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Electric parameters of Na^+/K^+-ATPase by measurements of the fluorescence-detected electric dichroism

Dietmar Porschke [a,*], Ernst Grell [b]

[a] *Max Planck Institut für biophysikalische Chemie, D-37077 Göttingen, Germany*
[b] *Max Planck Institut für Biophysik, D-60596 Frankfurt, Germany*

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Abstract

The electric parameters of Na^+/K^+-ATPase labeled by FITC have been characterized by measurements of the fluorescence-detected electric dichroism. The fluorescence emission was measured with polarizers at the magic angle and the light for excitation was usually polarized parallel to the field vector. The FITC-Na^+/K^+-ATPase preparations exhibit a negative electric dichroism at field strengths up to about 600 V/cm and a positive dichroism at higher field strengths. Pulse reversal experiments reveal a dominant permanent electric moment at low electric field strengths and an increasing contribution from an induced electric moment at higher field strengths. The dichroism rise curves and the transients upon pulse reversal show two relaxation processes with opposite amplitudes, whereas the dichroism decay curves in most cases can be represented by single exponentials at a reasonable accuracy. The amplitude A_2 associated with the slower of the rise processes is dominant at low field strengths and also approaches saturation already at low field strengths. The dependence of A_2 on the electric field strength is consistent with the orientation function for permanent dipoles and cannot be represented by the orientation function for induced dipoles. The fitted permanent dipole moment is in the range of $3.5 \cdot 10^{-24}$ Cm [$1 \cdot 10^6$ D] and shows only a relatively small decrease with increasing ionic strength. The stationary values of the electric dichroism up to field strengths $E \leq 800$ V/cm can be represented with high accuracy by an orientation function for disk-shaped particles with a permanent moment along the particle symmetry axis and an induced moment along the semi-major axis. The permanent electric moment determined according to this function is consistent with the one obtained from the amplitudes A_2. In summary, our measurements indicate that Na^+/K^+-ATPase is associated with a large permanent electric moment directed perpendicular to the membrane plane. The dipole moment per ATPase monomer unit is estimated to be $1.4 \cdot 10^{-27}$ Cm [430 ± 50 D].

Keywords: Dipole moment; Fluorescence-detected electric dichroism; Rotational relaxation; ATPase, Na^+/K^+-

1. Introduction

Using the chemical energy of ATP, the integral membrane protein Na^+/K^+-ATPase, which exists in all cellular membranes of higher organisms, actively pumps in a single turnover three Na^+ outward and two K^+ inward. These transport processes are thus associated with the translocation of a net charge across the membrane, which implies an electrogenic property of Na^+/K^+-ATPase and a dependence of its action on external electric fields. A major approach to the characterization of field-dependent properties has been the measurement of voltage-current relationships in cellular systems [1–7] or in model membrane arrangements [8]. These studies show that there is no simple linear current/voltage relationship: the membrane current decreases above a certain voltage, which indicates a voltage control of the pump activity. For an investigation of the electrogenic properties of Na^+/K^+-ATPase and an identification of field-dependent reaction steps, the action of the purified enzyme adsorbed to black lipid membranes has been studied by concentration jumps induced by photolysis [9,10]. These studies confirmed that Na^+ is essentially transported electrogenically, although other processes are considered to be influenced by the electric field too. In addition, several reports have been published on frequency-dependent effects of electric fields on the enzymatic activity [11–15]. Common to all these studies is the goal to elucidate the fundamental effect of the electric field on the functional properties of this membrane-bound protein in molecular terms.

* Corresponding author. E-mail: dpoersc@gwdg.de. Fax: +49 551 2011435.

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In the present investigation we describe an alternative approach, which is based on measurements of time-resolved spectroscopic parameters of Na^+/K^+-ATPase under the action of external electric fields. For this electrooptical study we preferentially used the highly purified, membrane-bound preparation of the renal pig enzyme [16,17] in the form of discs, where the natural orientation of the protein molecules is preserved. For fluorescence measurements in the visible spectral range, the enzyme was specifically labeled with the dye fluorescein isothiocyanate (FITC) similar to Karlish [18]. Labeling is considered to occur only within the ATP binding domain. Conditions were chosen where one dye molecule is covalently bound per $\alpha\beta$-monomer of the enzyme. This preparation already proved to be useful in the characterization of cation binding to the enzyme [18–23]. Now, in the course of the present study, the fluorescence properties of the covalently bound FITC have been used to characterize the field-induced orientation of the Na^+/K^+-ATPase preparation to deduce the electric parameters of the enzyme. We demonstrate that these membrane fragments containing Na^+/K^+-ATPase as the only protein component are characterized by an unexpectedly large permanent electric moment, which is related to the moments of the individual enzyme molecules that are arranged within the disc-shaped membrane fragments in a well-ordered direction.

