SAFRANINE AS A PROBE OF THE MITOCHONDRIAL MEMBRANE POTENTIAL

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1. Introduction

One of the most central postulates of the chemiosmotic hypothesis of oxidative phosphorylation is the existence of a large electrochemical proton gradient (protonmotive force) across the mitochondrial membrane [1,2]. This gradient, which is composed of a membrane potential and a pH differential, is postulated to be the obligatory link between respiration and phosphorylation in the process of oxidative phosphorylation. The membrane potential has been suggested to be the major component of the electrochemical proton gradient under most conditions, and measurements of this potential are therefore essential for the understanding of the mechanism of oxidative phosphorylation and mitochondrial ion transport.

The mitochondrial membrane potential has mainly been estimated from the distribution of K^+ or Rb^+ across the mitochondrial membrane in the presence of the ionophore valinomycin [3–8]. This method has given fairly consistent results and membrane potentials in the range 130–190 mV (positive polarity extramitochondrially) have generally been reported in coupled mitochondria supplemented with either substrate or ATP. The differences in the values reported by different workers may for the most part be attributed to the use of different values for the matrix volume. The use of a method of this kind, which requires partially artificial experimental conditions, makes confirmation by other independent methods most desirable. This has prompted several workers to search for suitable spectroscopic membrane potential probes. The fluorescent probe MC V has been used by Chance and collaborators [9,10] as a membrane potential indicator in mitochondria, and Laris et al. [11] have employed a cyanine dye for the same purpose. The latter workers calibrated the fluorescence with potassium diffusion potentials across the mitochondrial membrane and showed a linear relationship between fluorescence and membrane potential up to about 120 mV. Unfortunately, this value is much lower than the membrane potentials generally found by the ion distribution method. However, on the basis of their calibration curve, Laris et al. estimated the membrane potential of State 4 (for nomenclature, see [12]) mitochondria and arrived at values comparable to those found by the ion distribution method. Azzi et al. [13] and Jasaitis et al. [14] used anilinonaphtalene sulphonate (ANS) as a membrane potential probe and arrived at results comparable to those discussed above. However, Ferguson and co-workers [15] recently warned against the use of ANS as a membrane potential indicator in mitochondrial systems. Tedeschi, Harris and Pressman [16–20] have argued against the presence of a large mitochondrial membrane potential and have suggested the presence of a small potential difference independent of metabolism and with a polarity opposite to that expected on the basis of the chemiosmotic hypothesis.

Safranine is a positively charged dye which has been shown to reveal stacking associated with large spectral shifts in energized mitochondria [21], upon combination with polyanions [21] and in bacterial vesicles [22]. Åkerman and Saris [23] have recently shown that the stacking of safranine occurs upon induction of a diffusion potential of potassium across liposomal membranes.

In this communication it is shown that the induction of a diffusion potential of potassium or hydrogen ions across the membrane of isolated mitochondria (in the presence of valinomycin or proton-conducting

uncouplers respectively) and with positive extramitochondrial polarity, gives rise to a spectral shift in safranine due to stacking, which is identical to that observed upon energization of the mitochondria by respiration or ATP hydrolysis. The spectral shift is linearly related to the developed membrane potential up to at least 170 mV and is quantitatively the same irrespective of whether it is produced by a hydrogen or potassium ion gradient. The membrane potentials estimated in various states of isolated rat-liver mitochondria are similar or slightly higher than those found by the ion distribution technique. It is concluded that safranine may be a useful spectrophotometric probe of the mitochondrial membrane potential.

2. Materials and methods

Rat-liver mitochondria were isolated in sucrose-EDTA medium with final washes omitting the EDTA as described previously [24]. The standard reaction medium consisted of 0.2 M sucrose and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate (HEPES), pH 7.2 with or without a further addition of 20 mM KCl. In the experiments on potassium efflux, the mitochondria were used immediately after isolation, and in all cases during the same day.

Absorption spectra and time-dependent absorption changes were recorded with a Johnson Research Foundation scanning dual wavelength spectrophotometer (DBS-1) at wavelengths specified in the figure legends. This instrument allows and 'absolute' spectrum to be taken over a specified spectral interval with reference to the absorption at a set reference wavelength. This 'baseline' is memorized electronically. Following the experimental perturbation of the sample a new spectrum is recorded from which the initial baseline is automatically subtracted. Thus difference spectra of changes induced by specific perturbations may be conveniently recorded.

