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Influx and efflux kinetics of cationic dye binding to respiring mitochondria

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Abstract

We have investigated the kinetics of interaction of cationic fluorescent lipophiles (dyes) rhodamine 123, rhodamine 6G, tetramethyl rhodamine ethyl ester, safranine O, 1,1'-diethyloxacarbocyanine, 1,1'-diethyloxadicarbocyanine, and 1,1'-diethylthiadicarbocyanine iodide with isolated respiring rat-liver mitochondria (RLM). Dye flux across the RLM inner membrane was measured by following the kinetics of fluorescence signal change after mixing of dye and RLM. The time course of fluorescence was analysed in terms of a kinetic model of the binding and transport processes involved. The rate constants of dye influx and efflux were extracted from the observed effect on the apparent time constant of fluorescence change to equilibrium intensity upon mixing dye with increasing concentrations of RLM. From the influx rate constants obtained, the apparent permeability constants for dye influx (at zero potential) across the membrane were calculated and ranged from 3 to 140×10^{-4} cm/s. The influx rate constant was found to be linearly related to relative dye lipophilicity, as predicted by the model. As another test of the model, from the ratio of the influx and efflux rate constants, the apparent trans-membrane potential, Ψ, was calculated and found generally to agree with reported values, but to depend on the lipophilicity of the dye used. Not predicted by the simple model was a dissymmtry observed in the influx and efflux time constants for fluorescence change to equilibrium intensity. Inferences are made relating to the utility of these dyes as probes of Ψ.

Keywords: Fluorescent cationic dyes; Mitochondria; Flux kinetics

1. Introduction

Many water soluble aromatic cationic dyes have a tendency to dissolve in lipid. These lipophilic compounds interact strongly with biological membranes capable of creating an electrochemical potential across its bilayer. The mitochondria serve as an ideal model of such a biological energy transduction system. The process by which chemical energy is coupled to the creation of a electrochemical charge separation across the mitochondrial inner membrane and then recreated into production of ATP is understood in terms of the Mitchell chemiosmotic hypothesis [1]. This hypothesis describes a partition of energy, obtained from oxidation of available substrates by the electron transport chain, into an electrochemical potential component (trans-membrane potential, Ψ) and a proton diffusional potential component, ΔpH created by displacement of proton outward across the impermeable lipid bilayer. Cation movement across the inner mitochondrial

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membrane into the matrix volume is facilitated by interaction with Ψ. This active sequestration of lipophilic cations by mitochondria has led to several applications of the phenomenon. Colored or fluorescent cations have been used as specific histological stains of mitochondria in living cells [2,3]. They are able to distinguish certain cancerous cell types from normal cells on the basis of an enhanced sequestration of fluorescent cationic dyes [4]. They have been used as photosensitizers, concentrating preferentially into mitochondria of certain cancer cell types where, upon irradiation with light, they act as photodynamic chemotherapeutic agents [5,6]. If the cations themselves are drugs with biological activity in the mitochondria, they can be concentrated at their site of action [7–9,34]. Most importantly, they have served as probes of the intensity and change of Ψ [10–14]. When properly calibrated, these methods can be used to estimate the size of Ψ. Without calibration, changes in optical signal of the probe can be used to monitor the qualitative response of Ψ to perturbation by external chemical or physical agents.

Although a considerable literature exists recording the use of lipophilic fluorescent cations as optical probes of Ψ, little is published concerning the specifics of the dye–mitochondrial interaction, particularly in regard to the dynamics of optical probe response to changes in Ψ. Knowledge of the response kinetics of fluorescent optical probes towards following changes in Ψ is necessary to assure that the true change in Ψ is being followed [15,16]. In order to better understand the limits of cationic lipophilic dyes used as fluorescent probes of kinetics of changes in Ψ, we have investigated the kinetics of fluorescent signal change resulting from dye flux from respiring rat-liver mitochondria as the dye–mitochondria mixture equilibrates. The dyes studied were chosen from those commonly used as fluorescent probes of mitochondrial membrane potential. These dyes respond to concentration within the mitochondria by quenching of fluorescence, if not by a common mechanism, then to a common effect [16,17]. We attempt to interpret the observed kinetics of fluorescence in terms of a model of the processes involved, as adapted from a more complete theoretical treatment by Clarke [18]. We tested the model by measuring the correlation between dye flux rate constants and dye lipophilicity, predicted by the model. Relative dye lipophilicity was estimated by measuring the fraction of a constant amount of dyes added to non-respiring mitochondria. We also tested the model by applying it to the flux data measured to estimate the trans-membrane potential. Finally, the symmetry of the flux process was investigated by comparing the relaxation time constants of fluorescence signal change when equilibrium is approached by dye flux from without (initiated by addition of dye to suspensions of respiring mitochondria) to the fluorescence signal change when equilibrium is approached by movement of dye from within (initiated by dilution of pre-equilibrated dye–respiring mitochondria mixtures).

2. Materials and methods

2.1 Reagents

2.1.1 Dyes

The dyes investigated, their sources and purities are as follows: 1,1′-diethyloxadicarbocyanine iodide (DiOC2(5)), Molecular Probes Inc. (MP), Eugene, OR, > 95%; 1,1′-diethylthiadicarbocyanine iodide (DiSC2(5)), MP, > 95%; 1,1-diethyloxacarbocyanine iodide (DiOC2(3)), MP, > 95%; tetramethyl rhodamine ethyl ester (TMRE), MP, > 95%; rhodamine 123 dihydrate (Rh123), Aldrich Chemical Co. (A), Milwaukee, WI, > 99%; rhodamine 6G (Rh6G), A, > 99%; and Safranin O (SafO), A, 95%. A 1 mg/mL stock solution of each dye was prepared in absolute ethanol and stored in the dark at −20°C, except for the carbocyanines which were prepared at 0.1 mg/mL. Concentration of stock solutions were calculated from the absorbance of an ethanol-diluted aliquot using published extinction coefficients [19–21]. Just prior to use, working solutions of the dyes were prepared by dilution of ethanolic stock into sucrose–succinate buffer (SSB/EGTA, described below), to a final concentration of 100 μM.

