
Functional immobilization of biomembrane fragments on planar waveguides for the investigation of side-directed ligand binding by surface-confined fluorescence

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A method for the functional immobilization of Na,K-ATPase-rich membrane fragments on planar metal oxide waveguides has been developed. A novel optical technique based on the highly sensitive detection of surface-confined fluorescence in the evanescent field of the waveguide allowed us to investigate the interactions of the immobilized protein with cations and ligands. For specific binding studies, a FITC-Na,K-ATPase was used, which had been labelled covalently within the ATP-binding domain of the protein. Fluorophore labels of the surface-bound enzyme can be selectively excited in the evanescent field. A preserved functional activity of the immobilized enzyme was only found when a phospholipid monolayer was preassembled onto the hydrophobic chip surface to form a gentle, biocompatible interface. *In situ* atomic force microscopy (AFM) was used to examine and optimize the conditions for the lipid and membrane fragment assembly and the quality of the formed layers. The enzyme's functional activity was tested by selective K⁺ cation binding, interaction with anti-fluorescein antibody 4-4-20, phosphorylation of the protein and binding of inhibitory ligand ouabain. The comparison with corresponding fluorescence intensity changes found in bulk solution provides information about the side-directed surface binding of the Na,K-ATPase membrane fragments. The affinity constants of K⁺ ions to the Na,K-ATPase was the same for the immobilized and the non-immobilized enzyme, providing evidence for the highly native environment on the surface. The method for the functional immobilization of membrane fragments on waveguide surfaces will be the basis for future applications in pharmaceutical research where advanced methods for exploring the molecular mechanisms of membrane receptor targets and drug screening are required.

Introduction

Molecular interactions on biomembranes play a prominent role in the communication between cells and in signal transduction pathways.¹ Thereby, membrane receptors serve as the main

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targets,² able to recognize specific ligands selectively, which can trigger a cascade of functional cell responses such as the regulation of ion channel activity^{3,4} or induction of secondary messengers, *e.g.* release of intracellular calcium upon G-protein coupled receptor activation.^{5,6} Because of their regulatory mechanisms and their relevance in drug–target interactions, membrane receptors are, currently, the focus of detailed biophysical and biochemical investigations, to elucidate the relation between ligand binding and functional properties, or to resolve structure–activity relationships. In pharmaceutical research, membrane receptors are used as targets in the drug discovery process, where large numbers of new chemical entities are screened at an early phase.⁷ For that purpose, a number of physical sensing techniques, preferentially based on fluorescence detection and designed for high throughput screening, have been developed recently.^{8,9} Therefore, and due to the trend of integrating a larger and larger number of assays on a single detection plate,⁸ leading to increased surface/volume ratios in an assay well, surface-sensitive fluorescence techniques^{10–12} become increasingly important. However, detection of direct ligand–receptor interactions and of related protein functions is often limited, not only by the physical sensing technology, but also by the assay architecture, the non-specific binding, the loss of functionality upon immobilization of cells/receptors on surfaces and strong background signals from the bulk solution. Accordingly, the interfacing of biologically active receptor systems with such sensing surfaces, with preservation of the full functional protein activity and access to an optimum number of binding sites under controlled conditions, becomes more and more important, but still remains a major challenge.

Biosensor technology

Here, we present the advantageous combination of a highly sensitive technology for the detection of surface-confined fluorescence, excited in the evanescent field of planar waveguides,^{13,14} and a new method for the functional immobilization of biomembrane fragments, which contain the receptor target, on such sensor surfaces under defined conditions. The high assay performance of evanescent wave sensors is achieved by: (i) the strong spatial discrimination by the evanescent field, between specific binding signals and bulk fluorescence causing background signals (penetration depth of the evanescent field is typically of the order of a wavelength); (ii) highly oriented and functional biomolecules (receptor targets) on the surface and (iii) the use of fluorescent labels for signal generation, independent of molecular weight. Assays demonstrating the real-time monitoring of *e.g.* immunorecognition at working concentrations as low as several fM have recently been published.¹³

Here, we report, for the first time, applications using an integral membrane protein which was functionally immobilized in its native membrane environment on waveguide sensor surfaces. Na,K-ATPase was chosen for a systematic investigation of the immobilization process as well as for probing molecular interactions on an immobilized protein.

Na,K-ATPase

The enzyme Na,K-ATPase is a protein that exists in the plasma membrane of all higher organisms. Its principal function as an alkali-metal ion dependent ATPase was discovered by Skou.¹⁵ The free energy resulting from the hydrolysis of an intracellular ATP molecule is converted by this enzyme into the transport of 3 Na⁺ ions out of and 2 K⁺ ions into the cell. Both active cation transport processes occur against the existing alkali-metal ion concentration gradients of the plasma membrane. Furthermore, Na,K-ATPase exhibits electrogenic properties and thus contributes significantly to the regulation of the membrane resting potential. The protein itself is a heteromer and consists of the catalytic α -subunit, characterized by a molecular weight of *ca.* 100 kDa, and of the glycosylated β -subunit with a molecular weight of *ca.* 50 kDa. Besides its transporter function, Na,K-ATPase acts as the receptor for cardiac glycosides such as ouabain, which are bound to the extracellular side of the protein at very high affinity and lead to the inhibition of enzymatic activity.

Since the ionic composition of the medium differs between the cytoplasmic and the extracellular side and because the functional properties of the enzyme on both membrane sides are different, the structure of the protein must also be asymmetric. Consequently, the binding properties of ligands and cations are expected to be side-directed. For example, the binding affinity of a ligand or of an

alkali-metal ion to a site on one side of the membrane will depend on whether this ligand or cation is absent or present on the other side. Thus, if one wishes to understand the basic functional properties of the cation pump mechanism, such as that of Na,K-ATPase, the aspect of the sidedness is essential and calls for advanced investigation methods.

Na,K-ATPase was isolated as the major protein in nanoparticulate membrane fragments (discs of *ca.* 250 nm mean diameter) from specialized tissue such as kidney or salt glands. In contrast to solubilized systems, the protein is in its native membrane environment and retains its original biomembrane orientation (*cf.* Fig. 1). The isolated membrane discs, however, are surrounded by the same aqueous medium on both sides and are no longer capable of separating the two different aqueous cell compartments. Although such a preparation no longer allows the study of the aspect of sidedness, many relevant interactions, partial reactions and mechanistic aspects have been investigated. For example, a fluorescent marker, such as fluorescein-5-isothiocyanate (FITC) can be specifically bound to a single lysine residue, located within the ATP binding domain, for monitoring binding events by fluorescence quenching.^{16–18} The fluorescence of the labelled Na,K-ATPase changes characteristically upon binding of different ligands and cations and was also used here for the analytical detection.

