Na/K-ATPase and Related ATPases

Proceedings of the 9th International Conference on the Na/K-ATPase and related ATPases, Sapporo, Japan, 18–23 August 1999

Editors:

Kazuya Taniguchi
Division of Chemistry, Graduate School of Science,
Hokkaido University, Sapporo, Japan

Shunji Kaya
Division of Chemistry, Graduate School of Science,
Hokkaido University, Sapporo, Japan

2000

ELSEVIER

Amsterdam – Lausanne – New York – Oxford – Shannon – Singapore – Tokyo

Na/K-ATPase and Related ATPases
K. Taniguchi and S. Kaya (Editors)

Na,K-ATPase on a waveguide sensor: supramolecular assembly and side directed binding studies by surface-confined fluorescence

E. Grell[a], M. Pawlak[b], D. Anselmetti[c], E. Schick[a], E. Lewitzki[a] and M. Ehrat[b]

[a]Max-Planck-Institute of Biophysics, Kennedyallee 70, 60596 Frankfurt, Germany

[b]Zeptosens AG, Benkenstraße 254, 4108 Witterswil, Switzerland

[c]Solvias AG, Klybeckstr. 191, 4002 Basel, Switzerland

The functional assembly of FITC-Na,K-ATPase membrane fragments on a surface-modified Ta_2O_5 waveguide allows to investigate the directed binding of ligands by surface-confined fluorescence studies. The results allow to draw conclusions about the sidedness of interactions. The fluorescence intensity decrease observed upon the selective binding of K^+ is attributed to its coordination to a site accessible from the former intracellular membrane side.

1. INTRODUCTION

The side-directed functional properties of many integral membrane proteins are usually investigated by solubilizing the protein with detergents for the purpose of reconstitution in vesicular systems. In order to avoid detergents, a simpler method is desirable where small amounts of protein containing membrane fragments can directly be assembled in a side-directed manner on a surface due to supramolecular interactions. Based on sensitive surface-confined detection techniques, directed binding studies can then be performed. A realization of this concept (cf. [1]), consisting of nanoparticulate membrane fragments containing the cardiac glycoside receptor Na,K-ATPase, immobilized on a biocompatible planar waveguide, is discussed here. The planar arrangement offers the additional advantage that the topgraphy of the supramolecular assembly can be characterized by atomic force microscopy (AFM).

2. METHODOLOGY

Biocompatibility of planar, 150 nm thick Ta_2O_5 waveguides (surface modified with a C_{16}-phosphate) [2], attached to a 6 µl flow cell is achieved by a treatment with phospholipid vesicles (110 nm diameter), prepared by extrusion of 0.7 mM 1-palmitoyl-2-oleoyl-glycerophosphocholine in 10 mM sodium phosphate, 0.02% NaN_3, 150 mM NaCl, pH 7.5. This leads to the formation of a subsequent phospholipid monolayer (Figure 1a). After buffer exchange, the membrane fragments are functionally assembled (Figure 1a) by incubating the sensor surface with 1.5 µM FITC-Na,K-ATPase in 10 mM imidazole/HCl, 100 mM choline chloride (ChCl) pH 7.5 for 60 min at 20°C, as described in [1]. Purified Na,K-ATPase is

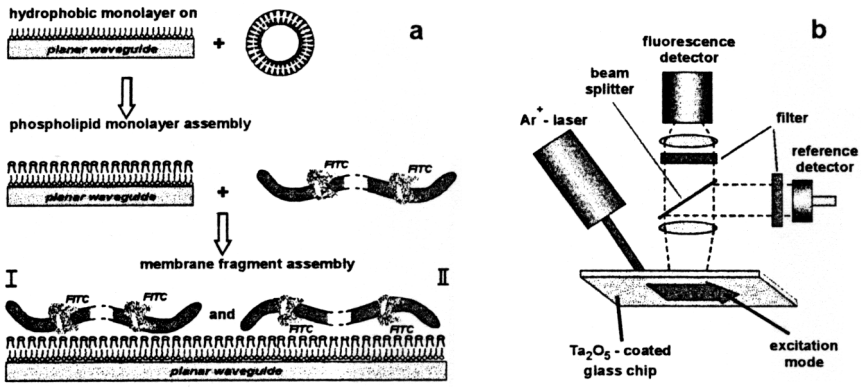

Figure 1. Schematic illustration of the supramolecular assembly of Na,K-ATPase (protomers) containing membrane fragments to the biocompatible waveguide surface (two possible orientations I and II) in a) and of the setup for surface-confined fluorescence measurements at the surface region of the waveguide (the arrow marks the propagating excitation mode); the waveguide is attached to a flow cell in b).