2.1.2 Other reagents

Buffer salts, sucrose, ethyleneglycol bis (aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), disodium succinate, rotenone, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co., St. Louis, MO.

Buffers were as follows. Mitochondrial isolation buffer (IB) contained 0.3 *M* sucrose, 1 m*M* EGTA, 0.2% (w/v) bovine serum albumin (BSA), 5 m*M* K_2HPO_4, and 10 m*M* 3-[*N*-morpholino] propane sulfonic acid (MOPS), adjusted to pH 7.4 with KOH. Mitoplast isolation buffer (MSH/BSA) contained 220 m*M* mannitol, 70 m*M* sucrose, 2 m*M* *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), 0.05% (w/v) BSA, pH 7.4. Respiration supporting incubation buffer (SSB/EGTA) contained 150 m*M* sucrose, 5 m*M* $MgCl_2$, 5m*M* disodium succinate, 5 m*M* K_2HPO_4, 2.5 μM rotenone, 1 m*M* EGTA, and 20 m*M* potassium-HEPES (pH 7.4). A Tris-buffered saline (Tris/saline) contained 150 m*M* NaCl and 10 m*M* Tris-HCl, pH 7.4.

3. Methods

3.1 Mitochondria isolation

Mitochondria from freshly excised liver of breeding-retired male Sprague-Dawley rats were isolated by differential centrifugation at 4°C into 0.3 *M* sucrose buffer by the method of Rickwood et al. [22]. Delipidated bovine serum albumin (final concentration 0.2 mg/mL) was added to the isolation medium during and subsequent to the second mitochondrial pelleting. Mitochondrial pellets were obtained by centrifugation at 12,000 g. Mitochondria were stored at 4°C at a concentration of ~ 20 mg/mL in IB. Mitoplasts were prepared following the method of Hovius et al. [23]. A suspension of 20 mg/mL of mitochondria in IB was centrifuged and resuspended in a volume equal to the initial volume of MSH/BSA. To this was added an equal volume of this MSH/BSA without BSA, and containing 6.0 mg/mL digitonin (for a final digitonin/protein ratio 0.3). After standing on ice for 15 min, the sample was diluted 10 times with MSH/BSA and centrifuged for 10 minutes at 12,000 *g*. The pellet was resuspended in MSH/BSA. Mitochondrial protein concentration was estimated using the Bradford method [24] with commercially available reagent and protocol (Pierce Chemical Co, Rockford, L) and BSA as standard. The integrity of the mitochondrial and mitoplast preparations was assayed by determining the respiratory control ratio measuring oxygen consumption with a Clark electrode [25] and by electron microscopy. Respiratory control ratios of preparations used for experiments were 4.4 ± 1.2 ($n = 5$) for mitochondria and 3.1 ± 0.9 ($n = 2$) for mitoplasts with succinate as substrate.

3.2 Spectroscopic methods

Fluorometric measurements were performed on a Farrand Optical (now Optical Technologies Inc., Elmsford, NY) Mark I spectrofluorometer using 10 nm band pass. A hard copy of the fluorometer signal output was recorded over time by a Kipp and Zonen (Delft, Netherlands) BD41 strip chart recorder. The fluorometer instrument response time was 0.3 s. Signal from the fluorometer was also acquired and digitized using a MetraByte (Taunton, MA) DAS-8 analogue to digital converter resident in an AT microcomputer, and programmed with Labtech Notebook software (Laboratory Technologies Corporation, Wilmington, MA) run in the strip chart mode. From raw fluorescence intensity data, the relaxation time of fluorescence signal equilibration, τ, was estimated from the slope obtained by linear least squares analysis of a plot of $\log[(F - \mathcal{F})/(F_0 - \mathcal{F})$ vs. time, using $\tau = -(\text{slope})/\ln 2$, where F_0 is the intensity of the dye mitochondrial mixture at zero time, and F is the intensity at time, t, and $\mathcal{F}$ is the fluorescence after equilibration has been reached.

3.3 Dye partition into non-respiring mitochondria

As a measure of relative dye lipophilicity, we measured the fraction of dye bound to mitochondria upon incubation of dye with mitochondria suspended in a non-respiration supporting

medium. To 1.0 mL of SSB/ EGTA, additionally containing 1 μM CCCP and 0.1 mM valinomycin, was added stock mitochondria and stock dye to bring the final concentrations to 1.0 mg/mL and 10 μM, respectively. The mixture was incubated for 30 minutes at ambient temperature and then 0.3 mL alliquoted into 600 μL micro-centrifuge tubes and spun at 12,000 g for 2.5 min. Of the supernatant 0.1 mL was removed and diluted into 1% (w/v) aqueous sodium dodecyl sulfate (SDS). The pellet was cut from the base of the tube using a razor blade and dissolved in SDS. The concentrations of dye in supernatant and pellet were determined by measuring fluorescence and comparing to standard curves of dye prepared in SDS. The fraction of dye bound (f_b) was calculated as the number of moles bound to mitochondria divided by the total number of moles of dye added. Coefficient of variance of measurement of f_b was 8% ($n = 3$).

