Surface Potential in Rat Liver Mitochondria: Terbium Ion as a Phosphorescent Probe for Surface Potential†

Ken Hashimoto and Hagai Rottenberg*

ABSTRACT: The binding and phosphorescence of Tb^{3+} in rat liver mitochondria and submitochondrial particles were investigated. Mitochondria were treated briefly with *N*-ethylmaleimide (NEM) to prevent phosphate leak and Tb^{3+} chelation. Up to 30 nmol of Tb^{3+}/mg of protein binds to mitochondrial membranes with high apparent affinity ($K_d \simeq 6$ μM). Generation of a membrane potential had no significant effect on the apparent affinity or capacity of Tb^{3+} binding in NEM-treated mitochondria. Mitochondrial bound Tb^{3+} phosphorescence can be induced selectively by excitation of aromatic amino acid residues. The decay of mitochondrial bound Tb^{3+} phosphorescence is biphasic. The phosphorescence of the slow phase ($t_{1/2}$ = 0.45–0.70 ms) is quenched by monovalent salts, indicating a negative surface potential at low salt medium of −5.4 ± 2.8 mV [10 mM 3-(*N*-morpholino)-propanesulfonic acid, pH 7.2, 5 μM Tb^{3+}]. In submitochondrial particles, a surface potential of −6.5 ± 2.7 mV was estimated under the same conditions. Energization did not affect the surface potential significantly in submitochondrial particles and only slightly in mitochondria. Analysis of the phosphorescence of mitochondrial bound Tb^{3+} reveals two binding sites with high (K_d = 1.5 μM) and low affinity (K_d = 29 μM). The high-affinity site is tentatively identified as the Ca^{2+} carrier. A fraction of the carrier-bound Tb^{3+} phosphorescence decays rapidly, presumably as a result of energy transfer to cytochromes in the membrane core. These intramembrane sites appear to move to the surface on the generation of a membrane potential. We conclude that the salt effect on the phosphorescence of the slow phase may serve as a reliable measure of delocalized surface potential in mitochondria and submitochondrial particles. Tb^{3+} binding to the high-affinity site may be useful as a probe for the mitochondrial Ca^{2+} translocator.

The mitochondrial inner membrane, as most other biological membranes, is negatively charged at neutral pH (Aiuchi et al., 1977; Quintanilha & Packer, 1977; Robertson & Rottenberg, 1983). The membrane surface charge may be an important factor in controlling electron transport, ion transport, and energy conversion (Searle & Barber, 1979; Itoh, 1980; Hashimoto et al., 1981). When mitocondria are "energized" by electron transport or ATP hydrolysis, a large membrane potential (negative inside) exists across the mitochondrial inner membrane (Mitchell & Moyle, 1969; Rottenberg, 1970). It was recently reported that the generation of a membrane potential in mitochondria increases the negative surface charge on the cytosolic surface of the mitochondrial inner membrane (Aiuchi et al., 1977; Quintanilha & Packer, 1977; Wojtczack & Nalecz, 1979; Archbold et al., 1980). This observation, if valid, would have profound implications concerning the mechanism of energy conversion in mitochondria (Williams, 1961; Rottenberg, 1978; Kell, 1979). In recent years a large number of studies in bioenergetics were devoted to the relationships between the proton electrochemical potential and energy conversion in mitochondria (Rottenberg, 1975, 1979). In many studies cationic hydrophobic probes are used to measure the membrane potential (cf. Skulachev, 1979; Bashford & Smith, 1979). However, these probes may also respond to changes in the membrane surface charge (cf. Matsuura et al., 1980). Therefore, if energization would produce changes in both surface charge and membrane potential, a reliable estimate of these effects would require probes that respond to only one of these parameters. The evidence that the development of membrane potential in mitochondria increases the negative surface charge of the membrane is based on (i) measurement of the electrophoretic movement of mitochondria (Aiuchi et al., 1977; Archbold et al., 1980), (ii) reinterpretation of the ANS response to energization (Aiuchi et al., 1977; Wojtczak & Nalecz, 1979), and (iii) the distribution of charged spin probes (Quintanilha & Packer, 1977). However, all of these measurements may reflect, indirectly, the formation of $\Delta\psi$ (Njus et al., 1977). In addition, the electrophoretic mobility of mitochondria may be affected by changes in the morphology of the mitochondria. The ANS response to energization of mitochondria was recently shown to result from the efflux of the anion and not from a change in the membrane surface charge (Robertson & Rottenberg, 1983). Similarly, we have concluded that the response of the charged EPR probes is due to potential driven uptake by the mitochondria and not to a change in the surface charge (K. Hashimoto, P. Angiolillo, and H. Rottenberg, in press). Nevertheless, it was not possible to conclude from these studies that there is no change in the surface charge, only that the potential induced signal is large and hence could mask any changes in the surface charge. It was therefore necessary to find a surface charge probe that does not permeate the inner membrane at any appreciable rate and thus does not respond directly to the membrane potential. Tb^{3+} is a phosphorescent cation of the lanthanide series and has been used as a probe for Ca^{2+} binding sites in many Ca^{2+} binding proteins (Martin & Richardson, 1979). The presence of aromatic amino acid residues in the vicinity of the binding site allows efficient energy transfer when these residues are excited (Brittain et al., 1976). Due to the positive charge of the ion, the extent of Tb^{3+} binding (at low concentration) will depend on the surface potential in the vicinity of the binding site. The binding can be conveniently monitored either by the phosphorescence of the protein-bound Tb^{3+} or by chelation of the free Tb^{3+} in the supernatant with an aromatic chelator after sedimentation of the mitochondria. If Tb^{3+} is not transported at an appreciable rate by the mitochondria, it may serve as a more suitable probe for the determination of surface charge. Moreover, because of the slow decay of the phosphorescence of bound

† From the Department of Pathology and Laboratory Medicine, Hahnemann University, School of Medicine, Philadelphia, Pennsylvania 19102. *Received May 3, 1983.* This work was supported by National Institutes of Health Grants GM-28173 and AAO 5662.

0006-2960/83/0422-5738$01.50/0

Tb^{3+}, it is an excellent donor for energy transfer which could be detected by the measurement of the decay time. In mitochondria, the most efficient acceptors from excited Tb^{3+} are the cytochromes (Doktor, 1982). The energy-transfer efficiency depends on the distance and relative orientation of the acceptor–donor pair and if both are charged also on electrostatic interactions (Strayer, 1978). Hence, the measurement of the lifetime of the phosphorescence of mitochondrial bound Tb^{3+} may provide information concerning local charges, distribution, and orientation of several membrane proteins. In this study, we show that Tb^{3+} is a sensitive probe of the mitochondrial surface charge. We also show that Tb^{3+} is not transported by mitochondria and hence allows estimation of the surface charge, regardless of the value of membrane potential. The changes of surface charge we detected on energization are small and do not appear to be significant. The decay of Tb^{3+} phosphorescence indicates the existence of several Tb^{3+} binding sites which differ in their distance from the heme groups of the cytochromes. The energetic state of the mitochondria appears to affect these distances and possibly the local electrostatic field within the membranes.