Mitochondrial protein was assayed by the Folin method [25] with bovine serum albumin as standard.

All experiments were performed with glass cuvettes of 1 cm light pass and at room temperature (23–24°C).

Safranine was purchased from E. Merck A. G., Darmstadt, and used without further purification by addition of small aliquots of a 5 mM aqueous solution of the dye. Carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) was a gift by Dr P. G. Heytler. All other reagents were commercially available products of highest available purity.

3. Results

Figure 1 shows a difference spectrum of the shift observed in safranine upon energization of the mitochondrial membrane by ATP (cf. [21]). In the experiments to be described below the various perturbations do all produce this shift or abolish a preexisting shift. This fact was carefully controlled in each single case.

As shown in fig.2, the shift is also observed upon induction of a diffusion potential of K^+ ions catalyzed by valinomycin. In the potassium-poor medium used here, the dye was found to be partially shifted already before addition of the ionophore and a further decrease of the absorption difference was induced by addition of potassium. The valinomycin-induced shift is insensitive to oligomycin and decays with a $t_{\frac{1}{2}}$ of approx. 4 min. Most of the shift was under these conditions abolished by addition of an uncoupler of oxidative phosphorylation. A further decrease occurred upon addition of potassium (see fig.2 and below). The induction of a diffusion potential of hydrogen ions across the mitochondrial membrane (with positive polarity in the extramitochondrial space) by a pulse of alkali in the presence of a proton-conducting uncoupling agent, also produced the spectral shift (see fig.3). Under these conditions the shift was abolished by the presence of valinomycin plus potassium.

The produced diffusion potential of K^+ and H^+ ions may be approximated by the use of the Nernst equation and the concentration gradients of the respective ions. In such calculations of the induced membrane potential the intramitochondrial potassium concentration of freshly isolated mitochondrial was assumed to be 120 mM [26]. An error in this estimate would cause only a very small error in the estimated membrane potential. Thus, if the intramitochondrial potassium concentration was 100 mM (see [5,27]) the potential would be overestimated by 5 mV. The results of series of experiments using the valinomycin and the pH transient techniques for induction of membrane potentials of various mag-

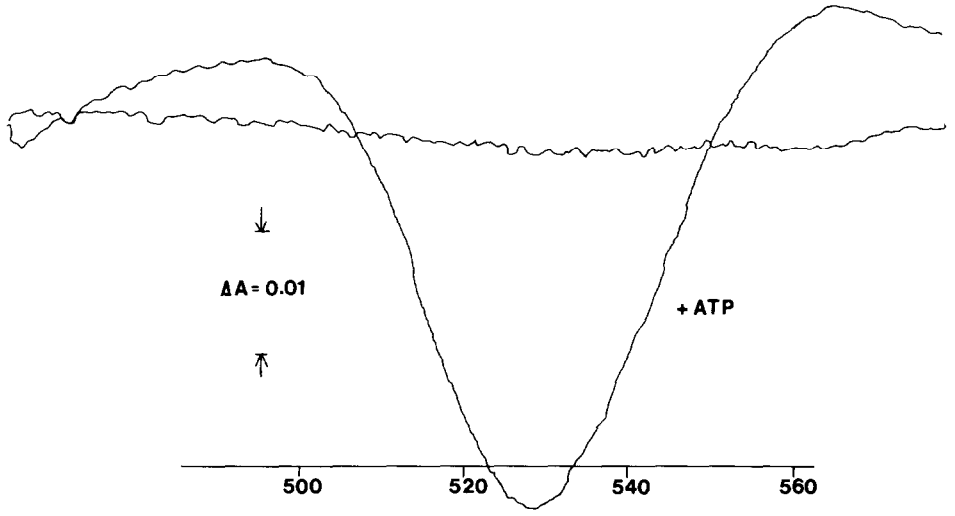


Fig.1. Spectral change in safranine upon energization of mitochondria. The sucrose-KCl-HEPES medium (see Materials and methods) was supplemented with 6 μM rotenone, 0.38 mM EDTA and 9.6 μM safranine. Rat-liver mitochondria were added to a concentration of approx. 1.7 mg protein per ml, and the mixture was incubated for 2 min. Then a baseline was recorded into the memory unit (see Materials and methods) and onto the chart (flat line in the figure). After addition of 1.5 mM ATP the difference spectrum was recorded (line marked +ATP). Reference wavelength 470 nm. The abscissa indicates wavelength in nm.

