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ARTICLE

V. Tychinsky · A. Kretushev · T. Vyshenskaja

Mitochondria optical parameters are dependent on their energy state: a new electrooptical effect?

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Abstract The membrane potential of mitochondria determines, to a large extent, their functional state and the response to the changing conditions of the environment. A correlation was found between the changes in the optical characteristics of isolated mitochondria and the composition of the incubation medium, which determines the potential of the inner mitochondrial membrane. The measurements performed by the method of coherent phase microscopy made it possible to establish a linear dependence of the phase height on the value of the membrane potential and to calculate the electrooptical constant, $K \approx (3–4) \times 10^{-7}$ m/V.

Keywords Coherent phase microscopy · Electrooptical effect in mitochondria · Mitochondrion · Transmembrane potential

Abbreviations CPM: coherent phase microscopy · MEOE: membrane electrooptical effect · MP: membrane potential · OP: optical path · PH: phase height

Introduction

The membrane potential (MP) is an integral parameter of the functional state of the mitochondrion (Skulachev1990; Hueser et al. 1998; Bernardi1999; Bernardi et al. 1999; Hueser and Blatter 1999; Nicholls and Budd 2000; Szewczyk and Wojtczak2002); it reflects the effect of the incubation medium on mitochondria (Hueser et al. 1998; Hueser and Blatter 1999; Szewczyk and Wojtczak2002) and determines the viability of mitochondria at large (Bernardi et al. 1999; SzewczykWojtczak2002). Studies on isolated (Hueser et al. 1998; Hueser and Blatter 1999), neuronal (Buckman and Reynolds 2001) and astrocyte (Diaz et al. 2000) mitochondria revealed fluctuations in the MP. Noteworthy, the MP of mitochondrial suspensions did not change under identical conditions. Fluctuations in the MP (Hueser et al. 1998; Hueser and Blatter 1999; Diaz et al. 2000; Buckman and Reynolds 2001; Thiffault and Bennett 2001) are a natural consequence of complex metabolic processes occurring in the cell and carry certain information, which has still no adequate interpretation. The recognition of individual ''voices'' in this complex ''chorus'' is a fundamental, albeit remote, problem of present-day biophysics. Its solution will yield a better insight into miscellaneous processes occurring in living cells in a real-time mode. The lack of algorithms relating individual dynamic parameters, e.g. spontaneous fluctuations in the MP (Hueser et al. 1998; Hueser and Blatter 1999; Diaz et al. 2000; Buckman and Reynolds 2001; Thiffault and Bennett 2001), to the functional state of mitochondria and the shortage of adequate methods for their measurement are the main obstacles on the way to the solution of this vital problem. Traditional techniques for the measurement of the MP (Bullen and Saggau1999; Zochowski et al. 2000) in isolated mitochondria do not meet the ever-increasing requirements for sensitivity, locality, non-invasiveness and calibration.

An alternative approach to the solution of this problem consists in analysis of the dependence between the optically measurable parameters of fluctuations in mitochondria and the functional state of mitochondria. The periodic changes in the phase height of mitochondria with a frequency of ~2 Hz observed during ATP hydrolysis (Tychinsky et al. 1998; Tychinsky et al. 2000; Vyshenskaja et al. 2002) provide an illustrative example of such dependence. However, the nature of this phenomenon has

V. Tychinsky (✉) · A. Kretushev
Moscow State Institute for Radioengineering, Electronics and Automation, Vernadsky Pr. 78, 117454 Moscow, Russia
E-mail: vladimir@tych.pvt.msu.su
Tel.: +7-095-4346792
Fax: +7-095-4348665