Materials and Methods

Preparation of Mitochondria. Liver mitochondria were isolated from male Sprague-Dawley albino rats (150–250 g) by homogenization in 0.25 M sucrose, 1 mM EDTA[1] (disodium salt) at pH 7.4, followed by differential centrifugation (Johnson & Lardy, 1967). The preparation was washed twice with a medium composed of 0.25 M sucrose and 1 mM Mops-Na^+ buffer (pH 7.4) to remove EDTA from mitochondrial suspension. *N*-Ethylmaleimide- (NEM) treated mitochondria were prepared as follows: NEM was added to mitochondrial suspension in the washing buffer at a final concentration of 40 nmol of NEM/mg of mitochondrial protein and was incubated for 1 min at room temperature. The mixture was diluted 5 times with cold washing buffer and washed twice in the same buffer. Finally, the sample was resuspended in a small volume of 0.25 M sucrose.

Submitochondrial particles were prepared by disruption of mitochondria in a Yeda press followed by differential centrifugation (Lindsay et al., 1972). Mitochondria (5–10 mg of protein/mL) were suspended for 10 min at 0–4 °C in 0.03 M sucrose, 5 mM $MgSO_4$, 5 mg/mL bovine serum albumin, 100 μM EDTA, and 5 mM Mops-Na^+ buffer (pH 7.4). After this hypotonic treatment, the mitochondria were broken by extrusion from the needle valve of a Yeda press under a pressure of 140 kg/cm^2 nitrogen. This suspension was centrifuged at 15500*g* for 30 min to remove mitochondrial debris. The supernatant was centrifuged at 106000*g* for 30 min. The pellet was washed once at 106000*g* with 0.25 M sucrose, 2 mM $MgSO_4$, and 5 mM Mops-Na^+ (pH 7.4) for 30 min. The resultant pellet was suspended in a small volume of 0.25 M sucrose (10–25 mg of protein/mL) and stored at −70 °C. Oxygen consumption was determined as described (Estabrook, 1967).

Estimation of Total Binding of Tb^{3+} to Mitochondrial Membrane. Binding of Tb^{3+} to NEM-treated mitochondria was estimated by measuring the free concentration of Tb^{3+} in the supernatant. The free concentration of Tb^{3+} was measured by using dipicolinic acid as a chelating luminescence sensitizer (Barela & Sherry, 1976). Mitochondrial suspension with Tb^{3+} (0–100 μM) were placed in 1.5-mL centrifugation tubes and spun at 10000*g* for 3 min in an Eppendorf centrifuge. The supernatant (0.6 mL) was diluted 5 times with a sucrose–Mes buffer (0.25 M sucrose, 10 mM Mes-Na^+, pH 5.8). Dipicolinate was added to this solution at a final concentration of 400 μM. A calibration curve for free concentration of Tb^{3+} was constructed in the same manner as described above, except for the addition of mitochondrial membranes.

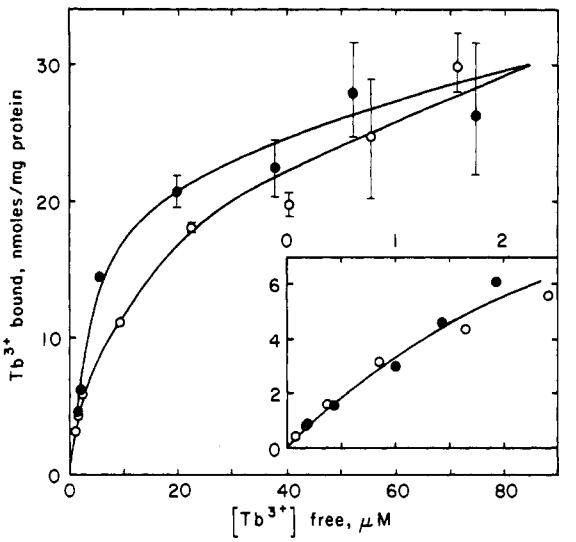

FIGURE 1: Binding of Tb^{3+} to NEM-treated mitochondria. Total binding of Tb^{3+} in the energized (○) and deenergized (●) state was determined as described under Materials and Methods. Tb^{3+} binding in low concentration range is shown in the inset. Mitochondria were suspended at a concentration of 1 mg of protein/mL in 0.25 M sucrose, 10 mM Mes-Na^+ (pH 5.8), 4 μM rotenone, 4 μg/mL oligomycin, 1 mM succinate, and up to 100 μM $TbCl_3$ in the absence (○) or the presence (●) of 0.33 μg of antimycin A/mg and 0.5 μM CCCP. The suspension was centrifuged 2 min after the addition of mitochondria. Dipicolinate was added to the supernatant, and the free Tb^{3+} concentration was determined as described under Materials and Methods. Temperature 23 °C.

Measurement of Tb^{3+} Phosphorescence Decay Kinetics. Decay kinetics of Tb^{3+} phosphorescence was measured by a Perkin-Elmer LS-5 luminescence spectrophotometer. In this instrument the excitation is by a repetitive xenon discharge lamp (9.9 W, 60 Hz, half-width duration 10 μs). In most cases the signal was averaged over 1 min (3600 flashes).

Phosphorescence decay was measured against the delayed time (t_d) at a fixed gating period (T_g). The kinetics was corrected for the gating distortion (see Appendix). All values were corrected for the suspension background phosphorescence. Values for all decay constants and extrapolated initial intensities of phosphorescence at zero time were estimated by using a linear regression curve-fitting analysis. All decay kinetic measurements were carried out at 15 °C.

Results

Tb^{3+} is a cation of the lanthanide series. In many enzymes, Tb^{3+} binds with high affinity to specific Ca^{2+} binding sites (Brittain et al., 1976). In mitochondria, La^{3+} inhibits Ca^{2+} transport, and it has been suggested that La^{3+} can also be transported by the Ca^{2+} transport system (Reed & Bygrave, 1974). If Tb^{3+} is also transported by the electrogenic Ca^{2+} carrier, we expect that generation of $\Delta\psi$ would increase the binding of Tb^{3+} by several orders of magnitude (Rottenberg & Scarpa, 1974; Scarpa, 1979).

Figure 1 shows Tb^{3+} binding curves calculated from the free Tb^{3+} concentration in the supernatant of mitochondrial suspension as described under Materials and Methods. In en-

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; ANS, 8-anilinonaphthalene-1-sulfonate.

