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Response of the Electrochromic Dye, Merocyanine 540, to Membrane Potential in Rat Liver Mitochondria

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Summary. Merocyanine binds extensively to rat liver mitochondria in spite of the presence of a sulfonic acid group which would suggest only limited penetration through the membrane. Passive binding shows both tight and weak binding components and is dependent on salt concentration and ionic strength in accord with the Gouy-Chapman theory. The binding of merocyanine to mitochondria is accompanied by both a fluorescence enhancement and a spectral shift. Induction of an electrical field by either respiration or K^+ diffusion potential results in a partial reversal of the spectral shift seen on dye binding. At low temperature, the merocyanine spectral response to an electrical field is biphasic, consisting of a fast phase with a $t_{1/2}$ of less than 1 sec at 15°C and a slower phase which may vary considerably in rate and extent with conditions. The spectral shift during the two phases appears similar, but differ in sensitivity to ionic strength and temperature. The spectral shift during the fast phase at 15°C indicates that the major component is a decrease in bound monomer and an increase in the aqueous dimer, indicating an "on-off" mechanism. It is suggested that the fast and slow phases of the merocyanine response may be due to two different populations of dye, possibly located at the outer and inner surfaces, respectively, of the mitochondrial membrane. The electrophoretic movement of the dye located in the membrane interior would result in the temperature-sensitive slow phase response. Demonstration of the proportionality of the fast phase response to the magnitude of the membrane potential suggests the usefulness of merocyanine in studies with mitochondrial systems.

Key Words merocyanine · membrane potential · mitochondria · electrochromic dyes · potential-sensitive dyes

Introduction

Transmembrane potentials ($\Delta\psi$) generated across ion-impermeable membranes are the major component of the proton electrochemical gradient, $\Delta\mu_{H^+}$ in energy-transducing systems, such as mitochondria and bacteria (Mitchell & Moyle, 1969). The assay of $\Delta\psi$ in mitochondria and similar energy-transducing systems may be most accurately performed by the equilibrium distribution of permeant ions across the membrane (Azzone, Pietrobon & Zoratti, 1984; Rottenberg, 1989). This methodology, however, requires that the system maintain a stationary state of constant $\Delta\psi$ during time required for the permeant probe to reach equilibrium and may involve time-consuming separation of the mitochondria from the reaction medium.

Membrane potential probes which respond with spectral or fluorescence changes, while less reliable quantitatively than the determinations made on the basis of permeant ion equilibrium distribution assays, allow the continual monitoring of these responses during changes in $\Delta\psi$ (Cohen & Salzberg, 1978; Smith, 1990). The mechanisms by which these charged probes respond to an electrical field have been divided by Waggoner (1976, 1979) into two general categories; namely, those which penetrate the membrane and redistribute across the membrane in response to a transmembrane potential, and those which do not readily cross the membrane but undergo a reorientation of the chromophore at the membrane surface in the presence of an electric field.

While the redistribution-type probes, such as the cyanine, safranine, and oxonol dyes, have been extensively used with various energy-transducing membrane systems, the second type has only rarely been utilized, probably because of the lower sensitivity of their response. The present investigation has examined the interaction of the electrochromic potential-sensitive dye, merocyanine 540, (merocyanine) with rat liver mitochondria. This dye has been used primarily in studies with excitable tissues and provides significant spectral and fluorescent responses upon membrane depolarization which are thought to be a result of dye reorientation in the membrane rather than redistribution across the membrane (Ross et al., 1974; Salama & Morad, 1976, 1979; Tasahi & Warashina, 1976; Ross et al., 1977; Waggoner & Grinwald, 1977).

Certain aspects of the interaction of merocyanine with mitochondria, chromatophores, and sub-

mitochondrial particles have been reported which suggest a response to changes in surface charge or changes in dye location (Aiuchi & Kobatake, 1979; Smith et al., 1980; Masamoto et al., 1981; Smith, Graves & Williamson, 1984); however, no definitive conclusions have been made as to the dye response to metabolic changes in intact mitochondria. The data reported here suggest that merocyanine, because of its negatively charged sulfonic acid group, is located at least partially in the mitochondrial membrane-aqueous interphases. The observed spectral response on initiation of respiration appears to be due to a rapid displacement of the dye off the outer surface of the mitochondrial membrane and a slow electrophoresis across the membrane from the inner surface, both of which appear closely linked to the generation of a transmembrane potential.

Materials and Methods

Rat liver mitochondria were prepared as described previously (Conover & Schneider, 1981). Liposomes were prepared from a mitochondrial phospholipid extract prepared by a procedure modified from Bligh and Dyer (Esfahani et al., 1979). The phospholipid extract was dried under nitrogen in a small test tube, and sonication medium was added to give approximately 20 to 30 mg phospholipid per milliliter. After vigorous mixing to suspend the phospholipid, the suspension was sonicated with the microtip of a Branson Sonifier to clarity which usually took 2 to 3 min. The sonication medium was generally 100 mM KCl, 10 mM TES (pH 7.5), and 0.5 mM EGTA. Immediately before use in K^+ diffusion potential studies, the liposome suspension was passed through a Sephadex G-25 column equilibrated with 0.21 M mannitol, 10 mM LiCl, 10 mM TES (pH 7.5), and 0.5 mM EGTA.

