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Coupling of proton translocation through ATPase incorporated into supported lipid bilayers to an electrochemical process [1]

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Coupling of proton translocation through ATPase incorporated into supported lipid bilayers to an electrochemical process [1]

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Abstract

H^+-ATPase is incorporated into solid-supported lipid bilayers separated from the gold support by a peptide spacer. The translocation of protons across the lipid film to the inner side is coupled to the discharge of protons at the gold surface. The overall process is investigated by square wave voltammetry (SWV) and double potential-pulse chronoamperometry (CA). As a result, the formation of a proton gradient is monitored by SWV whereas currents measured by CA monitor the stationary state when the enzyme activity is directly coupled to the charge transfer at the electrode. These currents markedly depend on the number of ATPases present in the bilayer. © 1997 Elsevier Science S.A.

Keywords: Solid-supported lipid layer; ATPase; Membrane proteins; Electrochemical coupling

1. Introduction

Model systems of biological membranes are liposomes [1], black lipid membranes (BLMs) [2], the interface between immiscible liquids (IIMLs) [3], and solid-supported lipid layers (SSLLs) [4]. The latter are the most recent ones investigated. SSLLs are prepared either as monolayers by chemisorption of lipids on mercury electrodes [5,6] or as bilayers on freshly cut metal electrodes [7]. Lipid bilayers are also formed by Langmuir–Blodgett techniques or by liposome fusion on top of hydrophillic surfaces [8,9]. SSLLs thus obtained, however, are not particularly designed to incorporate membrane proteins where an aqueous layer would be needed to separate the lipid layer from the solid support. In order to obtain such an aqueous layer, polyoxethylene [10,11] and other polymer spacer groups [12,13] were inserted between the hydrophillic head group of the phospholipid and the gold support. SSLLs with inserted peptide spacer groups have been shown to incorporate proton translocating F_0F_1 ATPases [14]. These ATPases were investigated earlier using other model systems such as liposomes and IIMLs. ATPases have been demonstrated in liposomes to transport protons at the expense of ATP hydrolysis from the outer to the inner aqueous phase [15]. The proton pumping activity has also been monitored across the IIML in the presence of lipids [16]. SSLLs with inserted spacer group can be seen as a hybrid between these two systems. They provide a lipid bilayer and, at the same time, two aqueous phases each connected to an electrode, as shown in Fig. 1. The peptide spacer is designed to mimic the inner aqueous phase of certain organelles such as chloroplasts and mitochondria. They also contain a protein network with many buffered and fixed-site groups. Fixed-site hydroxyl groups are introduced in our model system in order to accommodate an aqueous layer adjacent to the electrode. ATPases incorporated in this SSLL have been shown in a preliminary report to translocate protons from the outer to the inner aqueous phase where they are discharged at the gold electrode [14]. The principle of this process is illustrated in Fig. 1, the electrochemical properties of which will be described in greater detail in the present article.

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2. Experimental

2.1. Preparation of the lipid mono- and bilayers

The preparation of the tethered lipid mono- and bilayers with peptide spacers is described elsewhere [14]. In short the thiopeptide HS–$(CH_2)_2$–CO–Ala–Ser–Ser–Ala–Ala–Ser–Ala–COOH is obtained by solid phase peptide synthesis [17] using Fmoc(9–fluorenyl–methoxycarbonyl) strategy [18]. From this thiopeptide monolayers are formed on evaporated gold supports by self assembly. They are covalently linked to a phospholipid by in-situ coupling with DMPE (dimyristoylphosphatidyl ethanolamine) thus forming a lipid monolayer with peptide spacer. When these tethered lipid monolayers are exposed to a suspension of liposomes, they spontaneously start forming lipid bilayers as shown by surface plasmon resonance spectroscopy [14]. Liposomes with incorporated ATPases CF_0F_1 [15] and EF_0F_1 [19] from chloroplasts and *E. coli*, respectively, also fuse with the lipid monolayers to form lipid bilayers where the membrane proteins are incorporated. This is shown schematically in Fig. 2. Incorporation of the enzyme is indicated by an increased layer thickness as compared with the pure bilayers and also by different kinetics in real time of bilayer formation. Different from our previous work [14], bilayer formation is usually allowed to take place overnight while gold supports are incubated at 30°C in the liposome suspension. Electrochemical measurements are then carried out only after gentle rinsing of the support in the pure buffer solution. Previous measurements were performed with the liposomes still surrounding the electrode. Bilayers are shown to be stable for at least two days after rinsing.

