Biochimica et Biophysica Acta, 433 (1976) 674–683
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BBA 77304

BINDING OF FLUORESCENT LANTHANIDES TO RAT LIVER MITOCHONDRIAL MEMBRANES AND CALCIUM ION-BINDING PROTEINS

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(Received September 26th, 1975)
(Revised manuscript received December 16th, 1975)

SUMMARY

(1) Tb^{3+} binding to mitochondrial membranes can be monitored by enhanced ion fluorescence at 545 nm with excitation at 285 nm. At low protein concentrations (< 30 μg/ml) no inner filter effects are observed.

(2) This binding is localized at the external surface of the inner membrane and is unaffected by inhibitors of respiration or oxidative phosphorylation.

(3) A soluble Ca^{2+} binding protein isolated according to Lehninger, A. L. ((1971) Biochem. Biophys. Res. Commun. 42, 312–317) also binds Tb^{3+} with enhanced ion fluorescence upon excitation at 285 nm. The excitation spectrum of the isolated protein and of the intact mitochondria are indicative of an aromatic amino acid at the cation binding site.

(4) Further characterization of the Tb^{3+}-protein interaction revealed that there is more than one binding site per protein molecule and that these sites are clustered (< 20 Å). Neuraminidase treatment or organic solvent extraction of the protein did not affect fluorescent Tb^{3+} binding.

(5) pH dependency studies of Tb^{3+} binding to the isolated protein or intact mitochondria demonstrated the importance of an ionizable group of pK > 6. At pH < 7.5 the amount of Tb^{3+} bound to the isolated protein decreased with increase in pH as monitored by Tb^{3+} fluorescence. With intact mitochondria the opposite occurred with a large increase in Tb^{3+} fluorescence at higher pH. This increase was not observed when the mitochondria were preincubated with antimycin A and rotenone.

INTRODUCTION

Mitochondria bind and transport calcium in reversible, respiration dependent processes. Present evidence indicates that mitochondria can thereby control the concentration of free cytoplasmic Ca^{2+}, thus influencing such diverse cellular functions

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as gluconeogenesis (e.g. ref. 1) intercellular communication via tight junctions (e.g. ref. 2) and certain hormone mediated activities (e.g. ref. 3). Considerable effort has therefore been expended in describing the interaction of mitochondria with Ca^{2+} in terms of affinity constants, the action of uncoupling agents and respiratory inhibitors, and the isolation of soluble Ca^{2+} binding proteins of mitochondrial origin [4–7]. However, we still lack detailed information concerning the molecular mechanisms involved in the interactions of Ca^{2+} with mitochondrial membranes.

One experimental approach that has been applied to the problem is the use of the trivalent lanthanide ions as inhibitors of Ca^{2+} binding and transport [8, 9]. Binding studies with radioisotopes of La^{3+} show that, as with Ca^{2+}, there are two classes of binding sites that can be defined in terms of number and association constant [9]. Importantly, Reed and Bygrave [10] provide evidence suggesting that La^{3+} can also replace Ca^{2+} in a manner that is metabolically significant. For example, La^{3+} and Ca^{2+} can induce comparable changes in mitochondrial flavin fluorescence, cytochrome *b* spectra, and swelling. Moreover, these authors' studies with murexide as a Ca^{2+}/La^{3+} indicator, are suggestive of La^{3+} transport across the inner mitochondrial membrane, a result not observed in previous studies using La^{3+} isotopes [9].

We have earlier reported on the use of lanthanides as probes for the interaction of Ca^{2+} with the plasma membranes of erythrocytes and lymphoid cells [11, 13]. In this experimentation we took advantage of the spectroscopic properties (e.g. fluorescence) of the rare earths to identify and characterize membrane Ca^{2+} binding sites. Dramatic shifts in excitation maxima and greatly enhanced Tb^{3+} fluorescence documented that an aromatic amino acid, probably a tyrosine, is an integral component of high-affinity binding sites [11].

In the present publication we utilize lanthanides to characterize Ca^{2+} binding sites on mitochondrial membranes.