3.4 Kinetics of dye influx to equilibrium

Kinetics of dye influx were determined as follows. Prior to monitoring fluorescence, the wavelengths of emission and excitation of the dye were adjusted on the fluorometer to maximize the intensity of fluorescence relative to scatter. The excitation/emission wavelength (nm) pairs used were 490/535, 530/585, 550/580, 475/500, 530/550, 560/590, 640/670 for Rh123, SafO, TMRE, DiOC2(3), Rh6G, DiOC2(5), and DiSC2(5), respectively. To 3.0 mL of SSB/EGTA was added sufficient volume of mitochondria in isolation buffer to bring the final concentration of mitochondria to that desired and the mixture was allowed to stand for 5 min. Then, while the fluorescence was monitored, dye stock solution to bring the final dye concentration to 1 μM was added to initiate dye influx. Mixing was affected by repeated pumping of suspension into and out of a Pasteur pipette simultaneously with dye addition. The change in fluorescence intensity was recorded on the strip chart recorder or by computer and the time constant on of flux was determined as described above. Dependence of influx time constant on varying dye concentration at constant mitochondria concentration was performed similarly. To obtain a final concentration of dye over a range of 0 to 20 μM, 30 μL of a 100 times higher final concentration in SSB/EGTA was added to 3.0 mL of mitochondria suspended at a final protein concentration of 0.01 mg/mL in SSB/EGTA. Fluorescence change was monitored and time constant determined as described above.

To determine the effect of lipophilic counterions on cationic dye flux, Rh123 influx was determined while the final concentration of sodium tetraphenyl boron (TPB) was increased. Into 3.0 mL of SSB/EGTA sufficient mitochondria suspension was added to make the final concentration 0.1 mg/mL and sufficient ethanolic stock solution of sodium tetraphenylboron added to give the desired final concentration over the range 0.1 to 20 μM. After a 5 min wait for equilibration, sufficient Rh123 SSB/EGTA stock solution was added to give 1 μM final concentration. Change in fluorescence was recorded and the time constant of influx calculated as described above.

3.5 Kinetics of dye efflux to equilibrium

For dilution initiated efflux measurements, mixtures of 1 μM dye and 0.1 mg/mL mitochondria were prepared in SSB/EGTA and fluorescence of the mixture monitored, as above. After no further change in signal occurred, the sample was removed from the fluorometer and replaced with 3.0 mL of fresh SSB/EGTA. To initiate release of dye, 300 or 30, or 3 μL of the equilibrated dye-mitochondrial mixture was added to an appropriate volume of fresh buffer to yield 1:10, 1:100, and 1:1000 dilutions in a final volume of 3.0 mL. Raw data was obtained and the time constant of dye efflux was calculated as described above.

Efflux time constants in the presence of TPB was performed in a similar fashion with the exception that the medium into which the mitochondria were diluted additionally contained TPB at the same final concentration as present in the influx measurements.

4. Theory

4.1 Influx kinetics

The following scheme expresses the general features of the interaction of lipophilic cation with the mitochondrial inner membrane.

$$[D]_{ma} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} [D]_2 \underset{k_{out}}{\overset{k_{in}}{\rightleftharpoons}} [D]_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [D]_{cy} \quad (1)$$

Here the k's represent the rate constants for partition into and out of the aqueous–lipid phase at the two surfaces, the cytosolic, 1, and the mitochondrial matrix, 2. The rate constants for diffusion of dye between the lipid–aqueous interfaces, inward towards the matrix and outward towards the cytosol, are k_{in} and k_{out}, respectively. The values of k_{in} and k_{out} depend on the electrochemical membrane potential, Ψ, expressed across the bilayer. $[D]_{cy}$, $[D]_1$, $[D]_2$ and $[D]_{ma}$ represent the concentrations of dye found in each of the four compartments: cytosolic aqueous, cytosolic aqueous–lipid interfacial, matrix aqueous–lipid interfacial, and matrix aqueous, respectively. The ratios of rate constants, $k_1/k_{-1} = K_1$ and $k_2/k_{-2} = K_2$, define the aqueous-to-lipid partition coefficients of dye at the two surfaces.

We wish to have a mathematical description of the kinetics of fluorescence change upon addition of lipophilic dye to a lipid bilayer possessing an electrochemical potential maintained between its surfaces. The complete solution to this problem has been recently presented by Clarke [18] who modeled the fluorescence response of lipophilic fluorescent dye addition to synthetic lipid vesicles accross which a trans-membrane potential exists. The complete system description includes four differential equations which are numerically solved to yield the time course of fluorescence change after assumption of values for the rate constants, numbers of lipid bilayer internal and external binding sites, vesicle and dye concentration, electrochemical potentials, and general quenching mechanism. Although description of the kinetic data acquired here would be best performed with the complete kinetic model of Clarke, we chose to apply a simiplification. The simplification [26] results from assuming that dye binding to the inner mitochondrial membrane bilayer lipid faces is rapid, compared to subsequent diffusion across the membrane lipid. The strong lipophilicity of the dyes assures that the greater concentration of dye arriving at the inner motochondrial aqueous compartment will back partition into the inner membrane lipid, thus the greater portion of the potential dependant fluorescence signal change observed is expected to result from actions within the lipid compartment. Because a quenching of fluorescence quantum yield for the cations occurs only upon arriving at internal surface 2, under the rapid preequilibrium assumption, the rate of dye fluorescence change will be proportional to the rate of transfer, k_{in}, across the bilayer, represented by

$$R_{in} = -[D]_1 k_{in} = -k_{in} K_1 [Mito][D]_{cy}$$
$$= -k'[Mito][D]_{cy} \quad (2)$$

where [Mito] is the mitochondrial concentration, and $k' = k_{in} K_1$. The uptake of dye is counteracted by the rate of dye efflux, R_{out}

$$R_{out} = [D]_2 k_{out} = k_{out} K_2 [D]_{ma} = k''[D]_{ma}, \quad (3)$$

where $k'' = k_{out} K_2$.