General working concept

Our concept is based on the formation of a biocompatible, sensitized waveguide chip surface and subsequent assembly of membrane fragments on this support under defined conditions. The combination with surface-confined fluorescence detection by planar waveguides allows the simultaneous investigation of side-directed ligand binding and of functional properties of, for example, Na,K-ATPase (Fig. 1). The initial goal of this work was the stable and functional immobilization of ATPase-rich membrane fragments using surface-assembly techniques. The on-line monitoring capability of the planar waveguide (PWG) sensor allowed us to carefully examine each step of surface preparation. Additional knowledge on the surface structure was obtained by *in situ* AFM, which delivered important 2D-resolved information about surface coverage and surface morphology of the sensitized chip. After successful immobilization of the membrane fragments, the functional activity of the immobilized protein was probed by monitoring the fluorescence changes upon specific, side-directed binding of cations and ligands using the FITC-labelled ATPase. The described approach offers great advantages because no detergent solubilization of the protein is

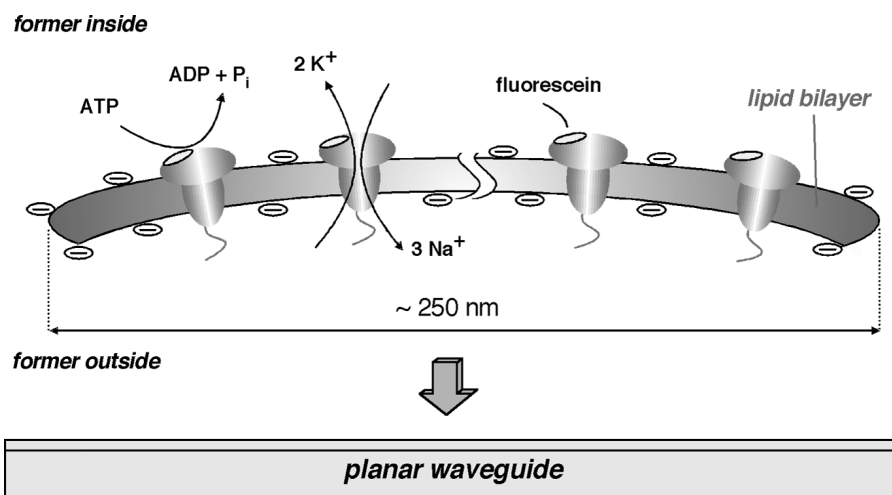


Fig. 1 Schematic illustration of a cross-section through a disc-shaped membrane fragment (diameter *ca.* 250 nm) containing FITC-labelled Na,K-ATPase, prior to adsorption onto a planar waveguide chip. The enzyme molecules consist of an α and β subunit. The whole protein is asymmetric with respect to function and protein moiety. The ATP binding side conserves a larger protein content and is located on the former, native inside of the biomembrane. Protein-to-lipid ratio of a membrane disc is typically *ca.* 1 : 250 (as determined for pig kidney membranes).

necessary, as is the case for other reconstitution procedures, because the assays can be optimized efficiently and because fast and reproducible measurements can be performed, due to the easy exchange of the aqueous media for the different assays. The orientation of the immobilized membrane fragments on the surface was finally concluded from the comparison of the fluorescence changes, measured with the PWG sensor, with those found with the same membrane sample in bulk solution.

Materials and experimental methods

Chemicals

Salts such as NaCl, choline chloride, imidazole and phosphates were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland) and were of the highest purity obtainable (Ultrapure or Microselect). Organic solvents such as propan-2-ol were also from Merck and of UV-spectroscopy grade (Uvasol). 1-Palmitoyl-2-oleoyl-glycero-phosphocholine (POPC) and fluorescein-lipid (PE) were from Avanti Polar Lipids (USA). Mouse monoclonal anti-fluorescein antibody (4-4-20) was from Molecular Probes (Netherlands).

Lipid vesicle preparation

Lipid vesicles were prepared by extrusion (100 nm polycarbonate filters, Avestin Corp., USA) with 0.75 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Avanti Polar Lipids), hydrated in 150 mM NaCl, 10 mM sodium phosphate buffer, 0.02% sodium azide at pH 7.5. The vesicle mean diameter was *ca.* 110 nm, as determined by dynamic light scattering (Zeta Plus, Brookhaven Instruments, USA).

Na,K-ATPase preparation

Na,K-ATPase-containing membrane discs were isolated from the dissected red outer medulla of pig kidney and from the rectal gland of dogfish (*Squalus acanthias*) in a two-step procedure. A microsomal fraction was prepared by tissue homogenization followed by differential-velocity centrifugations.¹⁹ This fraction consisted essentially of closed membrane particles, where the majority of the ATP binding sites were not accessible from the external medium. The microsomal fraction was activated by a short sodium dodecyl sulfate (SDS) treatment which led to an opening and partial solubilization of the microsomal membranes. After this treatment, the Na,K-ATPase discs remained intact but the enzymatic activity of the preparations increased markedly. The discs were separated by density-gradient centrifugation using sucrose.¹⁹ The pig kidney enzyme was kept in 25 mM imidazole in HCl, pH 7.5, containing 0.1 mM EDTA, and the dogfish enzyme in 30 mM histidine in HCl, pH 6.8, containing 25 wt.% glycerol at concentrations of *ca.* 2 mg ml⁻¹. The average Na,K-ATPase activity at 37°C was 30 μmol mg⁻¹ min⁻¹; it was reduced to less than 1% in the presence of ouabain. Protein content was determined according to a modified procedure of Lowry *et al.*²⁰ FITC-Na,K-ATPase was prepared as described in ref. 16, employing extended washing procedures²¹ and was kept in 10 mM imidazole in HCl at pH 7.5 on ice. Upon labelling the sample the Na,K-ATPase activity decreased to <1 μmol mg⁻¹ min⁻¹ but the 2,4-dinitrophenylphosphatase activity at 37°C was still about 19 μmol mg⁻¹ min⁻¹. The degree of labelling was calculated from the absorbance in the visible range, assuming that the molar absorption coefficient of FITC remained unchanged at the same pH. The concentrations of the enzyme given here were calculated on the basis of a molecular weight of 150 kDa.

Planar waveguide chips and surface preparation

Planar waveguide chips (16 mm × 48 mm) consisted of a 150 nm thin, waveguiding surface layer of Ta₂O₅ (refractive index = 2.3), deposited on 0.5 mm thick glass substrates (Balzers AG, Liechtenstein). The self-assembling chemistry, chemical metal oxide modification and physico-chemical analysis of the modified sensor surfaces is reported elsewhere.²² Briefly, the metal oxide surfaces were routinely cleaned by ultrasonication in organic solvent, followed by a UV ozone treatment. The cleaned, hydrophilic surfaces were further modified to reach a stable hydrophobic

character by self-assembling monolayers of mono-C₁₆-alkyl phosphates (Novartis Pharma AG) onto the freshly cleaned surfaces (3 day assembly at 1 mg ml⁻¹ in propan-2-ol). Chips were thoroughly washed in propan-2-ol, sonicated, dried and stored under nitrogen. Contact angles of water on the hydrophobic surfaces were determined to be in the range of 100–110°.

AFM

Each step of our surface preparation was carefully examined by *in situ* AFM. For AFM experiments under defined buffer environment we used a commercial instrument (Nanoscope IIIa, Bioscope, Digital Instruments, USA) with Si₃N₄-cantilevers (Digital Instruments or Olympus) with nominal spring constants of 0.02–0.58 N m⁻¹. The AFM was operated in the tapping mode in order to reduce unwanted tip–sample interactions due to the imaging process. Scanning was typically done at an imaging line frequency of 1 Hz. The surfaces carrying the lipid layers and biomembrane fragments were always kept under buffer during mounting onto the AFM sample stage and were prepared under conditions comparable to those used in the sensor experiments. For the chip preparation, a cell made from silicon allowed careful rinsing of excess lipid and/or membrane material as well as a reproducible exchange of the buffer solution.