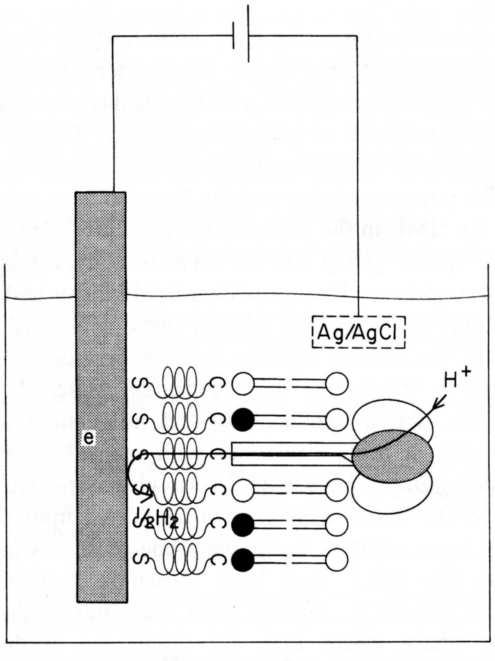

Fig. 1. Schematic representation of the coupling between the translocation of protons across the lipid bilayer catalyzed by the reconstituted ATPase and their discharge at the gold electrode.

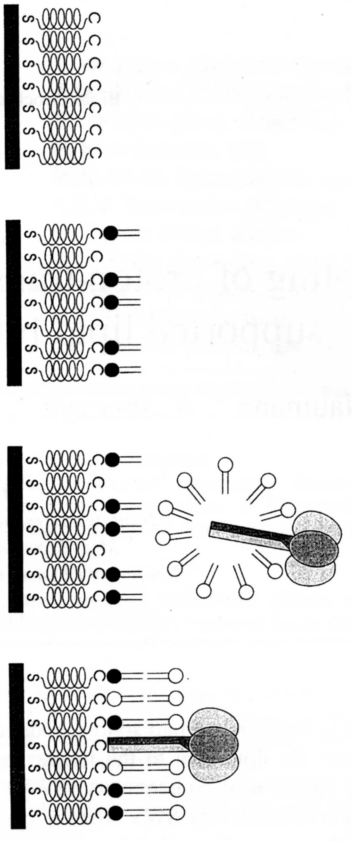

Fig. 2. Schematic representation of the formation of peptide monolayers, peptide-lipid monolayers and lipid bilayers with peptide spacer groups.

2.2. Preparation of the liposomes

Liposomes are prepared from phophatidylcholine and ATPase CF_0F_1 which is reconstituted into these liposomes by dialysis [20]. The concentration of the lipid is 8 mg ml^{-1}, while the protein concentration varied from 23 to 461 $\mu g\,ml^{-1}$ CF_0CF_1, thus varying the average number of enzymes incorporated corresponding to 1, 4, 10 and 20 CF_0CF_1 per liposome, respectively.

2.3. Electrochemical measurements

Electrochemical measurements are carried out using an Autolab instrument (ECO Chemie) and GPES3 software in

Table 1
Capacitance values of lipid layers measured by cyclic voltammetry

Layer	Capacitance $\mu F cm^{-2}$
Bare gold	18
Peptide-lipid, monolayer	3–5
Peptide-lipid, bilayer without protein	2–3
Peptide-lipid, bilayer with ATPase CF_0F_1	2–3
Peptide-lipid, bilayer with ATPase EF_0F_1	2–3

