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Monitoring of the Membrane Potential in Proteoliposomes with Incorporated Cytochrome-c Oxidase Using the Fluorescent Dye Indocyanine

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Abstract. A method has been developed to monitor changes of the membrane potential across vesicle membranes in real time. Using the potential-sensitive fluorescent dye indocyanine and on the basis of a water/lipid redistribution model, a calculation procedure has been introduced to estimate the membrane potential in vesicles with incorporated cytochrome-c oxidase. Physical parameters, such as vesicle size distribution and density of the lipid bilayer were estimated and used as calculation parameters. By extrapolation of the transient potential change to zero time, the initial rate of the potential change (dU/dt) could be calculated. It is also shown, that the initial potential change (dU/dt) may be used to study the proton/electron stoichiometry of cytochrome-c oxidase incorporated in the vesicles.

Key words: Cytochrome-c oxidase — Proteoliposomes — Membrane potential — Fluorescence — Electrogenic activity — Indocyanine dye

Introduction

Cytochrome-c oxidase is the terminal enzyme of the respiratory chain which catalyzes the four-electron reduction of molecular oxygen to water and couples this reaction with the uptake of protons from the mitochondrial matrix site. In this way, the free energy of oxygen reduction is stored in the form of a membrane potential (Mitchell, 1966). Since the proposal of Wikström, we know that cytochrome-c oxidase, in addition to the uptake of protons for water formation, is also capable of vectorial proton translocation and acts as an electrogenic proton pump (Wikström, 1977). However, the molecular mechanisms and the H^+/e^--stoichiometry of proton translocation by the cytochrome-c oxidase are still unknown. In earlier studies of the electrogenic activity of cytochrome-c oxidase, the changes of the proton concentration across the lipid membrane have been directly measured with a pH-microelectrode or a pH-indicator dye and related to the protein activity (Casey, Chappell & Azzi, 1979; O'shea et al., 1984; Papa et al., 1991; Capitanio et al., 1991). These measurements, however, are always accompanied with an uncertainty concerning the buffer capacity of the vesicle interface which should be taken into account when the real quantity of ejected protons have to be calculated (Proteau et al., 1983; Heberle et al., 1994). An alternative possibility of investigating the proton translocation is the measurement of the membrane potential developed during protein action. Methods to measure the membrane potential generated by cytochrome-c oxidase have been developed earlier based on indirect detection of the redistribution of lipophilic cations by ion selective electrodes (Shinbo et al., 1978; Brown & Brand, 1985; Steverding & Kadenbach, 1991). Because of slow response time of the ion-selective electrodes, the experimental setup presented in these works could be used under steady-state conditions, but it does not allow kinetic measurements of the membrane potential. The above considerations show that the investigation of electrogenic activity of the cytochrome-c oxidase and its ability of vectorial proton translocation requires an appropriate method with a fast response time, which could allow the recording of proton translocation events in real time.

The application of voltage-sensitive fluorescent dyes allows the estimation of the membrane potential of cell organelles and membrane vesicles (Bashford & Smith, 1978; Apell et al., 1985; Apell & Bersch, 1987). The mechanism of fluorescence response of the dye to an electrical potential difference across the lipid bilayer is still a matter of discussion (Sims et al., 1974; Beeler et al., 1981; Läuger, 1991). Under certain experimental

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conditions, the fluorescence alteration could be described by a simple redistribution model (Apell & Bersch, 1987). Fast response times of voltage-dependent fluorescent dyes makes them useful in investigations of the electrogenic activity of ion pumps reconstituted in vesicles (Waggoner, 1979; Beeler, Farmen & Martonosi, 1981). In this work, I present a method for real-time-monitoring of the membrane potential generated by the cytochrome-c oxidase using the potential sensitive fluorescent dye indocyanine.

Materials and Methods

Materials

Asolectin, L-α-phosphatidylcholine (type II from soybean) and cytochrome c (type VI from horse heart) were obtained from Sigma. Before use, asolectin was purified by the method of Kagawa and Racker (1971). Valinomycin was from Boehringer, Mannheim. Indocyanine[1] (NK 529) was purchased from Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan. The dialysis tubing (pore-radius 2.4 nm) was purchased from Serva, Heidelberg. Fluorescence experiments were carried out on a Perkin-Elmer 650–40 fluorescence spectrophotometer.

Buffers

If not otherwise indicated, the buffer A contained (in mM): 10 K-Hepes, pH 7.4, 1.7 NaCl and 200 KCl. Buffer B contained 10 K-Hepes, pH 7.4, 200 NaCl and 1.7 KCl. Buffer C contained 10 K-Hepes, pH 7.4, 10 NaCl and 140 KCl.

Isolation of Cytochrome-c Oxidase

Bovine heart cytochrome-c oxidase was prepared from isolated mitochondria as described in (Errede, Kamen & Hatefi, 1978).

Determination of the Cytochrome aa_3 Content

The cytochrome aa_3 content was calculated according to (Von Jagow & Klingenberg, 1972). The extinction coefficients for cytochrome aa_3 used were $\Delta A^{red\text{-}ox}{}_{605-630}$ nm = 24.0 mM^{-1} cm^{-1}, $\Delta A^{ox}_{603-650}$ nm = 40.0 mM^{-1} cm^{-1}, $\Delta A^{red}{}_{443-490}$ nm = 204.0 mM^{-1} cm^{-1}, $\Delta A^{ox}_{421-490}$ nm = 140.0 mM^{-1} cm^{-1}.

Measurement of Oxygen Consumption

Oxygen consumption was measured polarographically by using a Clark-type oxygen electrode attached to a thermostatically controlled cell (Brautigan, Ferguson-Miller & Margoliash, 1978). 10–20 μl vesicles with reconstituted cytochrome-c oxidase were introduced in 1.5 ml of buffer A with 25 mM potassium ascorbate and various concentrations of cytochrome c (1–50 μM). For measurements of the enzyme activity 0.05% laurylmaltoside and 0.1 mM EDTA were also added. If not otherwise indicated, the experiments were carried out at 20°C.

Vesicle Preparation

The enzyme was reconstituted into vesicles by the following procedure: purified asolectin (30 mg/ml) and 1.5% (w/w) Na-cholate was sonicated to clarity in buffer C. Cytochrome-c oxidase was added to the solubilized lipid to give a protein to lipid ratio of 1/50 (w/w). After brief mixing, the combined solution was transferred to 6 mm dialysis tubing and dialyzed for 48 hr at 4°C against a 500-fold excess volume of buffer C. During the dialysis, buffer C was replaced twice. To calibrate the membrane potential in control experiments, part of the vesicles was prepared in buffer A and then dialyzed additionally for 24 hr against buffer B to reach equal osmotic conditions but different inside/outside concentrations of K^+. Protein-free vesicles were prepared from purified asolectin by the same procedure without the addition of protein.

Determination of the Lipid Content

The lipid concentration in the suspension was estimated by determination of choline containing lipids using the phospholipid B test (Takeyama et al., 1977). The amount of choline containing lipids was related to that of all lipids in the asolectin composition. The concentration of choline containing lipids in the asolectin estimated in our laboratory (30%) was in a good agreement with published data (Kagawa & Racker, 1971).