Spectral studies were performed in temperature-controlled, stirred cuvettes using an Aminco DW2a dual-wavelength spectrophotometer. For studies on anaerobic-aerobic transitions, the medium was maintained at desired temperature under bubbling nitrogen prior to use. The cuvettes were equipped with fitted Plexiglass stoppers with a shaft sufficient to reduce the reaction volume to 2.0–2.5 ml and a 1–2 mm diameter opening to accept a syringe needle. After addition of the reaction medium and mitochondria, the stoppered cuvette was incubated with stirring at the desired temperature until completely anaerobic as determined by the reduction of cytochrome aa_3 monitored at 605–625 nm. Ten min were generally sufficient to obtain anaerobiosis and stabilization of the dye uptake. Aerobic transitions were initiated by the injection of air-saturated medium or calibrated hydrogen peroxide solutions into the cuvette. Hydrogen peroxide solutions were calibrated by polarographic measurement of oxygen released in the presence of catalase using air-saturated water as a standard. For fluorescence measurements a Spex Fluorolog 2 was used.

In the binding studies, merocyanine was equilibrated with mitochondria for 10 to 30 min. A 250-μl aliquot of equilibrated sample was spun down in an Eppendorf Microfuge for 3 min. After centrifugation, the pellet and a 0.1-ml aliquot of the supernatant fraction were mixed with 2 ml of 1% Triton X-100/10% NaCl. The optical densities of the pellets and supernatants were then read in an Aminco DW2a spectrophotometer.

Mitochondrial protein was determined by the biuret reaction using bovine serum albumin as a standard.

The dye, merocyanine 540, was obtained from Eastman Kodak Chemicals and was dissolved in dimethyl sulfoxide. Dimethyl sulfoxide concentrations never exceeded 0.1 mM, levels which show no observable effect on mitochondrial function (Conover, 1975). Solutions of the dye were generally prepared fresh daily, although their stability was probably good for several days if stored in the dark at 4°C.

Results

Binding of Merocyanine to Mitochondrial Membranes

Incubation of uncoupled mitochondria in a medium containing merocyanine results in an extensive binding of the dye. The absorption spectrum obtained on binding to mitochondria is similar to that seen on binding to phospholipid preparations (Waggoner & Grinwald, 1977) and is characterized by the appearance of a peak at 568 nm, due to the monomeric form of the dye in the nonaqueous environment of the mitochondrial membrane, and a smaller peak at 534 nm which is primarily due to the membrane-bound dimer (Waggoner & Grinwald, 1977). This latter peak increases markedly relative to the 568-nm peak with increasing dye concentrations. The binding is also accompanied by the disappearance of a peak at 504 nm which is characteristic of dimeric dye in aqueous solutions. The distribution between monomeric and dimeric forms in the membrane relative to the total dye bound was calculated from the absorption at 570 nm (Verkman & Frosch, 1985) and is shown in Fig. 1. These data show the shift towards the dimer with increasing amounts of dye which would be expected if the bound monomers and dimers are in equilibrium.

Merocyanine also shows a marked fluorescence enhancement in nonpolar environments relative to aqueous solutions of the dye (Aiuchi & Kobatake, 1979; Verkman, 1987). As shown in Fig. 2, the fluorescence enhancement when merocyanine binds to mitochondria increases to a maximum and then decreases as the dye bound rises above 2 nmol · mg protein^{-1}. This is presumably due to quenching of the monomer fluorescence by the nonfluorescent dimeric form of the dye which, as seen in Fig. 1, begins to increase markedly above 2 nmol · mg protein^{-1}.

A Scatchard plot of the dye binding when the dye bound is above 2 nmol · mg protein^{-1} indicates binding with a K_d of 37 μM and a maximum of 22 nmol bound · mg protein^{-1}, as well as extensive weaker binding (Fig. 3). At dye concentrations where less than 2 nmol · mg protein^{-1} are bound,

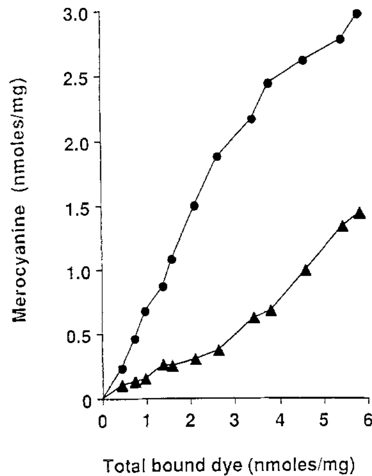

Fig. 1. Merocyanine monomer and dimer distribution relative to the concentration of dye bound. Rat liver mitochondria at 2 mg protein/ml were incubated in 0.18 M sucrose, 10.0 mM LiCl, 10.0 mM TAPSO, pH 7.4, 2.0 μM rotenone and 2.0 μM carbonylcyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) for 10 min with varied levels of merocyanine. The absolute absorption spectrum was measured, and the merocyanine bound was determined as described. The relative concentrations of monomeric and dimeric dye were determined as in Verkman and Frosch (1985) using the equation $A_{570} = \varepsilon_m$ ([Merocyanine]$_{total}$ − 2[dimer]) + ε_d [monomer]. The values, ε_m = 170,000 M^{-1} and ε_d = 60,000 M^{-1} were taken from Waggoner and Grinwald (1977). -●-, merocyanine as monomer; -▲-, merocyanine as dimer. Temperature was 25°C

