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Interaction of Two Different Types of Membrane Proteins with Model Membranes Investigated by FTIR ATR Spectroscopy

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Polarized FTIR ATR spectroscopy was used to investigate the interaction of mitochondrial creatine kinase (Mi-CK) and intestinal alkaline phosphatase (AP) with model membrane assemblies. Mi-CK was immobilized by adsorption to the negatively charged cardiolipin (CL) leaflet of a supported CL/DPPA bilayer. H-D-exchange of the enzyme and the stability under flowthrough conditions of the protein/membrane assembly were examined. AP, however, was bound to a DPPA Langmuir-Blodgett layer (LBL), followed by the completion of a bilayer-like structure by adsorption of POPC molecules from a vesicular solution. It turned out that the POPC adsorbate exhibited decreased molecular order compared to the POPC molecules on a supported POPC/DPPA bilayer. Enzymatic activity of immobilized AP was determined with p-nitrophenyl phosphate (p-NPP) as substrate and remained unchanged for at least 2 days.

INTRODUCTION

In vivo, there are several ways of how membrane proteins are attached to a lipid bilayer. We investigated two proteins with respect to their interactions with model membrane assemblies.

Mitochondrial creatine kinase (Mi-CK) is known to bind effectively to the surface of negatively charged bilayers like cardiolipin (CL) (1,2,3). Mi-CK catalyzes the phosphorylation of creatine by ATP and is therefore important for energy metabolism in cells of high and fluctuating energy requirements.

Alkaline phosphatase (AP) belongs to a wide group of enzymes which catalyze the non-specific hydrolysis of phosphate monoesters in an alkaline environment (4). AP is attached to the outer leaflet of the plasma membrane by the lipid moiety of a glycosyl-phosphatidylinositol (GPI) anchor (5). The hydrophobic hydrocarbon chains of this lipid moiety are responsible for the attachment of the enzyme to the lipid bilayer.

Our aim was to find experimental conditions enabling the formation of stable protein/membrane assemblies for both enzymes. The enzyme adsorption to the supported lipid matrices was monitored *in situ* by FTIR ATR spectroscopy, and the amount of bound protein was estimated.

MATERIAL AND METHODS

Chicken sarcomeric mitochondrial creatine kinase (Mi-CK, octamer with 340 kD) was obtained from T. Wallimann (Institute for Cell Biology, ETH-Hönggerberg, Zurich, Switzerland). Alkaline phosphatase (AP, dimer with 132kD) from bovine intestinal mucosa was provided by B. Roux (ICBMC, University Claude Bernard, Lyon, France). 1,2-Dipalmitoyl-*sn*-glycero-3- phosphoric acid (DPPA), Cardiolipin (CL), p-nitrophenylphosphate (p-NPP) and octyl-β-D-glucopyranoside (β-OG) were purchased from Fluka AG, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) from Sigma.

In case of Mi-CK, (Fig. 2, path 1: I - IV) as model membrane, a supported lipid bilayer was prepared using the Langmuir Blodgett (LB)/Vesicle-Method (6). With the help of a film balance, the first layer consisting of DPPA was transferred to the surface of a clean germanium plate. The second layer consisting of negatively charged CL was produced by spontaneous adsorption to the hydrophobic DPPA film from a vesicular solution. For the immobilization of Mi-CK a 0.55 mg/ml enzyme-solution in 10 mM phosphate buffer pH 7.0, 50 mM NaCl, was slowly pumped over the DPPA/CL bilayer at 25°C. The adsorption process could be monitored *in situ* because it occurred in an aligned cell in the sample compartment of the spectrometer by means of a peristaltic pump.

AP tends to precipitate in aqueous buffer solutions and needs some detergent for solubilization. Therefore, the transfer of AP from solution to the lipid bilayer is more critical than that of Mi-CK. At first, AP (50 µg/ml, 20 mM Tris buffer pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 50 µM $ZnCl_2$) solubilized with β-OG was directly adsorbed to DPPA, then a POPC vesicle solution was circulated through the ATR cuvette to reconstitute the bilayer (Fig.2, path 2: I,V,VI). Afterwards, the activity of the immobilized AP was measured by pumping substrate solution (p-NPP) (7) through a flowthrough cuvette. Enzymatic activity was determined from the rate of p-nitrophenol production by ester hydrolysis as detected by VIS spectroscopy at 420 nm.