T. Vyshenskaja
Physics Faculty, Moscow State (Lomonosov) University, Vorobjevy Gory, 119899 Moscow, Russia

not yet been finally elucidated. One of the hypotheses currently available postulates the dependence of the macroscopic optical parameters of a mitochondrion on its MP. To corroborate this hypothesis experimentally, it is necessary to establish the dependence of some optical parameter, e.g. the average refractive index of mitochondria on the MP, in incubation media of various compositions. The measurements performed on isolated mitochondria entailed the use of highly sensitive interference methods, which ensured high accuracy, spatial resolution and absolute calibration (Tychinsky et al. 1997; Weiss et al. 2000; Tychinsky2001). Interestingly, the average (by volume of mitochondria) refractive index of isolated mitochondria differed only slightly from that of the surrounding medium. Indeed, in the media with a refractive index n_0, isolated mitochondria with diameter d can be considered as a heterogeneity $n(x, y, z)$, which in the eikonal approximation (Solimeno et al. 1986; Tychinsky 2001) provides a local optical path (OP) difference:

$$h(x,y) = \int [n(x,y,z) - n_0]\mathrm{d}z \quad (1)$$

in the image plane of the optical system. This definition of maximum phase height (PH) ($h_{\max} = d[< n_m > - n_0] = d\Delta n$) was used for the study of the dependence:

$$\Delta n(\Delta\Psi) = h_{\max}/d \quad (2)$$

In this study, we report on the linear dependence of the increments $\Delta h_{\max}(\Delta\Psi) = \mathscr{K}\Delta\Psi$ in isolated mitochondria and provide the numeric value of the constant $\mathscr{K} \approx (3\text{–}4)\times 10^{-7}$ m/V for the postulated electrooptical effect which is now well established in crystal optics (Yariv1976).

Materials and methods

Isolation of mitochondria and solutions

Rat liver mitochondria were isolated using a standard procedure (Jonston and Lardy 1967). Mitochondrial suspensions were placed in a cell (0.3 mL) with a polished substrate and a cover glass separated by a 20-μm air gap. Change of samples was performed after each addition of new components (rotenone, ATP, oligomycin). The reaction buffer contained 0.2 M sucrose, 30 mM Tris, 10 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM KCl and 250 μM EDTA. Where necessary, the buffer was supplemented with 1 μM rotenone, 0.2 mM ATP, 1 mg/L oligomycin, 0.7 g/mg cyclosporin A and 1 μM rhodamine 123. All the reagents were purchased from Sigma.

Microscopy

The measurements were performed using coherent phase microscopy (CPM) (Solimeno et al. 1986; Weiss et al. 2000; Tychinsky2001), which is a convenient technique for the study of thin, transparent and unstained biological specimens. The CPM method was realized in an "Airyscan" coherent phase microscope; its optical arrangement represents a modification of the Linnikmicrointerferometer. A single-mode helium-neon laser (LG-207A, $\lambda = 633$ nm) was used as the coherent radiation source. The linear periodic modulation of the reference wave phase was achieved through the use of a mirror fitted with a piezoelectric actuator. An LI-620 coordinate-sensitive photo detector (a dissector image tube) and an electronic unit were used for the recording of the interference signal and its analog-to-digital conversion to local phase values. The dissector is an electrooptical converter with external photo effect (without charge storage) and a magnetic scanning of the electron beam image onto the plane of a small diaphragm.

The size and the position of the scanning field area were controlled using a computer program. The visual field of the microscope for the Olympus lens 50*/0.75 was 8 μm. The noise-limited sensitivity was $h_{\min} = 0.5$ nm; the sampling frequency and the image input rate were determined by the modulation frequency of 1 kHz (or 1 ms/pixel).

Mitochondrial suspensions were placed in a cell with a polished silicon substrate and a thin cover glass. The image of a single unstained mitochondrion was projected onto the display of the microscope, after which a 2- to 3-μm-long diametrical scan line was set on its image. PH values $h(x)$ were measured continuously along the scan line. The maximum PH values, $h_{\max}$, and the diameter d at the half-height level were determined from the PH profiles $h(x)$, where x is the position of the point on the scan line. The measurements of $h_{\max}$ were performed with nanometer accuracy (Weiss et al. 2000; Tychinsky2001); the error in diameter measurements did not exceed ± 50 nm. The images of liver mitochondria had variable transversal sizes, from 0.7 to 0.9 μm; their shapes differed only slightly from a spherical shape. The diameters of mitochondria varied within a 20% range under invariable environmental conditions; the variations in $h_{\max}$ values in normal media were in the range of 40–50 nm.