EXPERIMENTAL

Mitochondria were prepared from rat livers (Sprague-Dawley, Charles River) in 0.25 M sucrose according to Johnson and Lardy [14]. The mitochondria were washed three times in sucrose before further experimentation. The inner membranes (+matrix) were separated from the outer mitochondrial membrane by the digitonin (Fisher) method of Schnaitman et al. [15]. A final concentration of 1.1 mg digitonin per mg mitochondrial protein was employed. The inner membranes were pelleted at $9 \cdot 10^4\ g \cdot \min$ (Beckman J21B centrifuge: rotor JA21). The resulting supernatant solution was centrifuged at $4 \cdot 10^6\ g \cdot \min$ (Spinco L3 ultracentrifuge: rotor 50.1) to isolate the outer membranes. The purity of the different membrane preparation was monitored enzymatically with monoamine oxidase and succinic acid dehydrogenase activities representing respectively the outer and inner mitochondrial membranes. Monoamine oxidase was assayed as in [16] with 2-[^{14}C]tryptaminebisuccinate (specific activity = 40 mCi/nmol) obtained from New England Nuclear. Succinate dehydrogenase activity was determined as in [17] by the coupled reduction of oxidized cytochrome *c* (Sigma). The purity of the membrane separation was comparable to that achieved by Schnaitman et al. [15] (See Table I).

Corrected Tb^{3+} fluorescence spectra were obtained with a Perkin-Elmer MPF-3 spectrofluorometer with attached correction accessory as described in ref. 11.

Because changes in light scattering upon swelling or shrinking of mitochondria can distort fluorescent titration data, we titrate by scanning through the emission wave length region of 450–560 nm. This allows us to correct for scattering changes and to monitor the two emission wavelengths of Tb^{3+}, 488 nm and 545 nm, (see Fig. 1B). Although the excitation maximum for the Tb^{3+} · membrane complex is 285 nm (see Fig. 1A), the excitation wavelength commonly used in these titrations was 290 nm to minimize the influence of second order Rayleigh Scattering on fluorescence at 545 nm.

A water soluble Ca^{2+} binding protein was released from the mitochondria by aqueous extraction according to Lehninger [7]. After centrifugation at $4 \cdot 10^6$ *g* · min for 40 min, the supernatant solution was concentrated by ultrafiltration and dialyzed against 10 mM Tris · Cl pH 7.4. This extract was further purified by chromatography on Sephadex G-200 which had been previously equilibrated in 10 mM Tris · Cl buffer. The column was calibrated with bovine serum albumin (M_r 69 000), apoferritin (M_r 600 000) and γ-globulin (M_r 150 000).

Other methods utilized in experiments described below include lipid extraction with $CHCl_3/CH_3OH$ [18] and protein determination [19] with bovine serum albumin as standard.

The rare earths, Tb^{3+}, La^{3+}, Ho^{3+}, and Eu^{3+} were purchased as the chloride salts from Alfa Inorganics and prepared daily as 0.01 M stock solutions in water. Other chemicals include antimycin A, rotenone, neuraminidase, oligomycin and carbonylcyanide *m*-chlorophenylhydrazone and were obtained from Sigma Biochemicals.

RESULTS

With several Ca^{2+} binding proteins, e.g. concanavalin A [20] and transferrin [21], as well as plasma membranes from erythrocytes [11] and lymphocytes [13], Tb^{3+} binding can be measured by enhanced ion fluorescence at either 545 or 488 nm, exciting in the absorbance region of aromatic amino acids. As illustrated in Fig. 1A and 1B, similar spectral characteristics are observed with rat liver mitochondria at low concentrations of Tb^{3+}. The large shift in excitation maxima from 350 nm for the aqueous ion to 285 nm after complexing and the accompanying dramatic enhancement (several thousand fold) in ion fluorescence are indicative of intramolecular energy transfer from an organic ligand to the adjacent ion [22, 23]. In contrast to our previous studies with erythrocyte [11] and lymphocyte [13] membrane where maximal Tb^{3+} emission occurs upon excitation at 295 nm, the corresponding excitation maximum in the case of mitochondria is 285 nm.

Typical fluorescence titration curves with Tb^{3+} are seen in Fig. 2. "Inner filter effects" are avoided if the protein concentration is maintained at below 30 μg per ml. From the mitochondrial concentrations used, the amount of Tb^{3+} required to achieve saturation ($\sim 10^{-5}$ M) indicates that enhanced Tb^{3+} fluorescence is associated with the low affinity Ca^{2+}/Tb^{3+} binding sites. This binding is not the result of simple charge-charge attraction as opposed to chelation since the fluorescence is stable even at relatively high ionic strengths (> 0.3 M NaCl). Although not shown in this figure, Tb^{3+} binding as measured by fluorescence is completely abolished with 0.1 % sodium dodecyl sulphate or 8 M urea.