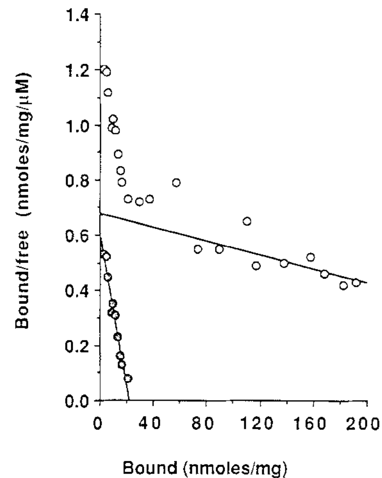

Fig. 3. Scatchard plot of merocyanine binding to rat liver mitochondria. Merocyanine was added to 0.1 M sucrose, 50 mM NaCl, 10 mM TAPSO, pH 7.4, 2 μM rotenone, 3 μM FCCP and 3 mg protein/ml rat liver mitochondria. Incubation was for 30 min before centrifugation. Bound and free dye were determined as described. -○- determined for dye concentrations from 13.2 to 990.0 μM; -●- derived by subtracting the binding of the dye to the low affinity site from the dye binding at the apparent high affinity site. Temperature was 25°C

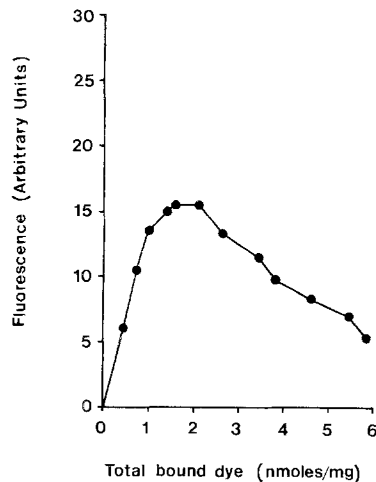

Fig. 2. Merocyanine fluorescence relative to the concentration of dye bound. Rat liver mitochondria were incubated as in Fig. 1, and the fluorescence was determined after 10 min. Excitation was at 534 nm and emission at 590 nm

the slope obtained is positive, suggesting a cooperativity in dye binding at low concentrations (*not shown*). This may result from the dye binding to the membrane in its monomeric form at low concentrations, but binding preferentially as a dimer with increasing concentrations.

The Spectral Response of Merocyanine Bound to Mitochondria to the Generation of $\Delta\mu_{H^+}$

The effect of $\Delta\mu_{H^+}$ generation on mitochondria-bound merocyanine may be seen in the transition from anaerobic to static head (state 4) condition upon addition of oxygen to mitochondria incubated anaerobically with merocyanine. This transition is accompanied by a blue shift in the dye spectrum seen in difference spectra as the disappearance of a peak at 574–576 nm and the appearance of a broad peak at approximately 500 nm (Fig. 4). The kinetics of the spectral changes at 15°C seen in Fig. 5 were monitored by using the wavelengths at 576 nm, corresponding to the membrane-bound monomer, as well as 534 and 504 nm, the absorbance peaks corresponding to the bound dimer and aqueous dimer, respectively. At 15°C, the response at 576 nm is markedly biphasic. There is a rapid absorbance decrease which is complete within 1 sec, while a slower phase (slower by two orders of magnitude) continues

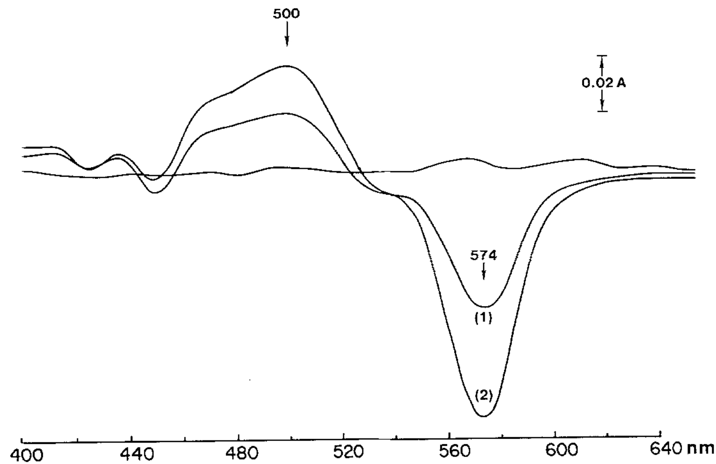

Fig. 4. Aerobic minus anaerobic difference spectra of merocyanine incubated with mitochondria. Cuvettes contained 0.21 M mannitol, 2 mM TES, pH 7.2, 0.2 mM EGTA, 10 mM LiCl, 10 mM succinate, 5 μM rotenone, 4 μg oligomycin, 10 μM merocyanine, 3 μg catalase, and 2 mg rat liver mitochondria in a 2.0-ml volume. Closed cuvettes were incubated approximately 10 min, during which anaerobiosis was obtained and dye uptake stabilized. H_2O_2 was added to the sample cuvette sufficient to maintain aerobiosis for 1 to 2 min, and the wavelength scans were taken immediately (*1*) and after 15 sec (*2*) to generate the curves shown. Temperature was 15°C

