-microscope image of membrane fragments containing Na,K-ATPase. Typical membrane fragments show sizes from 0.1 to $0.6\text{ }\mu\text{m}$. The membrane fragment on the left has crystallized domains with dimer and trimer rows of Na,K-ATPase. The unit cell of Na,K-ATPase has a typical size of $15 \times 6\text{ nm}$ and an inner angle of 65° . Groups of small dots in the uncrystallized areas correspond to oligomers of Na,K-ATPase. The electron-microscopic image was prepared by Dr. L. Dux (Konstanz, 1989). Reproduction with permission. Negative staining with 1% uranyl acetate. The bar corresponds to 100 nm.

SFM samples were prepared as follows: The solution containing membrane fragments was diluted in buffer [13] or in distilled water to obtain protein concentrations of 0.1 to 0.3 mg/ml . A drop of this solution was placed onto freshly cleaved muscovite mica [14] for 10 to 60 s, then the mica was rinsed carefully with buffer or distilled water and transferred to the SFM. Images have been taken mainly in buffer. Attention was paid to keep the sample always under liquid to avoid drying of the membrane fragments. If images were taken in air the samples were rinsed only with distilled water and then dried in a cooled exsiccator. Experiments were done at tem-

Fig. 2. Images of membrane fragments containing crystallized Na,K-ATPase at different resolutions. Scanning force about 5 nN, scanning at 4°C. (a) A typical image of membrane fragments adsorbed on mica. Already at this low resolution domains of two different heights are resolved. Fragment size varies between 0.25 and 1 μm. Image size: 7×7 μm. (b) Two different domains are clearly resolved: a lower, flat one in the upper left (lipid phase) and a higher, structured domain (protein phase) at the bottom. The protein phase shows an even higher border at the right. Height is given in arbitrary units and corresponds to 8 nm for the lipid phase and about 22 nm for the protein phase. Image size: 1×1 μm. (c) A further magnification of the protein phase is shown: structures with a diameter of about 15 nm, which are aligned in rows. Image size: 300×300 nm.

Fig. 2 (continued).

peratures between 0° and 4°C by operating the SFM inside an absorber refrigerator.

The SFM experiments were performed with an instrument using the optical-lever technique to detect the deflection of the cantilever. The cantilevers with microfabricated tips [15] had force constants ranging from 0.1 to 0.4 N/m, their resonance frequency was 66 kHz.

3. Results

At low resolution (image size: $7 \times 7 \mu\text{m}^2$) we find the mica substrate covered by membrane fragments with sizes ranging from 0.25 to $1 \mu\text{m}$ (fig. 2a). The average height of these fragments is about $22 \pm 2 \text{ nm}$ and quite uniform from fragment to fragment. These low resolution images are completely reproducible. At high scanning forces ($> 50 \text{ nN}$), the membrane fragments are not moved, but the surface structure is signifi-

cantly changed when the same area is scanned again. Therefore we conclude that the adhesion of the fragments onto the mica is rather high.

At medium resolution (image size: $1 \times 1 \mu\text{m}^2$) fragments show two different domains (fig. 2b). One type of domain is completely flat and approximately 8 nm thick (distance between mica and membrane surface). The second domain is 22 nm thick and frequently surrounded by structures which are another 20 nm higher. Similar observations have been made in electron microscopic images, where these highest structures at the border could be interpreted as up-folded membrane.

At high resolution (image size: $300 \times 300 \text{ nm}^2$) we find in the domain of 22 nm height, structures which are either non-ordered or, more often, arranged along rows (fig. 2c). The diameter of these structures varies between 10 and 24 nm, but is quite uniform on the same fragment. The spacing of the rows is 20 to 35 nm, their corrugation is

Fig. 3. Structural details of protein domains of membrane fragments at the smallest stable scanning force (1–2 nN). (a) Within the protein phase of membrane fragments rows are resolved with a spacing of 25–35 nm, their corrugation corresponds to 2 nm. Noise at the top of the image is caused by the cantilever snapping off the surface due to small imaging force. Image size: 300×300 nm. Compare to fig. 2b, taken at the same resolution but with a higher scanning force. (b) At highest resolution the rows shown in (a) reveal substructure: within the two larger rows several small rows with a spacing of 6–8 nm are resolved.

