USE OF THE COULOMBIC INTERACTIONS OF THE LANTHANIDE SERIES TO IDENTIFY TWO CLASSES OF CA^{2+} BINDING SITES IN MITOCHONDRIA

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SUMMARY: The degree of inhibition of respiration-dependent vs respiration-independent Ca^{2+} binding by rat liver mitochondria by different members of the lanthanide family was used to establish the existence of two different classes of Ca^{2+} binding sites. The distinction is based on the differences in cation:site interactions between the two classes of sites and the members of the lanthanide series. Lanthanide inhibition of respiration-dependent Ca^{2+} uptake suggests that the binding site is specific for the calcium ion. Those members of the lanthanide family whose ionic radii are nearer that of Ca^{2+} are the best inhibitors. The inhibition of respiration-independent Ca^{2+} binding is much different, indicating non-specific cation absorption.

It is well known that rat liver mitochondria can selectively transport Ca^{2+} and certain other bivalent cations against a concentration gradient by an energy-dependent mechanism. Ca^{2+} transport into mitochondria is inhibited by cations of the lanthanide series and by ruthenium red (1). Moreover, mitochondria have a rather high affinity for transporting Ca^{2+}. These observations have led to the concept that the mitochondrial inner membrane contains a specific transport system or carrier for Ca^{2+}. This carrier has been postulated to contain specific, high-affinity binding sites for Ca^{2+} to facilitate its Ca^{2+}-selective function and to account for the high affinity of mitochondria for Ca^{2+} (2,3).

Several years ago Reynafarje and Lehninger (2) reported the occurrence of high- and low-affinity Ca^{2+} binding sites in mitochondria and suggest that the high-affinity sites are associated with the energy-dependent translocation mechanism for Ca^{2+} while the low-affinity sites represent nonspecific absorption by bulk lipid and/or protein components of the membrane.

More recently, however, other groups have reported (4,5) that, if care is taken to ensure complete inhibition of respiration, isolated mitochondria do not show the presence of high-affinity Ca^{2+} binding sites. It was suggested that the previously observed high-affinity Ca^{2+} binding results solely from energy-dependent transport of very small amounts of Ca^{2+} into the matrix space.

This paper reports a new approach to this question and describes the occurrence of two different classes of Ca^{2+} binding sites in the inner mito-

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chondrial membrane which differ in their coulombic interactions with the Ca^{2+} ion. One class seems to be functionally coupled to energy-yielding respiration; the other class is energy-independent. This differentiation was established with use of the trivalent cations of the lanthanide series as isomorphous inhibitors and probes of calcium binding. Although no attempts have been made in this work to determine the relative concentrations of the two classes of Ca^{2+}-binding sites or their respective $K_{0.5}$ values, they can, however, be easily distinguished on the basis of their selectivity of inhibition by different lanthanide cations. The basic driving force underlying cation-binding selectivity of the transport system is the difference between the free energy change for the coulombic cation:site interaction and for the hydration of the cation.

MATERIALS AND METHODS

Liver mitochondria were prepared from Sprague-Dawley rats by the method of Schneider (6). They showed acceptor control ratios (succinate) greater than 5. The incubation medium was composed of 120 mM KCl and 3 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) adjusted to pH 7.4 with HCl. Chemicals used throughout were reagent grade without further purification. The lanthanides were obtained as oxides (99.8% pure) and dissolved in deionized water with small amounts of HCl.

Mitochondria (5 mg protein per 2.0 ml) were energized by incubation for 1 minute with 4 mM succinate in the presence of 250 nM rotenone at 37°C. $^{45}Ca^{2+}$ (40 nmoles per mg protein) was then added. All of the added Ca^{2+} was accumulated in 30 sec. Under these conditions, essentially all of the Ca^{2+} taken up was inaccessible to washing with 500 µM EGTA, indicating that the Ca^{2+} was transported into the matrix space. In all measurements of energy-dependent accumulation of Ca^{2+}, the mitochondria were treated with an excess of EGTA to remove any surface-bound Ca^{2+} (1). In other experiments carried out in the absence of respiratory substrate, but with antimycin A (225 nM) and an uncoupler (2.25 µM carbonylcyanide-p-trifluromethoxyphenylhydrazone) present, the mitochondria bound approximately 30 nmoles of Ca^{2+} per mg of protein in a respiration-independent process. Washing with EGTA removed all of the Ca^{2+} bound in the absence of respiration.

Inhibition of Ca^{2+} binding and uptake by the trivalent rare-earth ions was determined from the difference in Ca^{2+} accumulation or binding with and without the rare-earths. Detailed conditions are given in the legends of each figure.

THEORY

The approach employed in this work is based on the concepts developed by Eisenman and Sherry (7,8) on the role of coulombic forces in the cation selectivity of membranes (for a comprehensive review see Diamond and Wright (9)). In brief, there is a large body of evidence which shows that cation selectivity of group IA and IIA metals by biological membranes can be adequately described by coulombic forces involved in ion-ion or ion-dipole interactions. In the case of a negatively-charged site on a membrane exposed to an aqueous solution containing different cations, the cation preferred by such a site will be that which causes the greatest decrease in free energy of the system upon binding to the membrane site. Thus the relative affinity

of such a site for different cations will be governed by the difference between the free energies of hydration of the cations and the free energies of interaction between the cations and the negative site. The electric field strength of the membrane negative site thus plays an important role in determining the free energy decrease in the cation:site interaction and therefore controls cation selectivity. Membrane binding sites with very low field strength will not be able to effectively overcome the hydration energy of the more strongly solvated cations. Thus, the larger, more weakly hydrated ions will be bound preferentially. As an example, such sites would show selectivity for the group IIA cations in the order of $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$. In the opposite case, that of a strong-field binding site, the free energy decrease occurring on cation:site interaction is great enough to overcome the solvation energy of the ion. In this case cation selectivity will be determined by the ability of the cation to interact sterically with the membrane negative site. Smaller ions, despite their higher hydration energy, will be able to get closer to the negative site, thus maximizing cation:site interaction. Preference of strong-field sites for group IIA cations would then be $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$.