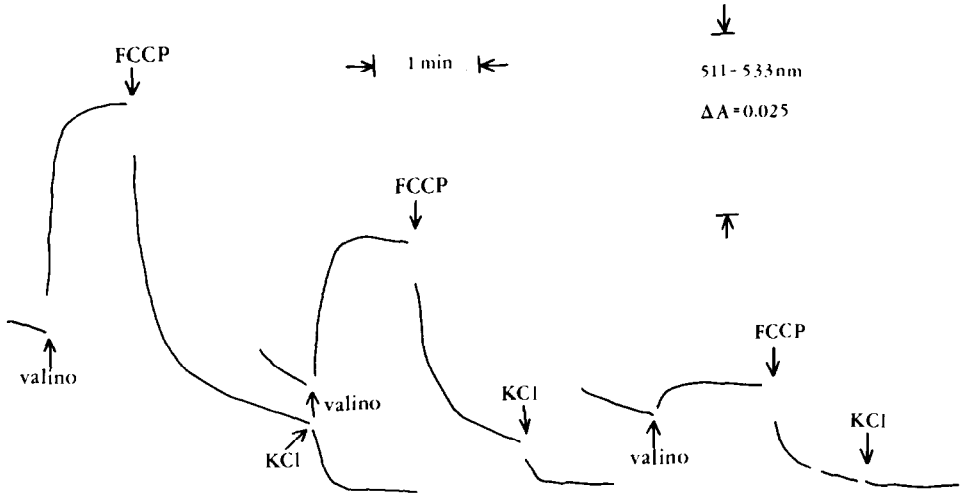


Fig.2. Spectral shifts in safranine induced by a diffusion potential of potassium. Rat-liver mitochondria (0.8 mg protein/ml) were suspended in sucrose-HEPES medium (see Materials and methods) containing 6 μM rotenone, 0.38 mM EDTA, 3.85 μg/ml oligomycin, 9.6 μM safranine and KCl in the following concentrations: left hand trace – no KCl added, middle trace 0.96 mM and right hand trace 3.8 mM. 38.5 ng/ml valinomycin and 0.48 μM FCCP were added as indicated in the figure, followed by 20 mM KCl.

nitude have been plotted in fig.3. A linear relationship was obtained between the extent of spectral shift and the induced membrane potential in the range 40–170 mV, and most important, the relationship was found to be identical within experimental error irrespective of whether the electrical potential difference was induced as a potassium or a hydrogen ion diffusion potential. This greatly supports the usefulness of the Nernst equation as applied on mitochondria under the present experimental conditions. Attempts to produce higher membrane potentials resulted in deviations from the straight line. For instance, a further decrease in the concentration of added potassium caused less increase in signal than expected from the straight line. This is presumably due to the fact that we estimated extramitochondrial potassium as *added potassium*. Thus, the membrane potential will be overestimated at the point where the concentration of added potassium approaches that of potassium that has leaked out from the mitochondria. With freshly isolated mitochondria, however, such an error became apparent only when the added potassium concentration was decreased below approx. 0.2 mM.