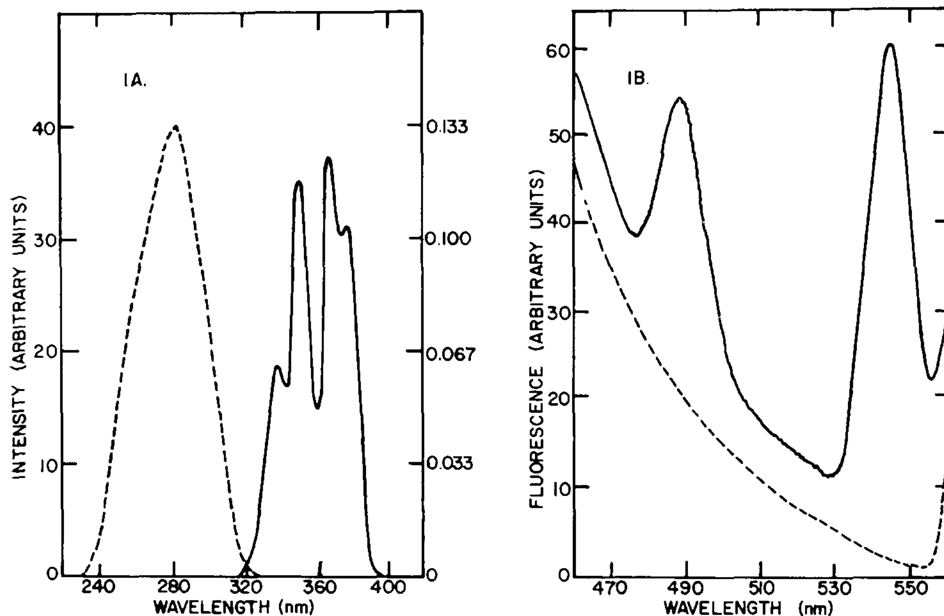

Fig. 1. Excitation and emission spectra of Tb^{3+} bound to rat liver mitochondria. (A) Excitation spectra with emission at 488 nm; solid line is free 0.02 M Tb^{3+} in 0.02 M Tris · Cl pH 7.4 plus 0.10 M NaCl; dashed line is a spectrum of Tb^{3+} ($5 \cdot 10^{-5}$ M) at a mitochondrial protein concentration of 74 μg/ml. in 10 mM Tris · Cl pH 7.4 plus 0.1 M NaCl. (B) Emission spectrum with excitation at 290 nm. Solid line is spectrum for Tb^{3+} ($5 \cdot 10^{-5}$ M) with mitochondria at 74 μg/ml protein in 10 mM Tris · Cl pH 7.4 plus 0.10 M NaCl. Dashed line represents scattering contribution to spectrum in absence of Tb^{3+}.

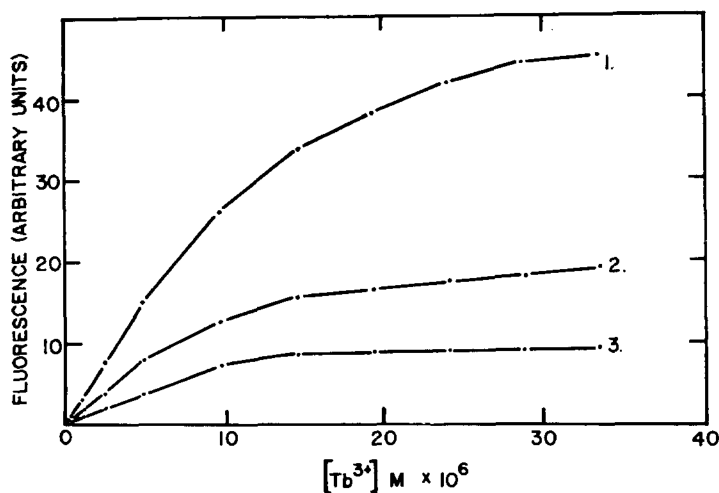

Fig. 2. Tb^{3+} titration of rat liver mitochondria. Mitochondria in 10 mM Tris · Cl pH 7.4 plus 0.1 M NaCl with excitation at 290 nm and emission at 545 nm. Protein concentration: 32 μg/ml (Curve 1); 15 μg/ml (Curve 2); 8 μg/ml (Curve 3).