Fluorescence measurements with planar waveguide sensor

An in-house developed waveguide sensor instrument was used for the detection of surface-confined chip fluorescence measurements.¹³ The schematic optical set-up is shown in Fig. 2. S-polarized (=transversal electric, TE-mode) excitation light at 488 nm from an Ar⁺-ion laser (model 5490ASL, 0–30 mW, Ion Laser Technology, USA) was coupled into the waveguiding layer by means of a diffractive grating at a distinct angle of incidence. Since excitation light is efficiently guided in the waveguiding layer, excitation light intensities could be kept low, in the range of 10–100 μW. Part of the emitted, surface-confined fluorescence, excited by the propagating wave, was collected at the substrate side with a 1 : 1 imaging optics (numerical aperture = 0.19) and spectrally discriminated by means of two six-cavity interference filters transmitting in the range

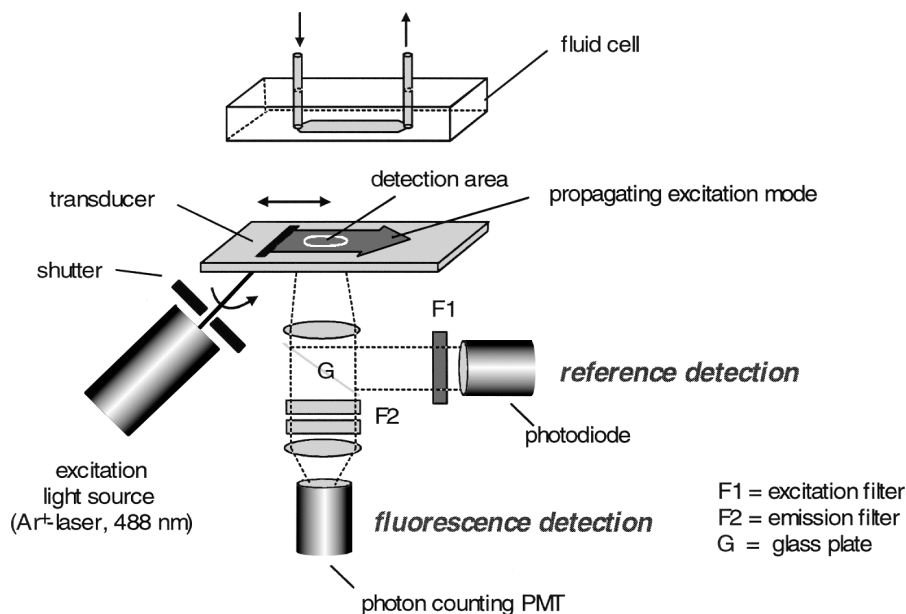


Fig. 2 Evanescent wave fluorescence measurement: surface-immobilized fluorophores are excited by laser light in the evanescent field of a planar Ta₂O₅ waveguide (propagating excitation mode). Fluorescence is detected to high sensitivity by a photon-counting photomultiplier (PMT) system. Fluorescence signals were referenced by measuring the excitation light within the detection area by means of a photo diode. Buffer and sample solutions were injected into the fluid cell with an automated fluid handling system (not shown).

515–545 nm (530DF30, Omega Inc., USA). Part of the surface-scattered light was used as a reference for the intensity of incoupled excitation light in the imaged area of interest. The field of detection was typically 5 mm × 1 mm, placed in the centre of the propagating beam by means of an aperture. A photon-counting PMT (H6240, Hamamatsu, Japan) in combination with a 225 MHz counter (model 53131A, Hewlett-Packard, USA) was used for the detection of fluorescence. Excitation and signal read-out was taken every 30 s with a sampling time of 1 s. Buffers and samples were applied to the sensor chip *via* a flow-through cell made from silicone elastomer (Sylgard 184, Dow Corning, USA). The cell volume was 6 mm³ (15 mm × 2 mm × 0.2 mm). Fluid supply was performed automatically using a dispensing pump–six-way valve combination (Cavro, USA) for handling different working buffers and a sample injector (231XL, Gilson, USA). Injection volumes were in the range 50–100 mm³ for stopped flow and up to 5 cm³ for continuous flow applications. The fluorescence detection and sample handling was fully automated and controlled by a PC. The chip holder and optical unit were temperature controlled. All experiments were performed at 20 °C. The experimental buffer conditions were: buffer I (150 mM NaCl, 10 mM sodium phosphate buffer, 0.02% NaN₃, pH 7.5), buffer II (100 mM cholineCl, 10 mM imidazole, pH 7.5) and buffer III (buffer II + 3 mM MgCl₂). Errors given for results are determined from at least three repeated measurements.

Fluorescence measurements in bulk cuvette

Bulk fluorescence measurements were performed on a Spex Fluorolog 222 instrument, equipped with a thermostatted cuvette holder. Measurements were performed under conditions comparable to those in the waveguide sensor using identical protein preparations. The temperature was 20 °C in all cases.

Results

The main goal was to establish an experimental procedure for the functional immobilization of protein-containing membrane fragments in a defined and controlled manner. The concept of surface immobilization was based on a two-step preparation protocol shown schematically in Fig. 3: the first step (left) is the transfer of the bare, non-sensitized physical, transducer into a bio-compatible interface, applying a self-assembly of a lipid monolayer to the hydrophobic metal oxide surface. The second step (right) comprises the stable physisorption of the membrane fragments onto the lipid monolayer interface, exploiting the negative surface charges and strong dipolar contributions²¹ of the biomembrane particles in a controllable physico-chemical environment. It is important to control the salt content and pH of the surrounding media, as tools for optimizing this process. The intention was to establish the basis for future assay applications by the development of a well defined and optimized surface-preparation protocol.

Lipid monolayer formation on waveguides

The process of a defined lipid monolayer formation (first step) was optimized by characterizing the surface topography with AFM. The non-sensitized waveguide chips (bare as well as hydrophobic) showed a homogeneous and flat surface with very little roughness (0.2–0.3 nm rms), thus being well suited for potential high-resolution studies. Lipid monolayer formation on the hydrophobic metal oxide chips was performed *via* lipid vesicle spreading. The spreading behaviour of POPC vesicles on the hydrophobic metal oxide surfaces was investigated by varying the preparation techniques of vesicles (extrusion, dilution) and buffer composition (ionic strength, different inorganic/organic salts). The whole process of optimizing lipid monolayer formation on planar waveguides is reported elsewhere.²³ Under optimum spreading conditions, almost complete surface coverage with lipid monolayers could be realized (Fig. 4) using vesicles prepared by extrusion (diameter *ca.* 110 nm, as determined) at relatively high inorganic salt conditions (150 mM NaCl, 10 mM sodium phosphate buffer, 0.02% NaN₃, pH 7.5). Lipid (POPC) concentration was always 0.5 mg ml⁻¹. Up to 10% of the surface area was occupied by small monolayer defects, homogeneously distributed over the chip as single spots of 10–20 nm in diameter (Fig. 4). From these defects, the mean thickness of the lipid monolayer could be easily determined (1.8 ± 0.6 nm),