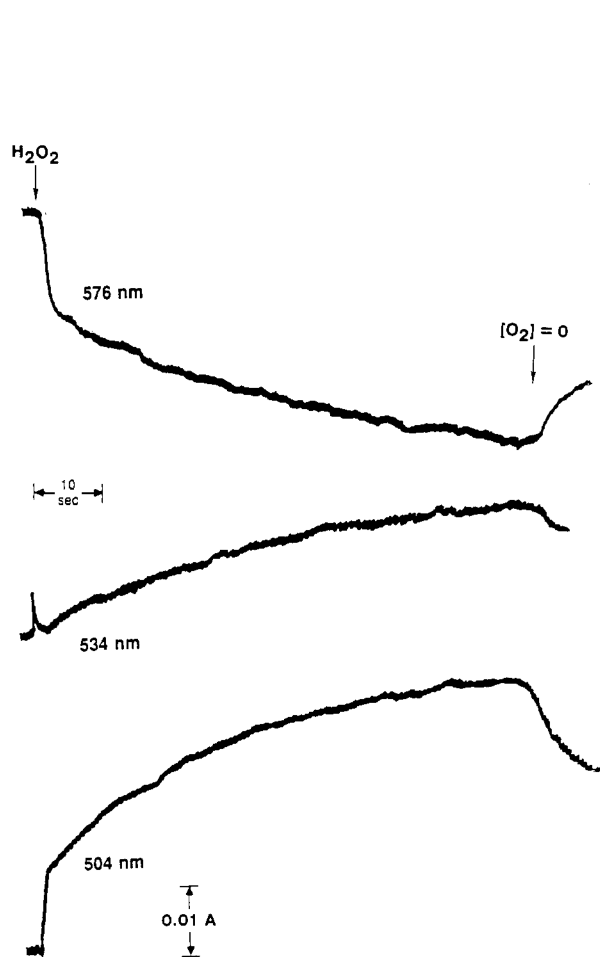

Fig. 5. Kinetics of the merocyanine absorbance response during an anaerobic-aerobic transition. The reaction medium contained 0.21 M mannitol, 10 mM LiCl, 2 mM TES, pH 7.2, 0.2 mM EGTA, 10 mM succinate, 2 μM rotenone, 4 μg oligomycin, 10 μM merocyanine, 3 μg catalase, and 2 mg mitochondria in a 2.0-ml volume. Temperature was 15°C. The responses were monitored at 576, 534, and 504 nm with a reference wavelength of 625 nm

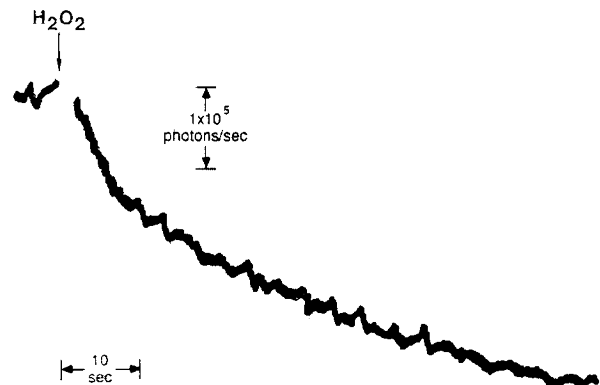

Fig. 6. Merocyanine fluorescence response to an anaerobic-aerobic transition. The reaction medium contained 0.18 M sucrose, 10 mM NaCl, 10 mM TAPSO, pH 7.4, 10 mM succinate, 2 μM rotentone, 4.7 μM oligomycin, 10 μM merocyanine, 3 μg catalase, and 2 mg/ml mitochondria and was incubated for 10 min before the addition of H_2O_2. Temperature was 15°C

until anaerobiosis and appears to approach a minimal absorbance with eventual slowing to a constant value. The absorbance rise on reaching anaerobiosis is also frequently biphasic, the extent of each phase being roughly proportional to that of the aerobic transition. The slow phase of the biphasic response, in particular, is temperature dependent, and as the temperature is increased, the rate of the slow phase increases until the biphasic nature of the response becomes difficult to observe.

The decrease in the bound monomer absorbance is mirrored exactly in the biphasic increase of the absorbance at 504 nm. On the other hand, the response followed at 534 nm gives a slow monotonic rise, suggesting a slow increase in the bound dimer, although the bound dimer and aqueous monomer (536 nm) cannot be distinguished from each other.