TABLE I

DISTRIBUTION OF Tb^{3+} BINDING AS MEASURED FLUOROMETRICALLY IN MITOCHONDRIA

Fraction	Total protein (%)	Total monoamine oxidase activity (%)	Tb^{3+} fluorescence* per mg protein (arbitrary units)
Intact mitochondria	100	100	54 (100 %)
Inner membranes + matrix	68	13	73 (93 %)
Outer membranes	4	61	12 (1 %)
Intermembrane material	29	20	6 (3 %)
Total recovery	101	94	96 %

* Fluorescence achieved at saturation with intact mitochondria (24 μg/ml), inner membranes (42 μg/ml) outer membranes, (41 μg/ml), and intermembrane material (105 μg/ml).

Similar fluorescence titration experiments have been performed with the outer and inner mitochondrial membranes which had been separated by the digitonin method [15]. The results of such experiments (Table I) clearly demonstrate that these fluorescent Tb^{3+} complexing sites are associated with the inner membranes. This is in accord with previous work suggesting that low affinity Ca^{2+} binding sites are localized in this membrane fraction. Moreover, although binding studies [12] with $^{45}Ca^{2+}$ and the mitochondrial membrane fraction indicate that the low affinity sites are equally distributed between the inner and outer membranes and have similar (but not identical) affinity constants, our results here suggest that these binding sites of the two membranes are qualitatively different. That the binding sites are on the external surfaces of the isolate inner membranes of the intact mitochondria is attested by our ability to erase Tb^{3+} fluorescence completely with addition of 1 mM EDTA. EDTA does not permeate the inner membranes [24].

We have examined the effect of adding respiration substrates, inhibitors and uncouplers on Tb^{3+} fluorescence. Antimycin A (0.1 μM), rotenone (0.1 μM), oligomycin (1 μg/ml), carbonyl cyanide *m*-chlorophenylhydrazone (1.0 μM), neutralized KCN (1.0 mM), succinate (0.5 mM) or β-hydroxybutyrate (0.5 mM) added singly or in combination have no positive or negative effects on the fluorescence of Tb^{3+} bound to mitochondria. Inorganic phosphate at micromolar concentrations markedly reduced Tb^{3+} binding, but this probably does not connote phosphate-facilitated transport of Tb^{3+} across the inner membrane, since mersalyl did not prevent the decrease in enhanced Tb^{3+} fluorescence. Moreover, other permeant anions such as acetate did not simulate the effect of phosphate. Thus under the conditions we employ the Tb^{3+} binding class monitored by enhanced ion fluorescence is similar to the low affinity Ca^{2+} binding in its independence from respiration.

Lehninger has been able to isolate a soluble Ca^{2+} binding protein from mitochondria by hypotonic aqueous extraction [7]. Utilizing the identical extraction procedure we are able to isolate a soluble protein fraction capable of binding Tb^{3+} as measured fluorometrically. Upon addition of Tb^{3+}, this soluble fraction yields the excitation and emission spectra that are identical to those obtained with intact mitochondria, maximal excitation occurring at 285 nm. The fluorescence of the Tb^{3+} · protein complex was stable to physiological salt concentration. When the extracted