The influence of the trans-membrane potential, Ψ, on inward migration of the ion is expressed through the rate constant k_{in} by [27]

$$k_{in} = (D_m A / V\lambda) \exp(bu/2) \quad (4)$$

where $u = F\Psi/RT$, which, at 20°C, is equal to $\Psi \times 0.0396$. Ψ(mV) is taken as positive if cation flow is aided by the potential. b is a parameter which reflects the position and shape of the energy barrier to ion trans-bilayer diffusion, which has been emperically established to be equal to 0.5 for mitochondrial transport of lipophilic cations [28]; D_m (cm^2/s) is the dye diffusion coefficient for transfer across the bilayer of width λ (cm); K_1 is the aqueous to lipid partition coefficient; A is the surface area of the mitochondria

(400 cm^2/mg mitochondrial protein [1]); and V is the incubation volume (1 cm^3). Likewise

$$k_{out} = (D_m A/V\lambda)\ \exp(-bu/2) \quad (5)$$

The rate of change in the concentration ($[D]_q$) of mitochondrial bound dye molecules within the quenching environment can be written [26]

$$d[D]_q/dt = k'[D][Mito] - k''[D]_q \quad (6)$$

where [D] and [Mito] are the total concentrations of dye and mitochondria, respectively. By introducing the terms $r = [D]_q/[Mito]$ and $r = [\mathscr{D}]_q/[Mito]$, where $[\mathscr{D}]_q$ is the concentration of bound dye at equilibrium, the following expression for the time change in r can be written [26]

$$dr/dt = -(k'[Mito] + k'')(r - r) \quad (7)$$

where r, and r are the fraction of total quenched binding sites are filled at time, t, or at equilibrium, respectively. Following mixing dye and respiring mitochondria in suspension, a change in r will result in a corresponding change in fluorescence of the mixture. The rate of fluorescence signal change resulting is related to the kinetic rate constants [26] by

$$dF/dt = -(k'[Mito] + k'')(F - \mathscr{F}) \quad (8)$$

where $\mathscr{F}$ is the fluorescence of the mixture at equilibrium. From eq. (8) it can be seen that the fluorescence signal should follow a single-exponential time function with a relaxation time, τ, given by

$$\tau^{-1} = k'[Mito] + k'' \quad (9)$$

A plot of τ^{-1} vs. [Mito] should give a straight line with slope k' and intercept k''. In comparing dyes, the relative value of k' should reflect the lipophility of dye partition (K_1) into mitochondrial lipid at the exterior face (eq. 2). Likewise, the relative value of k'' should reflect the lipophilicity of dye partition into lipid at the inner face, K_2. The ratio of k' to k'' estimates of the membrane potential from

$$k'/k'' = (K_1/K_2)\ \exp(bu) \quad (10)$$

From definition [27], the permeability constant, P, for dye transport across the bilayer may be calculated from the observed influx rate constant, k', using

$$P = (V/A)k'\ \exp(-bu/2). \quad (11)$$

4.2 Efflux kinetics

After a mixture of dye and suspended respiring mitochondria reach equilibrium, dilution of the mixture results in an instantaneous drop in the external dye concentration resulting in a net flux of dye out of the mitochondria. Equation (8) is equally suited [26] to describe the approach of fluorescence change to equilibrium under these initiation conditions. The model thus predicts that the dilution initiated fluorescence change should follow the same exponential function of time as when change is initiated by addition of dye to respiring mitochondria.

5. Results

5.1 Influx kinetics

After mixing a fluorescent cationic lipophile with respiring mitochondria, the mixture fluorescence changes over time, as represented by the

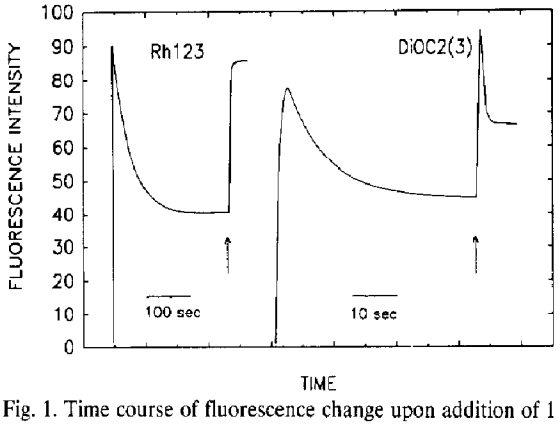

Fig. 1. Time course of fluorescence change upon addition of 1 μM Rh123 or DiOC2(3) to 0.1 mg/mL respiring rat liver mitochondria suspended in SSB/EGTA. Dye was added to mitochondria at the left most point of the trace. After equilibration of fluorescence signal, at the arrow, 5 μM CCCP was added to collapse membrane potential.