2. Materials and methods

Na^+/K^+-ATPase-containing microsomes were prepared from dissected tissue of the red outer medulla of pig kidney [16]. The enzyme was purified according to Jørgensen [16,17]. After sucrose density gradient centrifugation in the SW 28 swinging-bucket rotor (Beckman, Dreieich), fractions were collected from each tube and analyzed by determining the 2,4-dinitrophenylphosphate (DNPP) activity at a substrate concentration of 1 mM in 25 mM imidazole (microselect quality Fluka, Neu-Ulm)-HCl (pH 7.5) containing 20 mM KCl and 20 mM $MgCl_2$ at 37°C spectrophotometrically (420 nm). The protein concentration was determined by the Coomassie brilliant blue G-250 test (Bio-Rad, München) according to Bradford [24]. The selected fractions exhibiting the highest activity were combined, diluted with three volume parts of 25 mM imidazole-HCl (pH 7.5) containing 0.2 mM dithiothreitol (DTT; Aldrich, Steinheim) and centrifuged for 4 h at $74\,000 \times g$ and 4°C. The resulting pellet was resuspended in K^+-free 25 mM imidazole (microselect for luminescence quality Fluka, Neu-Ulm, which had been sublimed)-HCl (pH 7.5) containing 0.2 mM DTT (K^+-free means that the used buffer solution was not in contact with an electrode) to achieve a concentration around 2.5 mg/ml. For all the following steps only this particular quality of imidazole buffer was used. To remove trace components originating from the isolation media, the enzyme is centrifuged for 20 min at $200\,000 \times g$ at 4°C (TL-100, Beckman, Dreieich). After suspension of the pellet in a similar volume of the imidazole buffer, the sample was incubated for 20 min at 20°C with occasional, gentle shaking and recentrifuged at 20°C under the conditions mentioned before. This step, consisting of resuspending, incubating and centrifuging the enzyme, was repeated. The final pellet was taken up in the buffer suitable for the chosen investigation and the sample was kept in ice. Enzymatic activity was determined at protein concentrations around 1 ng/ml on the basis of spectrophotometric P_i determinations [17] at 37°C using the substrate 3 mM Tris-ATP (Sigma, Deisenhofen) in 30 mM histidine (microselect quality Fluka, Neu-Ulm)-HCl (pH 7.5) containing 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl with and without of 1 mM ouabain and in addition with the substrate DNPP, as indicated above. Protein concentrations were determined according to a modified procedure of Lowry et al. [25] and were calibrated with an enzyme reference sample that had been characterized by amino acid analysis (obtained from Prof. P.L. Jørgensen). The specific activities according to the ATP test were around 35 μmol P_i/mg per min and those according to the DNPP test around 40 μmol P_i/mg per min. The activities in the presence of ouabain were less than 1% of those in its absence.

For the fluorescence labeling a modified procedure of Karlish [18] was applied. The washed enzyme was centrifuged for 20 min at $150\,000 \times g$ and 4°C and resuspended in K^+-free 100 mM Tris (p.A. quality Merck, Darmstadt)-HCl (pH 9.2) containing 2 mM EDTA sodium salt (p.A. quality Merck, Darmstadt). Fluorescein 5′-isothiocyanate isomer I (FITC; Sigma, Deisenhofen) freshly dissolved in *N,N*-dimethylformamide (Uvasol quality Merck, Darmstadt) at a concentration of 1 mg/ml was added to the resuspended enzyme so that a total protein concentration of 0.5 mg/ml and FITC concentration of 0.06 mM resulted. After an incubation for 20 min at 20°C with gentle stirring, the pH of the labeling medium was reduced by adding the same volume of 25 mM imidazole-HCl containing 0.1 mM EDTA (Merck, Darmstadt) and 0.2 mM DTT (pH 7.5). The sample was centrifuged at 4°C for 20 min at $200\,000 \times g$ and the pellet resuspended in the same volume of EDTA containing imidazole buffer used before and incubated for 30 min at 37°C. In order to remove traces of non-covalently bound FITC, this procedure was repeated twice. Prior to the final centrifugation, the absorption spectrum between 350 and 650 nm was measured and, in addition, the protein concentration was determined. After subtracting the contribution assumed to be due to light scattering, the concentration of bound FITC is calculated, based on an extinction coefficient of $7.15 \cdot 10^4$ M^{-1} cm^{-1} at 490 nm. Usually 0.7 to 1.1 equivalents of bound FITC per Na^+/K^+-ATPase (assumed molecular weight 150 000) were found. After the final centrifugation under the same conditions the pellet of the FITC enzyme is suspended in the buffer chosen for the investigation to get

a protein concentration around 2.5 mg/ml. The sample was stored in ice. The specific activities of the final FITC enzyme preparations according to the ATP test were in the range between 0.5 and 1 μmol P_i/mg per min and those resulting from the DNPP test were around 20 μmol P_i/mg per min under the conditions specified above. The samples used for measurements of the electric dichroism usually contained $4 \cdot 10^{-7}$ M enzyme.