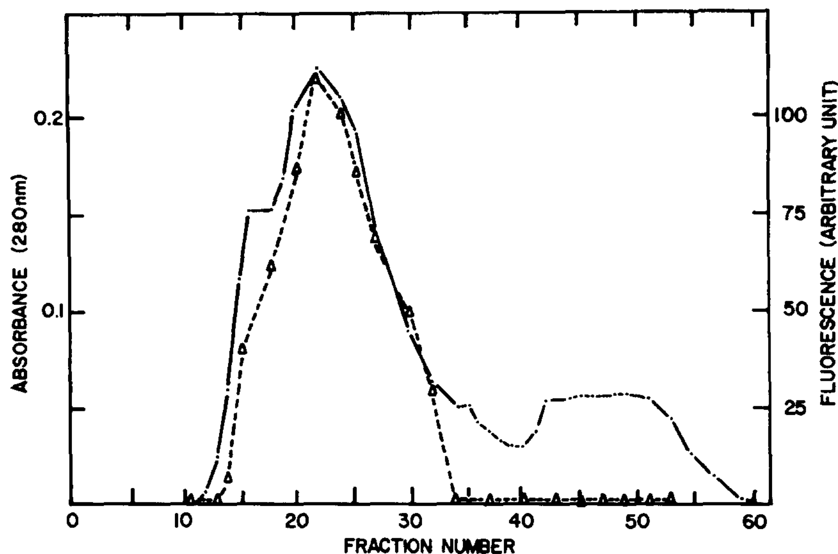

Fig. 3. Gel chromatography of Ca^{2+} binding protein as monitored by Tb^{3+} fluorescence. Sephadex G-200 column (1.5 × 23 cm) equilibrated with 10 mM Tris · Cl pH 7.4. Void volume is at Fraction 16 (apoferritin or blue dextran); fraction 31 (bovine serum albumin, M_r 69 000); Column volume (Fraction 50, phenol red). 0.9 ml volume fractions collected. Solid line is absorbance at 280 nm and dashed line represents fluorescence yield at 545 nm with excitation at 290 nm for 100 μl aliquots of each fraction and 10^{-5} M Tb^{3+}. 2 ml sample of the aqueous extract at a protein concentration of approx. 1 mg/ml was applied to this column.

protein is fractionated by chromatography on Sephadex G-200 a Tb^{3+} binding component elutes at a volume corresponding to a molecular weight of ~ 150 000 (Fig. 3). This agrees with the molecular weight determinations in [7].

Gomez-Puyou et al. [26] and Sottocasa et al. [6] have reported that their calcium binding fractions contain both lipid and carbohydrate. Moreover, Sottocasa et al. [6] observed that ruthenium red inhibits Ca^{2+} binding and therefore suggested that sialic acids might participate in binding. To test the possibility that either lipids and/or sialic acids contribute ligands for the Tb^{3+}/Ca^{2+} binding, we have examined the effect of extraction with chloroform/methanol (2 : 1) and sialidase treatment. Neither approach altered Tb^{3+} binding of the treated protein fractions as measured fluorometrically. Sialidase treatment also does not influence the Tb^{3+} fluorescence of inner mitochondrial membranes.

Qualitative conclusions about the relative proximity of the cation binding sites to each other can be derived by Tb^{3+} fluorescence with paramagnetic lanthanides [21]. The protein or membranes were titrated to 50 % saturation with Tb^{3+}, followed by equivalent amounts of Eu^{3+}, Ho^{3+}, or La^{3+}. If the rare earths have equal affinities for the binding sites and if the distance between binding sites is near the critical distance for energy (~ 10 Å, ref. 25), quenching of Tb^{3+} fluorescence by a dipole-quadrupole resonance transfer mechanism can be observed. The results of such an experiment with the soluble protein are presented in Table II and show considerable quenching of Tb^{3+} fluorescence by paramagnetic Ho^{3+} and Eu^{3+} but none with dimagnetic La^{3+}. Thus, these binding sites bear a close proximal relationship to one

TABLE II

DETERMINATION OF BINDING SITE PROXIMITY RELATIONSHIPS BY QUENCHING OF Tb^{3+} FLUORESCENCE

Ca^{2+} binding protein concentration was 56 μg/ml and titrations were performed at 0.1 M NaCl, 5 mM Tris · Cl pH 7.4.

[Lanthanide]	Fluorescence (arbitrary units)
$2.0 \cdot 10^{-5}$ M Tb^{3+}	30
$4.0 \cdot 10^{-5}$ M Tb^{3+}	60
$2.0 \cdot 10^{-5}$ M Tb^{3+} + $2.0 \cdot 10^{-5}$ M Eu^{3+}	15
$2.0 \cdot 10^{-5}$ M Tb^{3+} + $2.0 \cdot 10^{-5}$ M Ho^{3+}	8
$2.0 \cdot 10^{-5}$ M Tb^{3+} + $2.0 \cdot 10^{-5}$ M La^{3+}	28

another. Similar results were also obtained with intact mitochondria and with inner membranes*.