RESULTS AND DISCUSSION

Mi-CK is rapidly binding to the CL/DPPA membrane: within 45 min 99% of the process took place (Fig.1). Data should be comparable to those of Stachowiak et al. (3), who found a fast process (k_1=0.11s^{-1}) and a slower one (k_2= 0.008s^{-1}) within the first 250s. In our experiment we can prove the slower process to have a rate constant of 0.01s^{-1}. Furthermore we detected a third process with a rate constant of 7.10^{-4} s^{-1} observing the adsorption for about 50 min.

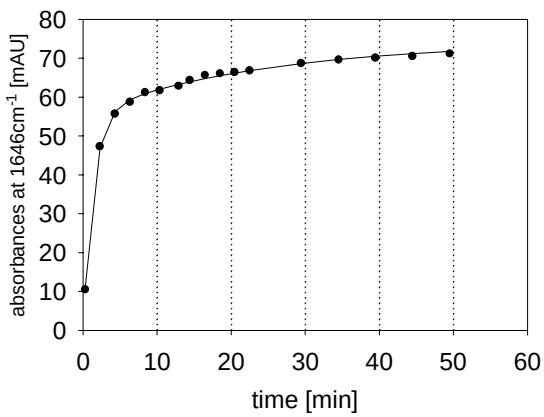

FIGURE 1. Absorbances at 1646 cm^{-1} during the adsorption of Mi-CK on a CL/DPPA bilayer: [●]... Data (A_{max} of amid I/I´-band) and [-]... kinetic fit of the adsorption of octameric Mi-CK on a DPPA/CL-bilayer.
Data were fitted with $f = A_0 - A_1 \cdot \exp(-k_1 \cdot t) - A_2 \cdot \exp(-k_2 \cdot t)$ using the Marquardt-Levenberg algorithm of Sigma-Plot. A_0 was estimated by extrapolation of a double reciprocal plot to be 74mAU. Results of the fit: A_1=55.4 ± 0.87 mAU, k_1=(1.23 ± 0.055)$\cdot 10^{-2}$ s^{-1}, A_2=18.6 ± 0.62 mAU, k_2=(7.05 ± 0.34)$\cdot 10^{-4}$ s^{-1} (15 iterations, Rsqr=0.999).

After 1 hour the medium was changed to D_2O (pH* 6.6) and the H-D-exchange was monitored. Analyzing the amide I/I´ and N-H stretching-bands indicates that Mi-CK is a rather stiff protein. About 20% of the H are persistent against the D-exchange within 20 hours. The quantification of the amide I/I´-band (1650 cm^{-1}) (8) revealed a surface concentration of about $1 \cdot 10^{-12}$ $mol \cdot cm^{-2}$, corresponding to a density of coverage of 60%. Fig. 3 shows FTIR ATR spectra of the Mi-CK and the outer leaflet of the bilayer consisting of CL. A weak dichroism of the amide I/I´ band points to a slight distortion of the Mi-CK structure as determined by X-ray crystallography (point group 422) (9).

The protein/membrane assembly was checked for stability under flowthrough conditions and found sufficient for 3 days. Furthermore, Mi-CK seems to stabilize the CL/DPPA membrane.

