Approaching determination of H^+-ATPase H^+/ATP stoichiometry

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Contents

1 Abstract

The plant plasma membrane H^+-ATPase was activated by the binding of a 14-3-3 protein. This complex can be stabilized by the fungal toxin - fusicoccin. During this activation H^+/ATP stoichiometry changed (1). This work presents approaching to find the new stoichiometry. The main part presents looking for the best preparation of plasma membrane vesicles for measurements. A new pH indicator – Glu^3 – was trapped inside vesicles in a few different ways such as Brij 58 treatment or sonication. Membranes were washed by ultracentrifugations or gel chromatography. The tight of prepared vesicles was examined by acid titration. The best result was achieved by sonication and gel chromatography. Brij 58 also affects the size of plasma membrane vesicles what was demonstrated. The average diameter decreases with increasing concentration of detergent.

2 Introduction

2.1 Plasma membrane H+-ATPase

Plasma membrane H^+-ATPase is a member of P-type ATPases found only in plants and fungi cells. It uses the energy released by ATP hydrolysis to pump protons out of the cell. This action generates an electrochemical gradient across the plasma membrane which consists of a potential of about -100 to -200 mV and a pH gradient of up to 2 pH units. The role of these gradients is to energize other transport proteins (channels, symporters, antiporters and uniporters).

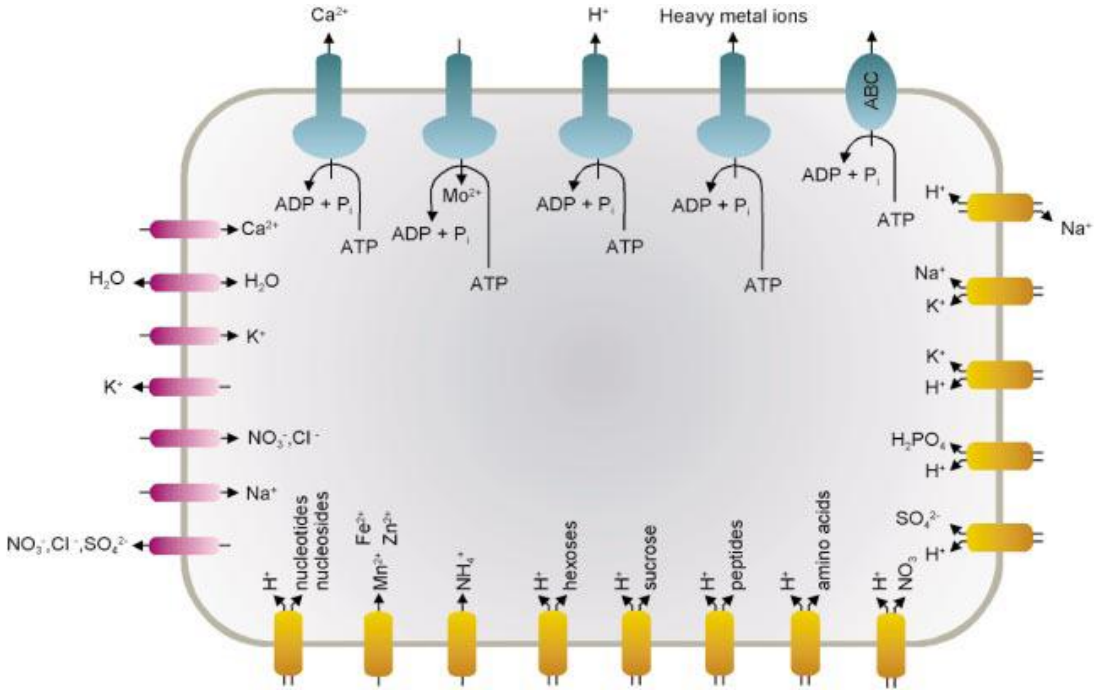

Figure 1. Scheme of the plant cell with transport proteins. Red – channels, orange – carrier proteins, blue – ATPases, in the middle the H^+-ATPase (2).

So indirectly the P-type plasma membrane H^+-ATPase has an influence on nutrients uptake from the soil, phloem loading, size of stomatal aparature and much more. Because of such an important role, the activity is strictly controlled. The C-terminal end, the R-domain plays an autoinhibitory role in binding to the catalytical part of the protein. The inhibition is stopped by phosphorylation on penultimate aminoacid (threonine) and binding regulatory protein from the 14-3-3 protein family. Then, the domain is released and protein activated. In the presence of fungal toxin – fusicoccin – phosphorylation is not required for the activation by the 14-3-3 protein. (2)

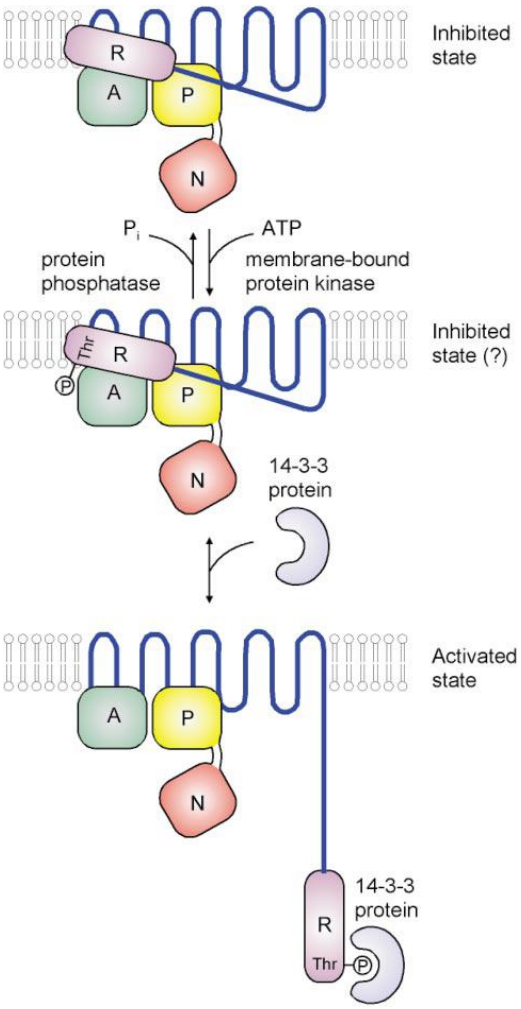

Figure 2. Activation of plasma membrane H^+-ATPase by 14-3-3 protein (2).

2.2 H^+-ATPase assay

The activity of plasma membrane H^+-ATPase can be measured on two ways: the rate of proton pumping or ATP hydrolysis. To measure the stoichiometry we need to control both processes at the same time. The best way to do that is by using H^+-ATPase assay proposed by Palmgren (3). ATP hydrolysis rate is measured as a decreasing of absorbance of enzymatically coupled NADH oxidation. Proton pumping as decreasing absorbance of acridine orange accumulated in plasma membrane vesicles. Unfortunately the proton pumping cannot be measured quantitatively using this dye.