Electron Microscopy of the Vesicles

The electron microscopic imaging of the vesicles was performed using the method of negative staining with ammonium molybdate $(NH_4)_6Mo_7O_{24}$ (Haschmeyer & Myers, 1972).

Fluorescence Measurements

Fluorescence experiments were carried out in a thermostatically controlled cuvette holder which was equipped with a magnetic stirrer. The excitation wavelength was set to 620 nm (slit width 20 nm) and the emission wavelength to 660 nm (slit width 5 nm). The indocyanine stock solution contained 5.76 mM dye in ethanol. From this solution dilutions were prepared daily by mixing with ethanol. Concentrations were chosen such that addition of 1 μl dye solution to the cuvette resulted in the desired final concentration (1–3 μM). All fluorescence data were normalized with respect to a fluorescence standard.

The cuvette was filled with 1 ml buffer and equilibrated in the cuvette holder to the desired temperature, then 1 μl of an indocyanine solution was added. After the fluorescence signal was constant, an aliquot of the vesicle suspension was added. Fluorescence changes, ΔF, caused by additions of reagents were determined as relative signal changes with respect to the fluorescence level, F, prior to the addition; they were corrected for the small dilution effect which was determined separately by adding a known amount of buffer solution. Independent experiments (*data not shown*) revealed that addition of 1–3 μl of ethanol had no effect on the fluorescence. At the beginning and at the end of each experiment the temperature in the cuvette was controlled. All experiments were carried out at 20°C.

[1] Indocyanine: 1,3,3,1′,3′,3′-Hexamethyl-2,2′-indodicarbocyanine iodide

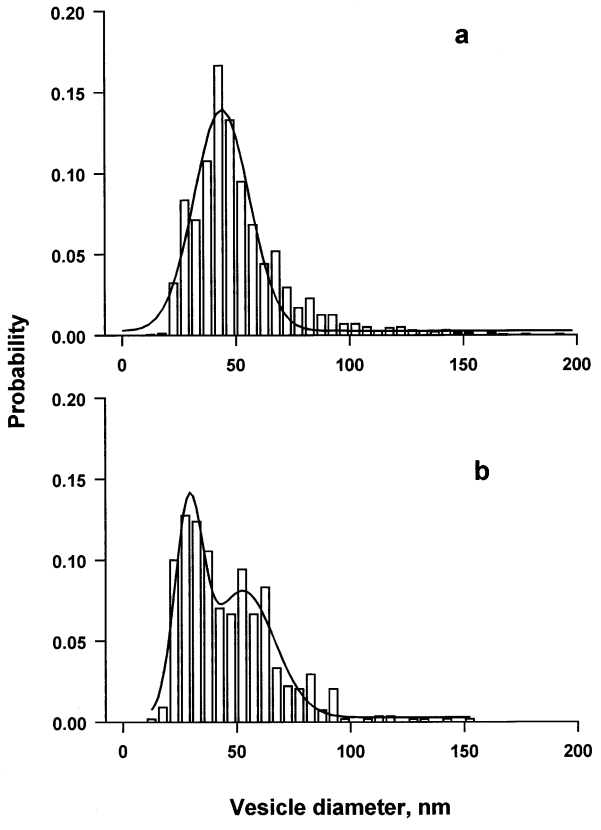

Fig. 1. Statistical analysis of the size distribution of asolectin vesicles without (*a*) and with cytochrome-c oxidase (*b*). The analysis of the size distribution has been done by measuring the diameter of each vesicle, followed by fitting of the data to the normal Gaussian distribution. The estimated average diameter for protein-free vesicles corresponds to 44 ± 12 nm, for protein containing vesicles 52 ± 14 nm.

Results

Physical Parameters of the Vesicles

The negative staining electron microscopy of the prepared vesicles revealed a rather narrow size distribution of mainly spherical vesicles. On the images of the vesicles an insignificant amount of nonspherical particles could be seen which were attributed to nonvesicular aggregates (*micrographs not shown*). The size distribution of the vesicle population was analyzed by measuring the diameter of each vesicle followed by fitting of the data to the normal Gaussian distribution. For the statistical analysis about 1,500 vesicle diameters were determined. By this method the nonvesicular fragments could be excluded from the analysis. Fig. 1*a* and *b* represents the probability function of the vesicle diameters in the range of 20–200 nm. The size distribution of vesicles without cytochrome-c oxidase, Fig. 1*a*, was in a good agreement with a Gaussian distribution, and the determined average diameter was 44 ± 12 nm. The size distribution of vesicles with incorporated cytochrome-c oxidase (Fig. 1*b*) clearly shows two vesicle populations, one of 29 ± 6 nm and the other of 52 ± 14 nm average diameter. The formation of two vesicle populations after protein reconstitution could be explained if we assume that not all vesicles in the population contain protein. This assumption has been supported experimentally by use of ion-exchange chromatography. The difference in diameter of protein-free and protein-containing populations could have resulted from the enlargement of vesicles due to protein incorporation (Madden, Hope & Callis, 1984). The formation of protein-free vesicles with an average diameter smaller than 44 nm is difficult to explain so far. One explanation could be that introducing the third component — protein to the lipid-detergent mixture could also influence the formation and size of protein-free vesicles. This subdivision of two vesicle populations could be analyzed statistically if we assume that the number of incorporated proteins is controlled by Poisson statistics. If we further assume that during the procedure of vesicle preparation most of the protein is incorporated into the vesicles, the protein-to-lipid ratio used in this work (*see v*esicle *p*reparation) predicts an average monomeric enzyme complex per vesicle of 1.9–2.2 According to the Poisson distribution of the enzyme, 40% of the vesicles should be protein free. This estimate is in a good agreement with the distribution represented in Fig. 1*b*. In our calculations, we used the molecular weight of the monomeric complex of cytochrome-c oxidase of 204,000 Daltons (Kadenbach et al., 1986). It is still not clear, however, whether the monomer or dimer is the functional unit of the reconstituted cytochrome-c oxidase.

To study the distribution of indocyanine between the water- and lipid phases, we need to know the volume of asolectin in the vesicle probe. This could be calculated if we know the density of the asolectin used. The size of the vesicle and the number of lipid molecules assembled in a vesicle give us the information about the average molecular weight and density of the asolectin. These calculations are based on data published earlier (Huang & Mason, 1978). From these data we assume that the radius of the lipid head group is 4.85 Å in the outer and 4.41 Å in the inner monolayer. Therefore, the area occupied by a lipid molecule in both monolayers can be calculated to be 74Å^2 and 61 Å^2, respectively. Protein-containing vesicles have an external diameter of 52 ± 14 nm (*see above*). If the lipid bilayer is assumed to be packed with the highest possible density of lipid head groups, a vesicle contains $8.273 \cdot 10^4$ lipid molecules with a deviation between $4.292 \cdot 10^4$ and $1.355 \cdot 10^5$ molecules due to the standard deviation of the vesicle diameter.