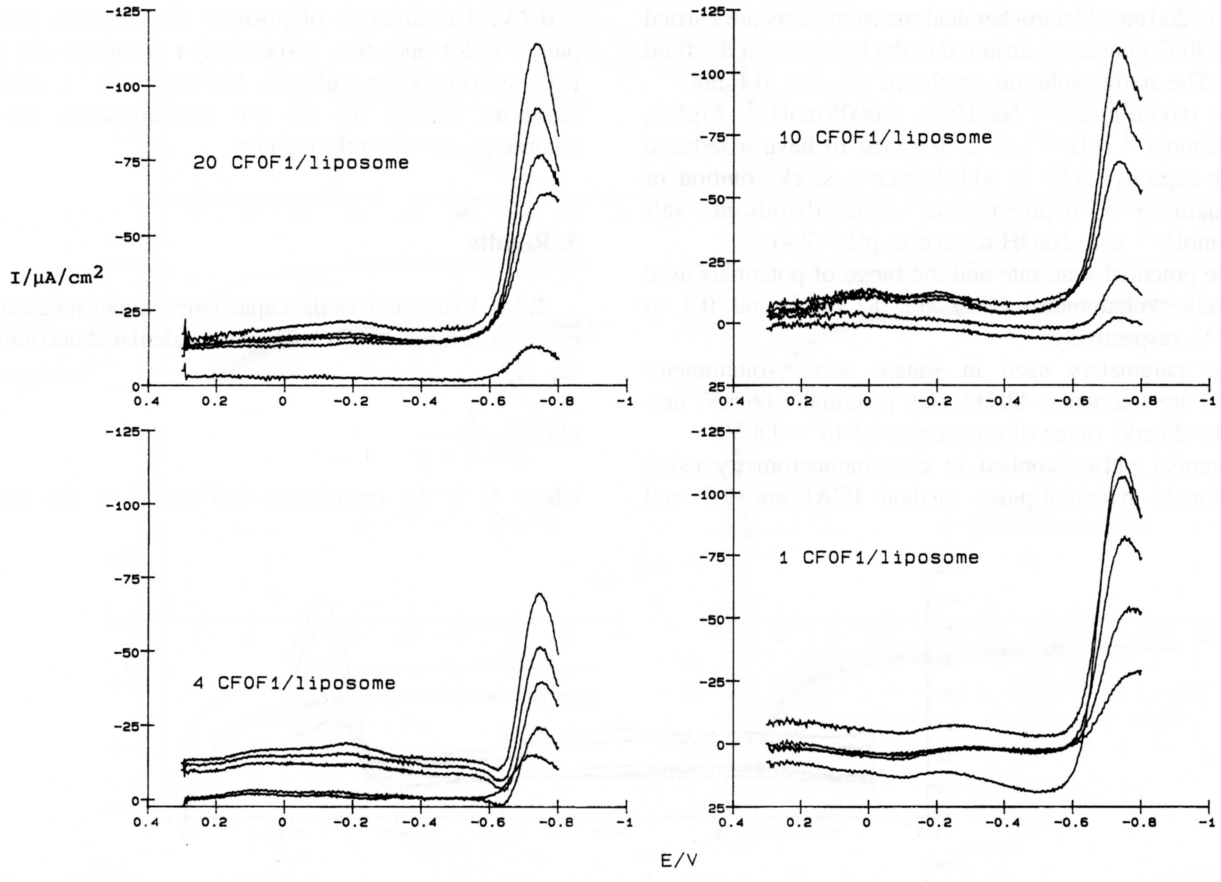

Fig. 3. Square wave voltammograms (80 Hz) at ATP concentrations of 2.5, 7, 29, 47, and 75 Mmol (normalized with respect to the background current with no ATP present) as a function of the number of ATPases per liposome.

a 5 ml cell equipped with a silver rod as counter electrode and an Ag|AgCl, sat. KCl as reference electrode. Gold supports having a circular active area of 0.2 cm^2 are used as working electrode. They are prepared by the deposition of gold on clean glass slides by electrothermal evaporation using a Leybold–Heraeus L 650 vapour deposition apparatus over a sublayer of 30 nm chromium at 300°C and a pressure of $10^{-5} \cdot 10^{-6}$ mbar. The thickness of the gold