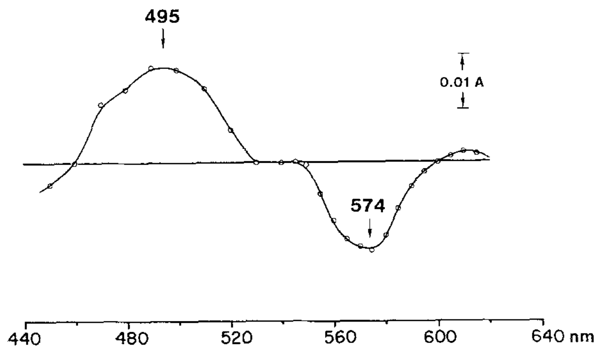

Fig. 7. Spectrum of the fast phase response of merocyanine upon oxygen addition to anaerobic mitochondria. Conditions were as in Fig. 5. Absorbance response was determined at each wavelength relative to nearest isosbestic point on identical oxygen additions. The fast phase response was complete at 1.0 sec and measured at 1.5 sec

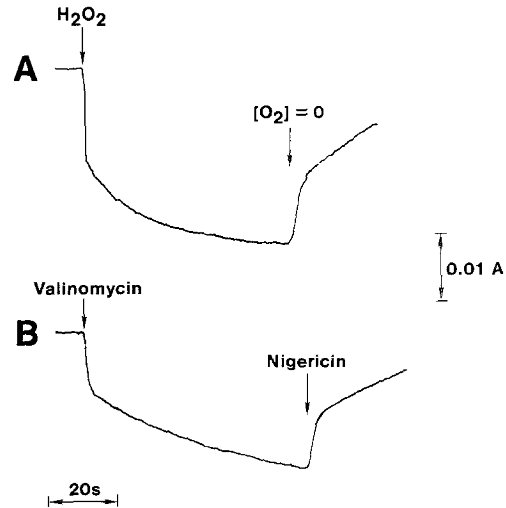

Fig. 8. Comparison of the merocyanine response during an anaerobic-aerobic transition and during the generation of a K^+ diffusion potential in mitochondria. (*A*) Membrane potential generated by oxygen addition to anaerobic mitochondria. Conditions for *A* were similar to those of Fig. 5. (*B*) Diffusion potential was generated by valinomycin addition to respiration-inhibited mitochondria. The reaction medium contained 0.21 M mannitol, 2 mM TES, pH 7.2, 0.2 mM EGTA, 10 mM LiCl, 5 μM rotenone, 1 μg antimycin, 4 μg oligomycin, 10 μM merocyanine and 2 mg rat liver mitochondria in a 2.0-ml volume. A diffusion potential was induced by addition of 5 nmol valinomycin and terminated by 5 nmol nigericin. Merocyanine response was followed 576–625 nm. Temperature was 15°C

As seen in Fig. 6, the fluorescence enhancement seen with mitochondria-bound merocyanine shows only a slow monotonic decrease on initiation of respiration with no apparent fast phase observable. This response, like that of the slow spectral responses, is very temperature dependent.

Both the spectral response and the fluorescence quenching are dependent on the ratio of dye to protein. The optimal responses are observed in the region of 2 to 4 nmol · mg protein^{-1} above which the responses decrease in magnitude.

In order to isolate the rapid phase spectral shift in its entirety, the response upon oxygen addition to mitochondria was followed by dual-wavelength measurements performed at varied sample wavelengths with a constant reference wavelength. The extents of the rapid phase response *versus* wavelength are plotted in Fig. 7. The spectrum obtained in this way closely resembles that obtained over the longer time periods (Fig. 4). This suggests that a similar mechanism may be involved in the major spectral changes of both rapid and slow phases of the response.

The Relationship between the Merocyanine Response and $\Delta\psi$

The responses observed with merocyanine in mitochondrial suspensions during anaerobic-aerobic transitions are sensitive to inhibitors of electron transport and to uncouplers of oxidative phosphorylation. Comparison of the sensitivity of the merocyanine spectral response at 576 nm to uncoupler concentration, with that of the spectral response of redistribution or "slow" dyes, such as safranine or cyanine dyes, under the same conditions, showed them to be similar, suggesting that the response of both types of dyes are dependent on the same uncoupler-sensitive parameter. Given the known response of the cationic permeant dyes to the $\Delta\psi$ component of $\Delta\mu_{H^+}$, it is probable that the merocyanine response too is dependent on $\Delta\psi$. This conclusion is supported by the sensitivity of the merocyanine response to the occurrence of ion movements which are known to dissipate $\Delta\psi$. Addition of Ca^{2+} or K^+ plus valinomycin results in a depression of the merocyanine responses. Furthermore, the extent of depression is proportional to the rate of cation transport (McKenzie, Azzone & Conover, 1991). Conversely, nigericin, a K^+/H^+ exchange ionophore which produces acidification of matrix space and suppression of the ΔpH component, shows no effect or gives a slight increase in the merocyanine responses.

Figure 8 compares the merocyanine response on the initiation of respiration with that resulting from the generation of a diffusion potential on addition of valinomycin to respiratory chain-inhibited mitochondria. Both responses are spectrally and kinetically very similar, and both fast and slow phases can be clearly seen.