To calculate the average molecular weight and den-

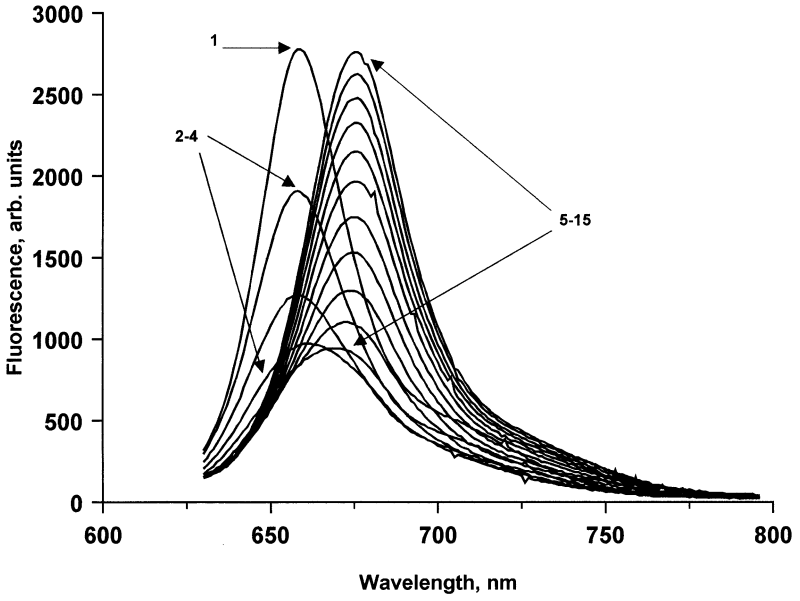

Fig. 2. Dependence of the fluorescence of indocyanine from the quantity of the vesicles in the water solution. 1, fluorescence spectra of the indocyanine (2.88 μM) in buffer A (in mM: 140 KCl, 10 NaCl, 10 Hepes, pH = 7.4). 2–15, consecutive addition of aliquots (15 μg asolectin) of the protein-free vesicle suspension. The excitation wavelength was 620 nm, slit width 20 nm and 5 nm.

sity of the asolectin we have measured the entrapped volume for a known quantity of lipid. Vesicles were prepared as described above with known concentration of K_2CrO_4, separated on a Sephadex G25 column and the lipid concentration was estimated. The whole entrapped volume was determined spectroscopically, assuming that the K_2CrO_4 concentration inside the vesicles remains constant. The average molecular weight and density of the asolectin estimated in such a way were 880 g/mol and 1028 mg/ml respectively.

Spectral Properties of Indocyanine

Indocyanine has its fluorescence emission maximum at 658 nm and its absorption maximum at 635.7 nm. The additional shoulders between 570–600 nm (absorption spectra) and 690–720 nm (fluorescence spectra) indicate the presence of higher aggregates of the dye in aqueous solution, as has been suggested by Sims et al. (1974). The fluorescence maximum of cyanine dyes shifts further to the red when one transfers the dye from water into nonpolar solvents. Consistent with this finding, the fluorescence maximum of indocyanine is shifted after interaction with lipid vesicles from 658 nm to 676 nm. This is shown in Fig. 2, which represents the fluorescence spectra of indocyanine as a function of the concentration of protein-free vesicles. Not only a red shift of the fluorescence spectra, but also a quenching effect could be seen during the vesicles addition, which is well-defined in the lipid concentration range between 0 and 60 μg/ml.

A fluorescence change similar to that seen after vesicle addition could be observed when a membrane potential (inside negative) across the lipid bilayer of the vesicles was generated. The exact mechanism which is involved in the fluorescence change during the polarization of the vesicle membrane is still unknown. It has become evident, however, that the behavior of many potential sensitive fluorescent dyes can be described by a distribution model (Sims et al., 1974; Apell et al., 1985; Apell & Bersch, 1987; Zouni et al., 1993). Normally the lipophilic cation indocyanine is distributed between the water and lipid phases according to its partition coefficient. Polarization of the lipid membrane by the generated membrane potential leads to redistribution of the dye molecules between the water and lipid phases because of electrostatic attraction between the positively charged indocyanine and the negatively charged vesicle interior.

As can be seen from Fig. 2, there were two fluorescence effects which appeared during the vesicle addition. First, there was a general red shift of the spectra of indocyanine (658 nm → 676 nm), and second, there was a pronounced quenching effect at the lipid concentrations 0–60 μg/ml (additions 2–4, Fig. 2). If we consider the distribution model, with an increasing vesicle concentration the amount of membrane-bound dye molecules also increases, and consequently the concentration of the dye molecules in water decreases. This would lead to a pure shift-effect of the indocyanine spectra with a constant quantum yield. The position of the peak maximum would then reflect the dye-bound/dye-free ratio. In fact, we observe not only the red shift of the peak maximum, but additionally there is a strong quenching effect which makes the analysis of the dye distribution more complex. It seems that the most appropriate description of the dye-vesicle interaction is a mechanism of dye binding to a vesicle with a discrete number of binding sites (Clarke,

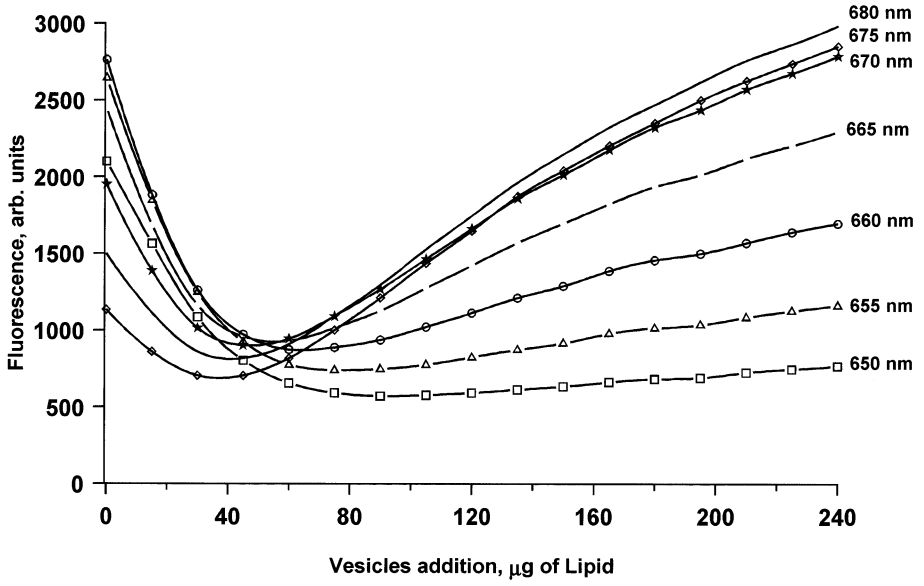

Fig. 3. Dependence of the fluorescence of indocyanine from the quantity of vesicles in the solution at various fixed emission wavelengths. Initial concentration of indocyanine was 2.88 μM in buffer A (in mM: 140 KCl, 10 NaCl, 10 Hepes, pH = 7.4).

