THE 2-(DIMETHYLAMINOSTYRYL)-1-METHYLPYRIDINIUM CATION AS INDICATOR OF THE MITOCHONDRIAL MEMBRANE POTENTIAL

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1. Introduction

The mitochondrial membrane potential ($\Delta\psi$) [1] is commonly determined from the distribution either of permeant radioactive labelled cations or of dyes yielding various optical signals (reviews [2–6]). Measuring ion distribution using the Nernst equation, requires sample collection techniques not well suited to monitor short time changes of the membrane potential. However, optical probes available do not distribute in agreement with the Nernst equation [7,8] and lack well defined calibration [7,9].

Styryl dyes, first used as indicators of the action potential of the giant axon [10], increase their fluorescence intensity drastically when incubated with energized mitochondria [11,12]. We show here that distribution of the 2-(dimethylaminostyryl)-1-methylpyridinium ion ($DSMP^+$) occurs between the external space and the matrix compartment of intact mitochondria in accordance to the Nernst equation. The resulting fluorescence signal is defined by its correlation to the amount of dye taken up by the mitochondria and is introduced as a quantitative measure of the membrane potential.

2. Materials and methods

Isolated rat liver mitochondria [13] were incubated in 250 mM sucrose, 10 mM Hepes (pH 7.3), 2 mM succinate and 8 μM rotenone at 25°C. All other concentrations were as indicated in the figure legends.

Fluorometry was performed with a Perkin Elmer MPF 44A spectrofluorometer (excitation at 479 nm, emission at 589 nm). For kinetic measurements the fluorometer was connected with a Tektronix 4051 calculator via an analog/digital converter (data output ~100/s). Velocity constants were calculated by regression analysis of the data.

$DSMP^+$ concentration in the extra- and intramitochondrial compartment was measured after addition of [^{3}H]DSMP-J (spec. act. 1–100 mCi/mmol) to the incubation medium. Mitochondria were spun down in an Eppendorf centrifuge (2 min, 0°C) and radioactivity was determined in the pellet (dissolved in 15% SDS) and in the supernatant by means of a Philips scintillation counter. Data correction for extramitochondrial fluid in the pellet and determination of the mitochondrial matrix volume was performed as in [14]. Distribution of $^{86}Rb^+$ (spec. act. 0.2–4 mCi/mg) (plus 0.5 μM valinomycin) and [^{14}C]$TPMP^+$ (spec. act. 14.5 mCi/mmol) was measured as described for [^{3}H]$DSMP^+$. Protein was determined by the biuret method.

DSMP-J was synthesized as in [15]. Purity was checked by IR- and NMR-spectroscopy and thin-layer chromatography with acetone/H_2O (50/50, v/v). For synthesis of [^{3}H]DSMP-J [^{3}H]CH_3J (spec. act. 100 mCi/mmol) was used. [^{14}C]TPMP-Br was synthesized from triphenylphosphin and $^{14}CH_3Br$ (spec. act. 14.6 mCi/mmol) [16]. All radioactive material was from Amersham Buchler GmbH (Braunschweig). All other reagents were of the highest commercially available purity.

3. Results and discussion

The fluorescence intensity measured with $DSMP^+$ depends very much on the polarity of the solvent and

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; $TPMP^+$, triphenylmethylphosphonium cation

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Table 1
Fluorescence intensity of 1 μM DSMP-J in different solvents and in aqueous albumin solutions

Solvent	Fluorescence intensity (arbitrary units)
Water	1.9
Ethanol	86.9
Chloroform	123.0
Acetone	14.5
Albumin 7.5%	28.2
15.0%	30.1

For details see section 2

is considerably increased in albumin solutions (table 1). We suggested therefore that fluorescence changes reflecting mitochondrial energization [11,12] were due to a reversible transfer of the dye into a more apolar microenvironment, i.e., the mitochondrial membrane or the matrix compartment with a high protein concentration. This could be proved by binding studies with [^{3}H]DSMP$^+$. As demonstrated in fig.1, the correlation of the fluorescence intensity displayed by rat liver mitochondria and the amount of DSMP$^+$ taken up by the organelles is strictly linear up to a certain amount of dye. It is also shown that within the linear range of this relationship, the fluorescence yield is virtually independent from the amount of mitochondria the dye is attached to. In other words, within the linear range the fluorescence depends only on the amount of DSMP$^+$ transferred into mitochondrial surroundings, no matter how concentrated the dye may be in this environment. This corresponds to the finding that 1 μM DSMP$^+$ yields practically the same fluorescence intensity in a 7.5% and 15% albumin solution (table 1). To explain these effects, mutual influences of styryl dyes and proteins in aqueous solution are now under detailed study.