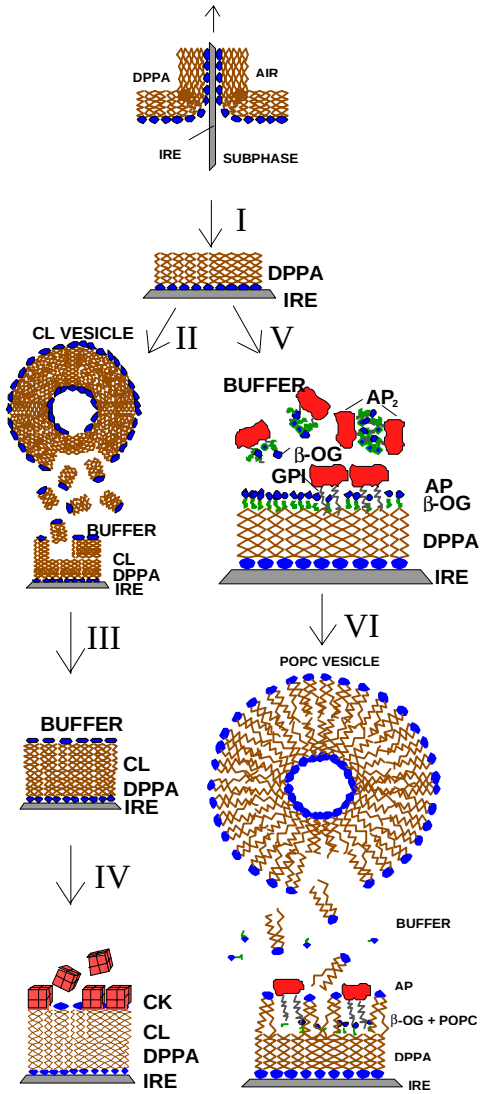

FIGURE 2. Schematic description of two pathways for immobilizing enzymes on lipid model membranes attached to an IRE-plate.
Path 1: Immobilization of Mi-CK. (I) Transfer of the inner IRE-attached DPPA-monolayer from the air/water interface of a film balance to an internal reflection element (IRE) by the Langmuir-Blodgett (LB) technique; (II) spontaneous adsorption of CL-lipids from vesicles energetically driven by the reduction of the unfavorable high energy of the hydrophobic surface of the DPPA monolayer in contact with the aqueous environment; (III) completed asymmetric CL/DPPA bilayer; (IV) adsorption of Mi-CK to the bilayer by electrostatic interactions.
Path 2: Immobilization of AP. (I) as described above; (V) spontaneous adsorption of AP (solubilized by b-OG) to the DPPA-Monolayer via its GPI anchor; (VI) reconstitution of a bilayer-like system by passing POPC vesicles over the AP-DPPA-assembly.

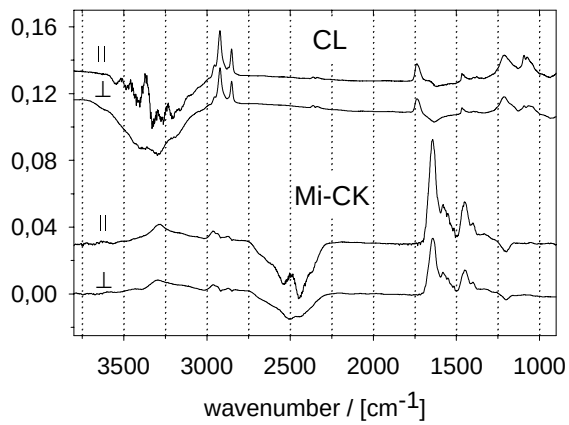

FIGURE 3. Polarized IR ATR absorbance spectra of CL and immobilized Mi-CK. *Top:* Cardiolipin from *E. Coli* (CL) assembled from a vesicle solution (0.67mg/ml CL) on a DPPA-layer. This bilayer was in contact with 20 mM phosphate buffer pH 7.0; T 18°C; reference, DPPA in phosphate buffer; dichroic ratio R, 1.16; surface concentration $\Gamma = 1.68.10^{-10}$ mol cm^{-2}. *Bottom:* Polarized IR ATR absorbance spectra of Mi-CK immobilized on a DPPA/CL bilayer in 10 mM D_2O phosphate buffer solution pH* 6.6; T, 25°C; reference DPPA/CL bilayer in D_2O buffer; dichroic ratio R, 1.85; surface concentration $\Gamma = 9.6.10^{-13}$ mol cm^{-2}; Both surface concentrations were estimated with the thin film approximation: angle of light incidence θ, 45°; number of active internal reflections N, 16.

In contrast to Mi-CK adsorbing to the negatively charged bilayer, AP exhibits a slower adsorption process. Saturation of adsorption to DPPA occurs after 7.5 h; the lipid-protein-interaction is taking place only via the GPI-anchor. Quantification of the amide I/I´-band (1650 cm^{-1}) (8) revealed a surface concentration of about $1.84 \cdot 10^{-12}$ $mol \cdot cm^{-2}$ (Fig.4) corresponding to a density of coverage of about 50%. It is supposed that AP is adsorbing to the DPPA LB layer as monomer (Fig. 2, V).