1991; Clarke, Schrimpf & Schöneich, 1992). If we assume that the initial concentration of dye in the water phase is sufficient for the saturating occupation of binding sites at small vesicle concentration and, because of that, the quenching of the fluorescence of the bound dye molecules occurs due to their aggregation, the fluorescence change in this case would be determined mainly by the change of the dye concentration in water. This is what we observe in Fig. 2. After the vesicle addition (2–4) the fluorescence intensity decreased and the position of the peak (658 nm) remained nearly unchanged. If after the addition of small amounts of vesicles to the appropriate concentration of dye not all of the binding sites are occupied, it would still be possible to apply the distribution model to describe the partition of the dye between water and lipid vesicles. The partition coefficient will then express the affinity of binding dye molecules to the vesicles (Zouni et al., 1993).

In Fig. 3 the fluorescence change of indocyanine is shown as a function of the lipid concentration at various fixed wavelengths. The wavelength at which linearity combines with a large fluorescence change in response to the change in vesicle concentration (dF/dC_{lipid}) would be the appropriate fixed wavelength to monitor the dye-vesicle interaction. In the following experiments a fixed wavelength of 660 nm was chosen because, in the range of lipid concentrations 5–30 μg/ml, the negative fluorescence change shows approximate linearity and the magnitude of dF/dC_{lipid} is comparatively high.

In the absence of lipid vesicles, the total fluorescence (F_0) is determined only by the amount of dissolved dye molecules. The fluorescence of indocyanine in water is a function of its concentration, ionic strength (and/or buffer composition) and aggregation state of the dye molecules. In Fig. 4 the fluorescence of indocyanine (F_0) at 660 nm can be seen as a function of its aqueous concentration. F_0 is referred to the fluorescence intensity measured with a fluorescence standard and expressed in dimensionless numbers. To describe this dependence we have used the following equation:

$$F_0 = Fm \cdot \left[1 - \exp\left(-\frac{c_w}{\beta} \right) \right] \tag{1}$$

where Fm is the maximum fluorescence, c_w is the concentration of indocyanine in water and β is a constant parameter which represents a value proportional to the magnitude of the inner filter effect of indocyanine. Because of the significant degree of overlap between the absorbance and fluorescence spectra of indocyanine (absorption max. at 636, fluorescence max. at 658), its fluorescence spectra is not linear. Equation (1) represents the solution of the differential Eq. (2):

$$\frac{dF_0}{dc_w} = Fm - \frac{F_0}{\beta} \tag{2}$$

As can be seen Fig. 4, Eq. (1) describes quite well the concentration dependence of the fluorescence of indocyanine in the range of 0–4 μM.

Estimation of the Partition Coefficient at Zero Membrane Potential

The membrane-water partition coefficient, γ, is defined by:

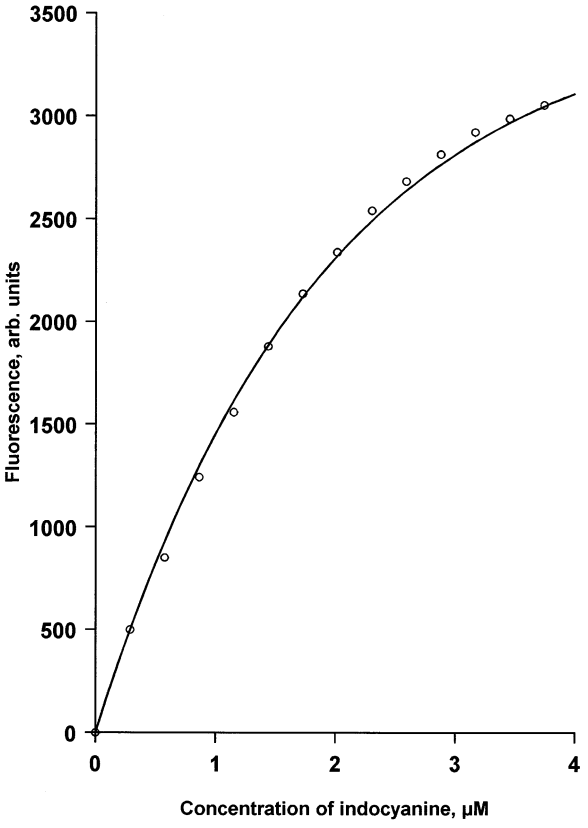

Fig. 4. Dependence of the fluorescence of indocyanine on its concentration in solution. Measurement buffer was (in mM) 140 KCl, 10 NaCl, 10 Hepes, pH = 7.4. Open circles are measured values; the smooth line is the fitted function Eq. (1) with parameters: $Fm = 3527$, $\beta = 1.883$.

$$\gamma = \frac{c_l}{c_w} = \frac{n_l/V_l}{n_w/V_w} \tag{3}$$

where c_1 and c_w are the concentrations of the dye in the lipid phase and in the water phase, n_1 and n_w are the amounts of dye in the lipid phase and in the water phase and V_l and V_w are the volumes of the lipid and water phases. If n_0 is the total amount of the dye, the amount of the dye located in the lipid bilayer can be calculated by:

$$n_1 = n_0 - n_w \text{ or } n_l = V_w \cdot (c_0 - c_w) \tag{4}$$

Where c_0 and c_w are the initial and postredistribution concentrations of dye in the water phase. Measuring the dye concentration before and after addition of vesicles we can estimate the membrane-water partition coefficient of indocyanine, which is defined by:

$$\gamma = \frac{c_l}{c_w} = \frac{(c_0 - c_w) \cdot V_w}{c_w \cdot V_l} \tag{5}$$

Rearranging Eq. (1) gives the following expressions for c_o and c_w:

$$c_0 = -\beta \cdot ln\left(1 - \frac{F_0}{F_m}\right) \tag{6}$$

$$c_w = -\beta \cdot ln\left(1 - \frac{F}{Fm}\right) \tag{7}$$

and the partition coefficient will be defined as:

$$\gamma - \frac{\left[\beta \cdot ln\left(1 - \frac{F}{Fm}\right) - \beta \cdot ln\left(1 - \frac{F_0}{Fm}\right)\right] \cdot V_w}{\left[-\beta \cdot ln\left(1 - \frac{F}{Fm}\right)\right] \cdot V_l} - \frac{ln(Fm - F) - ln(Fm - F_0)}{lnFm - ln(Fm - F)} \cdot \frac{V_w}{V_l} \tag{8}$$

where F_0 is the fluorescence of indocyanine in water in absence of lipid vesicles, F — the fluorescence of the dye after addition of a given amount of vesicles and Fm was obtained as a fitted parameter from Fig. 4.

Figure 5 represents the estimation of the partition coefficient at 0 mV for various vesicle concentrations in the solution. The volume of the lipid phase was calculated from the density of asolectin ($1.0276 \cdot 10^3$ mg/ml), *see above*, and the volume of the water phase (V_w) was 1 ml. The slope of the straight line in Fig. 5 defines the partition coefficient which corresponds to $\gamma = 68.5624 \cdot 10^3$.