traces of Fig. 1. Here, 1 μM Rh123 or DiOC2(3) were added to 0.1 mg/mL mitochondria suspended in respiration supporting medium, SSB/EGTA. The trace for Rh123 was typical to that seen for all dyes studied except the carbo and dicarbocyanines. The trace for DiOC2(3) is representative of the profiles for those dyes. Fluorescence intensity change arising from binding to the suspended mitochondria is expected to have two components. One component arises from the non-potential dependent partition of dye into the lipid of the mitochondria and results, as the dye partitions there, from environmental effects on the dye optical absorption and fluorescence quantum yield. The second component arises from the potential dependent quenching of the fluorescence by concentration forced aggregation of fluorescent cation after entering the mitochondrial inner volume. The change in dye fluorescence intensity resulting from non-potential dependent partition into mitochondrial lipid was measured by comparing the fluorescence intensity under identical excitation and emission wavelengths of 1 μM dye dissolved in either SSB/EGTA or in the aliphatic micelle interior of a buffered 3% Triton X-100 solution. The carbo- and dicarbocyanine dyes gave fluorescence enhancement in Triton X-100 over SSB/EGTA, while for the other dyes, a decrease in fluorescence was observed when in Triton X-100 micelles. Hence, the initial rise in Rh123 fluorescence appears monophasic, since both the membrane potential dependent and independent fluorescence changes are in the same direction. With DiOC2(3) and the other carbocyanines, the non-potential dependent fluorescence change is in the opposite direction to that of the potential dependent change, and a biphasic rise of fluorescence is seen. For all dyes, the time course of fluorescence due to the initial interaction with mitochondria is rapid compared to the slower course of quenching. The membrane potential independent fluorescence change due to lipid sequestration alone was determined by following dye fluorescence upon addition to non-respiring mitochondria suspended in SSB/EGTA containing 10 mM KCN to poison respiration and collapse Ψ. The time constants of fluorescence change after mixing for all dyes under these conditions were comparable or faster than the 0.3 s time constant of the instrument. Following these rapid initial changes, a slower potential dependent quenching dominates for all dyes. The time course of the slower quenching was found to be a monophasic exponential over 90% of the dynamic range of signal change. After equilibrium has been reached, as indicated by constant fluorescence signal, addition of 5 μM final CCCP to collapse Ψ results in return of the fluorescence intensity, as seen in Fig. 1.

The effect of the outer mitochondrial membrane on the potential dependent dye influx was determined by measuring the influx time constants, τ, of 1 μM Rh123 and Rh6G into 0.1 mg/mL SSB/EGTA suspensions of mitoplasts. A τ of 44 and 3.9 s for Rh123 and Rh6G was obtained (as compared with 36 and 3.2 s obtained with whole mitochondria), indicating that the effect of the external membrane on the potential dependent fluorescence signal was within the reproducibility of estimate.

The second order nature of the fluorescence change of a Rh123–mitochondrial mixture is seen in Fig. 2. The time constant for equilibration of fluorescence signal following mixing of dye and respiring mitochondria varied in a linear fashion when concentration of mitochondria to dye were varied while the other was held constant. Two pseudo first-order rate constants, k_{app}, and k'_{app} are related to the true second order rate constant at the limit of each reagent in excess: $k_{app} = k$[Mito in excess] and $k'_{app} = k$[D in excess]. k was obtained from the slope of a plot of k_{app} (equal to τ^{-1} observed) vs. [reagent in excess]. The conditions to satisfy the reagent excesses were based upon previous experimentation [29]. Figure 2A shows the effect on the inverse of the influx time constant of fluorescence change, τ^{-1}, at the mitochondrial excess limit, as Rh123 was held constant at 1 μM and the concentration of mitochondria varied from 0.025 to 0.5 mg/mL. A rate constant (at excess mitochondria) of 0.178 mL mg^{-1} s^{-1} was obtained from the slope of 2(A). Figure 2(B) shows a plot of τ^{-1} against Rh123 concentration in the dye excess limit. The mitochondria concentration was held constant at

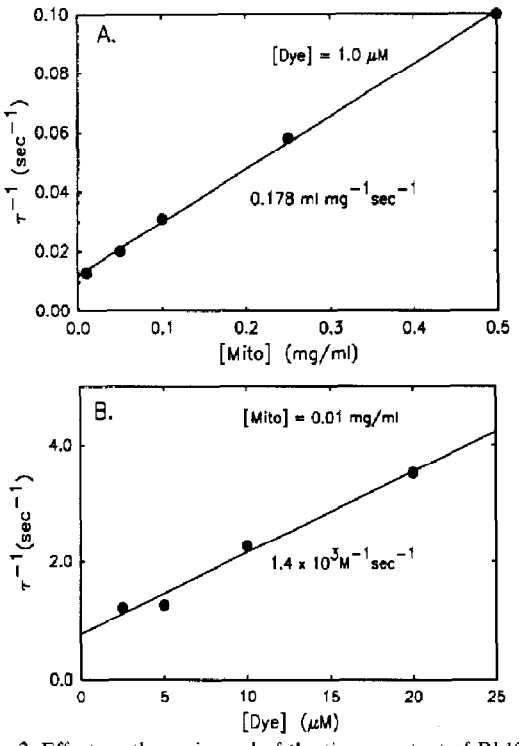

Fig. 2. Effect on the reciprocal of the time constant of Rh123 fluorescence change to equilibrium as a function of mixture component concentrations. (A) Varying amounts of mitochondria added to 1 μM Rh123 in SSB/EGTA. (B) Varying amounts of dye added to 0.01 mg/mL mitochondria in SSB/EGTA.