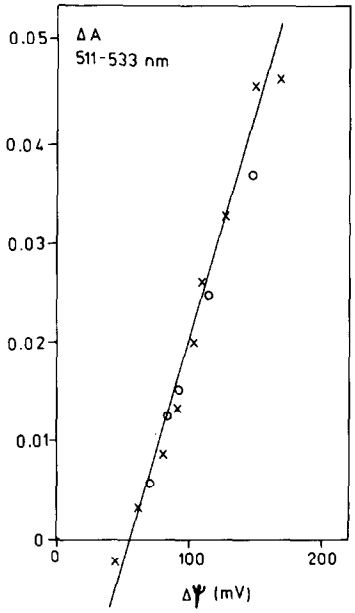
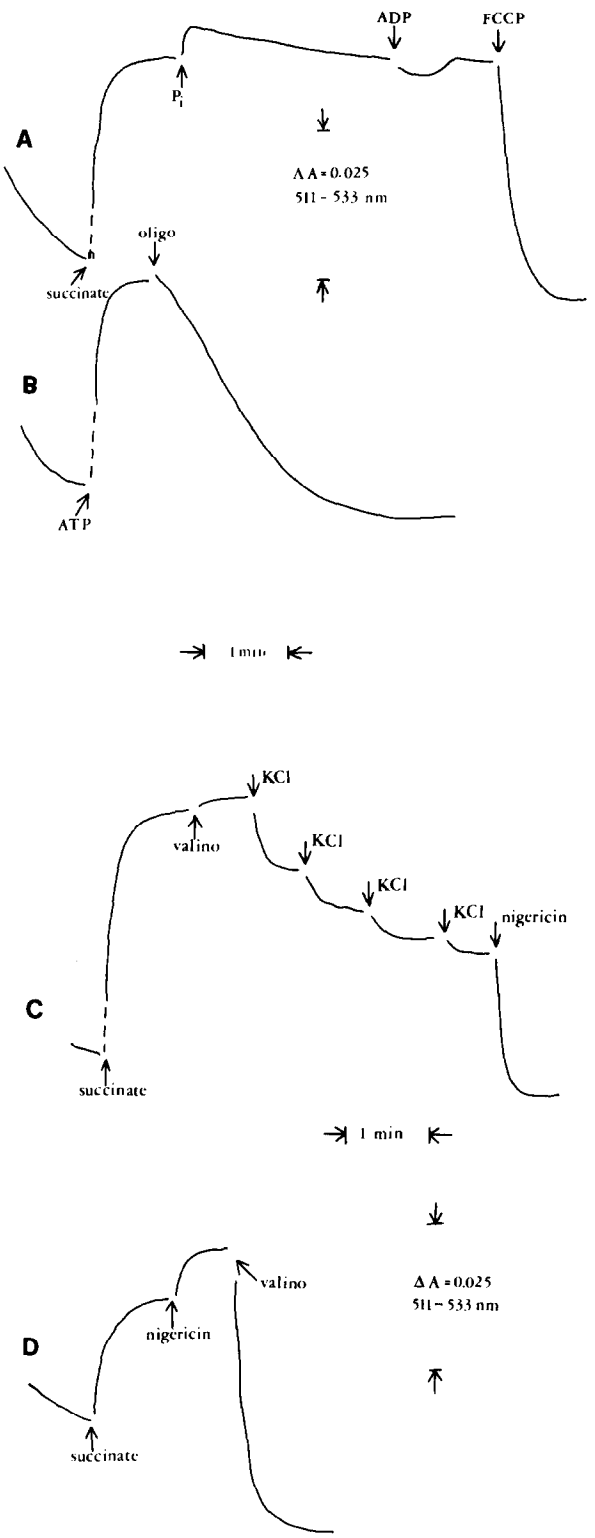


Fig.3. The dependence of the spectral shift in safranine on the diffusion potential of potassium and hydrogen ions. Conditions for potassium potential as described in the legend to fig.2. The potential was calculated according to the Nernst equation, $\Delta\psi = 60 \log \frac{(K^+_{in})}{(K^+_{out})}$, where (K^+_{in}) was assumed to be 120 mM (see text and (K^+_{out}) equal to added potassium. The extent of the signal was the difference in absorption after addition of valinomycin as compared to that after further addition of FCCP and KCl (to about 20 mM). The experiments were conducted exactly as shown in fig.2. The hydrogen ion diffusion potentials were generated as follows: Medium, sucrose-KCl-HEPES as described in Materials and methods, except that the pH was adjusted to 6.0 with HCl. The initial conditions were otherwise as described in the legend to fig.2, but with a further addition of 0.48 μM FCCP. The mitochondrial suspension was then incubated at room temperature for 10 min to allow pH equilibration across the membrane. This was followed by pulses of KOH that suddenly brought the pH of the external medium to various pH values in the range 7–8.5. The peak absorption change was measured and the membrane potential calculated according to the Nernst equation (see above) with pH_{in} 6.0 and pH_{out} = pH measured after KOH pulse. The drawn straight line is the best fit (least squares analysis) to the K^+ diffusion potential data (× – ×). The H^+ diffusion potential data is indicated with circles (○ – ○).