Calibration of the Fluorescence Signal as a Function of Membrane Potential

Membrane potentials may be generated by establishing a transmembrane K^+ concentration difference in the presence of valinomycin. At the beginning of the experiment the vesicle interior contains 200 mM KCl (buffer A); the extravesicular aqueous space contains 1.7 mM KCl (buffer B). The addition of valinomycin to the solution makes the vesicle membrane selectively permeable for the K^+ cations. The intravesicular concentration of K^+ is decreased by the release of K^+ ions, which builds up a Nernst potential of 120.4 mV (inside negative), according to

$$U = \Psi' - \Psi'' = \frac{RT}{F} \cdot ln\frac{c''}{c'} \tag{9}$$

ψ' and ψ'' are the intra- and extravesicular potentials, respectively, and c' and c'' are the corresponding K^+ concentrations; R is the gas constant, T the absolute temperature and F the Faraday constant.

After the inside-negative membrane potential is generated, further accumulation of indocyanine in the lipid phase occurs. According to the new equilibrium state, the partition coefficient changes. The new (apparent)

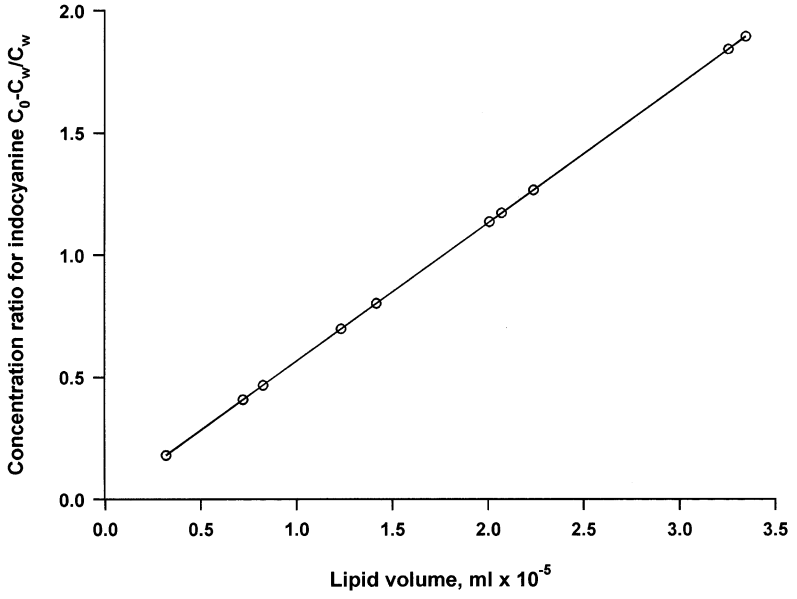

Fig. 5. Estimation of the partition coefficient at 0 mV voltage. C_0—is the initial concentration of indocyanine in the water phase, C_w—is the concentration of indocyanine in the water phase after redistribution. The slope corresponds to the partition coefficient at 0 mV voltage, $\gamma = 68.5624 \cdot 10^3$. The lipid volume was calculated from the estimated asolectin density 1028 mg/ml (*see text*), water volume (V_w) was 1 ml.

partition coefficient (γ_{app}) of indocyanine is related to the generated membrane potential and can be used for calculation of the membrane potential value (Apell & Bersch, 1987).

The accumulation of the dye in the lipid bilayer leads to a fluorescence decrease. The potential-induced fluorescence change was expressed by the relative fluorescence change, Fig. 6:

$$\frac{\Delta F}{F} \equiv \frac{F - F_u}{F} \tag{10}$$

F_u is the fluorescence estimated after the membrane potential generation.

Estimation of the apparent partition coefficient, γ_{app}, at several known membrane potentials and its relation to the membrane-potential dependent fluorescence change, $\Delta F/F$, revealed the functional dependence between membrane potential and the relative fluorescence change. The apparent partition coefficient could be defined analogously to the partition coefficient at zero voltage as

$$\gamma_{app} = \frac{ln(Fm - F_{app}) - ln(Fm - F_0)}{ln\, Fm - ln(Fm - F_{app})} \cdot \frac{V_w}{V_l} \tag{11}$$

where F_{app} is the fluorescence intensity at a predefined voltage value. The calibration of γ_{app} was carried out as shown in Fig. 6. Addition of indocyanine to a final concentration of 2,88 μM resulted in the initial fluorescence level (F_0). After that, the vesicle suspension (usually 1–3 μl) was added. The fluorescence decreased due to the dye distribution at zero voltage (in the absence of valinomycin) and reached the (F) level. Because of different K^+ concentrations in the intra- and extravesicular medium, upon the addition of valinomycin a membrane potential was generated according to the Nernst Eq. (9). When the K^+ concentration gradient was diminished by addition of small volumes (1–5 μl) of 3 M KCl solution, the membrane potential decreased and the fluorescence intensity increased. In this way the relative change of fluorescence intensity ($\Delta F/F$) and the apparent partition coefficient (γ_{app}) were determined for different fixed membrane potentials using Eqs. (10) and (11). The values of partition coefficients were determined separately for vesicle preparations with and without protein as shown in Fig. 7. The calculated curve was obtained by fitting the experimental data to the polynomial equation:

$$\gamma_{app} = \gamma + A_1 \cdot U + A_2 \cdot U^2 \tag{12}$$

where γ is the partition coefficient at 0 voltage, U is the membrane potential value, and A_1 and A_2 are fitting parameters ($A_1 = 1162.7\ mV^{-1}$, $A_2 = 11.29\ mV^{-2}$). Eqs. (11) and (12) allowed the calculation of the relative fluorescence change for any desired voltage and vesicle concentration in the range of 5–30 μg/ml lipid concentration. The relation between relative fluorescence change ($\Delta F/F$) and appropriate ΔU could be found by the interpolation of calculated voltage values.

Membrane Potential Generated by Cytochrome-c Oxidase

Cytochrome-c oxidase reconstituted in lipid vesicles formed by cholate dialysis exhibits two orientations. The right-side out orientation (cytochrome-c binding site oriented outside) however, dominates with 60–80% (Tihova, Tattrie & Nicholls, 1993). Addition of cytochrome-c to the extravesicular medium (in the presence