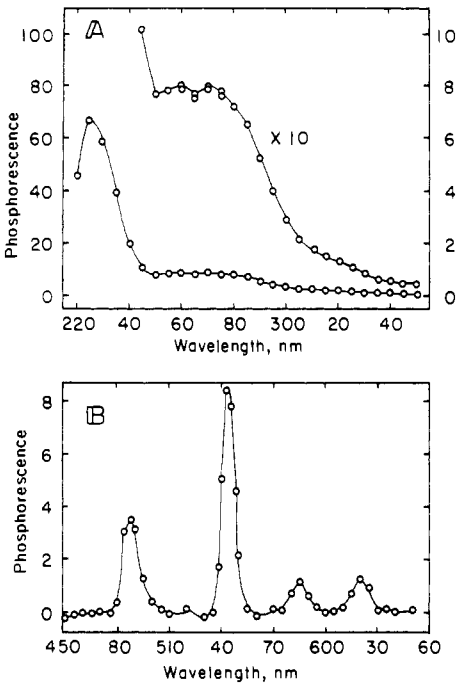

FIGURE 2: Phosphorescence excitation (A) and emission (B) spectra of bound Tb^{3+} in submitochondrial particles. The excitation spectrum was obtained by measuring luminescence at 543 nm. The emission spectrum was obtained by excitation at 280 nm. Phosphorescence between 0.5 and 9.5 ms after pulse excitation (10 μs) was measured. Submitochondrial particles were suspended at 0.1 mg of protein/mL of 0.25 M sucrose, 20 μM $TbCl_3$, and 10 mM Mops (pH 7.2). Upper curve in (A) was expanded (×10). All measurements were carried out at 15 °C.

ergized (succinate-driven) and deenergized (+antimycin A + CCCP) NEM-treated mitochondria, the binding affinity and capacity are of equal magnitude. The apparent binding capacity and apparent dissociation constant were estimated to be approximately 30 nmol of Tb^{3+}/mg of protein and 6 μM, respectively. In particular, there was no difference in the binding characteristics at low Tb^{3+} concentration (see inset of Figure 1). The results of Figure 1 indicate that there is no significant energy-dependent transport of Tb^{3+} in mitochondria, and hence Tb^{3+} must be bound only to the cytosolic surface of the inner membranes.

In Figure 1 as in all subsequent experiments, we used NEM-treated mitochondria to avoid phosphate leak from the mitochondrial matrix. Inorganic phosphate forms a strong complex with Tb^{3+}. Since mitochondrial membranes have a very active phosphate transport system, we decided to inhibit the phosphate efflux by *N*-ethylmaleimide (NEM) (Coty & Pederson, 1975). This treatment does not affect the generation of $\Delta\mu_{H^+}$ by succinate. The ADP-induced stimulation of oxygen consumption is inhibited through the inhibition of phosphate transport. However, the coupling, as indicated by the uncoupler stimulation of respiration, is not affected by NEM (data not shown).

The phosphorescence spectra of submitochondrial particles bound Tb^{3+} is shown in Figure 2. In the excitation spectra (A), two peaks were observed in the UV region. One is at 225 nm, and the other is at 265 nm. The latter broad peak corresponds to the Tb^{3+} emission which is sensitized by energy transfer from aromatic amino acid residues in mitochondrial proteins (Brittain et al., 1976). The maximum at 225 nm may correspond to the absorption maximum of free Tb^{3+} in aqueous solution (218 nm) and may show the direct excitation of total Tb^{3+}. The phosphorescence decay kinetics at 225 nm showed two phases corresponding to free and bound Tb^{3+} (not shown).

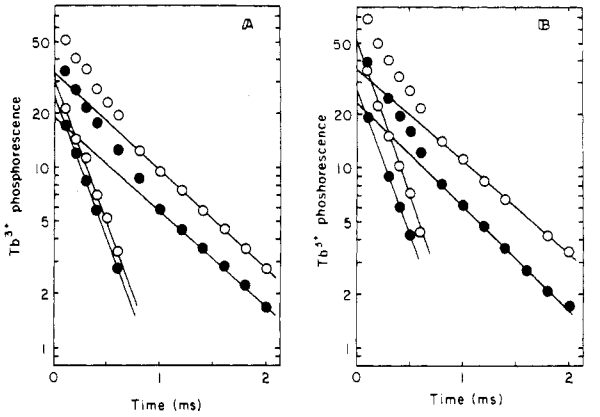

FIGURE 3: Salt effect on the phosphorescence decay kinetics of membrane-bound Tb^{3+} energized (A) and deenergized (B) states in submitochondrial particles. Fast phases were obtained by subtracting the extrapolated intensity of the slow decay phase from that of total intensity. Submitochondrial particles were suspended at a final concentration of 0.1 mg of protein/mL in 10 mM Mops-Na^+ (pH 7.2), 0.8 μM rotenone, 5 μM $TbCl_3$, and either 0.25 M sucrose (open symbols) or 0.125 M NaCl (closed symbols). Sodium succinate, 1 mM, was added in (A). The kinetics was measured at 15 °C.

The Tb^{3+} emission spectrum (B) which was sensitized by aromatic amino acid residues exhibited the typical fine quartet.

The decay kinetics of sensitized Tb^{3+} phosphorescence after pulse excitation in submitochondrial particles suspension is shown in Figure 3 in a semilogarithmic plot. In order to evaluate the effect of surface potential on Tb^{3+} binding, we examined the effect of high salt concentration on Tb^{3+} phosphorescence. Since Tb^{3+} is positively charged, high salt concentrations would increase binding if the membrane surface charge is positive and decrease the binding if the surface charge is negative. Both in energized (A) (succinate-driven) and in deenergized (B) state, salt quenches the phosphorescence, indicating that the surface charge is negative. In both low and high salt conditions, two phases in the decay kinetics were observed. The rate of decay of the fast phase was obtained by subtracting the extrapolated values of the slow decay phase from that of the total phosphorescence. The slow-phase phosphorescence was highly quenched by salt. The fast-phase phosphorescence was less sensitive to salt. In both energized and deenergized particles salt does not affect the decay constants but only the initial phosphorescence intensity, presumably due to decreased binding. The effect of salt on the apparent binding affinity of ion to membranes may be utilized to estimate the membrane surface potential (Haynes 1974; Castle & Hubble 1976; Quintanilha & Packer, 1977). We have recently applied this method to the estimation of surface potential in rat liver mitochondria from the fluorescence enhancement of ANS (Robertson & Rottenberg, 1983). At 125 mM NaCl and 10 mM Mops-Na^+ (pH 7.2), the surface potential in mitochondria (ψ_s) is almost completely screened (Robertson & Rottenberg, 1983). The surface potential (in mV, at 15 °C) at low salt medium, in the vicinity of Tb^{3+} binding sites, was therefore calculated as follows:

$$\psi_s = -RT/3F \ln (P/P_0) = -19.1 \log (P/P_0) \quad (1)$$

P represents phosphorescence intensity extrapolated to zero time in low salt medium, and P_0 at high salt medium in which the surface potential is vanishingly small. In this experiment, the estimated surface potential at low salt medium for the binding sites corresponding to the slow decay phase was −4.7 mV in the energized state and −3.8 mV in the deenergized state. At the binding site corresponding to the fast phase, the estimated surface potential was −1.3 mV in the energized and