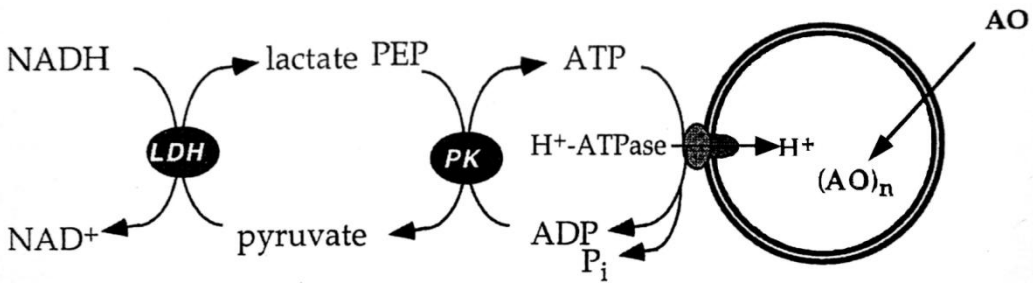

Figure 3. Scheme of H^+-ATPase assay. *LDH*- lactate dehydrogenase, *PK*-pyruvate kinase, AO – acridine orange.

2.3 Glu[3]

To develop a H^+-ATPase assay into a quantitative method acridine orange was replaced by Glu[3]. It is made of tetraarylporphyrin core with three generations of glutamic dendrites. This structure gives high water solubility, membrane impermeability and shifts of porphyrin pK_a to physiological pH range. These properties and also well divided optical spectra of protonated and deprotonated form make this dye useful to measure quantitatively differences of pH inside plasma membrane vesicles (4).

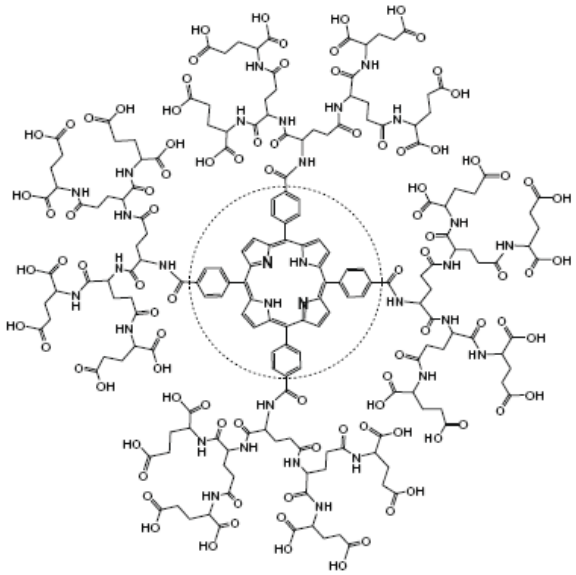

Figure 4. Structure of Glu[3].

2.4 Inverting vesicles

H^+-ATPase pumps protons outside the cell and hydrolysis of ATP occurs inside. The protein in plasma membrane vesicles has the same orientation. To expose ATP binding sites outside

and make the protein pump protons inside (where the volume is smaller, so it is easier to measure ΔpH), the vesicles must be flipped. To get it, detergent Brij 58 was used, which inverts all plasma membrane vesicles (5). The mechanism is not clear, the most likely mechanism is that the Brij 58 is incorporated into the membrane and changes its curvature, what flips the vesicles.

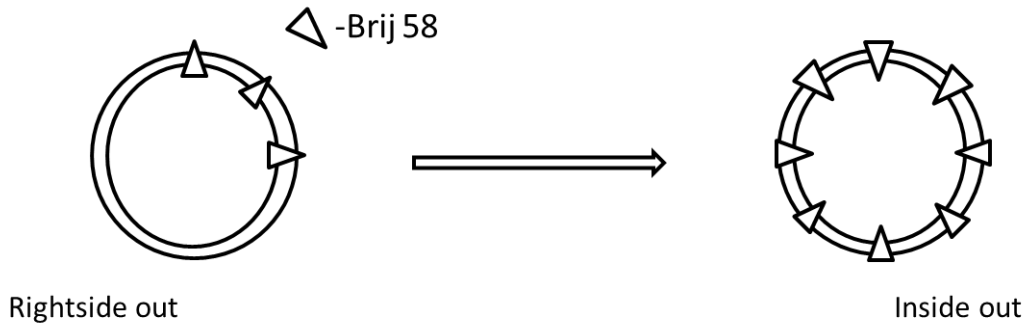

Figure 5. Plasma membrane vesicles flip in the presence of detergent – Brij 58.

3 Materials and methods

3.1 Plant material

Spinach (*Spinacia oleracea*) was grown in a greenhouse with supplementary light (210 µmol m^{-2} sec^{-1}, 350 to 800 nm, G/86/2 HPLR 400 W; Philips, Eindhoven, The Netherlands). Expanding leaves of 4- to 5-week-old plants were used (6).

3.2 Plasma membrane vesicles

Plasma membranes were purified from a microsomal fraction by two-phase partitioning (7). The final plasma membrane pellet was resuspended in 10 mM MOPS-BTP, pH 7.0, 20% (v/v) glycerol, 5 mM EDTA, and 1 mM DTT and stored at -196°C until required (6).

3.3 H^+-ATPase assay

ATPase activity was measured as described by Palmgren (7). In this assay, ATP hydrolysis is coupled enzymatically to oxidation of NADH by use of pyruvate kinase and lactate dehydrogenase. The rate of ATP hydrolysis is measured as the absorbance decrease of NADH at 340 nm. Proton uptake into the vesicles was monitored as the absorbance decrease of acridine orange at 495 nm. Proton pumping was determined from the initial slope. Addition of nigericin (7 µL 0,1 mM) removes electrochemical gradient across membrane, due to action of this antibiotic as ionophore for H^+ and K^+. The assay medium contained 10 mM MOPS-BTP, pH 7.0, 2 mM ATP, 4 mM $MgCl_2$, 140 mM KCl, 1 mM DTT, 0.25 mM NADH, 20 µM acridine orange, 1 mM phosphoenol pyruvate, 50 µg/mL of lactate dehydrogenase (in glycerol; Boehringer Mannheim, Mannheim, Germany), 50 µg/mL of pyruvate kinase (in glycerol; Boehringer Mannheim), 0.05% (w/v) Brij 58 (Sigma), 50 µg/mL of plasma membrane protein and 12.5 mM sucrose in a total volume of 1 mL. To determine the activation effect of 14-3-3 protein, the isoform µ12 to get 0.7 µM was added in the presence of 7 µM fusicoccin.