It should be noted from fig.3, that the straight line intercepts the abscissa ('zero' spectral shift) at a potential of about 50 mV indicating a membrane potential of this magnitude under our 'zero' conditions (uncoupler and 20 mM extramitochondrial KCl). As shown in fig.2, the valinomycin addition was followed by addition of an uncoupling agent which greatly reduced the spectral shift. The trace was however still slowly drifting downwards, but upon addition of potassium to a final concentration of 20 mM a further decay was induced followed by an essentially stable signal. This latter level was taken as 'zero' spectral shift. Our finding of a membrane potential of about 50 mV in uncoupled mitochondria suspended in the presence of 20 mM KCl is in excellent agreement with the recent data of Nicholls ([6], see also [3]), who measured a membrane potential of similar magnitude under comparable conditions using the ion distribution technique. Under these conditions the membrane potential is counteracted by a pH gradient, acid inside, resulting in zero electrochemical proton gradient [6].

The responses of safranine to various typical perturbations of isolated rat-liver mitochondria are summarized in fig.4.

The magnitude of the membrane potential in resting mitochondria respiring on succinate in the absence of P_i is about 165 mV (fig.4A) on the basis of the extent



of safranine shift and comparison to calibrations such as that of fig.3 with the same mitochondrial preparation. Very little variation (± 5 mV) was found in this value between different preparations of mitochondria, and the found membrane potential was very nearly the same irrespective of whether the substrate was succinate (fig.4A), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) plus ascorbate, or malate plus glutamate. Note that the addition of inorganic phosphate causes a small but consistent (about 10 mV) increase in $\Delta\psi$ under these conditions in agreement with the data of Nicholls [6] so that the State 4 potential is correspondingly higher. Upon transition to State 3 (addition of ADP) the membrane potential decreases only little (about 5–10 mV) and returns to its initial value on the subsequent transition to State 4. This cyclic effect is abolished by oligomycin, being again both qualitatively and nearly quantitatively in agreement with data obtained with the Rb^+ or K^+ distribution technique. The addition of ATP to non-respiring mitochondria (fig.4B) induces a spectral shift equivalent to about 160 mV in the absence of added P_i or ADP. This shift is not dependent on the concentration of ATP, but on the ratio ATP/ADP·P_i (i.e. upon the phosphorylation potential). The detailed dependence will be reported in a subsequent full paper.

Addition of valinomycin to respiring mitochondria (fig.4C) has little effect in the absence of added potassium, but subsequent addition of potassium decreases the signal, again a behaviour expected for the mitochondrial membrane potential which is broken down by the electrophoretic movement of potassium

Fig.4. The safranine response in various states of rat-liver mitochondria. The sucrose-KCl-HEPES medium (except in trace C where KCl was omitted from the medium) was supplemented with 6 μM rotenone, 0.38 mM EDTA, 9.6 μM safranine and rat-liver mitochondria (0.8 mg protein/ml). The following additions were made as indicated in the figure: 7.7 mM Tris-succinate, 1.5 mM ATP, 3.85 mM KH_2PO_4 (P_i), 3.8 μg/ml oligomycin, 0.19 mM ADP, 0.48 μM FCCP, 38.5 ng/ml valinomycin, 3.9 ng/ml nigericin and (in trace C) 4 additions of 0.96 mM KCl each. The induced membrane potentials in traces A and B (see text) were calculated on the basis of a calibration curve exactly as described in the legends to figs.2 and 3 with the same preparation of mitochondria. At a constant safranine/mitochondrial protein ratio, these calibration curves were found to vary very little from preparation to preparation.

catalyzed by valinomycin. The combination of valinomycin and nigericin (in the presence of potassium) abolishes the shift in much the same way as does an uncoupling agent.