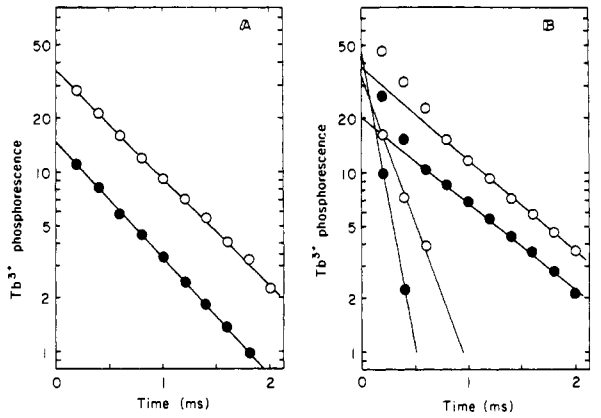

FIGURE 4: Salt effect on the phosphorescence decay kinetics of membrane-bound Tb^{3+} in energized (A) and deenergized (B) states in NEM-treated mitochondria. NEM-treated mitochondria were suspended to a final concentration of 0.1 mg of protein/mL in 10 mM Mops-Na^+ (pH 7.2), 1 mM sodium succinate, 0.8 μM rotenone, 5 μM $TbCl_3$, and either 0.25 M sucrose (open symbols) or 0.12 M NaCl (closed symbols) under aerobic (A) or anaerobic (B) conditions. All measurements were carried out a 15 °C.

Table I: Effect of Energy State on Surface Potential (ψ_s)[a]

	ψ_s (mV)			
	rapid decay phase		slow decay phase	
	energized	de-energized	energized	de-energized
NEM-treated mitochondria	b	+3.5 ± 7.9	−7.4 ± 0.6	−5.4 ± 2.8
submitochondrial particles	−2.7 ± 1.4	−5.9 ± 0.6	−5.6 ± 0.9	−6.5 ± 2.7

[a] Surface potentials at the two Tb^{3+} binding sites. Surface potential was calculated as described in the text. For the calculation of the intensity of Tb^{3+} phosphorescence at zero time we used the equations described in the Appendix. NEM-treated mitochondria (0.1 mg of protein/mL) or submitochondrial particles (0.1 mg of protein/mL) were suspended in 10 mM Mops-Na^+ (pH 7.2), 0.8 μM rotenone, 5 μM $TbCl_3$, and either 0.25 M sucrose or 0.125 M NaCl. Mitochondrial membranes were energized by the addition of sodium succinate (1 mM). In deenergized systems succinate was omitted or oxygen replaced with argon. In both systems of deenergized membrane, surface potentials were the same within the experimental errors. [b] Because of the very small contribution of the fast phase to the total kinetics, in energized NEM-treated mitochondria, we could not calculate the surface potential.

−5.3 mV in the deenergized state.

The decay kinetics of sensitized Tb^{3+} phosphorescence in NEM-treated mitochondria is shown in Figure 4. In the energized state (A), only a slow phase was observed at both low and high salt. Again, the phosphorescence was quenched by salt. In deenergized mitochondria both slow and fast phases were observed. Salt quenches the slow phase but appears to slightly enhance the fast phase. In this experiment, the estimated surface potentials in low salt medium for Tb^{3+} binding sites corresponding to the slow phase were −7.6 mV in the energized and −5.3 mV in the deenergized state. The apparent surface potential of the fast-phase Tb^{3+} binding site was estimated as +2.6 mV in the deenergized state.

Table I summarizes the results of several experiments similar to those shown in Figures 4 and 5. In the deenergized state the value of the estimated surface potential in low salt medium in submitochondrial particles and mitochondria is similar, −6.5 ± 2.7 and −5.4 ± 2.8 mV, respectively. Energization does not appear to produce significant change in submitochondrial particles (−5.9 ± 0.9 mV) but on the average produced a

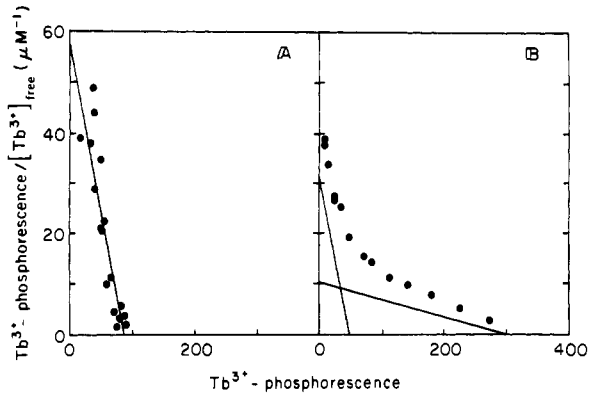

FIGURE 5: Determination of dissociation constants (K_d) for phosphorescent Tb^{3+} binding sites with different decay times [(A) $t_{1/2}$ = 0.13 ms; (B) $t_{1/2}$ = 0.53 ms] in NEM-treated mitochondria. Extrapolated values of fast (A) and slow (B) phases in Tb^{3+} phosphorescence at zero time were used as bound Tb^{3+} in the Scatchard plots. Tb^{3+} phosphorescence of slow decay phase (B) was analyzed to derive two classes of binding sites. Values for all slopes and intercepts were estimated by using a regression curve-fit program on a Hewlett-Packard HP-85 personal computer (solid lines). NEM-treated mitochondria were suspended to a final concentration of 0.1 mg of protein/mL in 0.125 M NaCl, 10 mM Mops-Na^+ (pH 7.2), and 0.8 μM rotenone. $TbCl_3$ (up to 100 M) was added, and the solution was allowed to incubate for 15 min before measurements of phosphorescence. The concentration of free Tb^{3+} was determined as in Figure 1.

slightly more negative potential in mitochondria (−7.4 ± 0.6 mV). This apparent difference is mostly due to the merging of the two phases in reenergized mitochondria and does not appear to result from increased charge. Both in mitochondria and in submitochondrial particles the kinetics and the initial intensity of the slow phase are relatively insensitive to energization, and the monovalent salt effects appear to be due solely to the screening of the membrane surface charge. The behavior of the fast phase is much more complex. In deenergized mitochondria salt enhances the initial phosphorescence, indicating that this binding site has positive surface potential. However, this binding site has high affinity (K_d = 1.5 μM) for Tb^{3+} (see Figure 5). Since we used 5 μM Tb^{3+} in this experiment it is possible that the Tb^{3+} already bound at this binding site renders the site positive. Indeed, we obtained negative values of surface potential at this site when 0.5 μM Tb^{3+} was used (see Discussion). Energization seems to slow the fast phase in mitochondria, presumably due to inhibition of energy transfer from Tb^{3+} to the energy-accepting chromophore by energization (see Discussion). Table II shows half-times of decay of the two phases in mitochondria and submitochondrial particles. Both fast and slow decay phases had fairly constant values of half-times in various conditions. In these experiments, the deenergized state was established by anaerobiosis or depletion of respiratory substrate. Addition of antimycin A and succinate caused a further decrease in the decay time of the slow phase in both NEM-treated mitochrondria or submitochondrial particles (Table III). Typical measurements gave 0.70 ms, at high salt, in NEM-treated mitochondria, and 0.62 ms, at high salt, in submitochondrial particles. Dissociation constants for Tb^{3+} binding sites which correspond to the fast and slow decay phases were determined by Scatchard plots in NEM-treated mitochondria (Figure 5). While there was a single binding site with high affinity (K_d = 1.5 μM) in the fast phase (A), two classes of binding sites were clearly resolved in the slow decay phase (B). One of these had the same high affinity (K_d = 1.5 μM) as the site for fast decay phase, and the other one had a much lower affinity (K_d = 29 μM). The ratio of the capacities of these binding sites