3.4 Influent of Brij 58 on the size of vesicles and activity of protein

Proton pumping and ATP hydrolysis were checked using H^+-ATPase assay with a series of Brij 58 concentrations: 0 µg/mL, 6,25 µg/mL, 12,5 µg/mL, 25 µg/mL, (in case of proton pumping: 31,3 µg/mL, 38,5 µg/mL),50 µg/mL, 100 µg/mL and 500 µg/mL. The size as average diameter of plasma membrane vesicles was done using dynamic light scattering based on

the samples directly after ATP hydrolysis measurements. Each result is an average of at least 90 scans in 20°C.

3.5 Trapping Glu^3

The main idea of trapping Glu^3 mechanism is shown in figure 6. Firstly the dye is caught and the membrane flipped. Then, the outside solution is washed away to get rid of Glu^3. Both steps may be done in several ways.

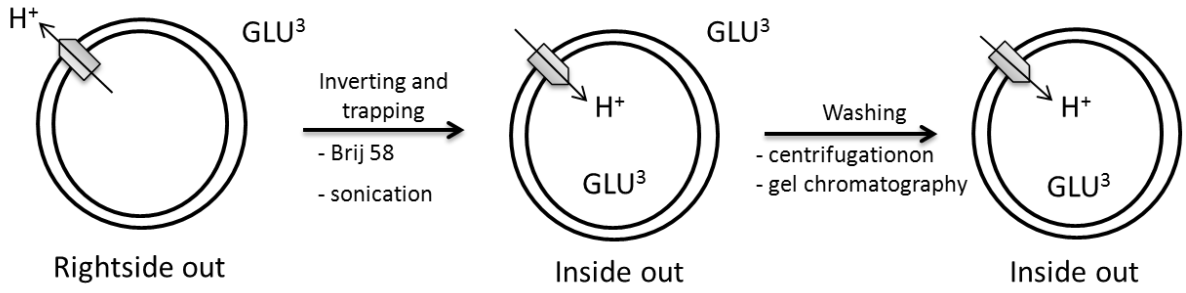

Figure 6. Scheme of vsicle's preparation.

3.5.1 Brij 58 treatment

Trapping solution contained: 50 µg/mL of plasma membrane protein, 12,5 mM sucrose, 0,67 mg/mL Glu^3, 1,5 mM EDTA, 0,36 mM DTT, 0,5 mg/mL Brij 58 and 20 mM MOPS-BTP (pH 7,0).

3.5.2 Freeze and thaw

Solution 5 mg/mL plasma membrane protein, 87,5 mM sucrose, 0,3 mg/mL Glu^3, 2,2 mM EDTA, 0,07 mM DTT and 20 mM MOPS-BTP (pH 7,0) was frozen in liquid nitrogen and thawed in room temperature water eight times.

3.5.3 Sonication

Ultrasonic treatment was performed on ice during five periods of 40 s separated by periods of 20 s for MOPS solution (1 mg/mL plasma membrane protein, 0,6 mg/mL Glu^3, 2,4 mM EDTA, 2,4 mM DTT, 20 mM MOPS-BTP (pH 7,0)) and six periods of 40 s separated by periods of 40 s for phosphate buffered solution (3 mg/mL plasma membrane protein, 1,5 mg/mL Glu^3, 2 mM EDTA, 50 mM K_2SO_4, 20 mM potassium phosphate buffer (pH 7,0)).

3.6 Cytochrome c

As a model cyt c was used instead of Glu^3. The sample consisted of 7 mg/mL cyt c (from horse heart), 1 mg/mL plasma membrane protein, 20 mM MOPS-BTP (pH 7,0), 10 mg/mL Brij 58, 10 mM KCl, 5 mM EDTA, 5 mM DTT in a total volume of 0,5 mL. In experiment with hydrophobic beads the concentration of cytochrome c was 5,4 mg/mL. The concentrations of cyt c were measured by in-house-constructed CCD-based spectrophotometer using the equation shown in the figure 6. Δε is equal to 19,6 $mM^{-1}cm^{-1}$ (8). To get the oxidized form, 5 µL of 0,1 M potassium ferricyanide was added, to oxidized 5 µL of 1 M potassium ascorbate was added.

$$[Cyt\ c]mM = \frac{A_{550(reduced)} - A_{542(reduced} + A_{542(oxidized)} - A_{550(oxidized)}}{\Delta\varepsilon_{550(reduced-oxidized)}}$$

Figure 6. Equation 3 for determining cyt c concentration, ε is a milimolar absorptivity of mammalian cytochrome c. (8)

3.7 Cleaning outside solution

3.7.1 Centrifugation

Pelleting was done by ultracentrifugation at 100'000*g* for 1,5h in 4°C. The pellet was washed three times by 20 mM MOPS-BTP (pH 7,0) with 100 mM KCl. In some cases the washing solution contains also 0,5 mg/mL Brij 58.

3.7.2 Ion exchange column

Sample was put on ion exchange column HiTrap QXL 1 mL, and eluted by adequate buffer.

3.7.3 Hydrophobic beads

The detergent was removed by adding hydrophobic beads (SM-2 Bio-Beads, Bio-Rad) in four portions of about 0,5 cm^3 with 20 minutes of gentle stirring between the additions. The beads were washed before usage (three cycles) with methanol and deionized water and then again with deionized water.

3.7.4 Size exclusion column

To remove Glu^3 from the bulk solution, the plasma membrane vesicles were also purified by size exclusion chromatography on a Sephacryl S-400 HR column (GE Healthcare).

3.8 Acid titration

Titration measurements were performed using an in-house-constructed CCD-based spectrophotometer with semiautomatic titration system. The same buffers which were used to prepare plasma membrane vesicles were used in this experiment. Gramicidin DMSO solution was added to a final concentration of approximately 1 µg/mL. 0,5 M sulphuric acid was used for acidification. Samples for these measurements were chosen from the richest of membranes fraction after gel chromatography.

3.9 Adapting proton pumping assay

Because the buffer of the assay was changed the proton pumping experiment was repeated in these new conditions. The new medium contained 10 mM potassium phosphate buffer (pH 7,0), 50 mM K_2SO_4, 90 mM KCl, 20 µM acridine orange, 50 µg/mL - plasma membrane protein, 2 mM ATP, 4 mM $MgCl_2$, 0,5 mg/mL Brij 58. The assay was run in the absence and presence of 0,5 µg/mL valinomycin. The reference contained 10 mM MOPS-BTP (pH 7,0), 140 mM KCl, 20 µM acridine orange, 50 µg/mL - plasma membrane protein, 2 mM ATP, 4 mM $MgCl_2$, 0,5 mg/mL Brij 58.