Electric field pulses were generated by a programmable arbitrary waveform generator AWG 5105 from Tektronix. The pulses were amplified by a precision power amplifier 5205A from Fluke. The samples were subjected to the electric field pulses in a cell constructed in close analogy to cells for temperature jump experiments used for fluorescence detection [26]. The lower electrode was made from platinum, the upper one from gold; the electrode distance was 13.6 mm. For our measurements we used an instrument designed for measurements of temperature jump relaxation [26,27]. The fluorescence was excited by light from a 600 W Hg-Xe lamp with a Schoeffel GM 250 monochromator; the standard wavelength for excitation was 436 nm; a Glan prism from Halle (Berlin) was used for polarization of the excitation light. The emitted light was collected behind 3 M polacoat 105 UVWR polarization filters oriented at 55° with respect to the electric field vector; the excitation light was suppressed by cut-off filters GG495 from Schott (Mainz). The fluorescence signal was processed by an analog unit, which reduces perturbations from lamp fluctuations by subtraction of the signal of a reference detector from that of the fluorescence detectors after amplification of both signals to the same absolute average level [27]. The measured fluorescence transients were digitized by a Tektronix DSA602. Stationary changes of the fluorescence intensity were evaluated by simple graphic routines on a PC. Transients were analyzed after transfer of the data to the facilities of the Gesellschaft für wissenschaftliche Datenverarbeitung, Göttingen, by a set of programs developed for analysis of chemical and physical relaxation, including programs for deconvolution [28].

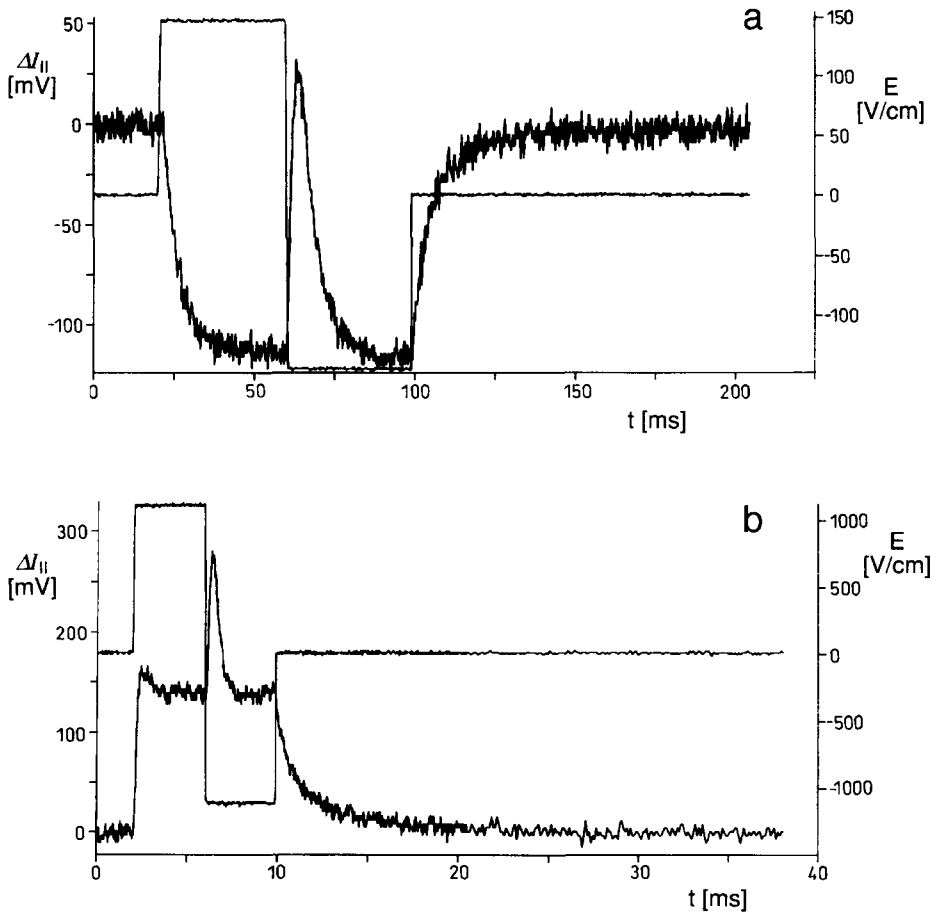

Fig. 1. Change of the fluorescence intensity $\Delta I_{||}$ of FITC-Na^+/K^+-ATPase induced by an electric field pulse with reversal of the field vector E; 20°C, 2.5 mM imidazole-HCl (pH 7.5), light for excitation polarized parallel to the field vector, emission polarizer at magic angle orientation, total signal in the absence of an external electric field 4 V. (a) Pulse of 'low' field strength; (b) pulse of 'high' field strength.