Results

Influence of incubation medium on phase height

We measured the PH ($\Delta h_{\max}$) of isolated mitochondria in incubation media with known values of $\Delta\Psi$ (Liberman1972; Skulachev1990; Hueser et al. 1998; Hueser and Blatter 1999; Diaz et al. 2000; Buckman and Reynolds 2001; Thiffault and Bennett 2001). The characteristic PH profiles of liver mitochondria after addition of rotenone and during stimulation of ATPase are shown in Fig. 1. As can be seen, after addition of rotenone, which blocks the activity of respiratory chain enzymes, the $h_{\max}$ value decreased nearly 30–50%. The

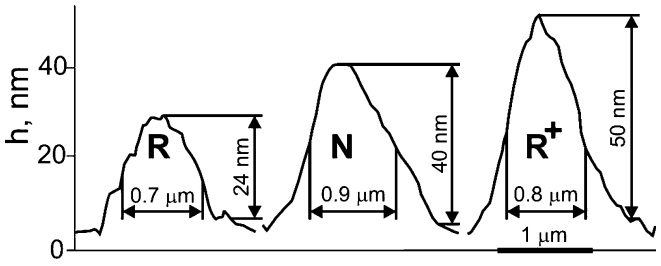

Fig. 1 The phase height profiles of mitochondria for three different media (N, control; R, rotenone; R^+, rotenone after addition of ATP). In the presence of rotenone, the phase height (h_{max}) decreased nearly 30–50%, while after addition of ATP it increased in comparison with the normal medium. The transverse sizes of the mitochondria remained practically invariable, while the phase height changed considerably

minimum values of h_{max} (10–20 nm) were observed in hypotonic buffers and in the presence of an uncoupler (FCCP), while after addition of ATP and succinate to rotenone-containing media this parameter increased in comparison with normal media. Comparison of recordings from about 300 mitochondria provided evidence that, dependent on solutions, functional height changes were 3- to 4-fold longer than the random individual ones. The maximum measured values of h_{max} reached 80–90 nm (data not shown). The effect of oligomycin manifested itself in a decrease of h_{max}, but to a lesser degree. The time-dependent changes in the mean values of $< h_{max} > (t)$ caused by sequential addition of rotenone, ATP and oligomycin to the reaction medium are shown in Fig. 2a (here, each point represents a mean from five measurements). Similar results were obtained previously during MP measurements by the electrode (Liberman1972; Skulachev1990) and fluorescent (Vladimirova et al. 1972) methods. Their measurements of $< \Delta\Psi > (t)$ are consistent with $< h_{max} > (t)$ in Fig. 2a and were used in Fig. 4c. The time-dependent changes in the fluorescence intensity after addition of rotenone, ATP and oligomycin to suspensions of submitochondrial particles have been observed previously (Vladimirova et al. 1972) (Fig. 2b). Obviously, the mean rates of changes in $< h_{max} > (t)$ and $< \Delta\Psi > (t)$ do not reflect the real time during which the changes in the state of isolated mitochondria take place (Hueser et al. 1998; Hueser and Blatter 1999). It seemed therefore important to examine whether or not the observed dependence was characteristic of faster mitochondrial processes. Addition of cyclosporin A, which markedly reduces the rate of MP decay (Hueser et al. 1998), enabled the observation of the delayed but rapid (~0.3 s) decline of PH from 55 to 15 nm (Fig. 3). Similar changes in the MP of isolated mitochondria under the action of cyclosporin A, which impedes the opening of transmembrane pores, were reported earlier by Hueser et al. (1998; Fig. 4B). A comparison of Fig. 3 with Hueser's results points to a correlation between the changes in $< h_{max} > (t)$ and $< \Delta\Psi > (t)$ in isolated mitochondria.