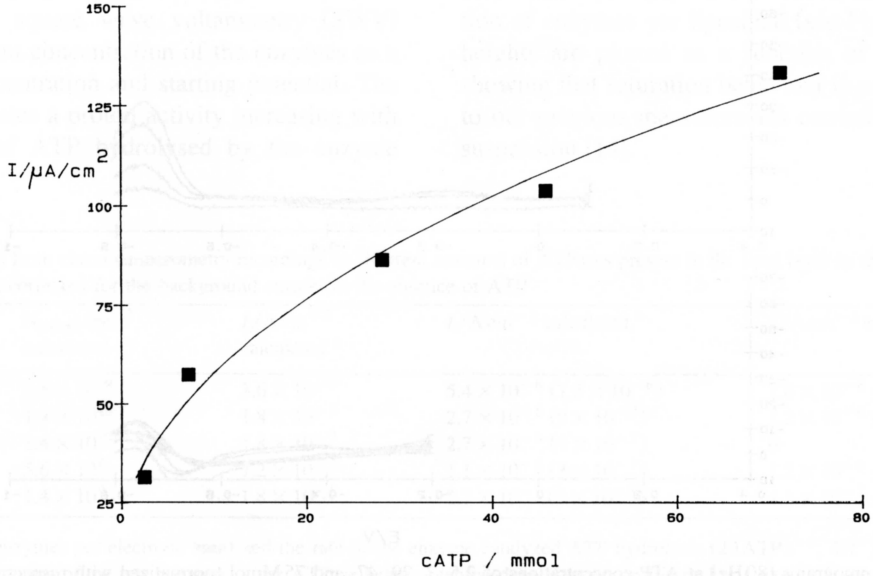

Fig. 4. Peak current at −0.7 V vs. ATP concentration of one of the SWV recordings shown in Fig. 3.

layer is 200 nm. Electrochemical measurements are carried out at 30°C in order to ensure that the lipids are in the fluid state. The buffer solution employed (K_2SO_4 0.1 mol l^{-1}, tricine 0.0005 mol l^{-1}, Na_2HPO_4 0.0005 mol l^{-1}, $MgSO_4$ 0.0002 mol l^{-1}, pH = 7.4) is designed to have a reduced buffer capacity. ATP is added from a stock solution of adenosine + 5′–triphosphoric acid disodium salt (0.25 mol l^{-1} with NaOH titrated to pH = 7.4).

The potential scan rate and the range of potentials used in cyclic voltammetry (CV) are 0.1 V s^{-1} and 0.3 to −0.2 V, respectively.

The parameters used in square wave voltammetry (SWV) are frequency: 80 Hz, step potential 3.66 mV, amplitude: 25 mV, range of potentials: 0.3 to −0.8 V.

Potential pulses applied in chronoamperometry using the double potential-pulse method (CA) are 0.3 and −0.7 V. The duration of positive and negative potential pulses is 2 s and 40 s, respectively. Potentials are given with respect to the Ag|AgCl, KCl(3.5 mol l^{-1}), reference electrode. Except for the CV measurements, the first recordings are taken throughout.

3. Results

Table 1 summarizes the capacitance values measured by cyclic voltammetry (CV). They are calculated according to Eq. (1)