0.01 mg/mL and Rh123 varied from 1 to 20 μM. From the slope of 4(B), a rate constant (at excess dye) of 1.4×10^3 M^{-1} s^{-1} was obtained. An estimate of the number of moles of Rh123 binding sites per milligram of mitochondria, n can be made [30] with the relation: $n = k$ (at excess mitochondria)/k (at excess dye). Thus a value of 129 nmol/mg was calculated for Rh123.

The kinetic model predicts that the reciprocal of the time constant of change of fluorescence approach to equilibrium, τ^{-1}, should be, at a fixed dye concentration, a linear function of the mitochondrial concentration (eq. 9). The slope and inercept of a plot of τ^{-1} against mitochondrial concentration estimate the apparent influx rate constant, k', and the apparent efflux rate constant, k", respectively. We prepared such plots (e.g. Fig. 2A) and estimated the rate constants by linear least squares analysis of the data. The results are tabulated in Table 1. The precision of estimate of k' was 10% ($n = 3$) while for k'' it was 30%. Using the estimate for k' and the assumption that the trans-membrane potential has a value of 200 mV (negative inside), the permeability constant, P, for import of dye across the mitochondrial inner lipid bilayer were calculated using eq. (11) and are tabulated in Table 1. These values of P can be compared with those previously reported for permeability of lipophilic anions across synthetic lipid bilayers: 2×10^{-3} cm/s for CCCP anion [36].

The kinetic model (eqs. 2 and 3) predicts that estimates of k' and k'' are linearly proportional to the dye–lipid partition coefficient, K_1 and K_2,

Table 1

Kinetic constants of cationic dye binding to respiring mitochondria [a]

Cation	k' (mL mg^{-1} s^{-1})	k'' ($\times 10^{-3}$ s^{-1})	k'/k''	Ψ (mV)	f_b	P ($\times 10^{-4}$ cm s^{-1})
SafO	0.132	3.5	37.7	186 [b]	0.25	3.2 [c]
Rh123	0.161	5.5	29.3	173	0.18	3.9
DiOC2(3)	1.11	40	27.8	167	0.32	27
TMRE	1.13	30	37.6	186	0.32	27
Rh6G	2.79	24	116	243	0.34	68
DiOC2(5)	4.94	41	121	245	0.54	120
DiSC2(5)	5.70	24	238	279	0.71	138

[a] Where k' and k'' are the forward and reverse rate constants obtained from the slope and intercept of plots of τ^{-1} vs. [Mito] for the dyes listed; Ψ is the mitochondrial membrane potential calculated; f_b is the fraction of 10 μM dye partitioned into 1 mg/mL non-respiring mitochondria; and P is the influx permeability coefficient.

[b] Calculated from k'/k'' using eqn. (10) and the assumptions that $K_1 = K_2$ and $b = 0.5$.

[c] Calculated from k' using eq. (11) with $V = 1$ cm^3, $A = 400$ cm^2/mg, $\Psi = 200$ mV, $b = 0.50$.

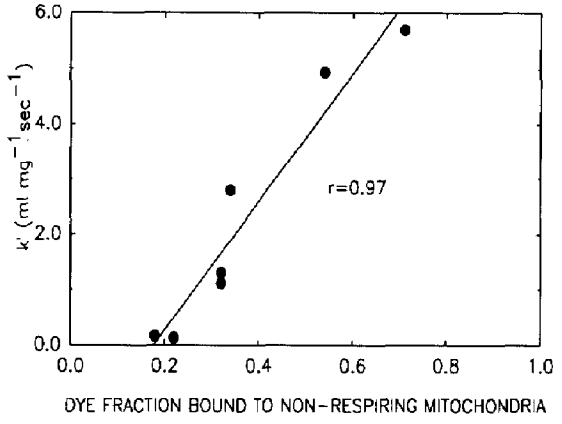

Fig. 3. Correlation of dye influx rate constants for fluorescence change to equilibrium with the fraction of 10 μM dye bound to 1.0 mg/mL non-respiring mitochondria.

respectively. To test this, one would ideally measure K_1 for each dye using non-respiring mitochondria following the methods described by Apell and Bersch [35] and then correlate their values with k' and k''. Because of limited resources, we estimated the dye relative lipophilicity by gravimetric determination of the bound fraction, f_b, upon addition of 10 μM of dye to a constant 1.0 mg/mL of non-respiring mitochondria (Table 1). Figure 3 shows a plot of k' against f_b, for which a least squares linear regression gave a correlation coefficient of 0.97. Likewise, a plot of k'' against f_b was linear, with a correlation coefficient of 0.76. The apparently lower degree of correlation is likely due in part to poorer estimate of the values of k''.

An interesting aspect of the kinetic model presented is that it allows calculation of the trans-membrane potential, Ψ, from estimates of k' and k'', using eq. (10) and the assumption that the dye–lipid partition coefficients at the two surfaces, $K_{1,2}$, are equal. Table 1 lists the results of this calculation, where it was assumed that the diffusion barrier geometric factor, b, is 0.5 [28]. For the dyes with lower relative lipophilicity (SafO, Rh123, TMRE), good agreement is found between the values of Ψ calculated (average value 182 mV) and reported in the literature [14] of around 160 mV. DiOC2(3) yields a lower estimate then expected of Ψ (157 mV), while the more lipophilic dyes (Rh6G, DiSC2(5), DiOC2(5)) appear to over estimate the membrane potential (average 256 mV), increasingly as relative lipophility increases.