The quantitative relationship of the fast phase response of merocyanine to membrane potential is

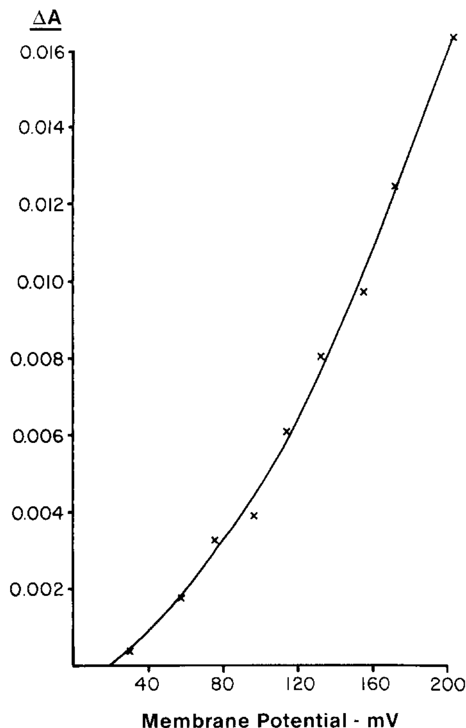

Fig. 9. Relationship between the magnitude of valinomycin-induced K^+ diffusion potentials and the spectral response of merocyanine. The reaction medium contained 0.21 M mannitol, 2 mM TES, pH 7.2, 0.2 mM EGTA, 5 μM rotenone, 1 μg antimycin, 4 μg oligomycin, 10 μM merocyanine and 2 mg rat liver mitochondria in a 2.0-ml volume. KCl concentrations were varied between 0.03 and 30 mM with the ionic strength maintained constant with LiCl. The internal K^+ concentration was assumed to be 100 mM. A diffusion potential was induced by addition of 5 nmol valinomycin. The response was followed at 576–625 nm and the extent of the fast phase response was used. Temperature was 15°C

shown in Fig. 9. The K^+ diffusion potentials generated on addition of valinomycin to respiratory chain-inhibited mitochondria was controlled by varying the concentration of K^+ in the medium relative to the internal K^+ concentration. In the range of 80 to 180 mV, the relationship appears to be quite linear.

Effect of Membrane Potential on Merocyanine Binding to Mitochondria

The spectral response to the generation of $\Delta\psi$ suggests that a displacement of bound merocyanine from mitochondria may be involved. Comparison of merocyanine binding in respiring, coupled mitochondria with that in uncoupled mitochondria showed that energization of mitochondria gives a small (16%) but reproducible ($0.05 < P < 0.10$) decrease in the merocyanine bound compared to un-

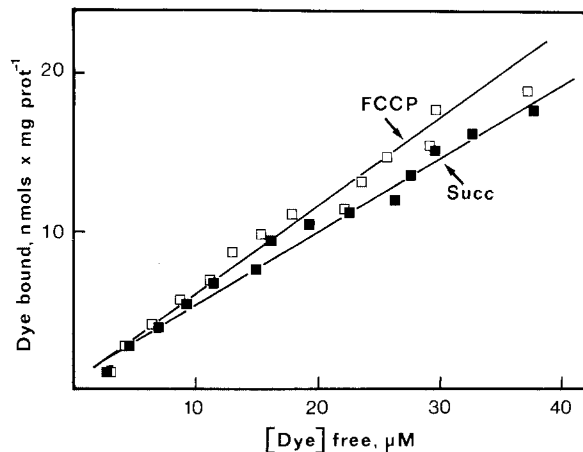

Fig. 10. Difference in merocyanine binding to mitochondria in coupled and uncoupled conditions. Reaction mixture contained 0.21 M sucrose, 10 mM Tris, pH 7.0, 20 mM LiCl, 5 mM succinate, 2 μM rotenone, and 1 mg rat liver mitochondria. Uncoupled mitochondria contained 2 μM FCCP in the reaction mixture. Mitochondria were separated from the reaction mixture by rapid centrifugation after 60-sec incubation, and the merocyanine determined on the supernatent fluid by spectral measurement at 568 nm after the addition of 2% Triton X-100

coupled mitochondria (Fig. 10). This can be observed at a wide range of protein, dye, and salt concentrations. The extent of energy-dependent decrease in dye binding increases linearly with the amount of dye bound, suggesting a displacement of the dye from one phase to another rather than a site-specific phenomenon. Scatchard analysis of such data indicates both an increase in K_d and a decrease in number of binding sites.

Interaction of Merocyanine with Liposomes

In order to eliminate the possible involvement of membrane protein in the mechanism of the merocyanine response, studies were made with liposomes prepared from mitochondrial phospholipids. The difference spectrum of merocyanine in the presence of a K^+ diffusion potential generated by addition of valinomycin to K^+-loaded liposomes (*not shown*) closely resembles that obtained with mitochondria during an anaerobic-aerobic transition (Fig. 4). This spectrum is also the mirror image of that seen on the binding of merocyanine to liposomes which supports the suggestion that induction of a K^+ diffusion potential in the liposomes releases phospholipid-bound merocyanine. The kinetics of the merocyanine response followed at 576–540 nm to the generation of K^+ diffusion potentials in liposomes shows the presence of both fast and slow phases, although the slow phase is diminished in extent due to the more

rapid decay of the diffusion potential as compared to that observed with mitochondria. It is therefore concluded that the merocyanine response to membrane potential is, for the most part, independent of the protein components of the mitochondrial membrane.

Effects of Ionic Strength on the Merocyanine Binding and Response

Due to the negative charge on merocyanine, the extent of dye binding is greatly influenced by the ionic composition of the medium. An increase in salt concentration increases the dye binding with an effectiveness of the cation being related to the valence, i.e., $La^{3+} > Mg^{2+} > Na^{+}$, as predicted by the Gouy-Chapman theory (McLaughlin, 1977).