$$C \equiv \frac{I}{2 \times A \times \nu} \tag{1}$$

where C is the capacitance ($\mu F\,cm^{-2}$), I the total of

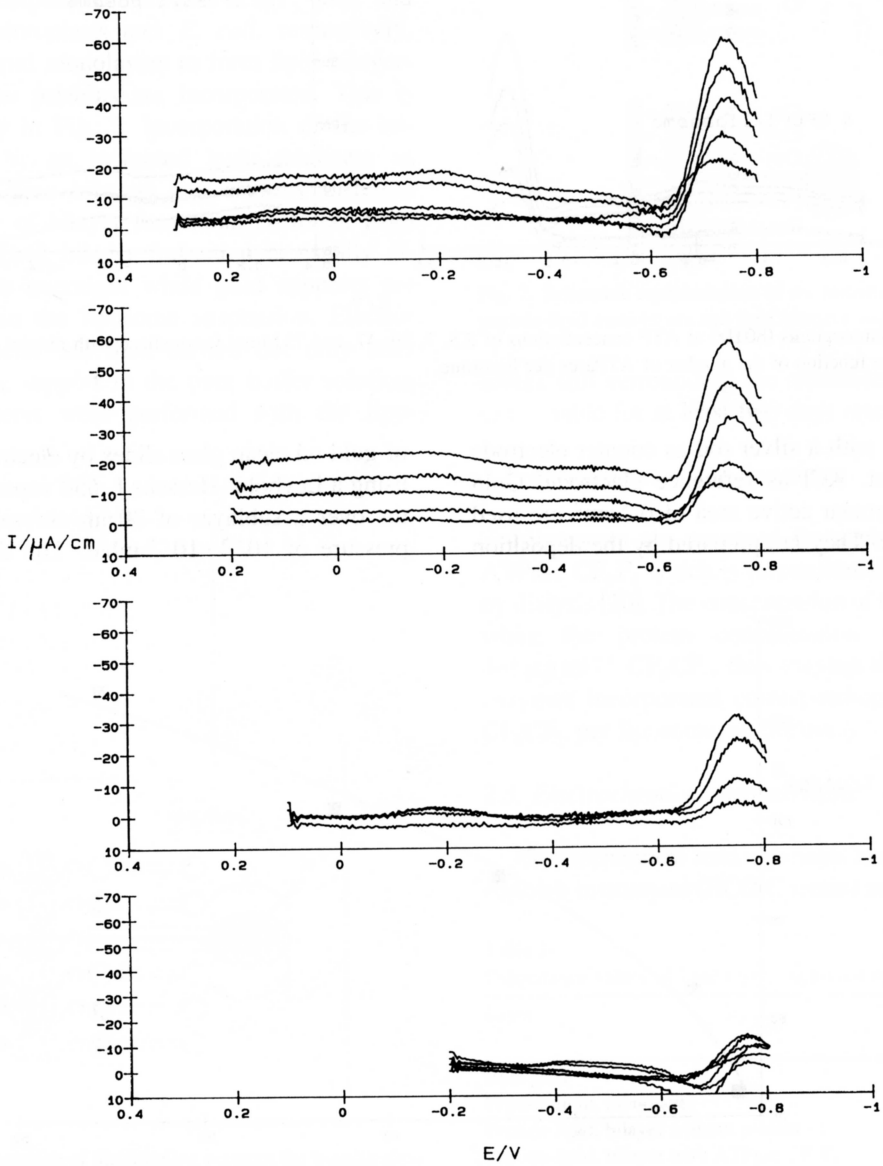

Fig. 5. Square wave voltammograms (80 Hz) at ATP concentrations of 2.5, 7, 29, 47, and 75 Mmol (normalized with respect to the background current with no ATP present) as a function of different starting potentials.