3. Fluorescence-detected electric dichroism

The electric dichroism is usually analyzed by direct measurements of the absorbance of polarized light [29]. In most cases this procedure may be applied without any problems. However, in the case of large membrane fragments the absorbance is often increased by contributions from light scattering. Such contributions may perturb the measured values of the electric dichroism. In our present investigation we have avoided complications resulting from light scattering by measurements of the fluorescence detected electric dichroism. In this procedure we measure the intensity of absorbed light by recording the fluorescence, which is proportional to the extent of absorbed light, provided that appropriate precautions are taken. It must be secured that the measured fluorescence signal is proportional to the total intensity of emitted light. For this purpose we have used polarizers in the emission light beam oriented at an angle of 55° with respect to the electric field vector. At this so called 'magic angle' orientation, the measured signal is independent of the orientation of the molecules and is proportional to the total intensity of the emitted light [30].

Under these conditions for the measurement of the fluorescence intensity, measurements of the electric dichroism simply require excitation with light of defined degree of polarization with respect to the electric field vector. For most of our measurements we used light polarized parallel to the electric field vector. Application of electric field pulses (may) induce changes of the absorbance of the light used for excitation, which are then reflected by changes in the intensity of the emitted light $\Delta I_{\parallel}$. The electric dichroism may be calculated according to

$$\frac{\Delta\varepsilon}{\bar{\varepsilon}} = \frac{\Delta\varepsilon_{\parallel} - \Delta\varepsilon_{\perp}}{\bar{\varepsilon}} = \frac{1.5 \cdot \Delta I_{\parallel}}{I_t} \quad (1)$$

where I_t is the total fluorescence light intensity observed in the absence of an external electric field. $\bar{\varepsilon}$ is the extinction coefficient of the sample at the wavelength used for excitation; $\Delta\varepsilon_{\parallel}$ and $\Delta\varepsilon_{\perp}$ are the changes of the extinction coefficient for light polarized parallel and perpendicular, respectively, under the influence of an external electric field; $\Delta\varepsilon/\bar{\varepsilon}$ represents the stationary electric dichroism according to the standard definition [29].

According to the standard relations valid for the electric dichroism, the field-induced change of the fluorescence light intensity excited by light polarized perpendicular to the field vector, $\Delta I_{\perp}$, should be in the opposite direction and fulfil the equation

$$\Delta I_{\parallel}/I_t = -2 \times \Delta I_{\perp}/I_t \quad (2)$$

Finally, an important test for field-induced conformation changes or field induced processes other than 'physical' orientation is possible by measurements using the magic angle adjustment of the polarizers both in excitation and emission [30].

4. Results

4.1. Electric parameters

In most of our experiments we have studied the field-induced alignment of Na^+/K^+-ATPase membrane fragments by measurements of the fluorescence emission with polarizers in the magic angle orientation, where the light used for excitation was polarized parallel to the field vector. Under these conditions electric field pulses in the range up to about 400 V/cm applied to solutions of Na^+/K^+-ATPase induced a decrease of the fluorescence intensity, indicating a negative electric dichroism (cf. Fig. 1a). When the light for excitation was polarized perpendicular to the field vector, the field-induced changes of the fluorescence are in the opposite direction in agreement with Eq. (2). Control experiments under magic angle conditions both in excitation and emission in some cases showed small relaxation effects, but the amplitudes were much smaller than those observed in corresponding experiments with the excitation light polarized parallel or perpendicular to the field vector. Thus, we did not observe any clear indication of field-induced conformation changes and we assign the fluorescence signals to field-induced alignment of the membrane fragments. The stationary changes of the fluorescence arrive at a maximum already at relatively low electric field strengths of about 200 V/cm. Pulses of higher electric field strength lead to a reduction of the amplitude and finally at field strengths above ≈ 600 V/cm to amplitudes with opposite sign (cf. Fig. 1b).

Reversal of the field vector induces a large transient in the fluorescence intensity (cf. Fig. 1), which indicates a dominant contribution from a permanent dipole or from a slow polarizability. More evidence for the existence of a permanent electric moment comes from an analysis of the stationary amplitudes measured at low electric field strengths up to 200 V/cm. The amplitudes measured in this range as a function of the electric field strength can be fitted at a satisfactory accuracy by the orientation function for permanent dipoles and cannot be represented by the orientation function for induced dipoles. The dipole moment obtained from these fits is in the range of about $3.5 \cdot 10^{-24}$ Cm [$1 \cdot 10^6$ D].