The kinetic model predicts a symmetry in the time constants of fluorescence change when the approach to equilibrium is by dye flow from outside inward (τ_{in}), as initiated by addition of dye to suspension of respiring mitochondria, or when approach to equilibrium is by dye flow from inside outward (τ_{out}) as initiated by dilution of pre-equilibrated dye–respiring mitochondrial mixtures. We tested this expected symmetry by measuring τ_{in} and τ_{out} for a collection of dyes. First, the influx time constant of fluorescence change to equilibrium was determined upon addition of 1 μM dye to 0.1 mg/mL respiring mitochondria. After equilibration, dye efflux was initiated by 1:100 dilution of an aliquot of the same sample into fresh SSB/EGTA, and the time constant of fluorescence change to equilibrium again determined. Separate experiments showed no appreciable change in time constant for dilutions of the equilibrated dye–mitochondrial mixtures at dilutions greater than 1:100. For the dyes studied, Table 2 lists the values of τ_{in} and τ_{out} obtained and the time constant calculated. In no case was the efflux time constant observed to be the same as the influx, and the ratio, τ_{out}/τ_{in}, varied from 1.9 to 112. The dyes having greater lipophilicity have the greater dissymmetry of flux. A linear regression of τ_{out}/τ_{in} against relative lipophilicity f_b, was linear with a correlation co-

Table 2

Influx vs. dilution initiated efflux time constants

Cation	τ_{in} [a]	τ_{out} [b] (s)	τ_{out}/τ_{in}
Rh123	55	105	1.9
SafO	34	65	2.0
DiOC2(3)	5.0	14	2.9
TMRE	4.2	20	4.7
Rh6G	3.8	24	6.2
DiOC2(5)	1.9	38	19.7
DiSC2(5)	1.3	146	112

[a] Obtained using 0.1 mg/mL freshly isolated mitochondria added into 1 μM Dye in SSB/EGTA to initiated influx.

[b] Obtained upon 1:100 dilution of the equilibrated influx sample into fresh SSB/EGTA.

efficient of 0.94 excluding, or 0.87 including DiSC2(5).

Finally, the model assumes that the cationic trans-membrane diffusion step is that which limits the rate of fluorescence change. If this is so, then addition of substances known to effect the lipophilic cation trans-bilayer diffusion coefficient should significantly effect the observed rate of fluorescence change. Studies have shown that addition to cell suspensions of lipophilic anions, such as tetraphenyl borate (TPB), increase the rate of mitochondrial uptake of fluorescent cations [31]. Co-addition of TPB, anilinonaphthalene sulfonate, or picrate to suspensions of respiring isolated mitochondria with cationic anesthetic, bipivacaine, accelerates both the rate of uptake and the uncoupling effect on respiration [32]. The acceleration in uptake has been proposed to be due to the formation of electrically neutral ion-pair complexes between the cationic and the added lipophilic anion enabling the cation to penetrate more rapidly through the lipid bilayer as part of a neutral lipophilic complex [33]. To elucidate the extent to which the cationic fluorescence time constants are effected by modulation of the trans-bilayer diffusion rate, we investigated the effect on the time constant of Rh123 fluorescence change upon pre-treatment of the mitochondria with lipophilic anion, TPB. Figure 4 shows a plot of the time constant of fluorescence change as a function of varied concentration of TPB, pre-equilibrated with constant 0.1 mg/mL mitochondria in SSB/EGTA prior to cation addition. The apparent rate constant increases linearly with TPB concentration with a slope of $6.0 \times 10^4\ M^{-1}\ s^{-1}$. A similar increase was seen with other dyes studied when anilinonaphthalene sulfonate or picrate were used as counter anion. As a control, pre-equilibration of mitochondria with similar concentrations of cationic tetraphenyl phosphonium ion did not significantly effect the rate of Rh123 influx. The effect of TPB on the symmetry of influx and efflux time constants was measured by determining the ratio of influx to efflux time constants of fluorescence change for Rh123 and Rh6G equilibrated with TPB pre-equilibrated respiring mitochondria. The effect of TPB was to shorten the time constants, but leave the dissymetry in the ratio of the time constants unchanged as compared to that obtained for untreated mitochondria.

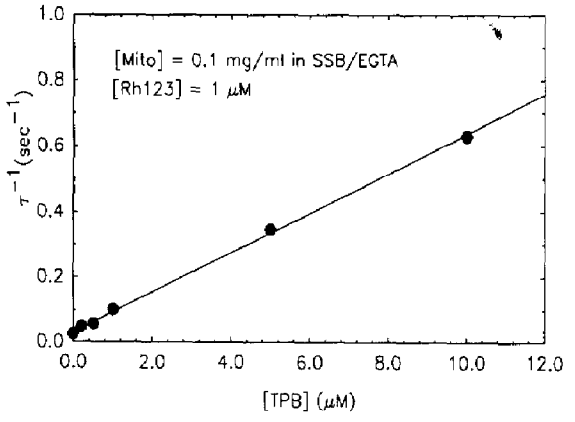

Fig. 4. Effect of addition of tetraphenylborate on time constant of fluorescence change to equilibrium for Rh123. Rh123 added to 1 μM to a preequilibrated mixture of 0.1 mg/mL mitochondria plus indicated concentration of TPB in SSB/EGTA.

6. Discussion

The purpose of this investigation was to obtain an understanding of the kinetics of fluorescence signal change upon addition of lipophilic cationic dyes to respiring mitochondria. We tested the fit of the observed fluorescence kinetics with a mathematical model of the processes involved (eq. 1), based on a simplification of a more general analysis recently reported by Clarke [18]. The model assumes that the rate determining step in generating change in fluorescence intensity is the trans-bilayer diffusion step, the rates of dye adsorption and desorption from the lipid being considerably faster. We find general agreement between the observed and the predicted kinetics of fluorescence signal change using the simplified model, with interesting deviations from prediction.