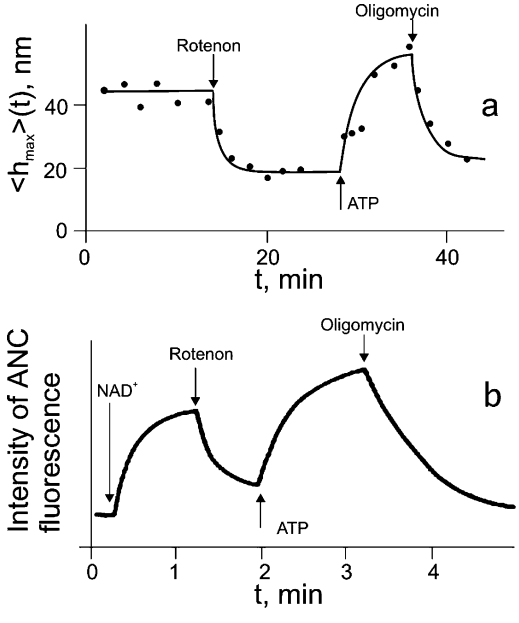

Fig. 2 (**a**) Time-dependent changes in the phase height of isolated mitochondria after sequential addition of rotenone, ATP and oligomycin. (**b**) Similar changes in the membrane potential (fluorescence intensity) measured in the submitochondrial particles suspension by the fluorescence microscopy method (Vladimirova et al. 1972)

Phase height dependence on membrane potential

The values of the maximal PH normalized for the diameter d ($\Delta n = h_{max}/d$) appeared to be very convenient for the quantitation of effects of the incubation medium and a decrease of the dependence of this value (Δn) on the diameter of a single mitochondrion. It is noteworthy that the dimensionless quantity Δn has a specific physical meaning and in the known approximation (Solimeno et al. 1986; Tychinsky2001) is equal to the difference of the mean (by mitochondrial volume) value of the refractive index $< n_m >$ and the refractive index n_0 of the external medium. Here:

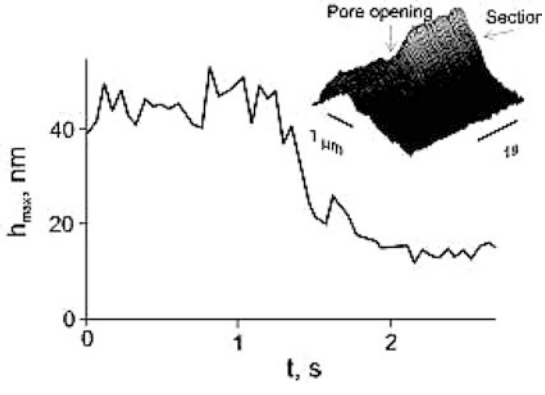

Fig. 3 Retardation of pore opening by cyclosporin A. Addition of cyclosporin A made it possible to follow the delayed drastic changes in the phase height profiles at the moment of pore opening (3D) and to measure the decrement in the phase height ($\Delta h_{max} \approx 40$ nm) in the section