It is already evident by visual inspection that the dichroism rise curves induced by pulses of more than ≈ 400 V/cm require two exponentials for fitting. A quantitative analysis shows that the rise curves induced by pulses with less than 400 V/cm also include two processes (cf. Fig. 2); the amplitude for the faster process A_1 remains relatively small at low field strengths. The amplitudes A_2 obtained for the slower process already approach saturation at field strengths around 200 V/cm (cf. Fig. 3),

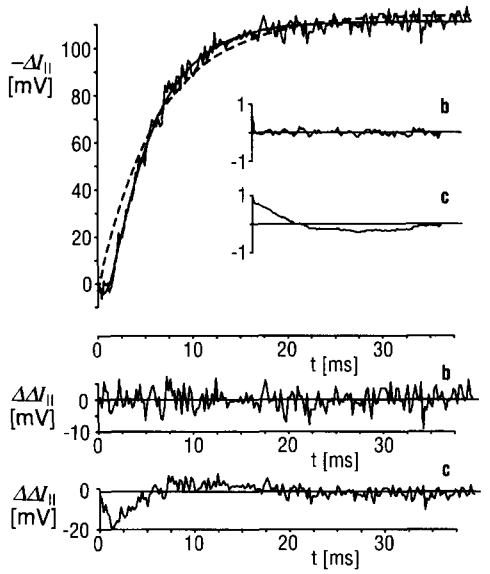

Fig. 2. Exponential fit of a rise curve of the fluorescence dichroism ($\Delta I = f(t)$), measured under the conditions of Fig. 1a. The experimental curve (line with noise) is the average of two shots. The solid line represents a least-squares fit by two exponentials ($\tau_r^1 = 0.87$ ms, $\tau_r^2 = 4.67$ ms, $A_r^1 = -47$ mV, $A_r^2 = 158$ mV) under the following conditions: the data recorded before application of the field pulse (cf. Fig. 1) were averaged, subtracted from the rise curve data and the ΔI-value of the fit at $t = 0$ was fixed to 0; the convolution of the data with the rise time of the detector (50 μs) has been included in the evaluation using the standard equations for convolution of exponentials [28,31]. The dashed line represents a fit by a single exponential under the same conditions. The lower panels, b and c, show the residuals of the fits ($\Delta\Delta I = f(t)$) with two and one exponentials, respectively; the insets b and c show the autocorrelation of the residuals of the two and one exponential fits, respectively.

whereas the amplitudes A_1 increase continuously in the range of applied field strengths. The dependence of the amplitudes A_2 on the electric field strength is consistent with the orientation function for permanent dipoles and cannot be fitted by the orientation function for induced

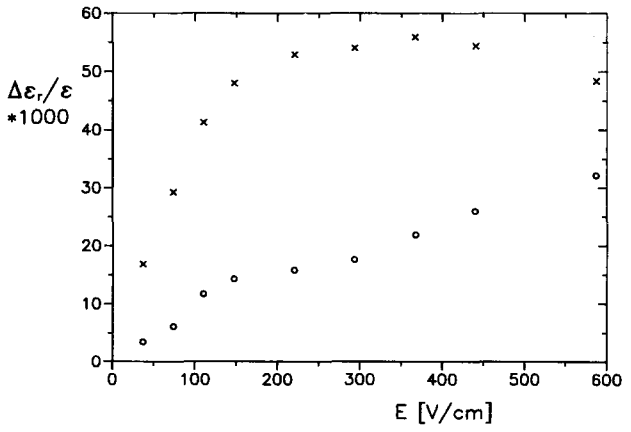

Fig. 3. Amplitudes associated with the relaxation processes of the dichroism rise curves of FITC-Na^+/K^+-ATPase as a function of the electric field strength E. Amplitude for the fast process $\Delta\varepsilon_r^1/\varepsilon$ (○) and for the slow process $\Delta\varepsilon_r^2/\varepsilon$ (×); 20°C, 10 mM imidazole-HCl (pH 7.5).