Linearity of the relationship between the fluorescence intensity and the amount of DSMP$^+$ taken up by mitochondria (fig.1) holds up to an intramitochondrial DSMP$^+$ level of ~3 nmol dye/mg mitochondrial protein. The relative decrease of fluorescence yield at higher concentrations is most likely due to aggregation of dye molecules, as indicated by a bathochromic shift of the maximum excitation wavelength. However, scattering effects may decrease the slope of the linear section with >4 mg mitochondrial protein/ml. Moreover, toxic effects on oxidative phosphorylation have been reported with >4 nmol dye/mg mitochondrial protein [12].

Because of its delocalized electrical charge [15] DSMP$^+$ appears likely to be taken up into the mitochondrial matrix space in analogy to other large organic cations, driven by the membrane potential. This uptake occurs independently of the dye concen-

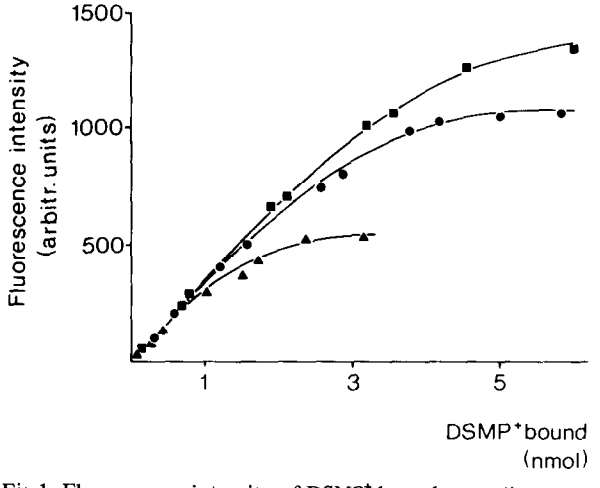

Fig.1. Fluorescence intensity of DSMP$^+$ bound to rat liver mitochondria: (▲) 0.66 mg, (●) 1.32 mg and (■) 1.86 mg mitochondrial protein/ml were incubated under state 4 conditions with varying amounts of [^{3}H]DSMP-J. Experimental details were as in section 2.

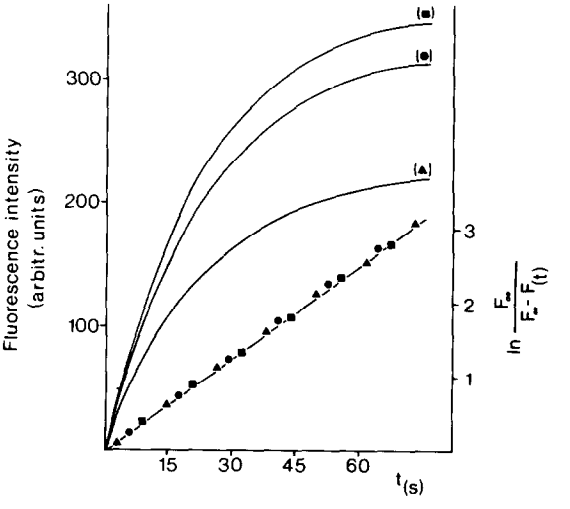

Fig.2. Kinetics of DSMP$^+$ uptake by liver mitochondria as indicated by the fluorescence intensity. The straight line results from evaluating the data in accordance to first order kinetics ($\ln F_\infty/F_\infty - F_{(t)} = kt$). Mitochondrial protein (1.32 mg/ml) was incubated under state 4 conditions with (▲) 0.73 μM, (●) 1.08 μM and (■) 1.16 μM DSMP-J.

tration in the incubate and is strictly subject to first order kinetics (fig.2). This shows that $DSMP^+$ permeates the membrane along the electrical gradient by simple diffusion. No specific binding is indicated of the dye on either side of the membrane and no primary active transport of $DSMP^+$ becomes obvious under the experimental conditions employed. Distribution of $DSMP^+$ would thus occur in a way permitting the determination of the membrane potential from the Nernst equation. It was further found that $DSMP^+$ per se has no effect on the membrane potential at dye concentrations necessary for membrane potential studies (up to 3 nmol $DSMP^+$/mg mitochondrial protein).

Membrane potentials of rat liver mitochondria as measured with $DSMP^+$ were from 190–205 mV under conditions of maximum energization. Similar results were obtained with [^{14}C]$TPMP^+$ (see also fig.3). Though considerably higher than most cited data [17] these results agree with [18,19].