isolated from dogfish rectal gland (*Squalus acanthias*) and pig kidney. The protein is labeled with FITC according to [3-4]. Fragments can assemble and thus be immobilized in two possible orientations: former intracellular side up (I) and former extracellular side up (II) in Figure 1a). Exchange of solutions is carried out by using a computer controlled pump and valve system. The fluorescence detection consists of excitation light from an Ar^+ laser (488 nm), coupled into the waveguide at a suitable angle of incidence via a diffractive grating, which leads to a propagating, evanescent wave field within an about 100 nm thick interface layer (Figure 1b). Only fluorophores located within this wave field are excited and their emission is monitored employing a high sensitivity photon counting system. Details are given in [1]. Each step of the assembly is separately studied by *in situ* AFM (tapping mode). The membrane fragment surface coverage ranges between 20 and 50% (Figure 2a).

3. RESULTS AND DISCUSSION

The assembly of the FITC-Na,K-ATPase containing fragments leads to an intense and stable fluorescence signal. The functional properties of the enzyme remain unchanged upon immobilization, which is concluded from the quantitative comparison of the fluorescence intensity changes induced by ligand binding under conditions of constant ionic strength with those observed for the same preparation in solution (both membrane sides accessible) [1]. Furthermore, the results provide information about the degree of side-directed assembly (orientation I relative to that of II in Figure 1a).

The anti-fluorescein mAb 4-4-20 only quenches the FITC fluorescence in the case of orientation I because the fluorophore is bound within the ATP binding region at the former intracellular membrane side. Also phosphorylation with P_i is only possible at this side and

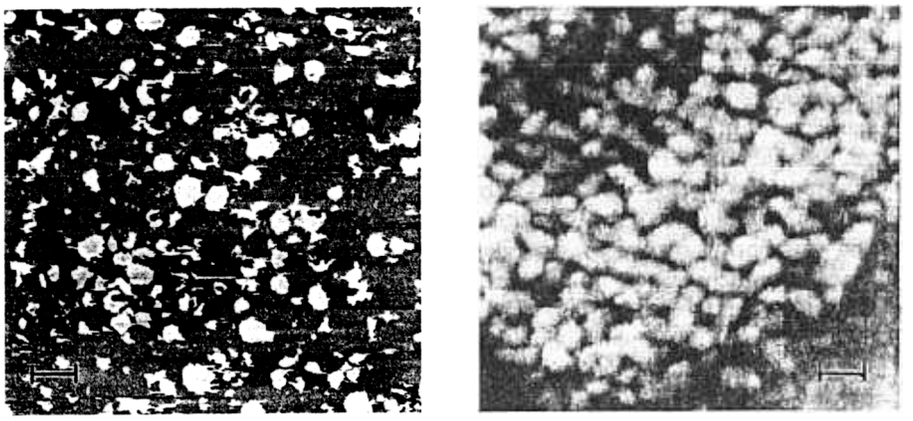

Figure 2. *In situ* AFM of dogfish FITC-Na,K-ATPase membrane fragments (bright spots, height about 12, mean diameter around 250 nm) assembled on the waveguide (with low resolution, left; bar: 500 nm) and of a part of one large fragment (with high resolution, right; bar: 50 nm). The dimensions of the particles rising by an average height of 3.9 nm above the surrounding membrane are indicative of the existence of protein clusters (≤ 10 protomers).

leads to a fluorescence decrease, too. On the other hand, the typical fluorescence intensity decrease observed in the presence of Mg^{2+} upon binding of the inhibiting cardiac glycoside ouabain, known to bind only to a site located at the former extracellular membrane side, can only be expected if the corresponding site is accessible in the immobilized state (orientation II in Figure 1a). The presence of millimolar concentration of Mg^{2+} in the Ch^{+} containing medium leads to a fluorescence intensity increase even if the ionic strength is maintained. This increase is not observed in solution. According to AFM studies, this is due to a Mg^{2+}-induced flattening of the membane fragments by a few nm, possibly as the consequence of a stronger interaction with the lipid surface. This flattening leads to an increase of the number of fluorophores located within the evanescent wave field for excitation and thus to the intensity increase.