about 1 nm. Structures as just described are very reproducible at our usual imaging forces [16] of 5–10 nN; subsequent images at the same spot do not show any appreciable difference apart from a small amount of drift. At the lowest stable imaging force of 1–2 nN we find a different structure: parallel rows of 25–35 nm spacing and 2 nm corrugation (fig. 3a). These rows can be further resolved into smaller rows of 6–8 nm width and 0.5 nm height (fig. 3b). No structure along either the small or the larger rows can be seen. These smaller rows are very unstable; a series of images taken at the same spot show already some differences, at higher forces than about 2 nN these rows are destroyed. By changing the direction of scanning in the x - y plane and verifying that the described structures correspondingly turned, we confirmed that all these structures were independent of scanning direction.

The lower, flat domains remain without structure also at high resolution (not shown).

Scanning the mica surface in buffer, we could not clearly resolve the typically structured surface between the fragments as one would expect from clean mica in air. Even in buffer without fragments the atomic lattice of the mica substrate was hardly observed. Addition of KCl to final concentrations of 1M and higher resolved the atomic lattice distinctly indicating electrochemical screening effects, as has also been observed by another group [17]. High ionic strength had no influence on the surface structure of membrane fragments.

We achieved the best images of crystallized Na,K-ATPase 1–2 days after dialysis, in buffer and with the sample cooled down to 0–4°C. The amount of debris in the preparation (presumably micells of lipoid material and phospholipase) increased drastically after 8 days and reduced the resolution of the images by covering the mica, membrane surface and also the tip of the cantilever.

Images scanned in air in the usual topographic mode of the SFM showed only the membrane fragments without inner structure; their height ranged from 12 to 16 nm. Fragments were also resolved with our newly developed scanning force friction microscope [18] in the normal topo-

graphic as well as in the friction mode [18]; the contrast is higher in the latter one, but no further details are resolved.

4. Control experiments

A series of control experiments have been performed to identify the material adsorbed to mica sheet as Na,K-ATPase containing fragments. For the first series of experiments we used the method of decorating enzyme with the fluorescence probe 5-IAF [12]. The enzyme was labelled with 5-IAF before crystallization. During crystallization the fluorescence intensity decreased to approximately 10% of the untreated preparation due to quenching effects. Labeled and crystallized fragments have been adsorbed to mica, and SFM experiments showed typical results as presented in fig. 2. These mica-adsorbed fragments have been investigated also by fluorescence methods. Upon successive addition of Na^+ , K^+ and ATP changes of fluorescence intensities have been observed in accordance with the pattern of noncrystallized membrane fragments [12]. The observed slow kinetics of the crystallized fragments is in accordance with the low residual enzymatic activity of the treated protein. Free 5-IAF dye does not adsorb to mica and does not contribute to fluorescence intensity.

In a second series of control experiments membrane fragments were scanned 1, 3, 5, and 8 days after the beginning of the crystallization. In accordance with results from electron-microscopy studies the structure of the fragments became more pronounced and the size decreased with time. From the third day on, two-dimensional structures were found on the membrane sheets as presented in figs. 2b and 2c. The crystalline domains increased with time, and in accordance with electron-microscopic observations, holes (to be seen in fig. 1) were found in the noncrystalline areas of the membrane fragments. After 8 days the surface of the remaining fragments was almost completely crystalline and fusion of fragments to large arrays was detected.

Since the height of the fragments measured by SFM is rather high, we calibrated the piezo tube

by optical interferometry and reproduced the values specified by the manufacturer.

5. Conclusion

Scanning force microscopy can be used to collect information on the topography of membrane-bound proteins with extramembraneous protrusions. In buffers of physiological composition and at moderate temperature, resolution is possible in the range of 2 nm. Comparison of electron-microscopic information which presents the density of matter against electron waves and the topographic information obtained with SFM show common features, but differ significantly in details. Looking at the surface instead of looking through the sample may be one reason; the absence of any fixation procedure on biological material in SFM another one.