To further characterize the Tb^{3+} binding sites of mitochondria, we have examined the effect of pH on Tb^{3+} fluorescence. A pH titration curve for Tb^{3+} binding to the soluble protein presented in Fig. 4A demonstrates that an ionizable group with a pK > 6 governs Tb^{3+} binding in this pH range. The titration curve in the pH range of 3–7 is completely reversible and is independent of the order of addition of base and cation. There is a gradual loss in Tb^{3+} fluorescence as the pH is raised

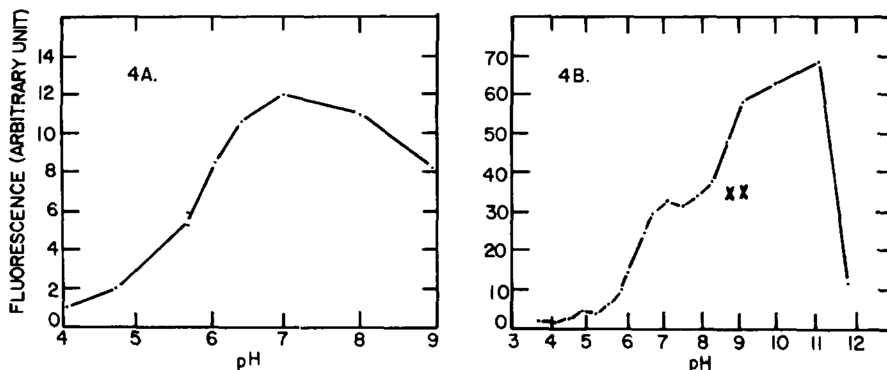

Fig. 4. pH Titration studies of Tb^{3+} binding to mitochondria and soluble Ca^{2+} binding protein A. Soluble Ca^{2+} binding protein at 27 μg/ml in 0.1 M NaCl. The order of addition of Tb^{3+} or base (NaOH) was reversible up to ~ pH 7.5. At pH > 7.5, addition of base resulted in a drop in Tb^{3+} fluorescence due to formation of insoluble Tb^{3+} · hydroxide complexes. $[Tb^{3+}] = 5 \cdot 10^{-5}$ M. B. Intact mitochondria at 70 μg/ml protein in 0.1 M NaCl. Tb^{3+} was added first followed by NaOH to achieve desired pH. Up to pH 7.5 order of addition of Tb^{3+} or base was reversible. Above pH 7.5, it was necessary to add Tb^{3+} prior to addition of base. At the two X's at pH 8.8 and 9.1, the mitochondria were pre-incubated in the presence of 0.1 μM rotenone and 0.1 μM antimycin A before adding Tb^{3+} or base. $[Tb^{3+}] = 5 \cdot 10^{-5}$ M.

* The assumption of random cation binding (i.e. equal affinity for Tb^{3+}, Ho^{3+}, Eu^{3+} and La^{3+}) probably has little influence on our qualitative interpretation of the experiments since the ionic radii of Ho^{3+} and Eu^{3+} are on either side that of Tb^{3+}.

above 7.5. This presumably is due to the successful competition by hydroxyl ion for Tb^{3+}. Likely candidates for an ionizable group with a p$K > 6$ that can also be a potential ligand for Ca^{2+} include histidine, cysteine, and tyrosine hydroxyls.

When similar pH titrations are performed with intact mitochondria, a group titrating with a pK near 6 is also observed. However, in contrast to the soluble protein when the pH is raised above pH 7.5 Tb^{3+} fluorescence continues to increase. Above pH 10.5, the fluorescence again drops. This substantial increase in Tb^{3+} fluorescence at alkaline pH is probably not due to generation of a new hydroxylated species of Tb^{3+} with a higher affinity for the membrane binding sites, because: (a) The increase in fluorescence above pH 7.5 is only observed when base is added after the Tb^{3+}; (b) enhanced fluorescence does not occur with the soluble protein above pH 7.5; (c) preincubation of the mitochondria with antimycin plus rotenone prior to addition of Tb^{3+} and base abolishes the enhanced fluorescence but not the fluorescence at pH 7.0 or below (Fig. 4B).

The last two arguments suggest that the increase in Tb^{3+} fluorescence induced in intact mitochondria at alkaline pH, derives from some structural, respiration-dependent property of the intact mitochondria. Previous studies on the effect of pH on low affinity Ca^{2+} binding showed that although there is no increase in the number of sites, the affinity constant at pH 9.0 is greater than at pH 7.4 [4]. Moreover, as will be discussed under certain incubation conditions known to induce super-stoichiometric Ca^{2+} binding, this change in affinity with pH is dependent on state IV respiration [30].