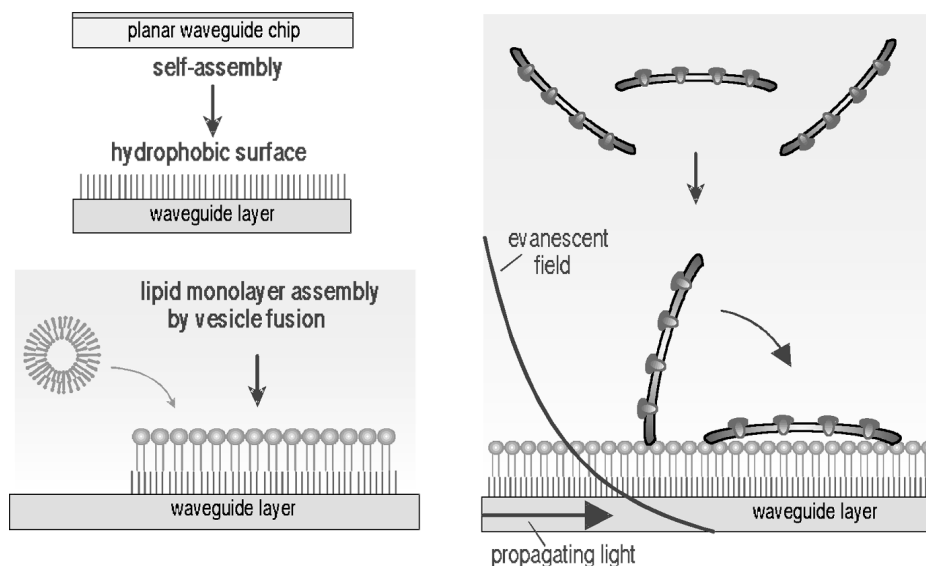


Fig. 3 Working concept for the functional immobilization of membrane fragments: first step (left), formation of a biocompatible interface by self-assembly of a lipid monolayer, after vesicle fusion, onto a hydrophobic waveguide surface; second step (right), controlled and stable physisorption of membrane fragments onto the preformed lipid interface on the waveguide. The penetration depth of the evanescent field of propagating excitation light is of comparable length to the size of the membrane discs. Upon surface-immobilization, the membrane discs can adopt two different orientations: former (cytoplasmic) inside surface up (as illustrated here) or former outside surface up. Drawn objects do not scale to real size.

corresponding well with the thickness of a single monolayer, as determined by other methods.²⁴ Since the diameter of the membrane discs for the second association process was found to be *ca.* 250 nm, 10–20 times larger than the size of the single defects (hydrophobic spots), the probability for contacts between biomembranes and such defects (leading to protein denaturation) in an otherwise closed lipid layer was considered to be negligible.

Membrane fragment immobilization

Having achieved a lipid-coated, biocompatible chip surface, surface-association of membrane discs, containing Na,K-ATPase, was carried out. Surface coverages and the morphology of the immobilized membrane fragments were investigated again by *in situ* tapping mode AFM. The kinetics of surface-association was monitored in real time with the fluorescence sensor using the FITC-labelled enzyme analogue. Typically, lipid monolayer formation and subsequent membrane fragment immobilization on a waveguide chip were performed in the PWG instrument and monitored in real time. The kinetics of these two processes is shown in Fig. 5 as fluorescence responses. After an initial phase of buffer equilibration (5 min at 0.5 ml min⁻¹ flow), lipid vesicles (first step) or membrane discs (second step) were injected and incubated under stopped-flow conditions for 1 h. In a subsequent buffer wash (10 min at 0.5 ml min⁻¹, *i.e.* almost a thousand times exchange of the cell volume) excess material was efficiently removed.

For the lipid assembly, no fluorescence increase was observed, as expected. Interestingly however, the process of lipid spreading could, nevertheless, be followed by a clearly detectable increase of the reference signal (excitation light). In the case of membrane disc association, a strong and fast increase of fluorescence intensity was observed due to the association of the labelled membrane discs onto the surface. After 1 h, the signal slowly approached a stationary emission at *ca.* 10⁶ cps. Only a minor portion of this signal disappeared on washing. This signal decrease was interpreted as the removal of loosely bound membranes in the vicinity of the surface. Final signal-to-noise ratios of the net fluorescence were 4000 ± 500. The established fluorescence signal, F_0 , in buffer II was checked for its long-term stability, a prerequisite for subsequent assay

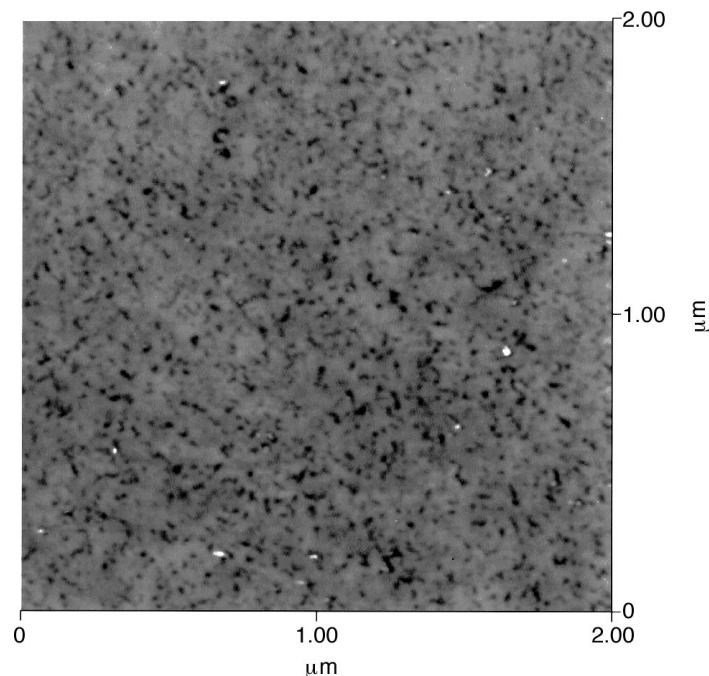


Fig. 4 Quality control of surface lipid assembly by *in situ* AFM: a lipid monolayer formed after spreading of POPC vesicles, prepared by extrusion (vesicle diameter 110 nm), to a hydrophobic Ta₂O₅ waveguide. The hydrophobic surface consisted of a self-assembled monolayer of mono-C₁₆-alkyl phosphate. Up to 10% of the total surface is covered by small-sized single defects. Preparation and imaging of the lipid monolayer were performed in 150 mM NaCl, 10 mM sodium phosphate buffer, 0.02% NaN₃ at pH 7.5. The gray scale of the image (black to white) corresponds to a height of 10 nm.

applications. Signals were stable as measured for up to 3 h in running buffer with signal readouts for 1 s every 1 min. Long-term drifts due to photo-bleaching were minimized using low illumination intensities (typically 40–100 μW before chip coupling). The concentration of membrane discs corresponded to a protein content of *ca.* 0.25 mg ml⁻¹. Assembly experiments have been carried out successfully with Na,K-ATPase prepared from both pig kidney and from dogfish rectal gland. However, in the following, the reported results refer only to the dogfish enzyme.

Membrane-surface association depends critically on the salt conditions of the surrounding buffer. In order to avoid an early loading of the cation binding sites of the enzyme, the use of Na⁺ and K⁺ cations in the assembly buffer was excluded. Therefore, surface immobilization was performed in 100 mM ChoCl, 10 mM imidazole, pH 7.5 (buffer II). High ionic strength strongly assisted the association of membrane particles to the surface and, in addition, provided a stable contact of the discs on the surface. Fig. 5 shows a representative experiment of lipid monolayer formation and membrane disc immobilization on the preformed lipid layer. Surface coverage and topology of such preparations were investigated under otherwise comparable conditions with tapping mode AFM in solution. Fig. 6 shows a representative AFM picture of membrane fragments immobilized flat on a lipid monolayer. The average coverages of such preparations ranged between 25% and 50%, depending on the scanning position on the chip and including chip to chip variation. In addition, coverages determined from fluorescence signals, with assembled membrane discs or with a phospholipid monolayer doped with fluorescein-labelled lipid (one label per 800 lipids), were in good agreement with the AFM results. This indicated the robustness of our preparation procedure.