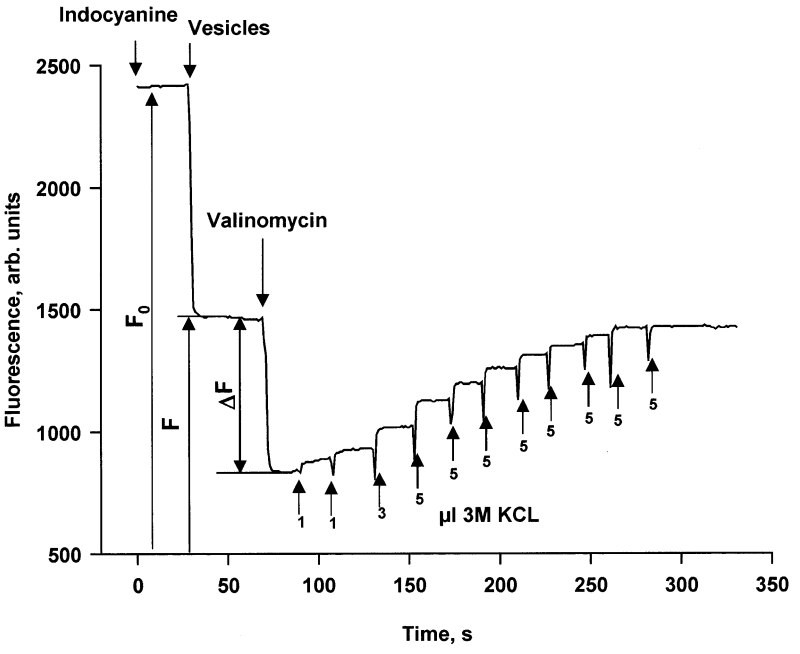

Fig. 6. Calibration experiment. Relationship between the fluorescence and the defined membrane potential. *F* is the fluorescence intensity in arbitrary units. At the beginning of the experiment 1 ml of buffer (in mM: 10 K-Hepes, pH 7.4, 140 NaCl and 10 KCl) was present in the fluorescence cell. Thereafter the following additions were made: 1 µl indocyanine solution (final concentration 2.88 µM) (F_0 is the initial fluorescence intensity in the cell); 1 µl (30 mg/ml lipid) vesicles with (in mM): 140 K^+, 10 Na^+, 10 K-Hepes and pH 7.4 inside (*F* is the fluorescence intensity at 0 mV); 1 µl valinomycin (final concentration 0.02 µM), ΔF — is the fluorescence change due to the membrane potential estimated (66,63 mV); consecutive additions of the 3 M KCl solution to change the defined voltage value.

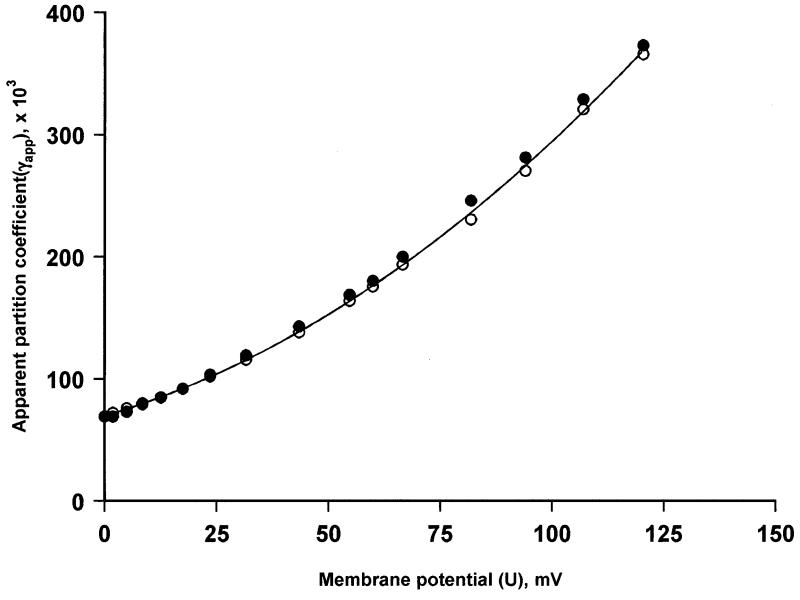

Fig. 7. Apparent partition coefficient, γ_{app}, as a function of the membrane potential. Open circles are protein free vesicles, solid circles are protein containing vesicles. The theoretical curve was obtained by fitting the experimental data to the polynomial equation (12), where γ is the partition coefficient at 0 voltage $\gamma = 68.5624 \cdot 10^3$, *U* is the membrane potential value, A_1 and A_2 are the fitting parameters ($A_1 = 1162.7\ mV^{-1}$, $A_2 = 11.29\ mV^{-2}$).

of K^+-ascorbate) resulted in activation of enzyme molecules with right-side-out orientation. Since the cytochrome-c oxidase works as a proton pump and reduces oxygen to water by the uptake of protons from the vesicle inside, activation of right-side-out oriented proteins generated an inside negative membrane potential. The electrogenic effect of cytochrome-c oxidase is represented in Fig. 8. After addition of 1–2 µl of vesicle suspension the fluorescence decreased and became stable at a fluorescence level which corresponded to the partition coefficient of the indocyanine at 0 mV voltage. Addition of K^+-ascorbate to a final concentration of 1 mM did not influence the fluorescence. Further addition of cytochrome-c to a final concentration of 10–50 µM, resulted in activation of the cytochrome-c oxidase and generation of a membrane potential which can be calculated from the change of the relative fluorescence ($\Delta F/F$) with time (Fig. 9).

Influence of Cytochrome-c and K^+-ascorbate Addition

In Fig. 8 it can be seen that addition of 1 µl of 1 M K^+-ascorbate solution to the vesicle-indocyanine suspen-

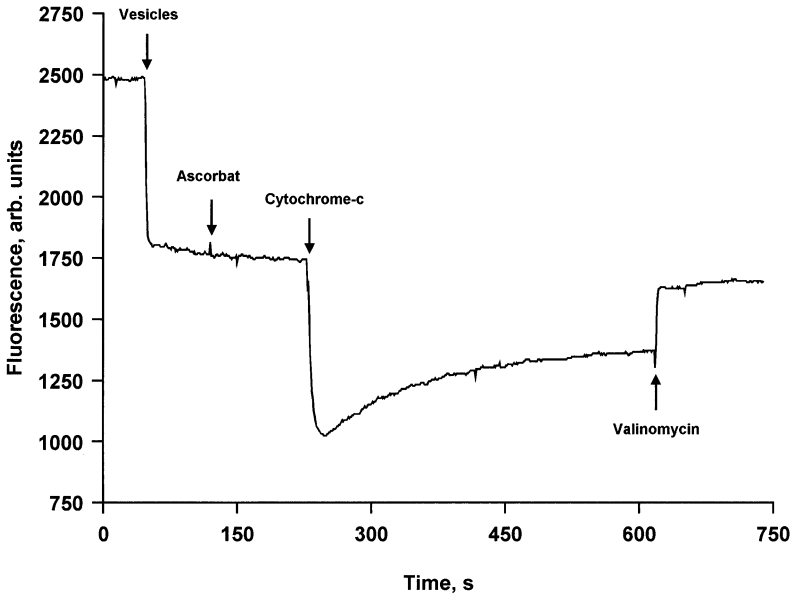

Fig. 8. Fluorescence change on the basis of membrane potential development by vesicles with incorporated cytochrome-c oxidase. Vesicles were reconstituted in buffer A (in mM: 140 KCl, 10 NaCl, 10 Hepes, pH = 7.4). The average amount of reconstituted proteins per vesicle was determined to be ≈2. The addition of 1–2 μl of vesicle, 1 μl of 1 M K^+-ascorbate solution to a final concentration 1 mM and 2 μl 10 mM cytochrome-c solution to a final concentration of 20 μM, results in activation of the cytochrome-c oxidase and generation of the membrane potential. At 620 sec valinomycin was added to decrease the membrane potential.