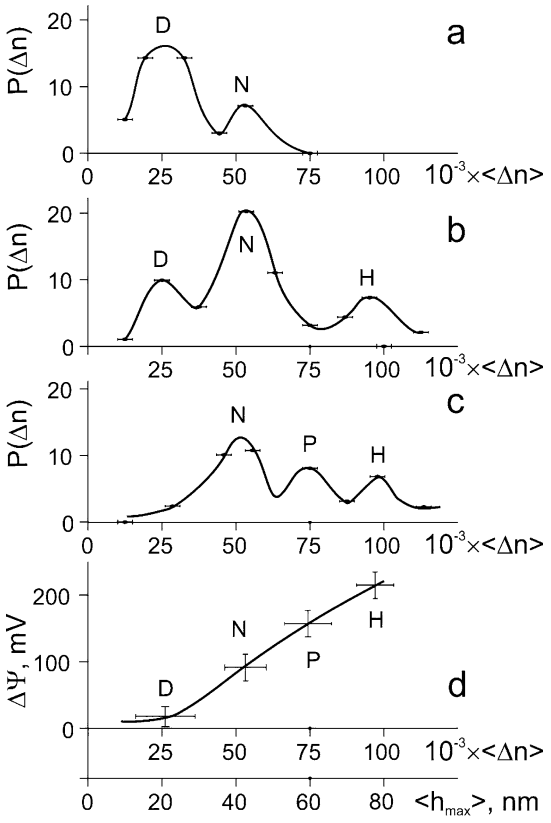

Fig. 4 The histograms of $p(\Delta n)$ for three different incubation media, where Δn is the mean difference between the refractive indices of the mitochondria and the incubation medium: (**a**) rotenone; (**b**) control; (**c**) rotenone after addition of ATP. Under the action of rotenone, some of the mitochondria remained in the normal (N) state. The more complex shape of the histogram (**c**) (rotenone + ATP) characterized by the presence of three maxima (N, P, H) can be attributed to the fact that in the course of ATP stimulation some mitochondria remained in the native state (N), while the rest passed to the polarized (P) or hyperpolarized (H) states. (**d**) The values of the mean refractive index $<\Delta n>$ and the phase height $<h_{max}>$ were determined from the histogram maxima (**a**–**c**). The almost linear dependence of the membrane potential $\Delta\Psi$ on Δn is considered as the main evidence for the electrooptical effect in mitochondria

$$\Delta n(\Delta\Psi) \approx <n_m>(\Delta\Psi) - n_0 \quad (3)$$

and $<n_m>$ correspond to the maximum value of the PH profile. At lower concentrations of the added components, $n_0 \approx 1.33$; therefore, any minor change in $<n_m>$ caused significant changes in h_{max} and, correspondingly, in Δn.

The histograms $p(\Delta n)$ for three different incubation media are shown in Fig. 4a–c. As can be seen, each histogram is characterized by several maxima. Their complex shapes can be attributed to the coexistence of different groups of mitochondria existing in different energy states. Thus, under the action of rotenone (Fig. 4a), the refractive indices of the majority of mitochondria (deenergized, D) were a minimum ($<\Delta n> \approx 0.02$–0.03), but some of the mitochondria were found to exist in the initial (normal) state. In ordinary buffers (Fig. 4b), some mitochondria were in the deenergized (D) state, while others were in the energized (N, H) states. The complex shape of the histogram characterized by the presence of three maxima for rotenone + ATP (Fig. 4c) can be explained by the fact that in the course of ATP hydrolysis the mitochondria existed in three different energy states, namely normal (N), polarized (P) and hyperpolarized (H) states. We determined the following approximated characteristic values of $<\Delta n>$ and $<h_{max}>$ for the D, N, P and H states from definite positions of the maxima: $<\Delta n>_D = 0.025$, $<h_{max}>_D = 20$ nm, $<\Delta n>_N = 0.05$, $<h_{max}>_N = 40$ nm, $<\Delta n>_P = 0.075$, $<h_{max}>_P = 60$ nm, $<\Delta n>_H = 0.1$, $<h_{max}>_H = 80$ nm. These values correspond to the approximate values of the MP designated as points D, N, P and H in Fig. 4d. Here, the values measured by electrode (Liberman1972; Skulachev1990) and fluorescent (Vladimirova et al. 1972; Hueser et al. 1998; Hueser and Blatter 1999; Diaz et al. 2000; Buckman and Reynolds 2001) methods were commensurate with the MP values measured under the conditions close to our experimental conditions.