Table II: Effect of Energy State on Half-Decay Times ($t_{1/2}$) of Tb^{3+} Phosphorescence[a]

		$t_{1/2}$ (ms)			
		fast decay phase		slow decay phase	
		energized	deenergized	energized	deenergized
NEM-treated mitochondria	sucrose	?[b]	0.19 ± 0.06	0.49 ± 0.04	0.52 ± 0.09
	NaCl (0.125 M)	?[b]	0.14 ± 0.05	0.52 ± 0.08	0.57 ± 0.12
submitochondrial particles	sucrose	0.17 ± 0.02	0.17 ± 0.02	0.59 ± 0.03	0.55 ± 0.03
	NaCl (0.125 M)	0.16 ± 0.03	0.18 ± 0.01	0.52 ± 0.01	0.56 ± 0.05

[a] Half-time of phosphorescence decay at the two Tb^{3+} binding sites. Experimental conditions are the same as in Table I. [b] We could not resolve the fast phase in energized NEM-treated mitochondria.

Table III: Effect of Antimycin A on Half-Decay Times ($t_{1/2}$) of Tb^{3+} Phosphorescence[a]

		$t_{1/2}$ (ms)	
conditions	additions	fast decay phase	slow decay phase
NEM-treated mitochondria	no addition	0.12	0.51
	+antimycin A	0.12	0.55
	+antimycin A + succinate	0.13	0.62
submitochondrial particles	no addition	0.17	0.60
	+antimycin A	0.17	0.58
	+antimycin A + succinate	0.18	0.70

[a] Effect of antimycin A on half-decay times of Tb^{3+} phosphorescence both in NEM-treated mitochondria and submitochondrial particles containing 0.1 mg of protein/mL, 0.8 μM rotenone, 5 μM $TbCl_3$, 0.125 M NaCl, and 10 mM Mops-Na^+ (pH 7.2). Additions were antimycin A (67 ng/mL) and sodium succinate (1 mM). Measurements were carried out at 15 °C.

was also determined (assuming that Tb^{3+} has the same quantum yield in all binding sites). Expressed as a fraction of total Tb^{3+} binding, the capacities of the three binding sites were 20% (K_d = 1.5 μM, fast decay), 10% (K_d = 1.5 μM, slow decay), and 70% (K_d = 29 μM, slow decay). Increasing the pH from 4.5 to 8.0 greatly enhances the phosphorescence. At low pH salt does not quench the phosphorescence, but the magnitude of quenching increases with pH (data not shown). These results are in agreement with our previous results which indicated that the mitochondrial surface charge increases with pH (Robertson & Rottenberg, 1983).

The salt concentration dependence of the phosphorescence of the slow phase in deenergized submitochondrial particles is shown in Figure 6. There is a clear cation valence dependency in Tb^{3+} phosphorescence quenching by salts. A similar valence dependency of the phosphorescence of the slow phase is also shown for deenergized NEM-treated mitochondria (Figure 7). These valence dependencies suggest that the multivalent salt-induced quenching is partially due to screening of the mitochondrial surface charge but also due to binding of multivalent cations to the mitochondrial surface. The valence dependency of the screening of the surface potential can be calculated from the Gouy equation (at 15 °C) (Mclaughlin, 1977)

$$\sigma = \frac{1}{135.8}\sqrt{c}\sinh(z\psi_0/49.6) \qquad (2)$$

where c is the salt concentration (M), σ is the charge density (e/A^2), and z is the ion valence. With the assumption of a residual surface potential of 5 mV in a medium of 100 mM monovalent salt, the calculated charge density is $^1/_{4255}$ A^2. The same surface potential is indicated in mitochondria in a medium of 10 mM divalent salt (Figure 7), but the calculated charge density for this condition is only $^1/_{6710}$ A^2. Thus, with

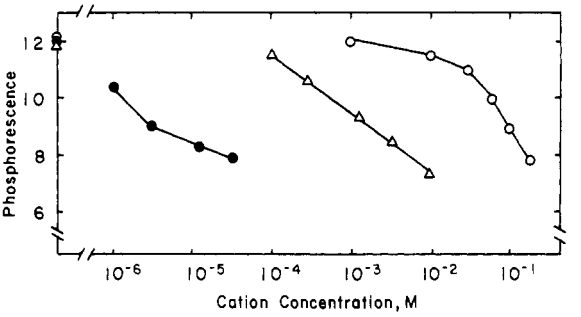

FIGURE 6: Effect of added salt concentrations on membrane-bound Tb^{3+} phosphorescence of the slow decay phase in submitochondrial particles. Reaction mixture was composed of submitochondrial particles containing 0.1 mg of protein/mL in 5 μM $TbCl_3$, 0.8 μM rotenone, 10 mM Mops-Na^+ (pH 7.2), and 0.25 M sucrose. Additions: (○) NaCl; (△) $MgCl_2$; (●) $LaCl_3$.

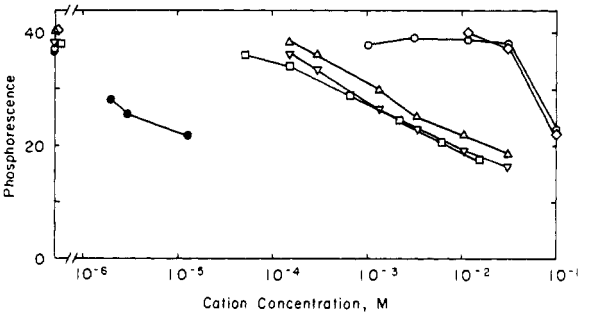

FIGURE 7: Effect of added salt concentrations on membrane-bound Tb^{3+} phosphorescence of the slow decay phase in NEM-treated mitochondria. NEM-treated mitochondria were suspended at a final concentratin of 0.1 mg of protein/mL in 5 μM $TbCl_3$, 0.8 μM rotenone, 10 mM Mops-Na^+ (pH 7.2), and 0.25 M sucrose. Additions: (○) NaCl; (◇) KCl; (△) $MgCl_2$; (▽) $CaCl_2$; (□) $SrCl_2$; (●) $LaCl_3$.

the assumption that the estimates of surface potential from eq 1 are reliable, the data of Figures 6 and 7 indicate that multivalent cations bind to mitochondrial membrane. This conclusion is in agreement with our previous studies on surface charge in mitochondria (Robertson & Rottenberg, 1983) and with previous studies of cation binding to mitochondria (Scarpa, 1979).