For about 7 of a total of 14 complete sets of experiments, the results are interpreted in terms of an approximatively equal distribution of orientations I and II on the chip. This is deduced from the observed fluorescence intensity changes which are typical for ligand binding on both membrane sides. The changes correspond only to about 50% of the magnitude observed for the free fragments in solution. Details are given in [1]. However, in the case of 6 sets of experiments, the fluorescence intensity decrease induced by K^{+}-binding comprises nearly up to 30% for the dogfish FITC-enzyme and reaches, in contrast to the results described before, the value observed for the non-immobilized fragments in solution. Spectrofluorometric KCl titrations in the ChCl containing medium under conditions of constant ionic strength lead to similar binding constants of about 5×10^{3} M^{-1} (stoichiometric coefficient assumed as 1) for both states of the fragments. After the dissociation of K^{+} achieved by washing with buffer, the fluorescence can be quenched upon treatment with the mAb 4-4-20. For fragment assemblies where such large K^{+}-effects are observed, the fluorescence change due to the specific

interaction with ouabain is comparatively small. The latter results suggest that the assembly of the fragments is side-directed under these circumstances and corresponds to orientation I in Figure 1a). This implies that under these conditions the K^+ binding site of the FITC-enzyme in its non-phosphorylated state is accessible from the side where the FITC-residue is located, namely the former intracellular side. If the site were located at the former extracellular side, only a small K^+ but a large ouabain effect would have to be expected (orientation II in Figure 1a). The same, unexpected conclusion about the location of the K^+ site has also been suggested from experiments with reconstituted FITC-Na,K-ATPase in a vesicular system [5]. The reason why not all assemblies provide a side-directed fragment orientation is not yet known and may depend on minor differences of waveguide layer properties and reproducibility of surface modification, fragment preparation and assembly. Also the introduction of charges in the phospholipid monolayer has not allowed to derive a conclusive explanation.

That FITC-Na,K-ATPase membrane fragments can be functionally assembled on the waveguide with the former intracellular side oriented upwards is confirmed by AFM. The height of the protein particles shown in the high resolution AFM of Figure 2b) is consistent with the expectation for an exposure of the intracellular side, which is concluded from the dimensions resulting from a low-resolution 2D-structure determination by electron crystallography [6]. In addition, the particle distribution visible in Figure 2b) implies that the protein cluster density is higher in the edge region of the fragment than in its central region.

In summary, the results show that a side-directed supramolecular membrane fragment assembly is achievable with very small amounts of material. If fluorescence detection can be applied, the directed binding of ligands to receptor proteins in membranes can be easily investigated without of isolating individual membrane constituents such as by detergent treatment, for example. Results about the sidedness of interactions can be obtained.

ACKNOWLEDGMENT

All sensor studies reported here have been carried out as a collaborative study with Novartis Pharma AG and Novartis Services AG, Basel, prior to the foundation of Zeptosens AG and Solvias AG. The support by Novartis as well as the help by Dr. J. Fritz is acknowledged.

REFERENCES

1. M. Pawlak, E. Grell, E. Schick, D. Anselmetti and M. Ehrat, Faraday Discuss., 111 (1998) 273.
2. G.L. Duveneck, M. Pawlak, M. Neuschäfer, D. Budach and M. Ehrat, SPIE, 2928 (1996) 98.
3. P.L. Jørgensen, Biochim. Biophys. Acta, 336 (1974) 36.
4. D. Poerschke and E. Grell, Biochim. Biophys. Acta, 1231 (1995) 181.
5. A. Rephaeli, D. Richards and S.J.D. Karlish, J. Biol. Chem., 261 (1986) 6248.
6. H. Herbert, E. Skriver and A.B. Maunsbach, FEBS Lett., 187 (1985) 182.