Discussion

A live isolated mitochondrion represents a complex dynamic object; its MP and structure change with time. The observed dependence of PH on the composition of the incubation medium might reflect the effects of numerous factors directly or indirectly coupled with the MP. It is still unknown to what extent the observed dependence:

$$\Delta n(\Delta\Psi) = h_{max}(\Delta\Psi)/d \quad (4)$$

reflects the structural changes in the mitochondrial matrix or is a manifestation of the direct effect of the transmembrane potential on the optical characteristics of the inner mitochondrial membrane. There is still no unambiguous answer to this question; therefore, the dependence $\Delta n(\Delta\Psi)$ should be regarded as an experimental result which requires further verification and analysis by independent methods. Notwithstanding, it is our opinion that an attempt to approach this problem from the biophysical standpoint is quite justified. The values cited in this study suggest that changes in the refractive index of the inner mitochondrial membrane may explain our results. The dependence of the refractive index on the MP gradient is well known in crystal optics and is commonly referred to as the "electrooptical effect" (Yariv1976). However, with regard to cell organelles and other cell structures, such phenomena have not been studied in sufficiently great detail.

A combination of high MP with a large surface of the inner mitochondrial membrane inside the cristae is a unique feature, which favors the registration and investigation of the membrane electrooptical effect (MEOE) in mitochondria. It should be noted that even minor changes in the PH of unstained isolated mitochondria

could be measured exclusively owing to the high sensitivity of the CPM method. The value of the electrooptical constant was calculated from the average (by volume of mitochondria) values of PH and the numerical values of the MP cited in the literature. The accuracy of measurements of the MP by fluorescent methods depends on the calibration procedure, fluorescent label, illumination source and other, sometimes uncontrollable, factors. Therefore, in performing quantitative estimates, we made some simplifying assumptions.

We proceeded from the assumption that the minimum MP values ($<(\Delta\Psi)_D> \approx$0–20 mV) correspond to the deenergized state of the mitochondrial membranes (e.g., pore opening, effects of rotenone and FCCP), while their maximum values ($<(\Delta\Psi)_H> \approx$200–210 mV) correspond to the maximum PH values observed during stimulation of the enzyme activity by the substrate. The MP values for the intermediate states (N, P) were determined with a lesser accuracy. The MP values for astrocyte mitochondria (Diaz et al. 2000) ($<(\Delta\Psi)_P> \approx$140 mV) should rather be associated with the energized P state, while lower MP values ($<(\Delta\Psi)_N> \approx$100–120 mV) are more characteristic of normal (N) states (e.g., oxidative phosphorylation of endogenous substrates). The linear approximation of the experimental dependences $<h_{max}>(\Delta\Psi)$ and $<\Delta n>(\Delta\Psi)$ can be presented as:

$$<h_{max}>(\Delta\Psi) \approx <h_{max}>(0) + K\Delta\Psi \quad \left(K \approx 3 \times 10^{-7}\mathrm{m/V}\right) \tag{5}$$

and:

$$<\Delta n>(\Delta\Psi) \approx <\Delta n>(0) + G\Delta\Psi \quad \left(G \approx 0.375\ \mathrm{V}^{-1}\right) \tag{6}$$

and reflects nominally the dependence of mean (by mitochondrial volume) optical parameters on the MP. For simplicity sake, this factor will further be termed as the membrane electrooptical effect (MEOE).

The designations used in Fig. 4 are as follows: D, fully deenergized mitochondria; H, mitochondria with a high degree of coupling between oxidation and phosphorylation; N, P, energized mitochondria with a lower degree of coupling. Further on, we shall focus our attention on concrete biophysical processes, which may contribute to MEOE. Since the structure of mitochondria is rather heterogeneous, the contribution to the PH can be made by miscellaneous optically indistinguishable factors, e.g. the state of the mitochondrial matrix, the number and sizes of the cristae, the polarizability of the inner membrane, etc. Also, it would be natural to suppose that these processes differ not only by their nature but also by temporal characteristics. We dispose of a vast body of experimental evidence that fast polarization changes in the inner mitochondrial membrane make a predominant contribution to the changes in the PH. Indeed, fluorescence microscopic analysis of MP fluctuations revealed the presence of two characteristic time responses to transient depolarizations (Hueser et al. 1998; Hueser and Blatter 1999). For fast fluctuations distinguished at low amplitudes, the repolarization time was relatively short ($\mathscr{T}_1 \approx$0.3–1 s), whereas in the case of slow recovery of the MP, e.g. in response to ADP, FCCP or pore closure, it was one or two orders of magnitude longer ($\mathscr{K}_2 \approx$10–30 s). Quite probably, a certain role in T_2 is played by slow structural changes; however, there is still no conclusive evidence on the structural changes associated with fast depolarization. The minimum time $\mathscr{K}_1$ is commensurate with the measured value of the time of the fast decline of the PH measured during pore opening (0.3 s) (see Fig. 3). Presumably, fast changes in the PH are functionally coupled with the least inertial processes, e.g. polarizability of the inner mitochondrial membrane, which is fully consistent with the MEOE hypothesis. It is known that the potential gradient of mitochondrial membranes can reach enormous (up to 10^7 V/m) values; therefore, it would be natural to suppose that they contribute a lot to the changes in the PH.