Distinct from other optical probes of membrane potential changes (reviews [3–6]), slow redistribution signals [3] as obtained with $DSMP^+$ are not based on quenching [20] or stacking effects [7]. The membrane potential can thus be determined directly from the optical signal by the Nernst equation when the correlation of fluorescence intensity and dye uptake has been defined (fig.1) and the matrix volume of the mitochondria has been measured:

$$\Delta\psi = -\frac{R \cdot T}{F} \cdot \ln \frac{[DSMP^+]_{in}}{[DSMP^+]_{ex}}$$

$$[DSMP^+]_{in} = \frac{F_I}{F_D \cdot \nu}; \quad [DSMP^+]_{ex} = \frac{D - [DSMP^+]_{in} \cdot \nu}{V - \nu}$$

F_I = Fluorescence intensity (arbitrary units) corrected for basis fluorescence (medium, mitochondria)
F_D = Fluorescence intensity/nmol $DSMP^+$ bound (arbitrary units)
D = Amount of $DSMP^+$ in incubate
V = Incubation volume
ν = Matrix volume of mitochondria in the incubate

Standardization of the measuring system can be approached in practice without circumstantial definition of the fluorescence intensity/nmol $DSMP^+$ bound (F_D) by the [^{3}H]$DSMP^+$ procedure (fig.1), if the mitochondria under investigation are capable of maximum membrane energization similar to rat liver mitochondria. In this case the fluorescence intensity displayed by the organelles with $DSMP^+$ under state 4 conditions is correlated to the theoretical amount of dye taken up by the mitochondria as calculated from an assumed membrane potential of 190 mV and the measured matrix volume. This data defines the linear range of the correlation between fluorescence intensity and dye uptake (fig.1) with good approximation, and membrane potential changes can thus be derived from the fluorescence intensity under the employed experimental conditions. There is little danger that the concentration of mitochondria and $DSMP^+$ used for standardization might not meet the linear section of the correlation (cf. above, fig.1).

For further control, membrane potential changes caused by increased extramitochondrial K^+ concentration in the presence of valinomycin [21] were measured with $DSMP^+$ and other indicators of the membrane potential. All monitors yield very similar results at high membrane potentials and a linear relationship is demonstrated with all indicators of the measured membrane potential and the negative log[K^+] (fig.3). Data recorded with decreased membrane potentials, however, are significantly higher with

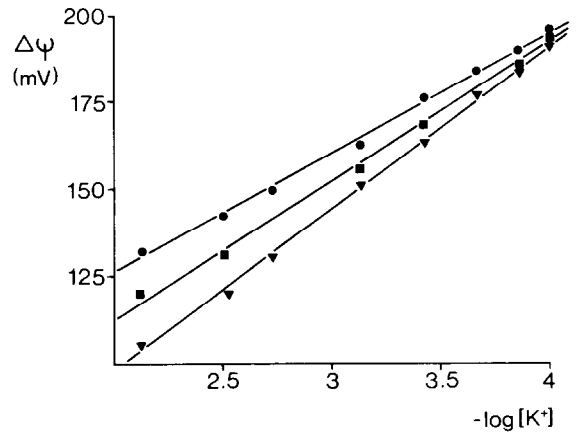

Fig.3. Decrease of the mitochondrial membrane potential by valinomycin-mediated potassium uptake as measured from the distribution of $DSMP^+$, $TPMP^+$ and Rb^+. Mitochondrial protein (0.86 mg/ml) was incubated with (●) 0.95 μM [^{3}H]-DSMP-J, (■) 2.05 μM [^{14}C]TPMP-Br and (▼) $^{86}Rb^+$-marked K^+ (plus 0.5 μM valinomycin) as indicated. Specific activities and experimental details were as in section 2. Data present means from 3 expt.

$DSMP^+$ than with Rb^+ and $TPMP^+$ (cf. below).

According to these results, determination of the membrane potential based on valinomycin-mediated distribution of $^{42}K^+$ or $^{86}Rb^+$ [2] is subject to extensive underestimates, when the employed K^+ (Rb^+) concentration is in the mM range [17]. Apart from affecting the membrane potential, valinomycin-mediated uptake of K^+ into the mitochondria has been shown to occur simultaneously with energy-driven proton extrusion [22,23], i.e., to be coupled to primary energy-driven processes. The Nernst equation is no longer applicable for membrane potential determination under these conditions. In practice the influence of K^+ uptake can be neglected at $<50\ \mu M$, as demonstrated with $DSMP^+$ and $TPMP^+$ in the presence of varying K^+ concentrations with and without valinomycin (not shown). On the other hand, there is no evidence that $DSMP^+$ or $TPMP^+$ are not subject to mechanisms similar to those demonstrated on valinomycin-mediated K^+ transport. However, $DSMP^+$ concentrations required are 100–1000-times smaller than K^+ (Rb^+) concentrations commonly used [17] and no practical violation of the Nernst distribution becomes obvious under conditions of high mitochondrial energization. This may be different at low energetic states. Varying properties of membrane potential indicators with respect to possible concomitant effects of their distribution could be a reason for diverse results as observed with different indicators at decreased membrane potentials (fig.3).

We may conclude that $DSMP^+$ accomplishes all conditions required for functioning as a membrane potential monitor under the employed experimental conditions. As a lipophilic cation it needs no ionophore that renders the mitochondrial membrane permeable for naturally abundant ions. As an optical probe it surpasses other dyes by well definable calibration and high sensitivity.

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