Concerning the topology and stability of surface-associated membrane particles, membrane discs preferentially associated flat on the surface. The stability of the physisorption of the membranes was concluded from the fact that the disc-like structures could not be removed with the AFM tip from their positions during the process of scanning. At comparatively high forces the

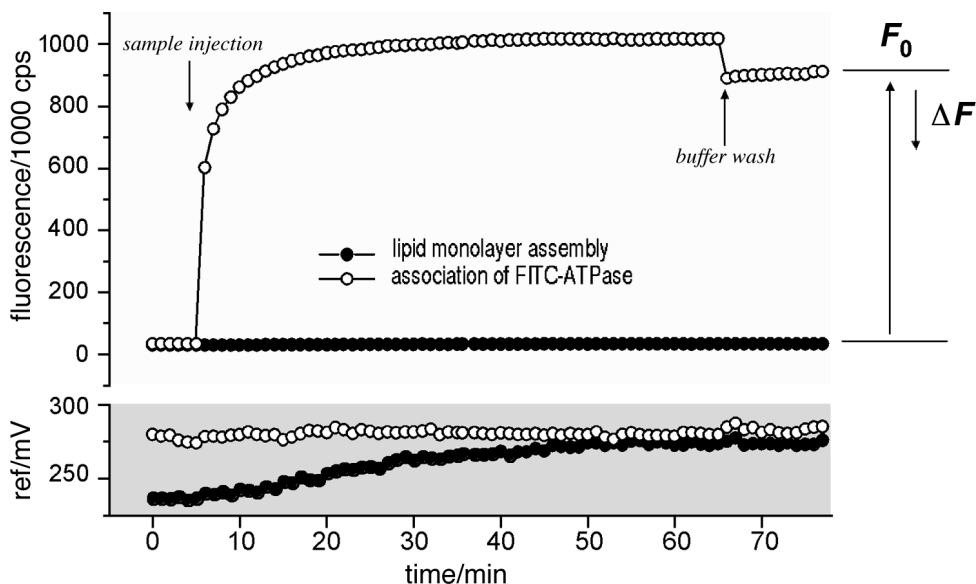


Fig. 5 Kinetics of lipid monolayer assembly (●) and subsequent surface-association of dispersed membrane fragments (○), containing FITC-Na,K-ATPase (protein concentration 0.22 mg ml^{-1} ; prepared from dogfish rectal gland), on a waveguide chip. Measurements were performed in 10 mM imidazole/HCl, 100 mM choline chloride, pH 7.5 (at 20°C). Fluorescence (in cps) and reference signals (in mV) were monitored after an initial 5 min of buffer exchange (under continuous flow), a 60 min sample incubation (under stopped flow) and final 10 min of buffer washing (under continuous flow). F_0 corresponds to the asymptotically reached emission intensity of surface-immobilized FITC-Na,K-ATPase, prior to the supply of samples containing interacting cations or ligands. Binding of the specific cations or ligands leads to the characteristic fluorescence changes ΔF .

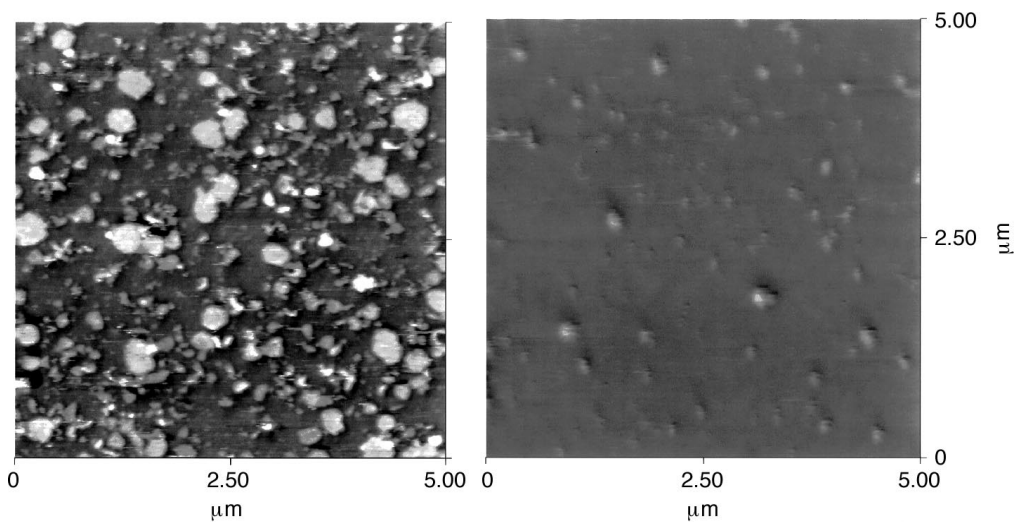


Fig. 6 AFM quality control of immobilized membrane fragments, containing FITC-labelled Na,K-ATPase, on sensitized waveguide surfaces. At high ionic strength (left) up to 50% surface coverage of assembled membrane discs was achieved; at low ionic strength (right) only few and loosely defined structures with lower contrast could be imaged. FITC-Na,K-ATPase was prepared from the dogfish rectal gland and used in concentrations of *ca.* 0.22 mg ml^{-1} . The gray scale of the image (black to white) corresponds to a height of 50 nm.

discs could even be dissected with the tip (data not shown). Further evidence for a stable membrane immobilization was the observation of a constant sensor fluorescence signal upon extensive washing with running buffer. The AFM experiments also indicated that the surface contact zone of a membrane disc was preferentially located close to the disc edge. The edges protruded an additional 4–5 nm into the solution. The central region of a membrane disc is assumed to be fairly flexible. This was concluded from the fact that the AFM resolution in the disc centre was lower than at the edge and that the height of the disc centre with respect to the substrate surface depended on the ionic composition of the buffer solution, for example by the addition of divalent cations. In the presence of 3 mM MgCl_2 , the disc centre height was reduced, typically, from 9 to 7 nm (–25%). When membrane particles were assembled at low ionic strength (10 mM imidazole, pH 7.5), a much lower surface coverage was obtained (Fig. 6, right). Under these circumstances, it was difficult to resolve the structures with high contrast in an AFM scan, indicating that a weaker interaction, in terms of a reduced contact area per disc, existed. This was also evident by the observation of larger fluctuations of the fluorescence sensor signal and, additionally, of lower base signals F_0 than at high ionic strength or in the presence of additional divalent ions.

On individual membrane preparations, AFM images with high resolution were recorded (Fig. 7). Within the central part of an immobilized membrane disc single protrusions with a density of about one structure per 1000 nm^2 were imaged. The structures protruded from the surface into the solution by an average height of 5–6 nm. The lateral size of the protrusions was in the range of 15–20 nm. Taking into account the size of the scanning tip (5–10 nm), the real size of the imaged structures must be *ca.* 10 nm. Such a size was interpreted as the membrane-external protein moiety of the ATPase. However, the lateral size of 10 nm is too large to represent an individual protein molecule. Therefore, we believe that the imaged structures correspond to aggregates of protein, equal or larger than a dimer. Considering aggregation, the final protein-to-lipid (P : L) ratio in the disc centre was determined to be in the range 1 : 100 to 1 : 1000. At the disc edge, P : L