The POPC reconstitution of the AP-DPPA-assembly (Fig. 2, VI) yields a "bilayer"-like structure where the AP exhibits a constant catalytic specific activity of 30 U/mg for at least 2.5 days (compared to 60 U/mg in the AP solution before adsorption). Furthermore, the adsorbed POPC ($\Gamma = 1.36 \cdot 10^{-10}$ mol cm^{-2}, density of coverage 50%) exhibits the same properties as a pure outer POPC layer adsorbed to DPPA, except for a diminished degree of order (Fig. 5). It seems that the presence of AP and/or of the tenside β-OG which is also bound to DPPA is disturbing the ordered adsorption of POPC molecules. Anyway, there has to be taken care about the amount of tenside present in the protein sample: If it is too large, AP will remain solubilized and will not adsorb to the DPPA layer; if it is too small, AP will aggregate from the beginning.

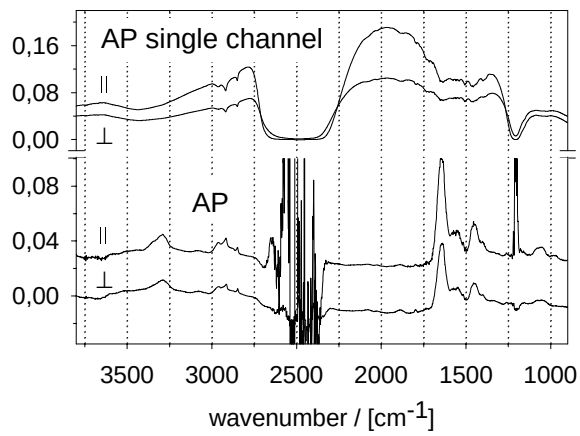

FIGURE 4. Polarized IR ATR spectra of AP. *Top*: Single channel spectra of AP immobilized on DPPA; 20 mM D_2O Tris buffer pH* 7.0; T 25°C. *Bottom:* Correspondent polarized IR ATR absorbance spectra; reference, DPPA in D_2O Tris buffer; dichroic ratio R, 1.62; surface concentration $\Gamma = 1.84.10^{-12}$ mol cm^{-2};angle of light incidence θ, 45°; number of active internal reflections N, 35.

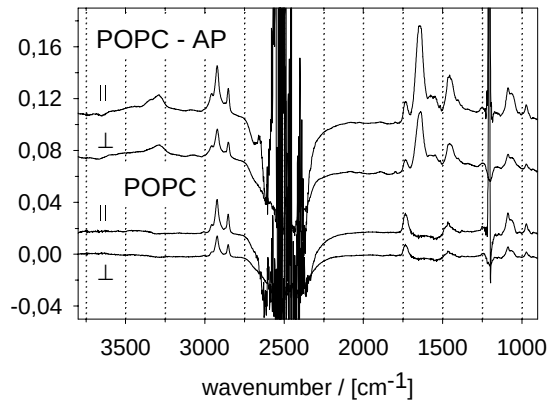

FIGURE 5. Polarized IR ATR absorbance spectra of POPC-AP and POPC. *Top:* POPC-AP assembled from a vesicular POPC-solution (0.67 mg/ml POPC) on a DPPA-layer with immobilized AP; 20 mM D_2O Tris buffer pH* 7.0; T 25°C; reference, DPPA in D_2O Tris buffer. *Bottom:* Polarized IR ATR absorbance spectra of POPC adsorbed on a AP-DPPA-assembly; T 25°C; reference AP-DPPA-assembly in D_2O Tris buffer; surface concentration $\Gamma = 1.36.10^{-10}$ mol cm^{-2}; dichroic ratio R, 1.59; angle of light incidence θ, 45°; number of active internal reflections N, 35.

Regardless whether the interactions between enzymes and lipid membranes are of electrostatic or hydrophobic nature, our results show that it is possible to build up well defined and stable protein/lipid-assemblies. The simultaneous application of FTIR ATR measurements with methods for determining the native enzymatic activity will give new insights into mechanisms of interaction between different types of membrane proteins and their lipid matrices.

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