The low- and medium-resolution images are in good agreement with electron-microscopy results: We interpret the lower domains as the double lipid layer and the higher domain as membrane parts with a high concentration of the transport protein Na,K-ATPase. The rather large values of the height of the fragments (8 nm for the lipid phase and 22 nm for the protein phase) differ significantly from values obtained by electron-microscopic studies (4–6 and 12–14 nm, respectively). Since calibration errors of the SFM can be excluded due to various checks, the deviations must have different reasons. Samples measured in air and in buffer were found to differ also in height. The dried preparation showed a thickness of 15–16 nm compared to 22 nm in buffer. The difference of 6 to 7 nm cannot be explained definitely up to now, but a variety of explanations are obvious and have to be considered and verified or ruled out: In aqueous phases lipid membranes are known to swell, a process which increases their thickness; the (extracellular) sugar trees of the glycosylated Na,K-ATPase might collapse when the preparation is dried, and since the sugars are assumed to face the mica surface the apparent height of dried fragments should be lower. At least up to now it cannot be excluded that some (soft) material from the solution in

which the membrane fragments were prepared adsorbed on the mica surface and formed the substratum on which fragments attach preferentially. Differences in electron-microscopic data and (the more corresponding) dried membrane fragments measured with SFM have to be handled with the view that electron microscopy detects densities of material layers (even of staining substrates) while SFM produces surface contour plots. Since the SFM is, in principle, the ideal instrument to measure surface corrugation on insulating surfaces, efforts to clarify the reason for this high membrane thickness are under progress.

At the highest resolution we do not resolve singular proteins but only rows of 6–8 nm spacing. These small rows seem to aggregate into groups of 4, 5 or more rows (figs. 3a and 3b). These rows have also been observed by electron microscopy on crystalline fragments, and are interpreted as domains of crystalline Na,K-ATPase. When rescanned with imaging forces above 1 nN these rows disappeared to form structures with diameters in the range of 10–25 nm. We propose that the observed pattern are oligomers of the protein as can be seen in fig. 1 outside the crystalline areas. However, no substructure within these areas could be resolved so far. These almost circular structures often arrange in a kind of superlattice with a unit cell between 12×20 and 20×30 nm. This process is irreversible and demonstrates impressively the influence of the scanning force on the image of soft biological samples.

The main problem for SFM with biological material is the softness of the samples. In our case the smallest possible imaging force is just small enough to resolve the native crystalline structure without destroying it. Further minimizing of the imaging force represents the main challenge for SFM on biological samples. SFM at lower temperatures (liquid nitrogen or even liquid helium temperatures) should stiffen the surface and thus improve image quality. However, observation of dynamic processes, one of the great advantages of SFM, would then not be possible. We believe that sharper tips and especially a better understanding of the interaction between

tip and sample at small distances (0–5 nm) should allow a better control of imaging force and thus lead to higher resolution.

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- [13] Buffer: Tris/HCl pH 7.3; 1mM CaCl₂, 1mM MgCl₂, 5mM H₃PO₄.
- [14] Potassium aluminosilicate 0.15 mm thickness, clear ruby from Goodfellow, Cambridge Science Park.
- [15] The scanning force microscope and microfabricated cantilevers were purchased from Park Scientific Instruments, 476 Ellis Street, Mountain View, CA, USA.
- [16] Since the tip is usually attracted towards the sample (due to the long-range Van der Waals interaction of all tip atoms within about 50 nm of the sample) even though the last few atoms of the tip feel a (strong) Coulomb repulsion we define the imaging force as follows: it is the difference between the force exerted by the cantilever (set by the feedback system) and the pullout force where the cantilever snaps off the sample (at this force the attractive Van der Waals force and the force exerted by the cantilever just balance; the net force on the sample therefore vanishes. This point is unstable).
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