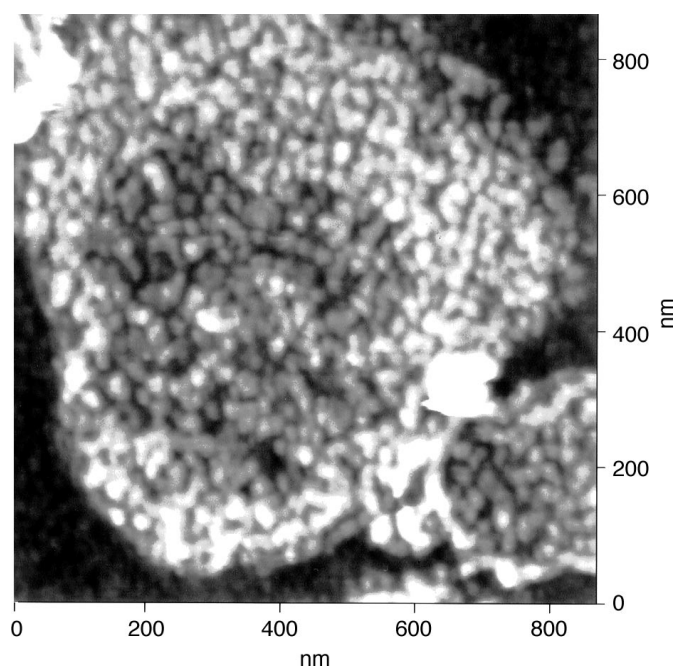


Fig. 7 High-resolution AFM picture of a membrane fragment, containing FITC-Na,K-ATPase (prepared from dogfish), immobilized on a sensitized waveguide chip. The central part of the disc shows individual protrusions with average lateral dimensions of 15–20 nm and an average height of 5–6 nm. Protrusions are interpreted as aggregated ATPase molecules (aggregation state > dimer). Protein : lipid ratios in the central part of the membrane disc were of the order of 1 : 100–1 : 1000, increasing towards and at the disc edge. The gray scale of the image (black to white) corresponds to a height of 20 nm.

ratios were still larger, leading to the assumption that, there, stronger aggregation or even first indications for protein crystallization occur.

Functional tests of immobilized Na,K-ATPase

Once the optimum conditions for a reproducible and stable immobilization of membrane fragments were identified, *i.e.* a stable and constant base fluorescence signal F_0 was generated on the chip, the functional activity of the Na,K-ATPase could be tested. This was done by using a set of complementary assays to probe the different binding sites of the protein. Each of these assays alters specifically, in a side-directed manner, the relative intrinsic fluorescence F/F_0 of the labelled enzyme. For a better overview, the characteristic fluorescence changes of the membrane discs in bulk solution upon variation of the buffer composition or additions of specific ligands are shown in Fig. 8. The specific binding of K^+ to the alkali-metal ion binding pocket was considered to be the most critical test for a preserved functional activity of the protein, since this binding is impaired with a characteristic change in protein conformation leading to a relatively large change of the fluorescein emission ($-\Delta F/F_0 \approx 30\%$).

Specific K^+ -binding and binding isotherm. In all preparations, the specific K^+ -effect was tested as a reference experiment. Small volumes of KCl in buffer II (maintaining the ionic strength constant) were applied under continuous flow. Upon K^+ -contact, the fluorescence dropped instantly to lower, stable values, dependent on the KCl concentration (*cf.* Fig. 9). This fluorescence effect was fully reversible, yielding reproducible fluorescence levels after washing in the absence of KCl and in subsequent repeats. Titrations over six orders of concentration (0.1 μ M to 100 mM KCl) were performed. From the specific fluorescence decreases ($-\Delta F/F_0$), a quantitative evaluation according to a binding isotherm was performed (Fig. 10). The data followed ideally according to a Langmuir binding kinetics, revealing a dissociation constant of $K_D = 180 \pm 20 \mu$ M. Saturation of binding was achieved above 2 mM K^+ , but only a $60 \pm 10\%$ fraction of the amplitude was reached when compared to the respective fluorescence changes in bulk solution (Fig. 8). On the other hand, the dissociation constant of the surface-confined measurements compared well to that found in bulk measurements. This indicates a preserved enzyme activity in the assembled state, but with a reduction of the number of K^+ -binding sites per assembled protein molecule. This may imply that not all available sites are accessible by the buffer medium.

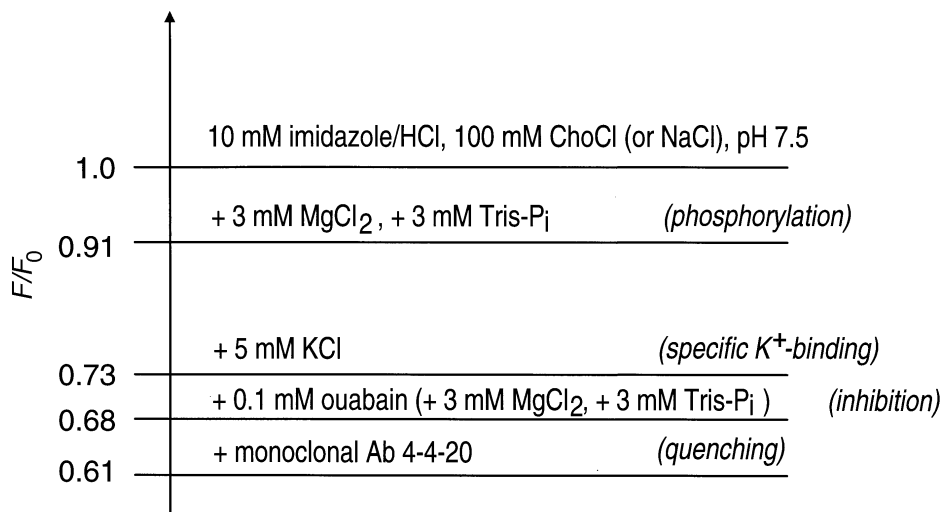


Fig. 8 Schematic illustration of the relative fluorescence intensity levels F/F_0 ($\lambda_{exc} = 488$ nm; $\lambda_{em} = 520$ nm) of FITC-Na,K-ATPase (prepared from dog fish rectal gland), as measured in bulk phase containing different cations or ligands (P_i = inorganic phosphate), relative to the base level F_0 in 10 mM imidazole in HCl, 100 mM choline chloride (ChoCl), pH 7.5 (buffer II) at 20 °C. The total ionic strength of all solutions was kept constant by adjustment with choline chloride (ChoCl).