Table 1
Electric parameters of FITC-Na^+/K^+-ATPase at different concentrations of the imidazole-HCl (pH 7.5) buffer at 20°C

Buffer [mM]	μ_a [Cm]	μ_b [Cm]	μ_c [Cm]	α_c [$Cm^2\ V^{-1}$]	$\Delta\varepsilon/\varepsilon$
1.25	$6.04\cdot10^{-24}$	$4.5\cdot10^{-24}$	$4.71\cdot10^{-24}$	$14.6\cdot10^{-29}$	0.0229
2.5	$5.27\cdot10^{-24}$	$3.5\cdot10^{-24}$	$3.41\cdot10^{-24}$	$9.20\cdot10^{-29}$	0.0306
5	$5.12\cdot10^{-24}$	$3.9\cdot10^{-24}$	$3.67\cdot10^{-24}$	$10.5\cdot10^{-29}$	0.0245
10	$3.04\cdot10^{-24}$	$2.7\cdot10^{-24}$	$2.53\cdot10^{-24}$	$5.97\cdot10^{-29}$	0.0279

μ_a, μ_b and μ_c are permanent dipole moments; α_c is the polarizability; $\Delta\varepsilon/\varepsilon$ is the electric dichroism of the FITC-enzyme. The parameters were determined as follows: μ_a from the stationary dichroism at low field strengths up to ≈ 200 V/cm by fitting to the standard permanent dipole function; μ_b from the amplitude A_2 associated with the slow dichroism rise process by fitting to the standard permanent dipole function; μ_c, α_c and $\Delta\varepsilon/\varepsilon$ from the stationary dichroism in the range of field strengths up to 700 V/cm by fitting to the orientation function for the disk model [32]. The estimated accuracy of the parameters is $\pm 10\%$.

dipoles. Again, the dipole moment obtained from the fits is extremely high (cf. Table 1).

The special dependence of the stationary dichroism on the field strength with the reversal of the sign corresponds to that of an orientation function designed by Shaw [32]. He has described the orientation function for disk-shaped particles with a permanent dipole along the particle symmetry axis and with an induced dipole along the semi-major axis. For this model the dichroism is given by

$$\Delta\epsilon/\epsilon = -2\cdot\Phi\cdot(\Delta\epsilon/\epsilon)_\infty \tag{3a}$$

$$\Phi = \frac{3}{4\gamma}\left[\frac{\beta^2}{2\gamma}+1 + \frac{e^{-\left(\frac{\beta^2}{4\gamma}+\gamma\right)}\left[\frac{\beta}{2\sqrt{\gamma}}(e^{-\beta}-e^{\beta})-\sqrt{\gamma}(e^{-\beta}+e^{\beta})\right]}{I}\right]-\frac{1}{2} \tag{3b}$$

where

$$I = \int_{t_1}^{t_2} e^{-x^2}\,dx$$

$$t_1 = -\sqrt{\gamma}-\beta/2\sqrt{\gamma}$$

$$t_2 = \sqrt{\gamma}-\beta/2\sqrt{\gamma} \tag{3c}$$

$$\beta = -\mu E/kT \tag{3d}$$

$$\gamma = -\alpha E^2/2kT \tag{3e}$$

$(\Delta\epsilon/\epsilon)_\infty$ is the limit value of the reduced dichroism, μ the permanent dipole moment directed along the symmetry axis of the discs, α the polarizability in the direction of the plane, E the electric field strength and kT the thermal energy. We have used this orientation function for fitting of our experimental data. As shown in Fig. 4, the data measured up to about 600 V/cm, where the sign is

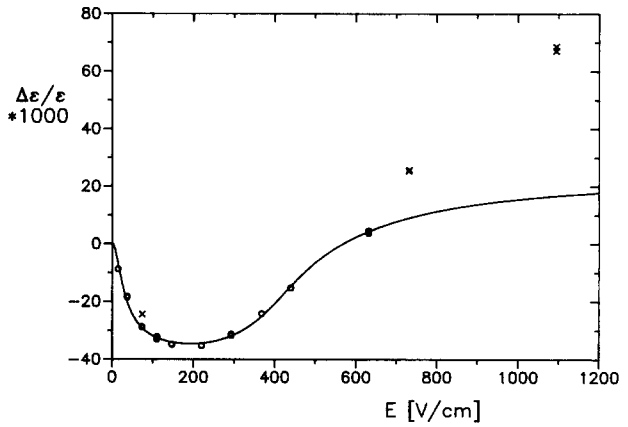

Fig. 4. Stationary values of the electric dichroism $\Delta\varepsilon/\varepsilon$ (×) of FITC-Na^+/K^+-ATPase as a function of the electric field strength, E. The continuous line represents the least-squares fit of the data points indicated by circles to the orientation function for the disk model with the following fitted parameters: a permanent dipole moment of $2.53 \cdot 10^{-24}$ cm along the symmetry axis, a polarizability of $5.97 \cdot 10^{-29}$ Cm^2 V^{-1} along the semi-major axis and a limiting value of the reduced electric dichroism of 0.0229. The data points indicated by × were not included in the least squares fit; the × at low field strength was measured *after* exposure of the sample to high field strengths; 20°C, 1.25 mM imidazole-HCl (pH 7.5).

reverted, can be fitted at a very satisfactory accuracy. When the data points with a large positive dichroism measured at higher field strengths are included in the analysis, the quality of the fits is decreased seriously. For this reason we have restricted the data analysis by the combined permanent/induced dipole model to the range of electric field strengths $E \leq 700$ V/cm. The permanent dipole moments obtained by these fits are again in the range of about $3.5 \cdot 10^{-24}$ Cm [$1 \cdot 10^6$ D] (cf. Table 1).