Discussion

Binding and Transport of Tb^{3+} in Mitochondria. Alkylation of sulfhydryl groups of energized mitochondrial membranes with a high dose of N-substituted maleimide derivatives was reported to cause the loss of membrane integrity and increase membrane permeability (Le Quoc & Le Quoc, 1982). However, in mild treatment, NEM did not cause significant changes in membrane permeability (Le Quoc & Le Quoc, 1982). We treated mitochondria with a limited concentration of NEM (40 nmol/mg of protein) in a short reaction time (1 min) and also in the deenergized state. We saw no significant reduction of the proton electrochemical potential ($\Delta\tilde{\mu}_{H^+}$) with this mild

NEM treatment [not shown; see also Ramachandran & Bygrave (1978)]. Addition of Tb^{3+} (up to 100 μM) to NEM-treated mitochondrial suspensions did not stimulate oxygen consumption. Also, no significant change in membrane potential or pH gradient (ΔpH) was observed on addition of Tb^{3+} (30 μM) to NEM-treated mitochondrial suspension (not shown). These data together with the data of Figure 1 suggests that Tb^{3+} was not transported electrogenically at a measurable rate. Reed & Bygrave (1974) reported that La^{3+} was accumulated by mitochondria, in an energy-dependent way. They also reported a stimulation of mitochondrial succinate oxidation by La^{3+} (in salt-free medium). However, no stimulation at low concentrations was observed (<75 μM). The requirement for high concentrations and the absence of stimulation in NaCl medium suggest that the effect on respiration is a nonspecific salt effect and not due to La^{3+} transport. Judging from our measurements of respiration, ΔpH, $\Delta\psi$, and Tb^{3+} binding, even at high concentrations of Tb^{3+} no measurable transport was indicated. Energization of mitochondria showed no significant difference in Tb^{3+} binding (Figure 1). Particularly at low concentrations, in which the pulse-excited phosphorescence measurements were carried out, there was no difference at all in the binding curves. We conclude that in our experimental conditions for pulse-excited phosphorescence measurements (5 μM Tb^{3+}, at 15 °C) Tb^{3+} is not transported. Oxalate and phosphate anion are strong chelators for Tb^{3+} and quench the Tb^{3+} phosphorescence. On addition of oxalate or phosphate, we could not observe any Tb^{3+} phosphoresence from mitochondrial suspension. Even if some Tb^{3+} was transported into the matrix during our measurements, it would be totally quenched because of the very high concentration of internal P_i. Hence, in mitochondria, transport of Tb^{3+}, if any, should not interfere with measurements of externally bound Tb^{3+}. Figure 1 indicates not only that Tb^{3+} is not transported into mitochondria but also that there is no pronounced change in the inner membrane surface charge. This conclusion is confirmed further by the measurements of the phosphorescence of mitochondrial bound Tb^{3+} as discussed below.

Microenvironment of Tb^{3+} Binding Sites in Mitochondria. The lifetime of Tb^{3+} phosphorescence is sensitive to the cation microenvironment (Horrock & Sudnick, 1979). Three kinds of deexcitation processes of Tb^{3+} can be expected: phosphorescence, radiationless transition via OH oscillators of coordinated water molecules (Horrock et al., 1977), and nonradiative energy transfer to an adjacent chromophore. The observed phosphorescence exponential decay constant, k_{obsd}, can be given by $k_{obsd} = k^0 + k_{OH} + k_{et}$, where k^0 is the intrinsic rate constant of Tb^{3+} phosphorescence, k_{OH} is the rate constant for the energy transfer to the O–H vibrations of coordinated water, and k_{et} is the rate constant for nonradiation energy transfer to the chromophore.

Horrock & Sudnick (1979) measured the lifetime of Tb^{3+} phosphorescence with a variety of ligands as a function of D_2O concentration and estimated the number of coordinated water molecules. In aqueous Tb^{3+} solution, Tb^{3+} had nine coordinated water molecules and a decay constant of 2.5 ms^{-1} (Horrock & Sudnick, 1979; our measurement gave 2.2 ms^{-1}). Dipicolinic acid and Tb^{3+} form a complex in which there are no water molecules in the first coordination sphere. The decay constant for this complex is 0.3 ms^{-1} (Horrock & Sudnick, 1979).

In this study, decay constants for the slow decay component of Tb^{3+} phosphorescence sensitized by mitochondrial protein were observed between 1.0 and 1.5 ms^{-1} (0.45 ms $\leq t_{1/2} \leq$ 0.70 ms) both in submitochondrial and in NEM-treated mitochondrial membranes. If there was no energy transfer except via OH oscillators of coordinated water, the slow decay constants would correspond to a rate constant of bound Tb^{3+} with two to four coordinated water molecules. The fast phosphorescence decay constants were between 3.6 and 5.3 ms^{-1} (130 μs $\leq t_{1/2} \leq$ 190 μs) in submitochondrial particles and between 2.8 and 8.0 ms^{-1} (87 μs $\leq t_{1/2} \leq$ 250 μs) in NEM-treated mitochondria. These fast decay rates, which are faster than that of fully hydrated Tb^{3+} (2.5 ms^{-1}; $t_{1/2}$ = 280 μs), certainly arise from another deexcitation process. It is possible that the energy of excited Tb^{3+} is transferred to a chromophore, the absorption spectrum of which overlaps with the emission spectrum of Tb^{3+}, by a Förster type dipole–dipole energy transfer (Thomas et al., 1978). Hemes of cytochromes, particularly of the *b* and *c* types, are the most plausible candidates for the energy-accepting chromophores in mitochondrial membranes (Doktor, 1982). Since protein-bound Tb^{3+} phosphorescence does not normally decay as fast as mitochondrial bound Tb^{3+}, it is reasonable to assume that considerable energy transfer occur even from the slow-phase fraction. This is compatible with our observation that high pH and antimycin A further increase the decay time of the slow phase.

Since the phosphorescence is induced by excitation of aromatic amino acids of the membrane-bound proteins, all the bound Tb^{3+} detected in the phosphorescence experiments is protein bound. The detection of three classes of binding sites, with fast decay and high affinity, slow decay and high affinity, and slow decay and low affinity suggests that these arise from at least two different proteins. The slow decay may arise from bound Tb^{3+} which is bound externally more than 20 Å away from the membrane chromophores. With the exception of cytochrome *c* the hemes of *all* the other cytochromes are buried in the membrane core, 20–50 Å from the surface. Since Tb^{3+} phosphorescence was not greatly modified by cytochrome *c* depletion (not shown), we concluded that cytochrome *c* does not contribute greatly to the energy transfer. Hence, the rapid decay may arise from bound Tb^{3+} which is internalized in the membrane core and is much closer to the intramembrane hemes. The high-affinity binding site (K_d = 1.5 μM) for Tb^{3+} may correspond to the Ca^{2+} carrier in inner membrane in view of its low capacity and high affinity which is similar to the affinity (K_d = 0.83 μM) for La^{3+} binding to the Ca^{2+} carrier (Lehninger & Carafoli, 1971). These sites may be distributed between the external face and the interior of the membrane, hence the existence of fast-decay and slow-decay fractions. The fact that the energization in mitochondria causes the disappearance of the fast phase suggests that the internalized sites are pushed to the surface on the formation of $\Delta\tilde{\mu}_{H^+}$ (Schuldiner et al., 1975; Cohn et al., 1981; Le Quoc et al., 1977). Because membrane proteins diffuse laterally at a rate which greatly exceeds the lifetime of Tb^{3+} phosphorescence but do not move across the membrane at all, we believe that in this case the only distance that matters for energy transfer is along an axis perpendicular to the membrane plane.