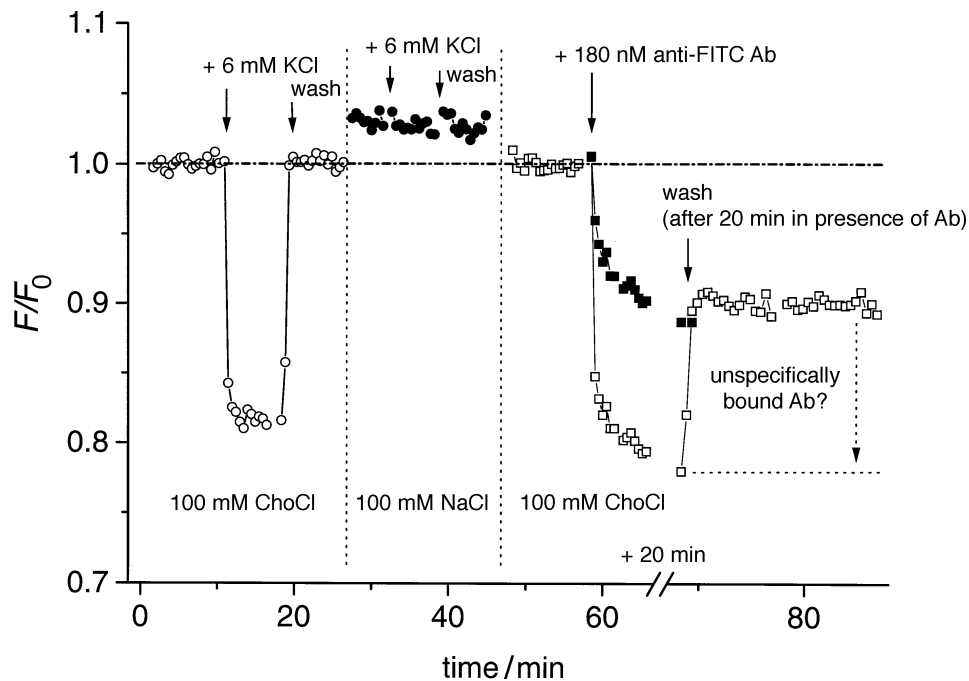


Fig. 9 Representative course of fluorescence signals in a typical assay sequence, measured after preparation of surface-immobilized, FITC-labelled Na,K-ATPase (dogfish) as shown in Fig. 5. (○) specific K^+ -binding after injection of 6 mM KCl, (●) competitive inhibition of specific K^+ -binding in the presence of high $[Na^+]$, and signal decrease upon addition of 180 nM anti-fluorescein antibody 4-4-20 [(□) measured signal; (■) signal corrected for unspecific offset].

Specificity of K^+ -binding. Further experiments were performed to check the specificity of K^+ -binding. When the immobilized membranes were saturated with high concentrations of NaCl (100 mM), no further signal decrease was observed upon addition of KCl (*cf.* Fig. 9). Thus, specific K^+ -binding is not observed in the presence of excess Na^+ , which is consistent with bulk measurements. Furthermore, with excess KCl, >10 mM, an additional K^+ -effect could be resolved, as visible from the observed deviations from constant saturation binding (*cf.* Fig. 10). This effect obviously suggests a weaker binding and was interpreted as an unspecific K^+ -binding to the protein or the membrane.¹⁸

K^+ -binding of membranes immobilized on bare, non-sensitized Ta_2O_5 surfaces. Control experiments were performed to clarify whether or not the preparation of a biocompatible interface, *i.e.* the chip covered with a lipid monolayer, is a necessary prerequisite for preserved ATPase binding characteristics. Therefore, membrane discs were immobilized on a non-sensitized, hydrophilic Ta_2O_5 chip. In this case, the typical signal decrease upon subsequent K^+ -addition did not occur, except for a minor effect of the order of -2% . It is interesting to note that much larger fluorescence signals could be observed upon assembly of the membrane fragments to the chip surface, indicating a *ca.* 10-fold higher association constant. This phenomenon was also verified in AFM experiments, in which *ca.* 10 times more diluted membrane suspensions reached comparable surface coverages (25%–50%) as in the case of the lipid-coated chips.

Binding of anti-fluorescein antibody. As an additional feature, the side of the fluorescein label, placed within the ATP-binding domain of the enzyme, was probed by fluorescence quenching upon specific binding of monoclonal anti-fluorescein antibody 4-4-20 (Molecular Probes). This was done in a typical assay sequence (in working buffer II) after the initial testing for the specific K^+ -binding as shown in Fig. 9. The antibody is able to quench the fluorescence of free fluorescein almost completely ($>90\%$).²⁵ In the case of the FITC-labelled ATPase-analogue, fluorescence in

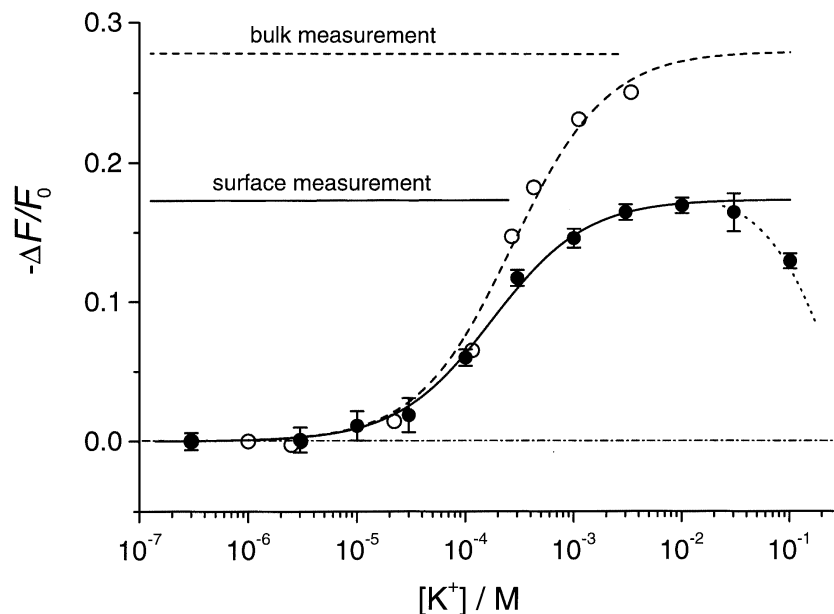


Fig. 10 Specific K^+ -binding of FITC-Na,K-ATPase (prepared from dogfish) upon additions of KCl over five orders of concentration, maintaining the ionic strength constant. Experiments were performed with surface-immobilized enzyme (\bullet) and with non-immobilized enzyme in bulk solution (\circ) in 10 mM imidazole in HCl, 100 mM choline chloride, pH 7.5 at 20°C. The surface-immobilized enzyme reached only *ca.* 70% of the relative saturation amplitude of enzyme in solution. The concentration dependence in both cases was well described according to a Langmuir binding kinetics, leading to dissociation constants of $K_D = 180 \pm 20 \mu\text{M}$ for the immobilized enzyme (—) and $K_D = 270 \pm 30 \mu\text{M}$ for the enzyme in bulk (---). The comparable K_D values indicate a preserved activity of the ATPase in the surface-immobilized state. A 70% relative saturation amplitude under a K_D comparable to solution suggests a 70% : 30% orientation of membrane fragments on the surface, with the specific K^+ -binding site exposed to the solution.

bulk is only quenchable to a maximum extent of 30–40%, depending on the method of preparation. We found that the fluorescence in the case of the surface-immobilized protein is only one half of the quenching effect in solution (20% quench) upon incubation in saturating concentrations of the antibody (180 nM). An initial fast and a subsequent slower kinetic quench response could be resolved. The initial fast one was reversible upon subsequent washing with buffer, whereas the quench representative of the slower phase (within minutes) remained stable for minutes at $F/F_0 \approx 0.9$ (10% quench). The later effect was interpreted as the specific (direct) antibody binding to the fluorescein label (slow dissociation), whereas the fast response may represent an unspecific interaction to the fluorescein emission.

Phosphorylation and binding of inhibitory ligand (ouabain). To investigate the specific binding of an inhibitory ligand (ouabain) and phosphorylation, the enzyme activity was studied by another set of assays. Phosphorylation probes especially the ATP-binding site of the protein. The immobilized protein was first equilibrated in the presence of 3 mM MgCl_2 (buffer III). The base fluorescence signals F_0 of the sensor were higher in this buffer by at least 5–6%. This effect was explained by the induction of a closer contact of the assembled membrane discs to the chip surface in the presence of divalent cations (see AFM results). Phosphorylation after incubation of 3 mM inorganic phosphate led to a minor decrease in F_0 in the range 2–6%. Specific binding of inhibitory ligand was observed by measuring the decrease in the fluorescence signal upon continuous addition of 2.5 mM ouabain in the presence of 3 mM MgCl_2 and 3 mM inorganic phosphate. The kinetics of ligand binding was as slow as in bulk membrane dispersions (*ca.* 20 min to reach a stationary signal). The final signal decrease stabilized at *ca.* 40% of the effect measured in bulk solution.