The deviation of the data measured at high field strengths from the orientation function calculated with the parameters valid at low field strength was observed in virtually all of our measurements in the same manner. Probably there is some nonlinearity of the induced dipole moment or some other complication resulting from the polyelectrolyte character of our samples. It should be mentioned that exposure of our samples to pulses of high electric field strengths lead to some irreversible or at least slowly reversible changes of the Na^+/K^+-ATPase preparation. This change is reflected by a deviation of the stationary dichroism measured at low field strengths for samples after exposure to high field pulses compared to that of the same samples before exposure to high field pulses (cf. Fig. 4).

The huge permanent dipole moment observed for our Na^+/K^+-ATPase preparations raises questions on the nature of this electric moment. Some polyelectrolytes simulate the existence of a large permanent dipole moment by a saturation of the ion polarization [33]. Usually the dipole moments resulting from saturation of ion polarization strongly decrease with increasing ion concentrations. Thus, it should be useful to check the dependence of the permanent dipole moment of our Na^+/K^+-ATPase preparation on the ionic strength. Measurements in the range from 1.25 to 10 mM buffer concentration revealed some decrease of the dipole moment with increasing ionic strength, but the decrease remains rather small (cf. Table 1). This relatively small dependence on the ionic strength suggests that the dipole moment of Na^+/K^+-ATPase is due to a real permanent asymmetry of its charge distribution.

4.2. Time constants of rotational diffusion

The dichroism decay time constants reflect the rotational diffusion of the membrane fragments. Most of the dichroism decay curves could be fitted by single exponentials at a satisfactory accuracy. This result demonstrates that the size distribution of membrane fragments is relatively narrow and that corrections of the experimental data for a size distribution are not necessary. The time constants obtained for most of the preparations were in the range around 6.5 ms, which corresponds to the hydrodynamic equivalent of a sphere with a diameter of about 370 nm. Determinations of the size of the particles by dynamic light scattering lead to similar values. The equivalence of the results obtained from the dichroism decay time constants and from dynamic light scattering shows that electric field pulses induce rotation of the entire membrane fragments and that the electric field effects do not involve any detectable changes of individual enzyme molecules or of other subunits of the membrane fragments. This result also shows that the electric parameters obtained in this investigation reflect the properties of whole membrane fragments.

A more appropriate hydrodynamic model of the ATPase membrane fragments are thin circular disks. Using an equation for the rotational relaxation time constant τ_d of thin circular disks [34], the diameter, b, of the disks may be calculated according to

$$b = \sqrt[3]{\frac{9kT}{2\eta}\tau_d} \tag{4}$$

where η is the viscosity of the medium and kT the thermal energy. From $\tau_d = 6.5$ ms we get a disk diameter of 490 nm.

The time constants associated with the dichroism rise are affected by the electric torque and, thus, are dependent on the field strength. An example for a set of rise time constants is given in Fig. 5 as a function of the reciprocal field strength $1/E$. In the case of the ATPase membrane fragments there appear to be limited ranges with a linear dependence. A linear dependence may be expected for permanent dipoles [35] (also, Porschke, D., unpublished material). Apparently, the coupling of the permanent and the induced dipole (cf. above) leads to a relatively complex

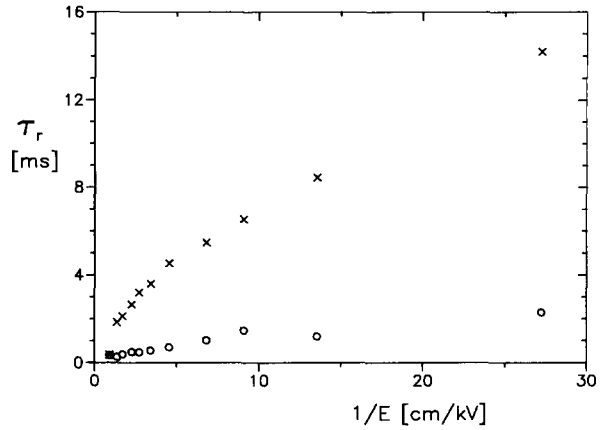

Fig. 5. Rise time constants τ_r^1 (×) and τ_r^2 (×) of FITC-Na^+/K^+-ATPase as a function of the electric field strength, E; 20°C, 10 mM imidazole-HCl (pH 7.5).

dependence, which may be analyzed by Brownian dynamics simulations, for example Ref. [36].