The assumption that the rates of sorption of dye at the aqueous–lipid interfaces are more rapid than the rate of dye trans-bilayer diffusion is supported by the data acquired and by reference to the literature. The rapid signal changes

(on the order of the instrumental time constant of 0.3 s) observed upon dye mixing with mitochondria and upon addition of CCCP to equilibrated dye–mitochondria mixtures indicate that the rates of these absorption and desorption processes have time constants less than 0.3 s. This is in agreement with previous reports [18,26,30] of k_{1} and k_1 of lipophilic dye partition into synthetic bilayers. Values of $> 1 \times 10^7\ M^{-1}\ s^{-1}$ and $\sim 10\ s^{-1}$ have been reported for k_1 and k_{-1}, which, for 1 μM dye, represent time constants of < 0.1 s or ~ 0.1 s), respectively. Comparison of these time constants to the 1.3 to 55 s influx time constants observed for membrane potential dependent fluorescence signal change (Table 2) demonstrates that trans-membrane diffusion process is 10 to 500 times slower than equilibration of dye with lipid.

The simplified kinetic model accurately reflects the observed dependence of the rates of fluorescence change on the relative lipophilicity of the dyes. It also predicts (eq. 10) that a direct calculation of the apparent trans-membrane potential (Ψ) can be made using the kinetic data, although in so doing we found the value calculated depended on the relative lipophilicity of the dye used as a probe. Not predicted or explained by the model is the dissymmetry observed between the influx and efflux time constants of fluorescence change (Table 2). This dissymmetry, as well as the observed dependence of the estimates of Ψ on dye lipophilicity, may be understood in light of the more complete analysis of Clarke [18] which takes into account the change in the electrostatic charge at the aqueous lipid interfaces as ionic lipophiles partition there. Clarke modeled the electrostatics of the lipid bilayer as three capacitances in series, one for each aqueous–lipid interface and one for the trans-membrane lipid interior. Binding of lipophilic cation to the external face of the inner mitochondrial bilayer will produce a surplus of positive charge on this surface, thereby enhancing the electric field across the bilayer, making it more negative than if no cation were absorbed. The subsequent diffusion of the cation across the bilayer will be effected by the increased field strength, resulting in a shorter time constant for diffusion across the bilayer. The more lipophilic the cation, the greater the deposition of charge at the outer surface, the higher the increase in trans-membrane potential and the shorter the trans-membrane diffusion time. Efflux of cation initiated by dilution of an equilibrated dye mitochondrial mixture will also be effected. The cation will experience a higher potential barrier against diffusion back across the bilayer with resultant increase in the time constant for fluorescence signal change. The greater the lipophilicity of the dye, the greater the concentration of dye at the exterior surface and hence the longer the relative time constant for efflux. With this reasoning, the cause of the dissymmetry in the time constants for influx and efflux is understood and the close link of the degree of dissymmetry with the dye lipophilicity would be expected. Alternatively, the Ψs were estimated from the kinetic data (Table 1) under the assumption that the partition constants of dye into the inner and outer surfaces of the inner membrane ($K_{1,2}$) are equal (eq. 10). This may not be a reasonable assumption, as it is known that there are differences in lipid content between the inner and outer surfaces of the inner mitochondrial bilayer [36,37] and also measured differences in $K_{1,2}$ obtained with non-fluorescent lipophilic potential probes [14]. A closer numerical agreement was seen between calculated and expected Ψ when the less lipophilic cations are used, such as Rh123, suggesting both that they perturb less the surface potential and that differences in $K_{1,2}$ are smaller than for the more lipophilic dyes. However, unless the ratio of K_1 to K_2 is known, the agreement of the kinetic method with equilibrium binding methods may be simply fortuitous.

Unfortunately, if the purpose of the fluorescence probe is to report the kinetics of change of the membrane potential, the less lipophilic dyes, which appear to perturb the Ψ being measured less, have the longest time constants of fluorescence signal response to rapid change in Ψ. The more lipophilic dyes are faster responding to dye influx, but are equally slow in efflux. Hence, there is no advantage in their use as fluorescent probes of the kinetics of alteration in Ψ. Of the dyes studied, safranine O is the best candidate as

a cationic fluorescent probe of the kinetics of Ψ, having the most symmetry of directional flux time constants, averaging about 50 s for mixtures of 1 μM dye and 0.1 mg/mL mitochondria. This response time constant can be improved by increasing the ratio of mitochondrial to dye concentration. Unfortunately, for the less lipophilic dyes the slopes of plots of τ^{-1} against mitochondrial concentration (Fig. 2) are shallow having values of about 0.2 mL mg^{-1} s^{-1}. Raising the mitochondria concentration a factor of ten only increases the response time by a factor of two, but also diminishes the dynamic range of fluorescence signal change upon equilibration [29], making the estimate of the response time less accurate. Thus the accuracy in the responsiveness of cationic fluorescent probes to changes in membrane potential is limited to potential changes which occur with time constants greater than about 30 s.

In conclusion this study has shown that the general aspects of the kinetics of fluorescence signal change associated with flux of cationic lipophilic dyes from respiring mitochondria are described by kinetic models, prepared and validated from previous experimentation on lipophilic dye interaction with synthetic lipid bilayer vesicles [18,26]. From the time constants of fluorescence signal change observed, it can be concluded that the ability of cationic dye fluorescence signal to follow change in mitochondrial inner membrane potential is limited to potential change with time constant slower than about 30 s.

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