4 Results and discussion

4.1 H+-ATPase activity

4.1.1 Proton pumping

This assay was done to ensure the reproducibility of the results for proton pumping from earlier works (3). The velocity of the reaction is similar, and for the control sample equals 0,083 ΔAbs at 495nm per minute per milliliter. In a presence of valinomycin the decrease of absorbance is almost twice faster (0,156 ΔAbs_{495nm}/min/mL). Valinomycin works as an ionophore for potassium cations, prevents establishing of a potential across the plasma membrane. The addition of 14-3-3 protein and fusicoccin, which should activate the proton pump, makes the pumping slower (0,037 ΔAbs_{495nm}/min/mL). The presence of imidazole in the 14-3-3 protein solution inhibits the enzyme.

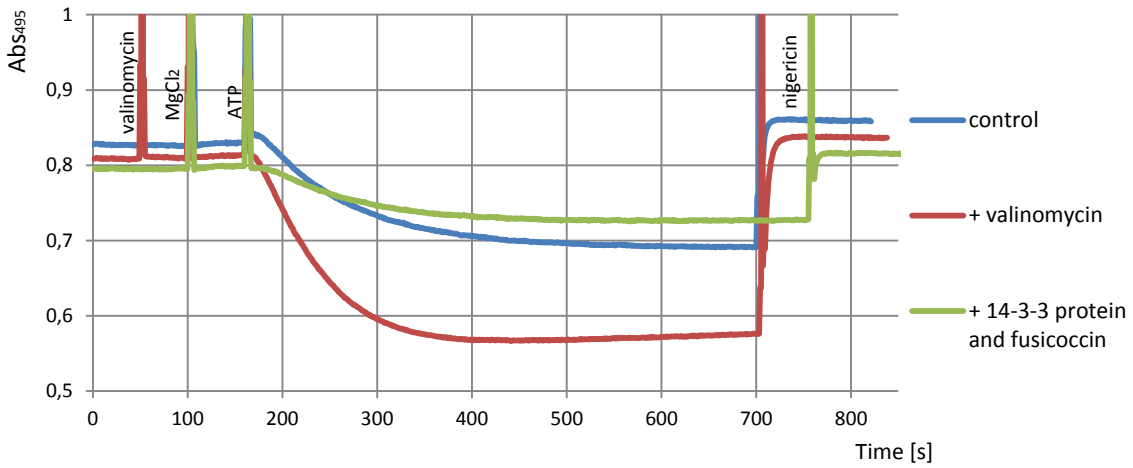

Figure 6. Effect of valinomycin (red) and 14-3-3 protein in the presence of fusicoccin (green) on H^+ accumulation.

4.1.2 ATP hydrolysis

Results of ATP hydrolysis are also similar to previous works (3) for control sample (0,57 µmol ATP/mg protein/min) and with addition of valinomycin (0,66 µmol ATP/mg protein/min), but addition of 14-3-3 protein in the presence of fusicoccin shows inhibitory effect (0,49 µmol ATP/mg protein/min) what was not expected.

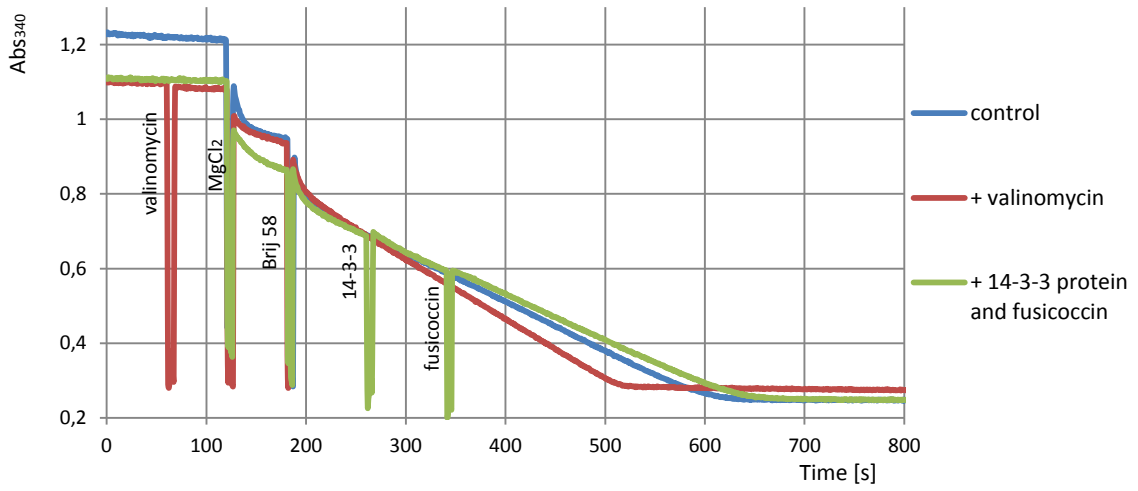

Figure 7. Effect of valinomycin (red) and 14-3-3 protein in the presence of fusicoccin (green) on ATP hydrolysis.

4.2 Influent of Brij 58 on assay

4.2.1 Proton pumping

Brij 58 increases proton pumping after reaching a critical concentration. Below 31,3 µg/mL there is no effect. Between 31,3 and 50 µg/mL appears big increase of activity. During a further increase of Brj 58 concentration the activity of the enzyme changes slightly (Figure 8.). This step is due to the plasma membrane vesicles are inverted (5) and become tight. At the beginning the vesicles are in some percentage inside-out because of the method of preparation – that's why the pumping is observed.

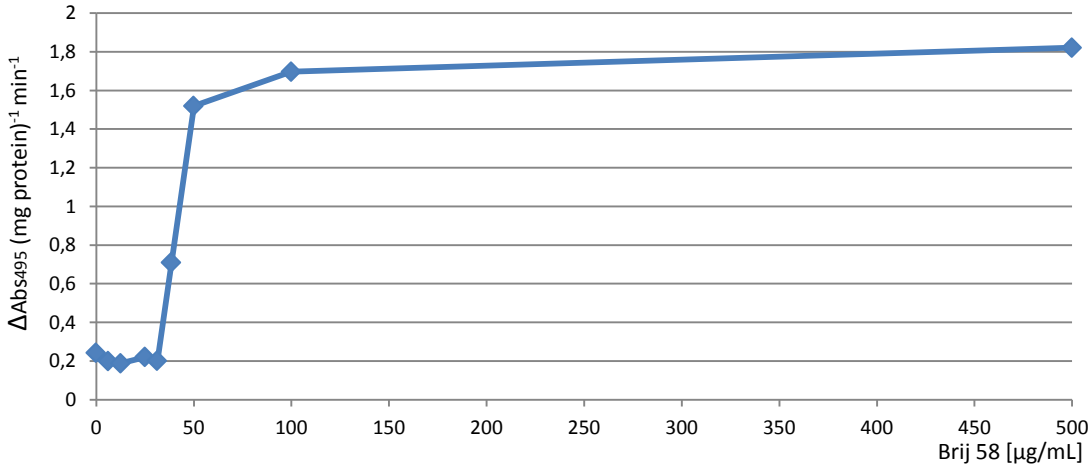

Figure 8. Effect of Brij 58 on H^+ pumping.