4.3. Various tests

For most of our measurements we have used the Na^+/K^+-ATPase labeled by FITC. As a check for a possible change of the sample response resulting from the labeling procedure, we have also analyzed a sample without label and have measured the fluorescence of the tryptophan residues of the protein. The tryptophan fluorescence intensity was not as stable as that of FITC because of some photoreaction. Nevertheless, it was possible to demonstrate that the tryptophan fluorescence reflects processes which are very similar to those indicated by FITC: a negative dichroism at low field strengths, a change to a positive dichroism at larger field strengths and large transients upon reversal of the field vector in particular at low field strengths. Another independent check was based on measurements of the birefringence, which does not require any label. Again, the birefringence data showed effects closely corresponding to those indicated by the FITC fluorescence. The bi-refringence is negative at low field strengths, changes to a positive one at higher field strengths and shows large transients upon reversal of the field vector. Thus, we conclude that the electric parameters derived in the present investigation are characteristic of Na^+/K^+-ATPase and are not perturbed by introduction of the fluorescence label.

Measurements of the electric dichroism of the FITC-enzyme in the presence of different cations such as KCl (up to 1 mM), 1 mM NaCl, 1,5-bisguanidinium-*n*-pentane dichloride (up to 2.6 mM) or of 0.5 mM ouabain provided results similar to those obtained in the standard buffer. In the presence of KCl, the electric dichroism was, in some of our experiments, slightly larger than in the presence of other ions. A detailed analysis of the influence of various cations on the electric parameters of Na^+/K^+-ATPase remains for future investigations; this analysis requires a more detailed control of the Na^+/K^+-ATPase preparation.

5. Discussion

The results obtained in our present investigation on the electric properties of Na^+/K^+-ATPase are similar in some respects to results obtained previously for bacteriorhodopsin from *Halobacterium halobium*. Bacteriorhodopsin has been investigated by measurements of the linear dichroism, the birefringence and light scattering under electric field pulses [37–42]. In all cases it has been concluded that the data indicate a substantial permanent dipole moment associated with the bacteriorhodopsin membrane fragments. The permanent dipole moment is directed perpendicular to the membrane plane. A polarizability in the membrane plane leads to a reversal of the sign of the electrooptical effects at higher field strengths. Usually the authors have separated the contributions of the permanent and the induced dipole by measurements using AC pulses of low (e.g., 0.5 Hz) and high frequency (e.g., 2 kHz). Some authors have also used an expansion of the orientation function for disks developed by Shah [32]. Our present evaluation, using the orientation function without approximations, should be more accurate than previous evaluations. The permanent dipole moments obtained for bacteriorhodopsin by different authors are quite different, which may be partly due to differences in the preparations and the experimental conditions, but also partly due to different approximations used in the evaluations. The permanent dipole moment per protein unit has be estimated to be in the range from 2 to $4.7 \cdot 10^{-28}$ Cm [60 to 140 D]. It has been suggested that part of the dipole moment of bacteriorhodopsin results from 'movements of charges in the close vicinity of the particle surfaces' and may be an 'interfacial' moment [43].

The present data obtained for Na^+/K^+-ATPase fragments may also be used to estimate the dipole moment per ATPase unit. From the size of the membrane disks derived from the dichroism decay time constant (cf. above) and the density of monomer $\alpha\beta$ enzyme particles of 0.0125 per nm^2 of the membrane plane [44,45], we get an estimated value of 2300 ATPase molecules per disk and an estimated dipole moment of about $1.4 \cdot 10^{-27}$ Cm [430 ± 50 D] per protein unit. The relatively small dependence of this dipole moment on the salt concentration suggests that it is not mainly of an 'interfacial' character and that it is not due to some saturation of a polarizability. However, we cannot exclude the possibility that the asymmetry of the charge distribution may be partly due to a non-symmetric distribution of charged lipid residues. The large dipole moment associated with Na^+/K^+-ATPase raises some interesting possibilities for the regulation of the enzyme function by the membrane potential. At present we do not have sufficiently clear evidence for field-induced conformation

changes of Na^+/K^+-ATPase from the available electrooptical experiments. In the presence of large 'physical' orientation effects it is not simple to detect field-induced changes of the conformation or of ligand binding unambiguously. However, other spectroscopic labels may be more sensitive to potential field-induced conformation changes than the labels used in our present investigation. Probably electric fields also affect the binding of some of the ligands involved in the enzyme action. Further electro-optical investigations should be useful for elucidation of these reactions, for characterization of the enzyme action under electric fields and for the identification of the electric parameters of the enzyme frozen into particular states like E_1 or E_2.

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