Brittain et al. (1976) have shown that the maxima in the excitation spectra of Tb^{3+} phosphorescence at 259, 280, and 295 nm correspond to energy transfer from aromatic rings of phenylalanine (PheA), thyrosine (Tyr), and tryptophan (Trp). It is possible that Trp may dominate as an energy-donating species in many cases because of the more favorite Trp (fluorescence) – Tb^{3+} (absorption) spectral overlap. In fact, Brittain et al. (1976) have reported that Trp appeared as a donor 23 times out of 32 cases. We observed the 259- and

280-nm peaks but not the 295-nm peak in the excitation spectrum of submitochondrial particles (Figure 3).

Surface Charge and Tb^{3+} Binding in Mitochondria. Since Tb^{3+} is a trivalent cation, the apparent affinity of its binding is very sensitive to the surface charge. When the binding is measured at free concentrations which are lower than the dissociation constant, the binding is proportional to the apparent affinity which is a function of the surface charge (McLoughlin, 1977; Robertson & Rottenberg, 1983; Haynes, 1974; Castle & Hubble, 1976). The binding of Tb^{3+} would itself perturb the surface charge; hence, for meaningful measurements the Tb^{3+} concentration must be kept low. In Figure 1 there was no difference in binding of Tb^{3+} between energized and deenergized mitochondria. This indicates not only that there is no appreciable uptake of Tb^{3+} but also that there is no significant change in the membrane charge. We studied the phosphorescence of bound Tb^{3+} in order to observe more clearly the effect of surface charge on Tb^{3+} binding. As Figures 6 and 7 clearly demonstrate, the phosphorescence of mitochondrial bound Tb^{3+} is quenched by all salts. This is expected since the negative charge of the mitochondrial membrane will be screened by salt in accordance with the Gouy–Chapman theory. However, the dependence of the quenching on the valence of the cations indicates that in addition to screening, multivalent cations bind to the membrane, thereby changing the net charge. These observations indicate that Tb^{3+} phosphorescence may be used to estimate surface potential in mitochondria from the quenching by monovalent salts. Because of the limited sensitivity of our instrument we have conducted our experiments initially at about 5 μM external Tb^{3+} which reduces the surface charge by 10–20% as judged by Figures 6 and 7 and independent estimates with ANS (Robertson & Rottenberg, 1983). Hence, our estimated values of ψ_s (Table I) are 10–20% less than those estimated by ANS. A more correct estimate can be obtained with improved sensitivity at Tb^{3+} concentrations less than 1 μM. Indeed, recently we have obtained a more negative value of surface potential using 0.5 μM Tb^{3+}. Nevertheless, Tb^{3+}, even at 5 μM, is preferable for detecting small changes in ψ_s because of its higher sensitivity and the absence of interference from potential-dependent transport. Moreover, the accuracy in the estimation of changes of ψ_s is increased since phosphorescence measurements are largely free of light-scattering artifacts which interfere with fluorescence measurements in mitochondria and other membranes. In Figures 6 and 7 we have used the quenching of the slow-phase phosphorescence as a measure of ψ_s since this phase is insensitive to the metabolic state and appears to reflect the delocalized charge of the mitochondrial membranes. Also, the low affinity of this site reduces the effect of bound Tb^{3+} on the measured ψ_s. It is difficult to measure the accurate surface charge near the fast decay site, since this site has a high affinity for Tb^{3+} (k_d = 1.5 μM). However the salt dependence of the phosphorescence of the fast phase yields interesting information. Measurements at 5 μM Tb^{3+} gave a positive local surface potential in NEM-treated mitochondria but a negative value in submitochondrial particles. This indicates that two sites, presumably the matrix and the cytoplasmatic sites of the Ca^{2+} carrier, are different in charge and/or affinity for Tb^{3+}.

The absence of significant change in the delocalized surface potential of mitochondria and submitochondrial particles on energization is in agreement with our interpretation of ANS fluorescence in mitochondria (Robertson & Rottenberg, 1983) and is incompatible with previous reports suggesting a 10–20 mV more negative ψ_s in energized mitochondria than deenergized mitochondria (Aiuchi et al., 1977; Archbald et al., 1980; Quintanhila & Packer, 1977; Wojtczak & Nelecz, 1979). Recent experiments with the charged spin probe cat_{12} indicate that this probe is internalized by energized mitochondria which obscures any changes that might occur in ψ_s. In submitochondrial particles this spin probe indicated no transport and no change in ψ_s (K. Hashimoto, P. Angiolillo, and H. Rottenberg, in press). Thus, all previous reports on energy-dependent modulation of surface charge rely on probes which respond to both membrane potential and surface charge, leading to erroneous interpretations. Our estimates of surface potential in nonenergized mitochondria, in low salt medium, are in fair agreement with previous estimates of 10–20 mV obtained from the electrophoretic mobility of mitochondria (Aiuchi et al., 1977; Archbald et al., 1980), from charged spin probe distribution (Quintanhila & Packer, 1977; K. Hashimoto et al., unpublished results), and from the salt effects on ANS fluorescence (Robertson & Rottenberg, 1983).

In summary, we conclude that Tb^{3+} binding, as measured by time-resolved phosphorescence from protein-bound Tb^{3+}, may be used to estimate the membrane surface potential. These measurements do not indicate a significant change in the surface potential of energized mitochondria or submitochondrial particles.

Acknowledgments

We thank Dr. Jan Hoek for critical reading of the manuscript.

Appendix

Measurement of Phosphorescence Decay Kinetics by the Gate-Shift Method. When the phosphorescence decay kinetics ($P(t)$) is a single exponential with first-order decay constant, k, we obtain

$$P(t) = P_0 \exp(-kt) \tag{A1}$$

where P_0 is the intensity of phosphorescence at zero time. We measured the decay kinetics of Tb^{3+} phosphorescence by varying the delay period (t_d) from the beginning of the excitation flash to the opening of the gate for a fixed time period (T_g). Since the data are collected over a period which is of the same order of magnitude as the decay time, a considerable decay occurs during sampling. The resultant kinetics as a function of t_d (at a constant value of T_g) can be expressed as follows:

$$P_{T_g}(t_d) = \int_{t_d}^{(t_d+T_g)} P(t)\, dt \tag{A2}$$

$$P_{T_g}(t_d) = (1/k)[1 - \exp(-kT_g)][P_0 \exp(-kt_d)] = P_0' \exp(-kt_d) \tag{A3}$$

Equations 1 and 3 show that the first decay constant obtained by the gate-shift measurement (eq 3) is the true decay constant, k, but the extrapolated intensity P_0' must be divided by $(1/k)[1 - \exp(kT_g)]$ to obtain the correct P_0.