As seen in fig.4D, nigericin when added alone causes an increase in the safranine shift of about the same magnitude as the effect of inorganic phosphate (cf. fig.4A). The effects of P_i and nigericin are not additive and are consistent with the idea that an abolition of the existing pH gradient under conditions of respiration (by P_i^-/OH^- exchange [28], or by K^+/H^+ exchange [29]) will tend to increase the membrane potential in an attempt to keep the electrochemical proton gradient at a constant value.

4. Discussion

The present results strongly suggest that safranine responds with a large spectral change upon induction of an electrical potential difference across the mitochondrial membrane (fig.3) similar to what is found in liposomes [23]. It is of particular importance that the spectral change is observed upon creation of a diffusion potential of protons under which conditions the electrochemical proton gradient (protonmotive force) is zero. Thus 'energization' of the mitochondrial membrane by reversal of a hypothetical proton pump can be specifically excluded as the basis for the spectral change in safranine. In fact, under conditions of the diffusion potential of hydrogen ions, the mitochondria are totally 'de-energized' from the point of view of oxidative phosphorylation. It is also significant that a good quantitative agreement was found between safranine responses induced by potassium and hydrogen ion diffusion potentials.

Colonna et al. [21] have suggested that stacking of safranine in mitochondria may be the result of binding of the dye to 'fixed' negatively charged groups in a hydrophobic environment of the mitochondrial membrane. If so, the rearrangement of these charges and/or their environment that favours the stacking phenomenon seems to coincide with the development of an electric field across the mitochondrial (and liposomal, see [23]) membrane. The observations of safranine stacking in liposomes [23] would thus suggest that these charges originate in the head groups of the phospholipids. Preliminary studies (M. K. F. Wikström and K. E. O. Åkerman, unpublished data) have shown that the energization of sonicated submitochondrial particles by coupled respiration or ATP hydrolysis, as well as the induction of a diffusion potassium of opposite polarity as compared to the mitochondrial system (positive inside the vesicles), produces a safranine shift qualitatively identical to that in intact mitochondria. This indicates that stacking of the dye indeed seems to occur in the membrane proper (or on the inner surface in mitochondria) rather than in the matrix space, in agreement with the conclusions of Colonna et al. [21].

It is clear that although the spectral shift in safranine can certainly be associated with the development of an electric field across the mitochondrial membrane under some conditions (fig.3 and [23]), the identical spectral response upon energization of the mitochondria by coupled respiration or by ATP hydrolysis need, in principle, not necessarily be accompanied by an electrostatic potential difference between the bulk aqueous phases on each side of the mitochondrial inner membrane. This must be pointed out especially as the stacking effect appears to be due to binding of the dye to the membrane proper. On the other hand, the remarkable qualitative and quantitative agreement between the potentials estimated from the safranine shift and those calculated with the aid of the ion distribution methods (bearing in mind that the safranine shift is calibrated on the basis of true membrane potentials), strongly indicates that the energized state of the mitochondrion is indeed associated with a fairly large membrane potential of some 170 mV (State 4), which is positive extramitochondrially and is dependent on mitochondrial metabolism (contrast 16–20).

With Nicholls [6] and Wiechmann et al. [7] we would like to stress, however, that the electrochemical proton gradient across the mitochondrial membrane (of which the membrane potential is the major component) is not sufficiently large to balance an extramitochondrial phosphorylation potential of approx. 15 kcal/mol under State 4 conditions if a H^+/ATP stoicheiometry of 2 [30–32] is assumed for the proton-translocating ATPase. When the pH gradient of State 4 mitochondria is taken into account [6,33] and added to the found membrane potential to yield the electrochemical proton gradient, the minimum H^+/ATP stoicheiometry that would be required

for equilibration of the protonmotive force with an extramitochondrial phosphorylation potential of 15 kcal/mol [34,35] is close to 3 (see also [6,7]). However, it is obvious that a fixed stoicheiometry of 3 H^+/ATP for the mitochondrial ATPase/ATP synthetase enzyme is not compatible with the present formulation of the chemiosmotic hypothesis (see e.g. [2]) and further studies are clearly required to resolve this problem. The role of the partially electrogenic adenine nucleotide translocator [35] is one of the key problems in this respect, but the possibility of a variable H^+/ATP stoicheiometry of the proton-translocating ATPase also offers interesting possibilities.

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