Since it has been suggested that the merocyanine responses in mitochondrial suspensions were related to surface charge changes (Aiuchi & Kobatake, 1979), the effects of varying salt concentrations on both merocyanine binding and the merocyanine response to anaerobic-aerobic transitions are compared in Fig. 11. While increasing concentrations of Na^{+} or Mg^{2+} cause some depression of the merocyanine response, this effect is considerably less than the large increase in dye binding. For example, addition of 50 mM NaCl reduces the merocyanine response to an aerobic transition by only 20%, but increases the binding of merocyanine to mitochondria several-fold. It is probable that this observed decrease in merocyanine spectral response is the result of the increase in bound dye/protein ratio and is similar to the decrease in spectral response observed when higher levels of dye are used at low ionic strengths.

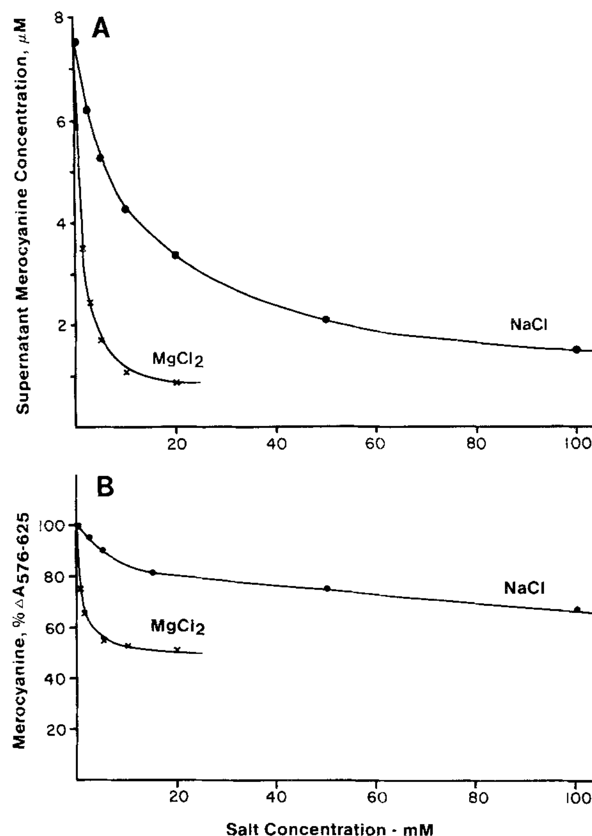

Fig. 11. Effect of ionic strength on the interaction of merocyanine with mitochondria. (*A*) Effect of ionic strength on the binding of merocyanine to mitochondria. Incubation mixture contained 0.21 M mannitol, 2 mM TES, pH 7.2, 0.2 mM EGTA, 5 μM rotenone, 5 μM FCCP, 10 μM merocyanine and 1 mg rat liver mitochondria in 1.0 ml. Osmolarity was maintained constant at differing salt concentrations by varying the mannitol concentration. After 2-min incubation, the mitochondria were centrifuged in a microfuge and the merocyanine determined on the supernatant fluid by spectral measurement as described. (*B*) Effect of ionic strength on the rapid phase merocyanine response to anaerobic-aerobic transitions in mitochondria. Conditions were as in Fig. 5. Osmolarity was maintained constant by varying the mannitol concentration

Discussion

The advantages of a measure of membrane potential that responds rapidly and can be monitored continuously are obvious. In mitochondrial and bacterial systems, the membrane potential probes most frequently used for this purpose have been membrane-permeant cationic compounds of the type classified by Waggoner (1976, 1979) as redistribution probes. Such probes undergo a redistribution between the compartments separated by the energy-transducing membrane which may result in concentration differences of several orders of magnitude in the case of mitochondria where $\Delta\psi$ is about −180 mV at static head. The redistribution of the dye is accompanied by H^{+} extrusion and increased oxygen consumption by mitochondria in a manner which depends on the extent of dye translocation (Colonna et al., 1973). These properties of redistribution probes limit their use in following rapid $\Delta\psi$ transients, especially when proton movements are a concern.

Merocyanine presents a distinct contrast to the above probes. It is an amphipathic molecule with hydrophobic properties in the region of the chromophoric groups, but possessing a localized charge in the form of a sulfonic acid residue attached to a side chain. The most probable localization of the probe in membrane systems is at the membrane-water interface with the chromophoric residue in the hydrophobic phase (Verkman, 1987). Because of the sulfonic acid group, translocation across the mem-

brane should be limited (Smith et al., 1984; Verkman & Frosch, 1985; Verkman, 1987).

Induction of an electric field by initiation of respiration in anaerobic mitochondria or generation of a K^+ diffusion potential by addition of valinomycin results in a spectral shift involving a decrease in absorbance at 576 nm due to the membrane-bound monomeric form of merocyanine. This response appears to have two kinetic phases at 15°C, an initial rapid phase with a $t_{1/2}$ of less than 1 sec and a slow phase of a somewhat variable rate. The increase in absorbance at 504 nm, with similar biphasic kinetics, suggests that the decrease in membrane-bound monomer is accounted for primarily by an increase in dimer in the medium aqueous phase.