When the decay kinetics $[P(t)]$ is composed of two exponentials with different first-order decay constants (k_1, k_2), then

$$P(t) = P_{01} \exp(-k_1 t) + P_{02} \exp(-k_2 t) \tag{A4}$$

where P_{01} or P_{02} is the intensity of phosphorescence of each exponential at zero time. The resultant kinetics by gate-shift measurements $[P_{T_g}(t_d)]$ can be expressed as follows:

$$P_{T_g}(t_d) = \int_{t_d}^{(t_d+T_g)} P(t)\, dt = (P_{01}/k_1)[1 - \exp(-k_1 T_g)] \exp(-k_1 t_0) + (P_{02}/k_2)[1 - \exp(-k_2 T_g)] \exp(-k_2 t_d) = P_0'_1 \exp(-k_1 t_d) + P_0'_2 \exp(-k_2 t_d) \tag{A5}$$

When eq 4 and 5 are compared, the ratio of intensities between two exponentials (P_{01}, P_{02}) in the real kinetics becomes

$$P_{0'1}/P_{0'2} = (P_{01}/k_1)[1 - \exp(-k_1T_g)]/[(P_{02}/k_2)[1 - \exp(-k_2T_g)]] \quad \text{(A6)}$$

Thus, first-order decay constants of gate-shift measurements are the same as the true decay constant. In extrapolating to initial phosphorescence in our experiments (Figures 4 and 5) we have used eq 7 to correct for the true intensities of the phase

$$P_{01}/P_{02} = (P_{0'1}/P_{0'2}) \frac{(1/k_2)[1 - \exp(-k_2T_g)]}{(1/k_1)[1 - \exp(-k_1T_g)]} \quad \text{(A7)}$$

in the inserts of these figures, as well as in calculation of ψ_s.

Registry No. Tb, 7440-27-9; antimycin A, 1397-94-0.

References

Aiuchi, T., Kamo, N., Kurihara, K., & Kobatake, Y. (1977) *Biochemistry 16*, 1626–1630.

Archbald, G. P. R., Farrington, C. L., Lappin, S. A., McKay, A. M., & Malpress, F. H. (1980) *Biochem. Int. 1*, 422–427.

Barela, T. P., & Sherry, A. D. (1976) *Anal. Biochem. 71*, 351–357.

Bashford, C. L., & Smith, J. C. (1979) *Methods Enzymol. 55*, 569–586.

Brittain, H. G., Richardson, F. S., & Martin, R. B. (1976) *J. Am. Chem. Soc. 98*, 8255–8260.

Castle, J. D., & Hubbell, W. L. (1976) *Biochemistry 15*, 4818–4831.

Cohn, D. E., Kaczorowski, G. J., & Kaback, H. R. (1981) *Biochemistry 20*, 3308.

Coty, W. A., & Pedersen, P. L. (1975) *Mol. Cell. Biochem. 9*, 109–124.

Doktor, M. E. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 749.

Estabrook, R. N. (1967) *Methods Enzymol. 10*, 41–47.

Hashimoto, K., & Nishimura, M. (1981) *J. Biochem. (Tokyo) 89*, 909–919.

Haynes, D. H. (1974) *J. Membr. Biol. 17*, 341–366.

Horrocks, W. D., Jr., & Sudnick, D. R. (1979) *J. Am. Chem. Soc. 101*, 334–340.

Horrocks, W. D., Jr., Schmidt, G. F., Sudnick, D. R., Kittrell, C., & Berheim, R. A. (1977) *J. Am. Chem. Soc. 99*, 2378–2380.

Itoh, S. (1980) *Biochim. Biophys. Acta 591*, 346–355.

Johnson, D., & Lardy, H. (1967) *Methods Enzymol. 10*, 94–96.

Kell, D. B. (1979) *Biochim. Biophys. Acta 549*, 55.

Lehninger, A. L., & Carafoli, E. (1971) *Arch. Biochem. Biophys. 143*, 506–515.

LeQuoc, K., & LeQuoc, D. (1982) *Arch. Biochem. Biophys. 216*, 639.

LeQuoc, D., LeQuoc, K., & Goudmer, Y. (1977) *Biochim. Biophys. Acta 462*, 131.

Lindsay, J. G., Dutton, P. L., & Wilson, D. F. (1972) *Biochemistry 11*, 1937–1943.

Martin, R. B., & Richardson, F. S. (1979) *Q. Rev. Biophys. 12*, 181–209.

Matsuura, K., Masamoto, K., Itoh, S., & Nishimura, M. (1980) *Biochim. Biophys. Acta 592*, 121–129.

McLoughlin, S. (1977) *Curr. Top. Membr. Transp. 9*, 71–144.

Mitchell, P., & Moyle, J. (1969) *Eur. J. Biochem. 7*, 471.

Njus, D., Ferguson, S. J., Sorgato, M. C., & Radda, G. K. (1977) in *Structure and Function of Energy-Transducing Membranes* (Van Dam, K., & Van-Geler, B. F., Eds.) p 237, Elsevier/North-Holland, New York.

Quintanilha, A. T., & Packer, L. (1977) *FEBS Lett. 78*, 161–165.

Ramachandran, C., & Bygrave, F. L. (1978) *Biochem. J. 174*, 613–630.

Reed, K. C., & Bygrave, F. L. (1974) *Biochem. J. 138*, 239–252.

Robertson, D. E., & Rottenberg, H. (1983) *J. Biol. Chem. 258*, 11039–11048.

Rottenberg, H. (1970) *Eur. J. Biochem. 15*, 22.

Rottengerg, H. (1975) *J. Bioenerg. 7*, 61.

Rottenberg, H. (1978) *FEBS Lett. 94*, 295–297.

Rottenberg, H. (1979) *Methods Enzymol. 55*, 547.

Rottenberg, H., & Scarpa, A. (1974) *Biochemistry 13*, 4811.

Scarpa, A. (1979) in *Membrane Transport in Biology* (Giebisch, G., et al., Eds.) Vol. II, p 263, Springer-Verlag, Berlin.

Schuldiner, S., Kerwar, G. K., Kaback, H. R., & Weil, R. (1975) *J. Biol. Chem. 250*, 1361.

Searle, G. F. W., & Barber, J. (1979) *Biochim. Biophys. Acta 545*, 508–518.

Skulachev, V. P. (1979) *Methods Enzymol. 55*, 586–603.

Strayer, L. (1978) *Annu. Rev. Biochem. 47*, 819.

Thomas, D. D., Carlson, W. F., & Strayer, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5746.

Williams, R. J. P. (1961) *J. Theor. Biol. 1*, 1.

Wojtczak, L., & Nalecz, M. J. (1979) *Eur. J. Biochem. 94*, 99–107.