4.2.2 ATP consumption

The influence of increasing Brij 58 concentration on ATP hydrolysis started appearing during the first addition of the detergent. It is correlated to the amount of active sites of H^+-ATPase exposed to outer solution.

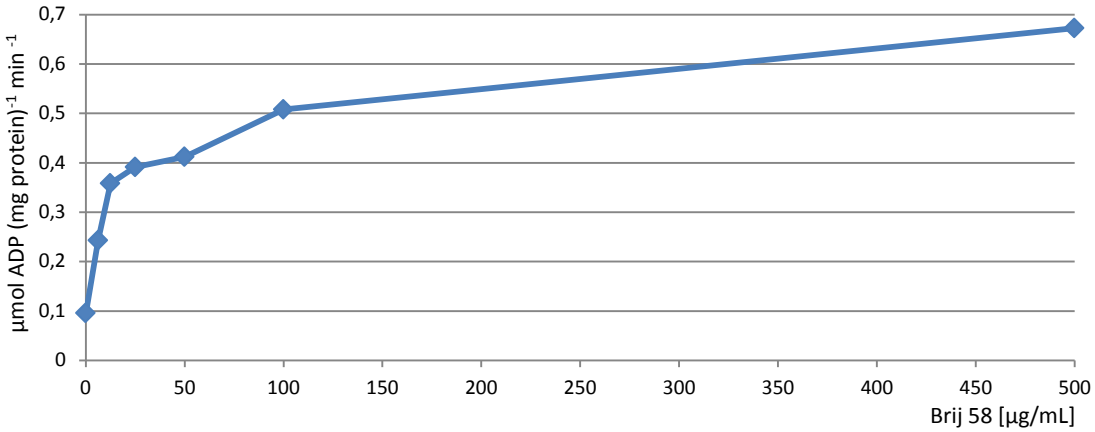

Figure 9. Effect on ATPase activity of Brij 58.

4.2.3 Size of vesicles

As we can see (Figure 10.) the size of plasma membrane vesicles is decreasing, when the concentration of Brij 58 increases. The initial average diameter of vesicles equals to 470 nm shrank almost two-fold to 240 nm.

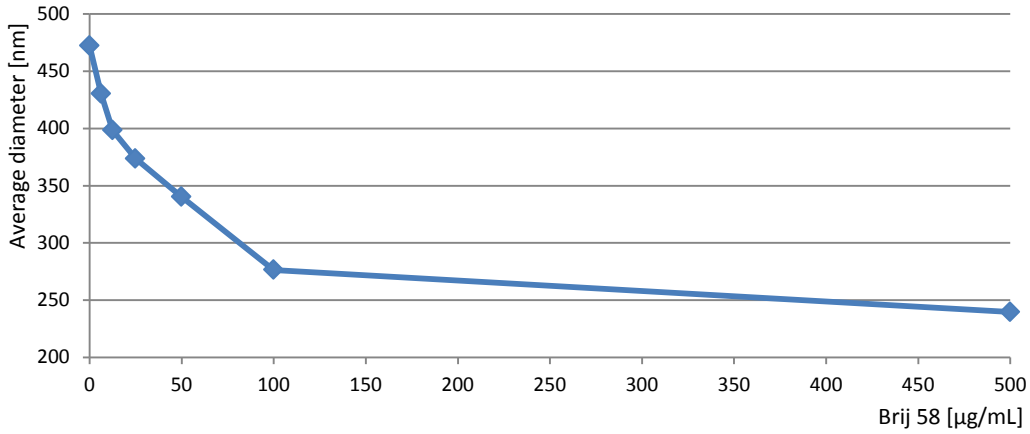

Figure 10. Effect of Brij 58 on size of plasma membrane vesicles.

4.3 Trapping Glu^3 inside plasma membrane vesicles

4.3.1 Brij 58 treatment

This method gave Glu^3 inside (or somehow bound to) vesicles, but after each centrifugation the concentration of the dye decreased (Figure 11, graph A and B), which meant, that it had leaked. Small changes of parameters or method did not make it better – maintaining the same concentration of Brij 58 during all preparation (Figure 11, graph B), or keeping the temperature around 4°C (Figure 11, graph C). In a case of using ion exchange column to get rid of the Glu^3 from outside solution (after one centrifugation), the dye was no longer present in the sample. To avoid using ultracentrifugation (g force might damage vesicles treated by detergent) size exclusion column was used to get rid of the Glu^3 from outside solution. The result was quite similar to the rest of cases, when Glu^3 appeared, but membranes were very diluted (Figure 11, graph D).