DISCUSSION

We have been able to monitor Tb^{3+} binding to mitochondria by enhanced ion fluorescence. Evidence that the binding sites involved are the so-called "low affinity Ca^{2+} sites" can be summarized as follows:

(a) The concentration of Tb^{3+} required to achieve saturation as measured fluorometrically is appropriate for these sites. To inhibit high affinity Ca^{2+} binding only requires 0.1 nmol La^{3+} per mg mitochondrial protein [27, 9].

(b) Fluorescent Tb^{3+} binding is localized on the external surface of the inner membrane as are low affinity sites [4].

(c) Both "fluorescent" Tb^{3+} binding and low affinity Ca^{2+} binding at standard conditions are independent of metabolic inhibitors such as antimycin A and uncouplers [4].

(d) Soluble protein fractions extracted from mitochondria demonstrate not only low affinity Ca^{2+} binding [7] but also enhanced Tb^{3+} fluorescence.

(e) We are also able to observe an increase in Tb^{3+} fluorescence by increasing the pH above 8 which is consistent with the known increased affinity of low affinity sites for Ca^{2+} with alkalinization [31].

The similarities in excitation maximum and pH dependency of the fluorescence (at pH < 8) for the Tb^{3+} complexes with intact mitochondria and isolated Ca^{2+} binding proteins [7] give support to the proposal that the low affinity Ca^{2+} bindings sites are associated with these proteins. Moreover, the excitation spectrum for the mitochondrial · Tb^{3+} complex, and the absence of any effect of organic solvent extraction on Tb^{3+} fluorescence indicate that phospholipids do not contribute to low

affinity binding. Previous evidence based on acetone extraction of whole mitochondria suggested these lipids as of dominant importance in this binding [28, 29].

The use of Tb^{3+} as a fluorescent probe for Ca^{2+} allows for a partial molecular characterization of these complexing sites. Specifically:

(a) The excitation maximum at 285 nm is diagnostic for either tryptophan or tyrosine at the cation binding site. One cannot now decide between these amino acids, but the availibility of the soluble protein should allow use of technique such as Raman spectroscopy to differentiate between the two possibilities.

(b) Although the pH titration studies suggest that a histidine directly participates in Ca^{2+}/Tb^{3+} binding other interpretations cannot be excluded. However, the paramagnetic properties of the lanthanides and the well resolved proton magnetic resonance spectra of histidine in soluble proteins allows for experimental resolution of this question.

(c) The neuraminidase experiments with inner membranes and soluble protein confirm the work of Gomez-Puyou et al. [26], who could not detect sufficient sialic acid in the soluble protein to account for the Ca^{2+} binding.

(d) That there is more than one fluorescent Tb^{3+} binding site per 150 000 molecular weight protein is apparent from the quenching experiments with lanthanide couples. Moreover, these binding sites must be in close proximity to account for the considerable quenching observed.

In conclusion, the high pH experiments deserve some attention since their results suggest that our fluorescence approach described might serve to elucidate the relationship between Ca^{2+} binding and mitochondria energization. In this regard Reynafarje and Lehninger [30] have proposed a rationale for the super-stoichiometric H^+ ejection and Ca^{2+} uptake induced by state 4 respiration of $pH > 8.0$. They postulate that state 4 energization at the membrane results in decreased affinity for H^+ and an increase Ca^{2+} affinity. The Ca^{2+} sites involved are thought to be the low affinity sites. The enhanced Tb^{3+} fluorescence observed with $pH > 8.0$ is consistent with similarly observed increased affinity for Ca^{2+} by these sites at high pH. Moreover, the enhanced fluorescence is dependent on the metabolic state of the mitochondria since preincubation with antimycin A and rotenone inhibits their response. Because the number of low affinity sites does not increase with alkalinization and we do not observe corresponding change in Tb^{3+} fluorescence with the isolated soluble protein (Fig. 4A), a conformational change in the mitochondrial membrane appears to be involved.

ACKNOWLEDGEMENTS

This research was supported by National Science Foundation (GB-42041), American Cancer Society (DFHW) and the Damon Runyon Cancer Memorial Fund (RBM).

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