It has been reported that merocyanine bound to phospholipid membranes occurs as an equilibrium mixture of monomers which are oriented either parallel or perpendicular to the membrane surface and dimers which are oriented exclusively parallel to the membrane surface. It has been further suggested that the electrochromic fluorescence responses of merocyanine in artificial membrane systems involve a rate-controlling reorientation of perpendicular monomers to the parallel position followed by a rapid aggregation of the monomers to the nonfluorescent dimer (Dragsten & Webb, 1978; Verkman & Frosch, 1985; Verkman, 1987). Such a monomer-to-dimer transition does not appear to be responsible for the rapid responses observed here with mitochondria, since in this case the response should show a rapid rise in the 534-nm absorption peak of the bound dimer corresponding to the decrease in absorption of the monomer at 576 nm, rather than the observed rapid rise in the 504 nm peak assumed to be due to the aqueous dimer.

Comparison of the difference spectra of both fast and slow phases with those obtained during dye binding by either liposomes or mitochondria indicates that the induction of a membrane potential results in a release of merocyanine from the phospholipid bilayer of the mitochondrial membrane in both cases. This "on-off" response to membrane potential is not generally thought to be the mechanism involved in merocyanine responses, although it has been suggested in studies on *Rhodopseudomonas sphaeroides* (Masamoto et al., 1981). It has recently been reported that an "on-off" mechanism is involved in the electrochromic response of impermeant oxonol dyes, which have considerable structural similarities to merocyanine although carry an additional negative charge (George et al., 1988).

The biphasic nature of the dye response must, then, reflect the presence of two distinct monomer populations within the membrane, both of which respond to $\Delta\psi$, but which are operationally distinguishable on the basis of their rate of movement from the membrane to the aqueous phase and the temperature sensitivity of this movement. The two populations of dye may result from the slow distribution of merocyanine between the inner and outer surfaces of the mitochondrial inner membrane during incubation. Generation of a potential across the membrane, negative to the inside, would result in a rapid displacement of monomeric dye off the outer surface, while the dye at the inner surface or dimers within the membrane would be limited by the relatively slow, temperature-dependent electrophoresis to the outer surface prior to release. The amount of dye displaced in the rapid phase, calculated from the ε_m of monomer absorbance (Verkman & Frosch, 1985) was 0.02–0.03 nmol · mg protein^{-1} or about 1% of the total bound dye. The difference between this estimation of the monomeric dye displacement and the dye release observed from total dye binding studies (Fig. 10) is related to the time difference in the procedures and the probable loss of both monomeric and dimeric forms of the dye during the binding studies.

In the proposed mechanism, it would be expected that rapid loss of the fluorescent monomer should be seen as a rapid quenching of the fluorescence. The absence of such a fast phase in the fluorescence quenching (Fig. 6) may be attributed to the use of a dye/protein ratio near the maximum for fluorescence enhancement (Fig. 2) where a change in dye bound would effect little change in fluorescence. Fluorescence has frequently been utilized to monitor the merocyanine response to membrane polarization (Ross et al., 1974; Aiuchi & Kobatake, 1979). From the observations reported here, it would appear that the fluorescence response is not a useful measure of $\Delta\psi$ in mitochondria.

Another question to be resolved is whether the driving force for the dye response is a change in surface charge, as previously proposed (Aiuchi & Kobatake, 1979), or a change in transmembrane potential. This question has long been debated in the case of 8-anilino-1-naphthalene sulfonate, which also moves from the mitochondrial membrane to the aqueous phase upon energization of the mitochondria. Recently, arguments have been made in favor of the view that the predominant cause of the fluorometric response of 8-anilino-1-naphthalene sulfonate to energization of mitochondria is due to the change in $\Delta\psi$ and not to a change in surface charge (Robertson & Rottenberg, 1983). Some of these arguments may also apply to the merocyanine response. In particular, large changes in ionic strength have rather small effects on the anaerobic-aerobic transition-induced spectral response of merocyanine. These relatively small effects are most likely

accounted for by the large changes in dye/protein ratio due to the increased ionic strength and do not support the suggestion that changes in surface charge are measured by merocyanine during mitochondrial energization. Furthermore, the merocyanine spectral response at constant ionic strength shows a quantitative linear relationship to K^+ diffusion potentials varied over the range 80–180 mV.

Disadvantages in the use of merocyanine in the measurement of membrane potential are the low sensitivity of the dye response, i.e., the extent of the fast phase absorbance change is much smaller than that of the redistribution probes and the necessity of using low temperature to minimize transmembrane dye movement. On the other hand, an advantage of merocyanine is that the fast phase response occurs with a $t_{1/2}$ of less than a second and does not depend on massive dye translocation. In view of the rapidity of its response, merocyanine is a useful probe for rapid metabolic transitions involving $\Delta\psi$ generation (McKenzie et al., 1991). Furthermore, merocyanine would not perturb the generation of $\Delta\psi$ in membranes by creating significant charge movement during its response. The small fraction of the total dye which undergoes change during the fast phase and the relatively slow movements of the bulk of the bound dye indicates that the effects on the membrane capacitance would be small.

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