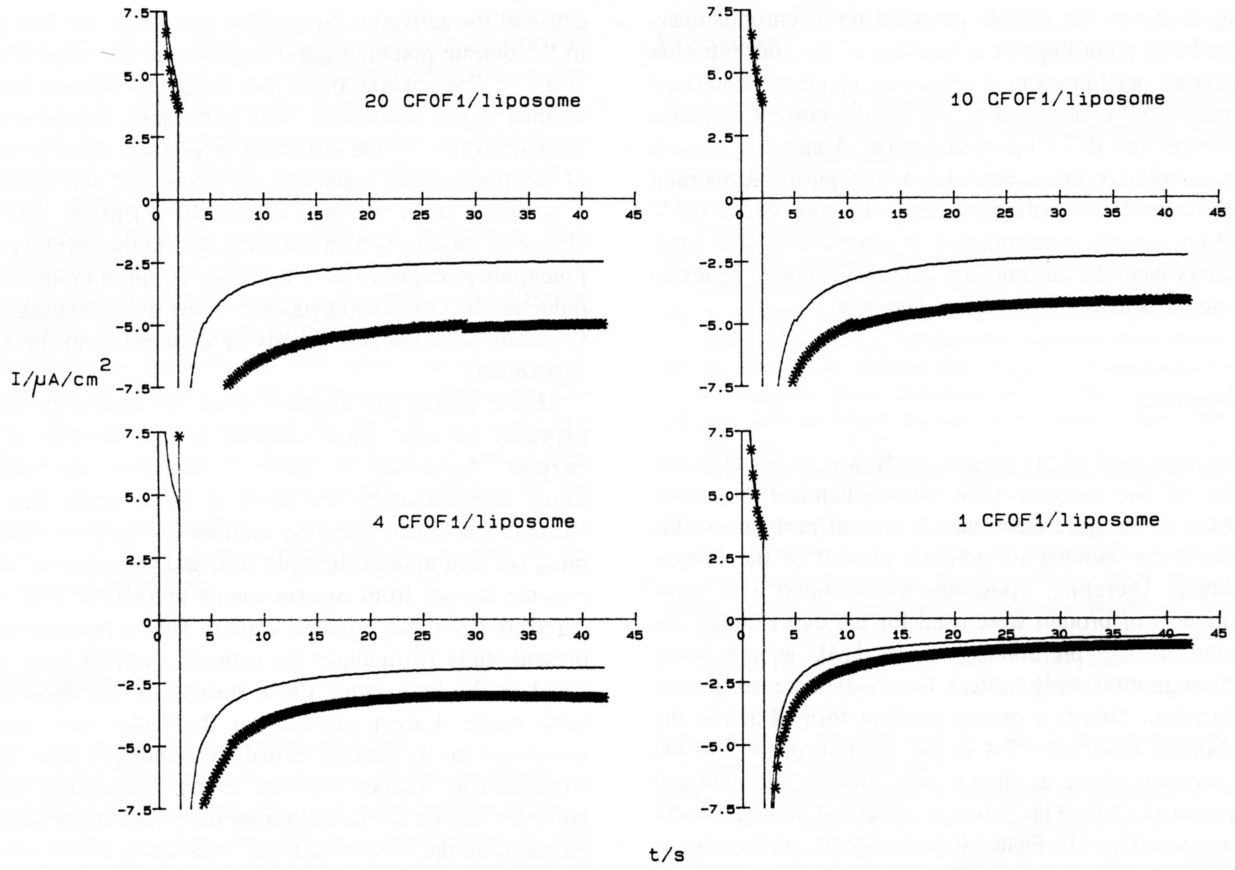

Fig. 6. Examples of double potential chronoamperograms as a function of the number of ATPases per liposome in the presence (————) and absence (· · · · · ·) of 5 Mmol ATP.

anodic and cathodic currents (μA) taken numerically at a potential of 0 V, A the electrode area (cm^2) and ν the scan rate ($V\,s^{-1}$). The capacitance of a perfect lipid layer is reported to be $0.5\,\mu F\,cm^{-2}$ [10]. Hence the values shown in Table 1 indicate imperfect lipid layers with and without incorporated ATPases [14].

Fig. 3 shows the square wave voltammetry (SWV) recordings at a constant concentration of the enzymes as a function of ATP concentration and starting potential. The peak at −0.7 V indicates a proton activity increasing with increasing amounts of ATP hydrolysed by the enzyme while protons are translocated to the inner side. As the starting potential becomes more negative, peak heights decrease, eventually vanishing when the starting potential becomes as negative as −0.2 V.

At the same starting potential SWV recordings do not show much variation as a function of different concentration of enzymes per liposome (see Fig. 4). In Fig. 5 peak heights are plotted as a function of ATP concentration, showing that saturation behaviour is not obtained, contrary to our previous measurements carried out in the liposome suspension [14].