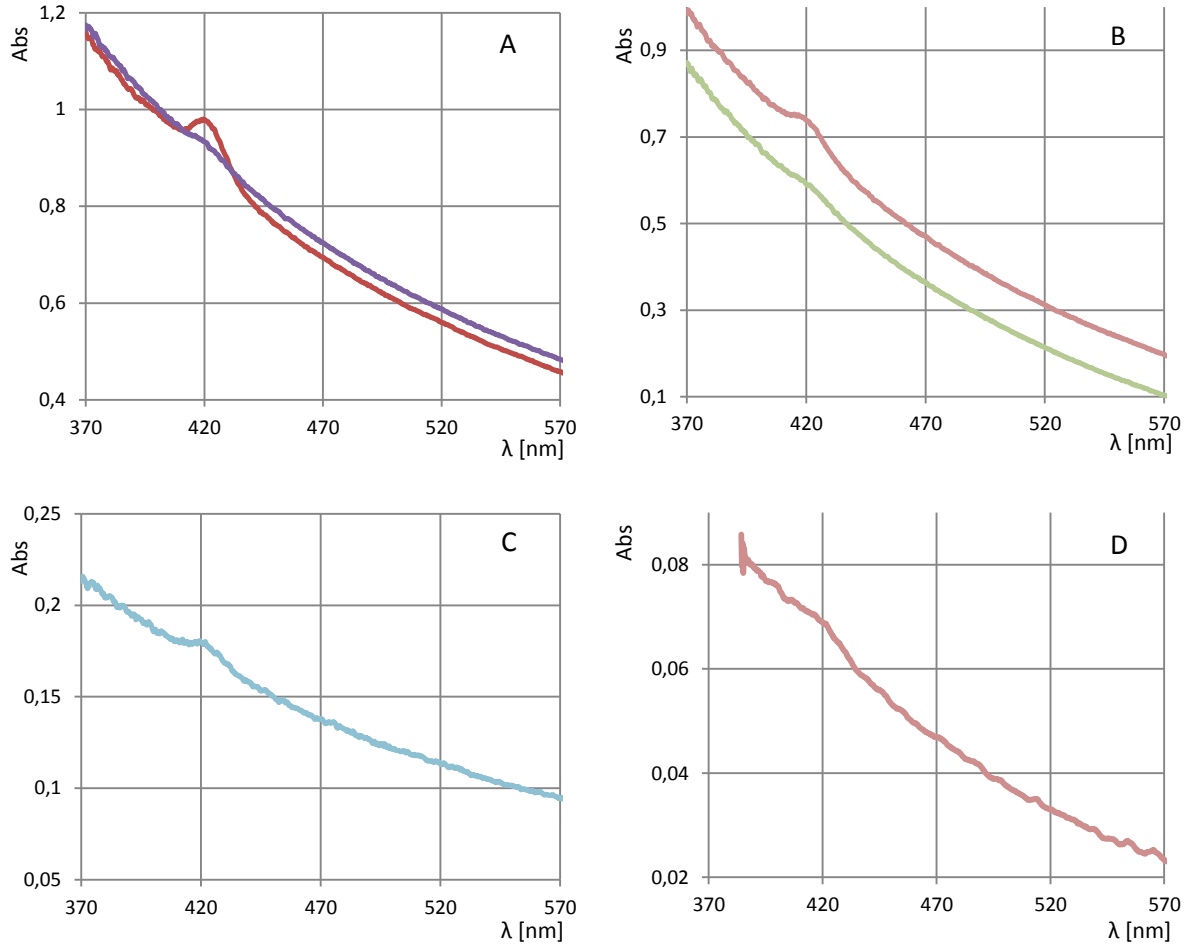

Figure 11. Spectrum of plasma membrane vesicles solutions with trapped Glu^3 (the Soret peak close to 420 nm). In graph **A** the curves show the presence of Glu^3 after second (red) and third (violet)

centrifugation. Graph **B** presents the same experiment, but with stable Brij 58 concentration during the whole washing procedure. Curve pink – the result after second centrifugation, green – the third. Graph **C** shows the same method (presence of Brij 58), but in cool conditions. The sample after the third centrifugation, diluted 33,3 fold. Graph **D** presents the absorption of the fraction from the size exclusion column with the highest concentrated membranes. Slopes are in all cases caused by scattering.

4.3.2 Freeze and thaw

After three centrifugations there was no Glu^3 in the plasma membrane vesicles' solution.

4.3.3 Cytochrome c

This experiment was done to check how much of different compounds is possible to be caught inside the vesicles. As it is shown (Figure 12) amount of cyt c which remained in membranes was very low and decreasing after each pelleting. It could mean that putting the force during centrifuging squeezes cyt c out of the vesicles. One of the reasons of instability might be Brij 58 incorporated with plasma membrane. To get rid of the detergent hydrophobic beads were used. After second spin, the concentration of cyt c approaches to the limit of determination, so the measurements are flawed (Figure 13), that's why the amount of cyt c of membrane's solution (blue bar) from previous centrifugation was not equal the sum of amounts of cyt c in the membrane's solution and the supernatant (blue and green bar) of following centrifugation. Leaking is still present. Cyt c was also adsorbed by hydrophobic beads.

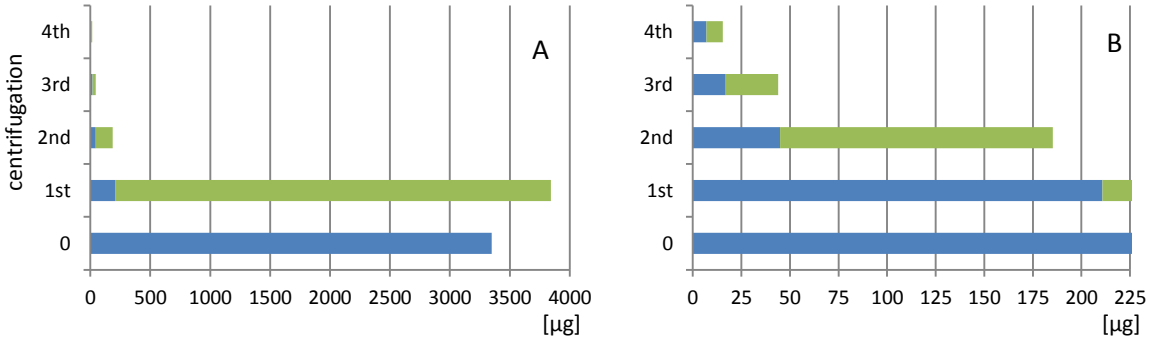

Figure 12. Mass distribution of cyt c during centrifugations. Plot B is a zoom of plot A. Blue bars represent the amount of cyt c in membrane's solution; green bars – in supernatant. *0* is a sample before centrifugation.

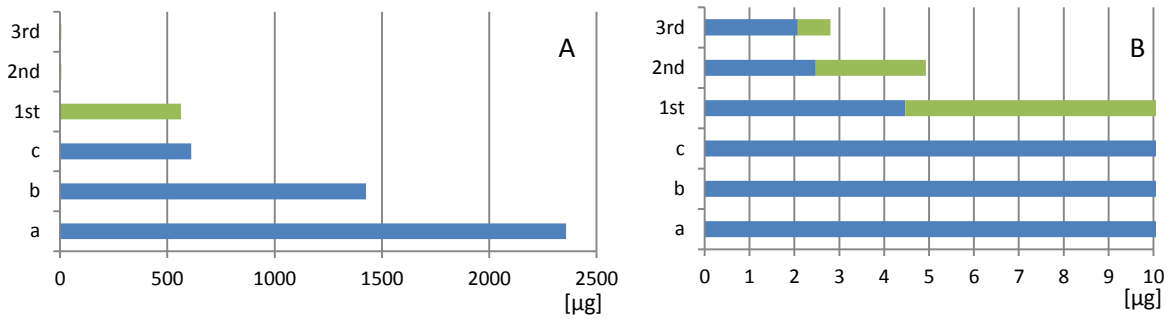

Figure 13. Mass distribution of cyt c during hydrophobic beads treatment and centrifugation. . Plot B is a zoom of plot A. Blue bars represent the amount of cyt c in membrane's solution; green bars – in supernatant. Bar *a* represents initial amount of cyt c; *b* – in the middle of beads treatment; *c* – after beads treatment, before centrifugations; *1st*, *2nd* and *3rd* are the numbers of consecutives spins.