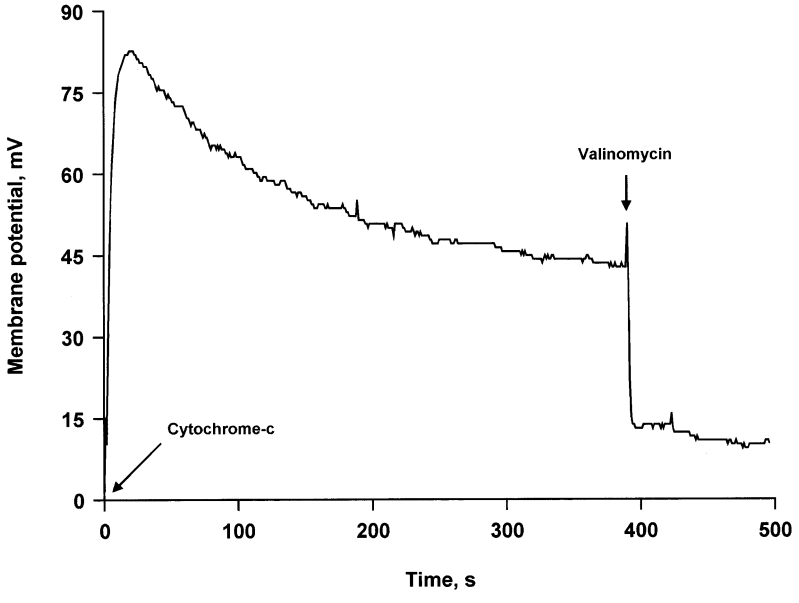

Fig. 9. Membrane potential development calculated from the transient change of the relative fluorescence ($\Delta F/F$) from the experiment in Fig. 8. Time point zero is related to the moment of addition of the cytochrome-c.

sion has no influence on the fluorescence. A final concentration between 10–25 mM caused a fluorescence change ($\Delta F/F$) in the range of only 0.5–1%.

Due to its absorbance at 550 nm, addition of cytochrome-c decreased the intensity of the fluorescence. This effect was measured in experiments with protein-free vesicles. The dependence revealed linearity in the concentration range between 0 and 100 μM cytochrome-c, and corresponds to a fluorescence change, $\Delta F/F$, of 0.5% per 1 μM cytochrome-c added (*data not shown*). This fluorescence change has been taken into account in further calculations of the membrane potential by subtraction of this fluorescence change from the registered transient data of $\Delta F/F$, produced by the generated membrane potential. The cytochrome-c effect was estimated by analogous measurements with the protein-free vesicles.

Time Resolution

One of the restrictions of this experimental setup is the time resolution of the initial fluorescence change upon the enzyme activation. The time necessary to reach homogeneity after addition of a component, or the ''stirring time,'' was measured as follows. To the cuvette containing indocyanine (final concentration approx. 2.88 μM) in buffer C, 1–2 μl of the vesicles (without protein) were

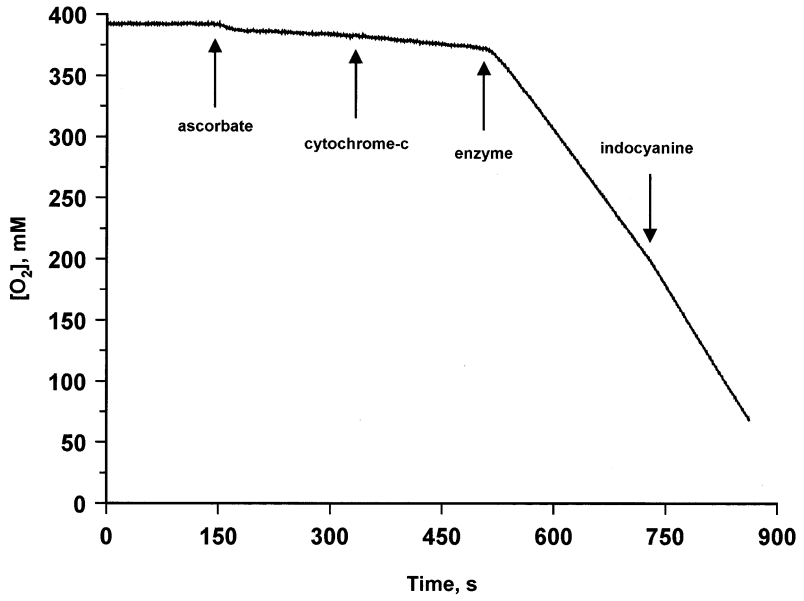

Fig. 10. Influence of indocyanine on the activity of cytochrome-c oxidase. Oxygen consumption was measured as has been described in Materials and Methods. The enzyme activity was measured (in mM): 40 KCl, 10 Hepes, 0.05% laurylmaltoside, 0.1 EDTA, 25 K^+-ascorbate and 50 μM cytochrome-c. The turnover number (TN) is expressed in $e^- \cdot sec^{-1} \cdot$ heme aa_3^{-1}. Before indocyanine addition the TN was 95 sec^{-1}, after indocyanine addition the TN was 106 sec^{-1}.

added. After the dye redistribution equilibrium was reached, various portions (1–10 μl) of a 10 mM cytochrome-c solution were added and the time until the fluorescence reached a constant value was measured. Since the vesicles were protein free, the solution of cytochrome-c produced a reduction in fluorescence intensity caused only by absorption at 550 nm. Therefore, the fluorescence equilibration time after the cytochrome-c addition is the ''stirring time'' of our experimental setup. This stirring time was estimated to be 400–500 msec from an average of 10 measurements. The redistribution time of the dye molecules between water and lipid phase, however, is much shorter and lies between 100 μsec and 10 msec (Ross et al., 1977). For real time measurements it is short enough to detect the membrane potential change even after one enzyme turnover.

Influence of the pH on the Fluorescence of Indocyanine

The change of pH value induced by the activity of the cytochrome-c oxidase may influence the fluorescence of indocyanine and/or its ability to interact with the lipid bilayer. To test this suggestion we studied the effect of the pH change on the fluorescence. The relative fluorescence change ($\Delta F/F$) corresponds to 4.7% per pH unit. Due to an appropriate buffering capacity (10 mM Hepes), a pH change during the action of cytochrome-c oxidase could not be more than 0.001 pH units. Therefore, the change of relative fluorescence due to pH change is negligible and can be ignored in our calculations.

Influence of Indocyanine on the Function of Cytochrome-c Oxidase

In Fig. 10 the influence of indocyanine addition on the activity of cytochrome-c oxidase is shown. After addition of cytochrome-c, the turnover rate measured corresponded to 96 e^- per sec and heme aa_3. Addition of indocyanine (final concentration 2.88 μM) increases the turnover rate by 10–15%. Normally, the standard deviation of the oxygen consumption measurements lay between 5–10%. Therefore, for more accurate measurements this deviation should be taken into account but could be ignored for the present studies.