Table 2
Stationary cathodic currents from chronoamperometry recordings at different amounts of ATPases present in the lipid layer in the presence of 5 mmol l^{-1} ATP in the buffer solution, corrected for the background current in the absence of ATP

Number of CF_0F_1 per liposome	N_{CF0F1} cm^{-2} calculated	$I/A\,cm^{-2}$ calculated [a]	$I/A\,cm^{-2}$ calculated [b]	$I/A\,cm^{-2}$ experimental
20	2.8×10^{10}	3.6×10^{-7}	5.4×10^{-6} (1.8×10^{-6})	$1.8\times10^{-6}\pm1.0\times10^{-6}$
10	1.4×10^{10}	1.8×10^{-7}	2.7×10^{-6} (9×10^{-7})	$1.2\times10^{-6}\pm5.5\times10^{-7}$
10 + tentoxin	1.4×10^{10}	1.8×10^{-7}	2.7×10^{-6} (9×10^{-7})	0
4	5.6×10^{9}	7.2×10^{-8}	1.1×10^{-6} (3×10^{-7})	$1.2\times10^{-6}\pm6.4\times10^{-8}$
1	1.4×10^{9}	1.8×10^{-8}	2.7×10^{-7} (9×10^{-8})	$4.1\times10^{-7}\pm1.3\times10^{-7}$

[a] calculated from N_{CF0F1} (enzymes per electrode area) and the rate of the enzyme catalyzed ATP hydrolysis ($20\,ATP\,s^{-1}$, $4H^+/ATP$).
[b] calculated under the assumption of the rate constant of the ATPase being potential-dependent according to $k = k\exp[-(1-\alpha)F\phi/RT]$ ($\phi = -0.7$ V, $\alpha = 0.9$), in brackets: the current calculated from the maximum turnover of the enzyme ($400\,H^+\,s^{-1}$).

Fig. 6 shows the double potential-pulse chronoamperometry (CA) recordings as a function of the concentration of enzymes per liposome at otherwise identical conditions including ATP concentration. An anodic current response follows the first (0.3 V) potential pulse. A cathodic current response follows the second (−0.7V) pulse. A marked dependence of the cathodic current (during the −0.7 V pulse) on enzyme concentration is observed in this case. Stationary cathodic currents are listed in Table 2. Tentoxin is shown to suppress the current response.

4. Discussion

The main goal of the present study was to correlate the activity of the enzyme with electrochemical processes occurring at the gold electrode. A critical problem in this context is the number of proteins present in the bilayer membrane. Therefore, liposomes reconstituted with varying amounts of protein were used for the experiments. As expected in the preliminary report [14], square wave voltammogramms only reflect the final state of proton translocation. This is a proton gradient formed across the lipid bilayer from pH = 7.4 in the outer to pH ≅ 5 in the inner aqueous phase as shown with ATPase reconstituted in liposomes [15]. The same is observed in our model system, see Fig. 3. Peak shaped signals increasing in height as a function of the ATP added indicate an increase in proton concentration in the thin aqueous layer adjacent to the electrode while the pH of the bulk aqueous phase remains unchanged (pH = 7.4). With a $K_m = 320\,\mu M$ the enzyme would be saturated at all ATP concentrations used in the experiments, where K_m is the Michaelis–Menten constant of ATP binding to ATPase. However, the addition of larger amounts of ATP also creates higher ratios [ATP]/[ADP]/[Π], thus shifting the chemical driving force, the free enthalpy of ATP hydrolysis