4.3.4 Sonication

The final method of trapping Glu^3 was sonication and size exclusion column to wash the plasma membrane vesicles. The amount of caught dye was still very low, but peak was well expressed and could be used to measure changes in pH (Figure 14). Further purification of the sample on an ion exchange column eliminates all Glu^3, which means that the dye was outside vesicles, or vesicles were unstable in the column.

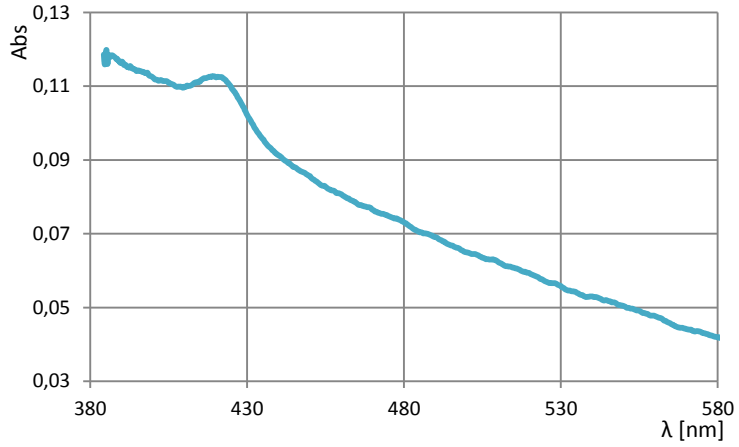

Figure 14. Spectrum of plasma membrane vesicles solutions with trapped Glu^3 (the Soret peak close to 420 nm). Slope is a result of scattering.

4.4 Acid titration

To be able to measure proton pumping, it was necessary to know how the Glu^3 behaved in the plasma membrane vesicles. It was done by acid titration in a presence of gramicidin. In a buffer from H^+-ATPase assay – MOPS-BTP pH 7.0 – Glu^3 trapped with vesicles worked

in totally different way (Figure 15) than the free dye in this buffer without membranes (Figure 16). The basic peak (419 nm) was decreasing, but the acidic peak did not increase (439 nm). Moreover the decreasing peak shifted. The titration of the free dye in MOPS-BTP buffer also showed, that in this conditions pK_a of Glu^3 was equal to 5,05 – lower than expected (more then 6,0) (4).

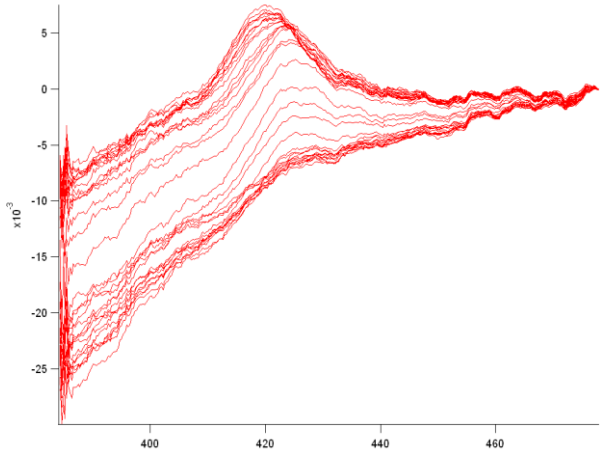

Figure 15. Acid titration of Glu^3 in plasma membrane vesicles in a presence of gramicidin in MOPS-BTP buffer (pH 7,0).

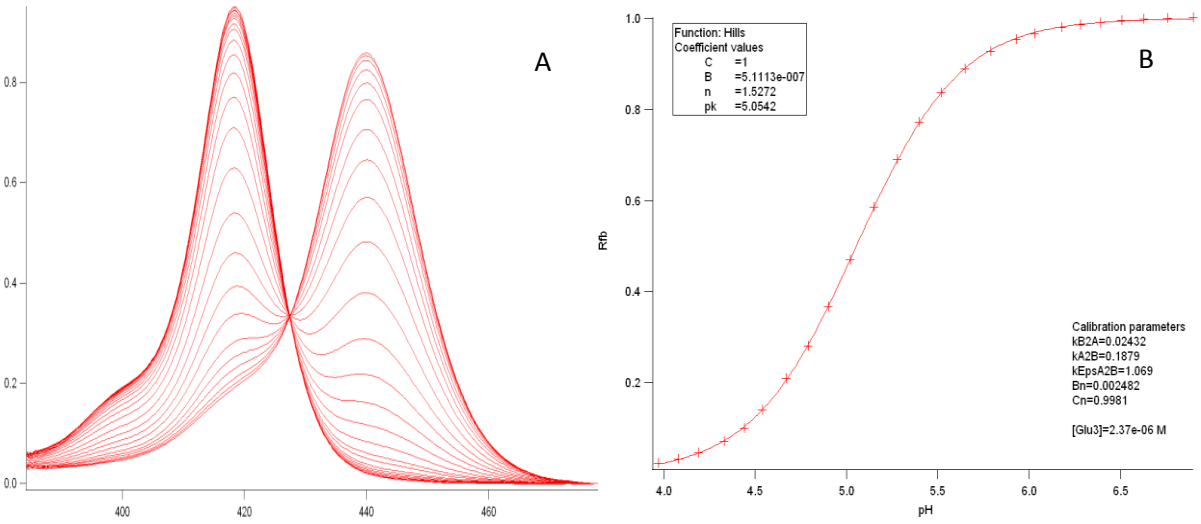

Figure 16. Acid titration of Glu^3 in MOPS-BTP buffer (pH 7,0). pK_a=5.05.

Buffer was changed into potassium phosphate buffer (pH 7,0) and titrations were repeated in presence (Figure 17) and absence (Figure 18) of gramicidin to check if the dye was inside or outside the vesicles. Gramicidin works as an ion channel for monocations (H^+, K^+), so when Glu^3 was trapped inside vesicles, spectra should have shifted in the presence

of gramicidin during titration but not in its absence. Plots show that Glu^3 was inside the vesicles. The peak of diprotonated form appears in the sample without gramicidin because of very low pH, close to 3, in which vesicles are no so tight. The pK_a of Glu^3 calculated based on the sample with gramicidin was much lower than in the same conditions but with Glu^3 trapped in the artificial liposomes (Figure 19). The pK_a 3,1 meant that the dye was degraded because of low pH (at the end of titration the pH was below 3) or, less likely, glutamic dendrons were digested by some proteases left in the vesicles.