Together with the K^+ -binding studies (60% of the solution effect) and the antibody-induced

quenching studies (40% of the solution effect), the results obtained with specific binding of ouabain (40% of the solution effect) are consistent with the assumption that the two orientations of membrane fragments upon immobilization on the waveguide (former inside up and former inside down) establish almost equally on the surface (*cf.* Fig. 3).

Discussion

We present a new method, showing that transmembrane proteins, embedded in natural biomembrane fragments, can be immobilized with well preserved activity, in a rationally defined and controlled manner, on a planar, metal oxide waveguide transducer. The optimization of the individual preparation steps for a defined and functional immobilization of membrane fragments was the main issue of this work. First examples are presented as to how the functional activity and the specific binding sites of immobilized Na,K-ATPase can be probed by surface-confined fluorescence. A new optical technique, based on the sensitive detection of fluorescence, excited in the evanescent field of thin planar waveguides, was applied for these investigations.

Evanescent field fluorescence detection

The detection of evanescent-field excited, surface-confined fluorescence has the inherent advantages of high sensitivity at large signal-to-noise (S : N) ratios with an almost complete suppression of background signals from the bulk environment. In the present case of immobilized fluorescein-labelled biomembrane particles, S : N ratios of up to 5000, at only partial surface coverages, were obtained. This implies that, under optimal sensing conditions, functional probing of membrane proteins in very small detection fields, approaching sizes similar to the scanning areas of the presented AFM pictures, may become possible. On the other hand, even in the case of macroscopic detection fields ($\geq 1 \text{ mm}^2$), consumption of only low volumes of receptor samples or the use of very dilute samples, *e.g.* at low receptor yields, are required. Compared to bulk measurements, biological receptors immobilized on surfaces offer the great advantage that different buffer media and/or specific ligands or inhibitors can be sequentially applied to the same preparation and, subsequently, exchanged in a fast and easy manner. Thereby automation enables a high degree of reproducibility. A fast assay development and optimization,²⁶ and finally a high assay performance are the consequences, as demonstrated in the present case.

Functional surface immobilization

The surface immobilization of membrane proteins, under preservation of full protein activity and with presentation of a maximum number of accessible binding sites, is the key challenge for the investigation of protein functions with surface-sensitive detection schemes. We have pursued the concept of immobilizing intact membrane fragments where the proteins are embedded in their natural environment. There, a high likelihood consists that the protein function is maintained during the whole process of surface preparation. This is different to other methods, which employ the almost random immobilization of membrane receptors in purified and/or detergent-solubilized form on the surface²⁷ or, in a more defined way, the immobilization of solubilized receptors on functionalized surfaces *via* chemical affinity tags (His-Tag approach), positioned at distinct protein locations.¹⁰ With the latter method, the ligand-binding activity was established as for the native receptor, but it has to be critically monitored for each preparation step, for changes in the protein environment (*e.g.* change of detergent) and for each reaction partner. The presented method is the preferred one to immobilize membrane receptors in their native environment under retention of the natural biomembrane orientation. However, in contrast to individual receptor molecules, the demands for the surface immobilization of nanoparticulate membranes in combination with surface-sensitive detection schemes are much higher. Especially, the stability of surface contacts seems to be very important, since the dimensions of the membrane fragments were of the same size as the penetration depth of the evanescent field (few hundred nm). Instabilities in the orientation of surface-associated membranes with respect to the evanescent field, especially when exposed to flowing buffer solution, would result in large fluctuations of the sensor signal (as observed in our first experiments or when applying too low ionic strength). We have achieved a stable and functional membrane fragment immobilization in a flat configuration.

Necessity for a biocompatible interface

The experiments clearly showed that the presence of a gentle, biocompatible interface is needed for a functional immobilization of Na,K-ATPase. The enzyme lost its specific cation binding activity completely when immobilized directly on the bare chip surfaces, although the protein was still embedded in its native membrane environment. The biocompatible interface was formed by covering the waveguide chip with a self-assembled phospholipid monolayer. Such a biomimetic layer protects the sensitive membrane proteins from potential denaturing contact with the bare, non-sensitized transducer surface. In addition, the chemical composition and the physico-chemical properties of such adlayers can be well controlled, since many natural and synthetic lipids and cofactors for their formation are available. The physico-chemical properties of the lipid surface and the diffusion properties of the monolayer can easily be modified and anchor groups for affinity binding can be introduced.²⁸ In addition, lipid layers are able to suppress the interaction of soluble substances with the surface thus reducing the non-specific binding.²⁴

Stability of membrane fragment immobilization in the evanescent field

The conditions for a stable immobilization of the membrane fragments on the preformed biocompatible interface have been optimized by varying the physico-chemical properties of the surrounding buffer media. Buffer solutions at high ionic strength, as used in the course of this study, are known to overcome the long-range, repulsive electrostatic interactions (membranes as well as oxide surfaces are preferentially negatively charged) and to favour the strongly attractive van der Waals forces.²⁹ In the presence of the lipid monolayer, the membrane affinity to the coated surfaces was much lower compared to the non-sensitized metal oxide surfaces. However, a fully preserved protein activity established in the case of the preformed biocompatible surfaces was concluded from the comparable assay characteristics and binding constants of surface-assembled enzyme and 'free', non-immobilized enzyme in solution (see specific K^+ -binding).

Aspect of sidedness

Besides the aspect of establishing a general procedure for the functional immobilization of membrane fragments, the other important interest in the investigation of a surface-immobilized Na,K-ATPase was the aspect of sidedness of the specific binding sites. To address this aspect, there are literature reports of electrophysiological measurements carried out with Na,K-ATPase, either with intact cells such as ventricular myocytes³⁰ or oocytes,³¹ where only the extracellular medium can be changed extensively or the enzyme can be reconstituted in vesicular systems.^{32–34} Such reconstitutions imply that the membrane-bound protein has to be solubilized with a suitable detergent with retention of its full enzymatic activity. These are obviously conditions in which the original orientation of the protein molecules in the disc membrane has been lost. The solubilized enzyme, which forms a mixed protein-detergent micelle, is then reincorporated in the lipid membrane of the vesicular systems. Besides the fact that it is difficult to find suitable detergents which do not alter irreversibly the structure and functional properties of the protein, the reconstituted enzyme can adopt two different orientations (inside-out and inside-in) in the vesicular lipid membrane. In addition, it can be adsorbed onto the lipid surface without being incorporated. In order to study side-directed properties of such systems it is necessary to introduce discriminations biochemically, for example by inactivating one state of orientation.

Two recently developed techniques provide information about directed properties of electrogenic as well as of electroneutral steps that are functionally coupled. These techniques are related to electrical measurements of Na,K-ATPase discs adsorbed onto or incorporated into black lipid membranes^{35,36} and also employ the patch method on excised membrane fragments originating from cellular systems. Because the transport currents of transporter proteins are very small compared with those of ion channels, the development of the giant membrane patch method, which permits the inside-out and the outside-out membrane orientation, has led to interesting studies.^{37,38}

In the case of a surface-immobilized ATPase, the sidedness of specific binding sites can be determined under conditions in which all membrane fragments are homogeneously oriented on the surface. Having achieved a functional immobilization of membrane fragments in a sensor

configuration, under control of membrane fragment orientation on the sensor surface, the aspects of sidedness may be investigated in a comparatively simple and straightforward manner using the planar waveguide approach.

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