$$\Delta G = \Delta G^0 + RTln\frac{[ADP][\,\Pi]}{[ATP]} \qquad (2)$$

to numerically larger values ($\Delta G° = -36\,kJ\,mol^{-1}$, the standard free enthalpy of ATP hydrolysis). This is taken as a tentative explanation for the formation of increasing proton gradients as a function of ATP concentration where a saturation is not observed. It would be in line with the observation that the effect appears not to depend appreciably on the number of enzymes incorporated under otherwise identical conditions. Another feature of the ATPase of chloroplasts can be observed in the SWV measurements: According to Fig. 4 the magnitude of the proton gradient depends on the starting potential. Positive potentials on the inner side of liposomes reconstituted with CF_0F_1 ATPase are necessary to activate the enzyme [21]. This would account for the smaller proton gradients formed with increasingly negative potentials indicating that the enzyme senses the potential applied to the electrode. Because of the activation by positive potentials, the first pulse in the double potential pulse experiment was chosen to be +0.3 V. The positive pulse also brings the system back to defined initial conditions. This is because, except for hydrogen formed by the reduction of protons, other products of electrochemical reduction are reoxidized and hydrogen is removed from the gold surface by diffusion. The discharge of protons is then observed during the second pulse. Potentials as negative as −0.7 V are required to induce the reduction of protons to hydrogen at the gold electrode. The reduction potential can already be deduced from the SWV experiment.

Hence during the negative pulse the discharge can be expected to take place coupled to the activity of the enzyme. According to Table 2, however, the currents found experimentally are about 8 times larger than the currents calculated from the number of enzymes incorporated per unit area of the lipid film and the turnover of the enzyme known from measurements in vesicles [14] compared to 100 times reported earlier. This is because in the present study particularly the cathodic currents were measured in the long range (40 s) instead of the short range (ms) mode. Larger currents in the latter case can be accounted for by protons already accumulated prior to the measurement. Larger currents in the long range mode, however, can be accounted for by the effect of the negative potential on the enzyme activity. According to the work of Läuger [22], the rate constants of the proton pumps strongly depend on potentials, particularly those applied externally. The potential dependence is given by Eq. (3)

$$\tilde{k} = k\exp\frac{-(1-\alpha)F\phi}{RT} \qquad (3)$$

where k is the overall rate constant (s^{-1}), since single-step rate constants of the ATPase are not known [15], α the symmetry factor, ϕ the applied potential (V) and F, R and T having the usual meanings. As for the symmetry factor of 0.9, it is considered that hydrolysis takes place in the F_1 part of the ATPase and protons have to transfer almost the entire width of the lipid membrane along the F_0 channel. Taking into account the turnover of the enzyme measured by the stopped flow technique in liposomes without an applied potential the calculated currents are too small compared with those found experimentally, see the third column in Table 2.

However, using Eq. (3) and assuming the enzyme senses the externally applied potential (−0.7 V), the currents would be limited only by the maximum turnover of the enzyme also measured in liposomes. Taking into account these effects, the calculated currents given in column 4 of Table 2 are in fairly good agreement with those found experimentally. This applies to stationary currents measured by CA. A full analysis of the chronoamperograms, e.g. with respect to the rate limiting step could not be performed. The reason is an unspecific background current at the gold electrode [23]. As for the number of enzymes

per unit area of the lipid film, according to the currents measured, they are not very well correlated to the number of enzymes incorporated in the liposomes. This might be caused by the fact that proteins accumulate in lipid layers reconstituted with a lower average number of enzymes. Currents measured with higher amounts of enzyme, however, exhibit markedly larger standard deviations. This could be as a result of either an uneven distribution of the enzymes or to a different fusion behaviour of the different populations. Further investigations are under way to clarify these questions. An optimum with respect to reproducibility was found between 4–10 ATPases per liposome. Cathodic currents are suppressed by DCCD, an unspecific inhibitor of the enzyme as shown earlier [14], and also by tentoxin, an extremely species-specific inhibitor of the ATPase from chloroplasts. This is taken as a further indication of the enzyme activity being coupled to the electrochemical process.

5. Conclusion

From these results it is concluded that

- due to the activity of the enzyme proton gradients are formed across the lipid film and monitored by SWV.
- taking into account the potential dependence of rate constants, steady state cathodic currents recorded by CA in the long range mode appear to be directly coupled to the translocation of protons catalyzed by the enzyme.
- the concentration of enzymes per unit area of the lipid bilayer is affected by the concentration of the enzymes in the liposomes.
- the enzyme senses the potential applied to the electrode either for activation of the enzyme or for discharge of protons.

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