It should be noted in conclusion that all the numerical estimates in the Appendix represent purely illustrative values. In addition to polarization, contributions to the low-inertia processes can be made by phase transitions in protein molecules, changes in membrane elasticity under the action of coulomb repulsive forces (flexoelectricity) and electrostriction (Hianik and Passechnik1995).

We fully realize that the results presented herein are only the first step towards the interpretation of various functional states of isolated mitochondria. The main finding of this study is the correlation between changes in the PH and MP in mitochondria, which is attributed to MEOE. This result is especially useful in that it provides additional information about the dynamics of intracellular processes independently of the offered interpretation.

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Appendix

Here we shall restrict here our consideration to the evaluation of the role of the inner membrane polarizability in MEOE, where:

$$<h_{max}>(\Delta\Psi) \approx <h_{max}>(0) + K_I\Delta\Psi \tag{7}$$

and K_I is linked with changes of the refractive index $n_{cr}(\Delta\Psi)$ of the inner mitochondrial membranes. The constant K_I can be quantified from the data shown in Fig. 3 ($<h_{max}>(\Delta\Psi) - <h_{max}>(0) = 40$ nm), assuming that $(\Delta\Psi) \approx$130 mV. Then, for the linear dependence of

the increments in the PH and MP, $K_I \approx 3\times10^{-7}$ m/V. Stipulating that in this approximation the main contribution to $\Delta h_{max}(\Delta\Psi)$, i.e.:

$$\Delta h_{max}(\Delta\Psi) \approx \Delta n_{cr}(\Delta\Psi) H_\Sigma = H_\Sigma CE \quad (8)$$

(where $E = \Delta\Psi/H_0$ is the MP gradient, H_0 is the thickness of the inner membrane and H_Σ is the total thickness of the cristae membranes) is made by the cristae membranes, for the common definition (Yariv1976) of the electrooptical constant we obtain:

$$C = \Delta n_{cr}(\Delta\Psi), \qquad C = KH_0/H_\Sigma \quad (9)$$

Assuming that H_0 is the genuine thickness of the inner mitochondrial membrane, equal to 8 nm, and the total thickness of the mitochondrial cristae H_Σ at the point Δh_{max} projection on the scan line is equal to 150 nm (which corresponds to the relative thickness of the cristae membranes being $H_\Sigma/\langle d\rangle \approx 0.2$ at the mean diameter $\langle d\rangle \approx 750$ nm), we obtain $C \approx 1.2\times10^{-8}$ m/V. This value is commensurate with the corresponding values for nematic liquid crystals [$(1–5)\times10^{-8}$ m/V (Brown and Wolken1979)], which share many structural features with the inner mitochondrial membranes and cells (Sonin1983). The corresponding value $\Delta n_{cr}(\Delta\Psi) \approx 0.4$ is commensurate with the experimentally determined value $\Delta n(\Delta\Psi) \approx 0.5$ of the potential-dependent component of the optical anisotropy in nematic liquid crystals, which is attributed to the changes in the orientation of the dipole moments.

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