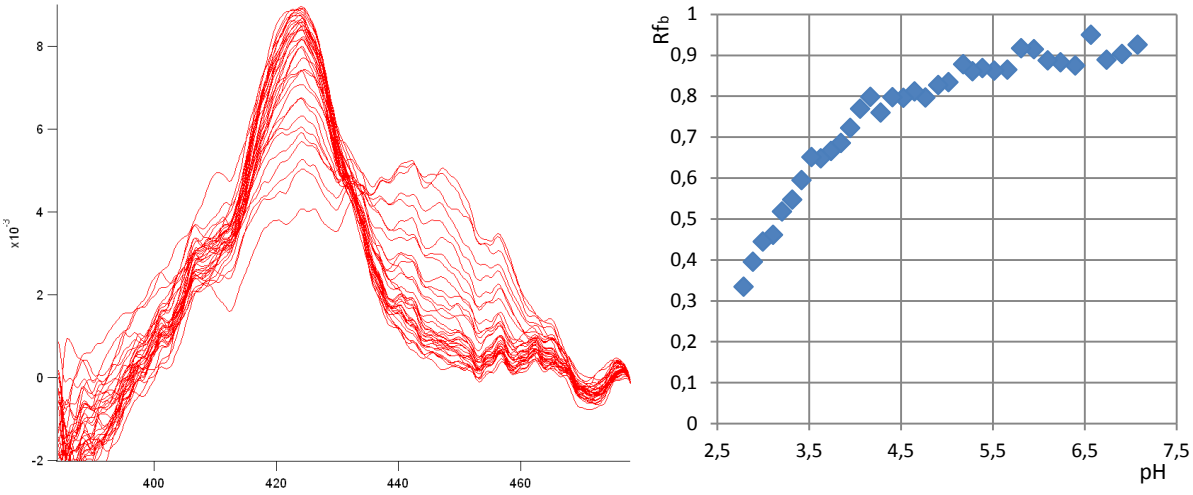

Figure 17. Acid titration of Glu^3 with plasma membrane vesicles in a presence of gramicidin in phosphate buffer (pH 7,0). pK_a=3.1.

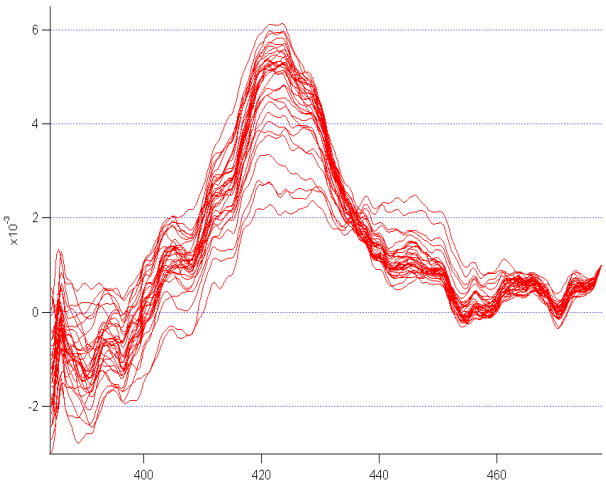

Figure 18. Acid titration of Glu^3 with plasma membrane vesicles in phosphate buffer (pH 7,0).

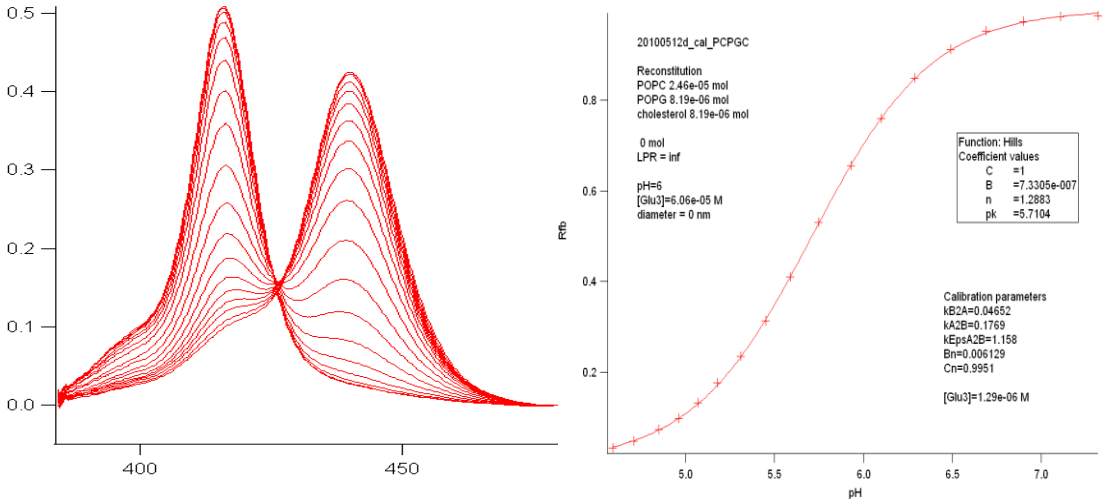

Figure 19. Acid titration of Glu^3 in artificial liposomes in the presence of gramicidin in phosphate buffer (pH 7,0). pK_a=5,7

4.5 Adapting pumping assay to phosphate buffer

The rates of proton pumping in this simplified assay were lower and for the MOPS-BTP solution were equal to 0,016 ΔAbs_{495nm}/min/mL. In the phosphate buffer decreasing of absorbance was slightly lower – 0,011 ΔAbs_{495nm}/min/mL, but in the presence of valinomycin it increased to 0,027 ΔAbs_{495nm}/min/mL what makes it useful for further measurements.

5 Conclusions

H^+-ATPase assay was successfully reproduced except the activation by the 14-3-3 protein. In case of the proton pumping due to presence of imidazole which works as an uncoupler, but the inhibition effect on ATP hydrolysis remains incomprehensible.

With the increasing concentration of Brij 58 the rates of proton pumping and ATP hydrolysis were increased. This trend was due to flipping and shrinking the vesicles.

Brij 58 affected the size of plasma membrane vesicles. The average diameter of vesicles shrank from 470 nm for native vesicles, down to 240 nm after detergent treatment.

Based on optical spectra of the vesicles containing Glu^3 solutions, the best method of preparing these vesicles turned out to be the sonication and gel chromatography. Brij 58 treatment gave less trapped dye and the centrifugation caused leakages.

The structure of Glu^3 was not stable inside the plasma membrane vesicles. pK_a of the dye decreased significantly which might be due to destruction of dendrons by low pH or proteases.

pK_a of Glu^3 in MOPS-BTP buffer was lower than in phosphate buffer. It was recommended to lead H^+-ATPase assay in phosphate buffer to have a higher resolution of proton pumping.

Activity of H^+-ATPase in phosphate buffer was lower than in MOPS-BTP, but still useful for the experiment.

6 Future work

Because natural plasma membrane vesicles affect Glu[3], and the yield of trapping the dye inside vesicles was low in the next step purified H^+-ATPase should be reconstituted in artificial liposomes. Experiment should be redo based on this new material.

7 Acknowledgments

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