The Initial Rate of Membrane Potential Change

A biological membrane can be described physically by two parameters, the specific membrane capacitance, C_m, and (leak) membrane conductance, Λ_m. The change of the membrane potential, dU/dt, combines these parameters and the electrogenic activity by the following relation:

$$\frac{dU}{dt} = \frac{n_p \cdot e_0 \cdot k_s \cdot v}{A \cdot C_m} - \frac{\Lambda_m \cdot U}{C_m} \tag{13}$$

where n_p is the number of outside-out (functionally) oriented protein molecules per vesicle, e_0 the elementary charge, k_s is the stoichiometry factor (translocated protons per turnover), v the turnover rate of the pump and A the average area of the vesicle membrane. Since immediately after enzyme activation the membrane potential is close to 0, leak current term, $(\Lambda_m U)/C_m$, can be neglected and Eq. (13) reduces to (Apell & Bersch, 1987; Ivashchuk-Kienbaum, Riek & Apell, 1993; Ivashchuk-Kienbaum & Apell, 1994):

$$\frac{dU}{dt} = K \cdot k_s \cdot v \tag{14}$$

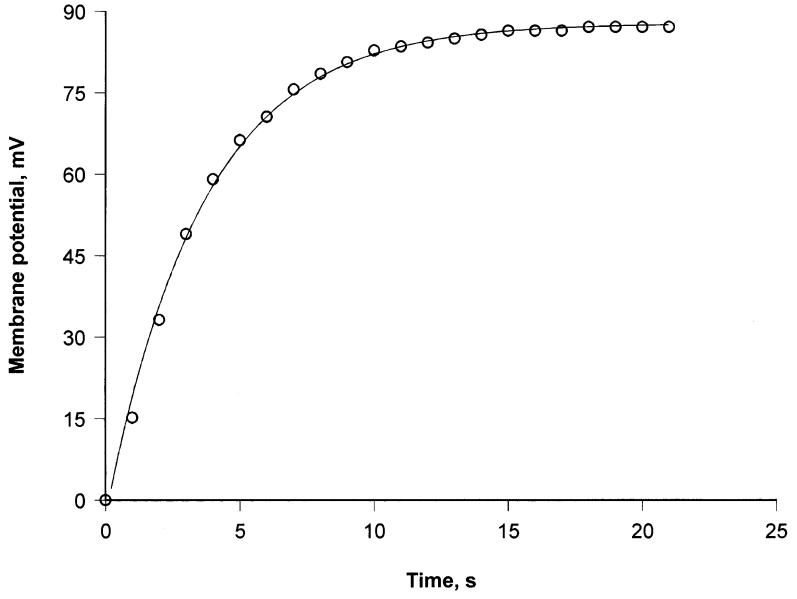

Fig. 11. Time resolved membrane potential change. Time resolution was performed in the range of 0–25 sec from the measured and calculated membrane potential data (Fig. 9). Open circles are measured data, smooth line are fitted data to the function $U = U\max(1 - \exp(-t/\tau))$, where t is time in sec, $U_{max} = 79.05$ mV and $\tau = 3.28$ sec are fitting parameters. From these data the calculated dU/dt corresponds to 24.1 mV/sec.

where K contains all substrate independent factors of Eq. 14:

$$K = \frac{n_p \cdot e_0}{A \cdot C_m} \tag{15}$$

This analysis shows that under standard conditions the initial rate of membrane potential change depends only on the stoichiometry factor (k_s) and the turnover rate of cytochrome-c oxidase incorporated in the vesicle membrane (v). Figure 11 shows a complete time course of an experiment. For the calculation of the initial rate of the membrane potential generation we have used the data from Fig. 9 in the range of 0–25 sec, fitted to the function $U = Umax(1 - \exp(-t/\tau))$, and differentiated with respect to t to obtain the initial rate (dU/dt) at time point 0. The greatest calculated potential value reached was 79.05 mV and the initial rate of potential change corresponded to 24.1 mV/sec. The variation of these two parameters could be used further in the study of proton/electron stoichiometry for cytochrome-c oxidase.

Discussion

The ability of lipophilic fluorescent dyes to change their spectroscopic properties according to the membrane potential applied across the lipid bilayer is well known (Sims et al., 1974; Waggoner, 1979; Beeler et al., 1981). The mechanism of voltage response of some potential-sensitive dyes, on the other hand, is not understood so far (Waggoner, 1979; Clarke et al., 1992). But in many cases, especially for cyanine or oxonol dyes, the response mechanism could be adequately described by a distribution model (Apell et al., 1985; Apell & Bersch, 1987; Zouni et al., 1993). Depending on the polarity of its environment, the indocyanine dye displays different fluorescence emission spectra. The fluorescence maximum shifts to red wavelengths when the dye molecule moves from water into the lipid phase (Fig. 2). The dye redistribution events can be detected by measuring the fluorescence change in the range 650 nm–690 nm. In this paper, the use of the indocyanine dye to monitor membrane potential in vesicles with cytochrome-c oxidase has been shown. Although it seems that a binding model with a discrete number of binding sites is more suitable for the description of the dye-vesicle interaction, I show here that at high dye/vesicle ratios the simple distribution model could be used. The restriction of this method is the concentration of the vesicles, which should be in the range of up to 30 μg/ml lipid at dye concentrations of about 2.5–2.9 μM. At 660 nm we could register a relative fluorescence change, $\Delta F/F$, of 7% per 10 mV of membrane potential change.

The time resolution of this experimental setup is determined mainly by two time constants. First, the ''stirring time,'' which is the time until the solution reaches its homogeneity in the cuvette and second, the time response of the fluorescent dye. The stirring time was the main time limiting factor and corresponded to 400–500 msec. The time response of the indocyanine dye is considerably shorter. Ross et al. (1977) observed a biphasic fluorescence signal from nerve axons stained with indocyanine after application of a voltage step, consisting of a fast component with a time constant below 100 μsec and a second slower component with a time constant of about 10 msec. Since the turnover number of cytochrome-c oxidase is in the range of 50–100 sec^{-1}, the

response time of the indocyanine is short enough to detect the change of the membrane potential without deformation even after one enzyme turnover.

The time course of the relative fluorescence change after the addition of cytochrome-c was measured and used to calculate the membrane potential change. Extrapolation of this voltage course to the time point 0, gives us a function of the membrane potential development with time. By differentiation of this function, the initial rate of potential change was determined. The initial potential change is a parameter independent of the permeability of the lipid membrane. The permeability of the liposome membrane to protons is rather high (comparable to K^+ and Na^+ ions) (New, 1990). Therefore, estimation of the initial potential change would be helpful in investigations of the electrogenic activity of cytochrome-c oxidase. The further improvement of this method to reduce the ''stirring time'' will allow the recording of rapid kinetics of the membrane potential development and, consequently, the resolving of proton translocation events induced by cytochrome-c oxidase.

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