At intermediate field strengths the selectivity of cation binding would correspondingly depend on the relative free energies of the relative coulombic interactions. This simple treatment of the coulombic interaction of point charges has succe-sfully predicted the cation selectivity of many different biological membranes, as reviewed by Wright and Diamond (9).

One point not considered in detail by Wright and Diamond (9) is the possible contribution to field strength and the free energy decrease provided by multiple functional group attachment to the cation at the membrane binding site. Evidence has been presented in the case of cation binding by deformable macrocylic compounds that the energy change as the functional group assumes the conformation of minimum energy allowing maximum coordination with a given cation may contribute significantly to the total free energy change on interaction with cation (10,11). The greatest total free energy decrease will occur when the cation can achieve maximum coordination with the least unfavorable deformation of the binding site. This concept may be extended to interactions between a cation and a membrane binding site. In solution, the coordination sphere of the cation is occupied by water molecules. Maximum site interaction would be achieved as these water molecules are replaced by the functional groups at the binding site. If the dimensions of the binding site are not infinitely variable, then large cations would be excluded by steric forces, while smaller cations would not be able to achieve coordination to all of the possible binding groups. The maximum decrease in free energy of interaction could then be achieved only by one specific cation.

RESULTS AND DISCUSSION

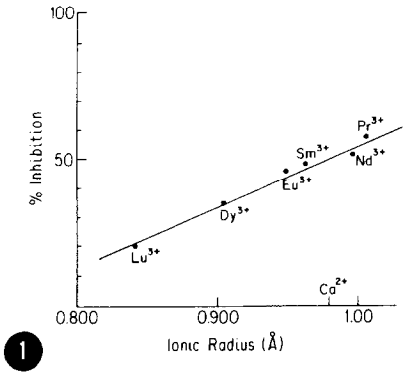
If, as has been suggested (2), mitochondria do contain distinctly different high- and low-affinity binding sites for calcium, these sites may have significantly different field strengths. Distinction between two such classes of binding sites could be made by differences in the free energy changes of cation:site interaction. High-affinity sites would exhibit the greatest decrease in free energy when binding with Ca^{2+}, while the low affinity sites would show a smaller free energy change upon Ca^{2+} binding. It is extremely difficult to measure directly free energy changes in such systems. However, by measuring the selectivity of binding of a group of

related cations, the relative field strengths and relative specificity of two classes of binding sites can be assessed. Thus if high and low affinity Ca^{2+} sites exist in mitochondria, they should be readily distinguishable on the basis of their respective cation selectivity patterns.

The trivalent members of the lanthanide family provide a convenient series with which to assess whether there is more than one class of Ca^{2+} binding sites in mitochondria. These cations are a much better choice as probes of coulombic interaction, than the members of the group IIA metals. The alkaline earths span a large range of chemical and physical properties, whereas all of the trivalent lanthanides have similar chemical properties and their ionic radii and free energies of hydration vary in a sequential manner throughout the series (12). These ions have been given much attention as isomorphous substitutes for Ca^{2+} in biological systems (13). The lanthanide ions are very potent competitive inhibitors of mitochondrial Ca^{2+} binding and transport, while not affecting respiration or oxidative phosphorylation (14,15). By employing these ions as competitive inhibitors of respiration-dependent and respiration-independent Ca^{2+} binding and uptake in mitochondria, the site-specificity of the Ca^{2+} binding sites for the rare-earth ions can be assessed. Those lanthanide ions which produce the largest decrease in free energy upon ion:site interaction will be bound to the greatest extent and show the largest inhibition of Ca^{2+} binding and/or accumulation. The relative activity of these ions in inhibiting respiration-independent Ca^{2+} binding vs respiration-dependent Ca^{2+} uptake can thus be the criteria for determining the selectivity sequence of binding.

In the first set of experiments the inhibition of respiration-independent binding of Ca^{2+} by mitochondria by different lanthanide cations was determined. As shown in Figure 1 the relative inhibition of Ca^{2+} binding by the members of the lanthanide family shows a linear decrease in inhibitory effectiveness, in the sequence Pr^{3+} to Lu^{3+}. The first members of the group, which have the largest ionic radii are the best inhibitors. The smaller ions, which have the largest hydration energies (12) are the weakest inhibitors of Ca^{2+} binding and have the smallest equilibrium binding constant; they show least decreases in free energy upon binding. A lyotropic series such as this has been shown by Wright and Diamond to be a useful indication that the membrane binding site has a low electric field strength, so that ion:site interactions are weaker than hydration energies (9).

On the other hand, the inhibition profile of respiration-dependent Ca^{2+} transport (Fig. 2) shows a much different pattern. Inhibition of Ca^{2+} binding becomes maximal near the middle of the lanthanide series, with Sm^{3+} as the most effective. In these experiments any effects due to differential rates of



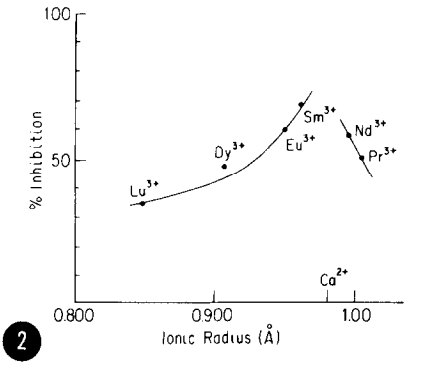


Fig. 1 Lanthanide inhibition of respiration-independent Ca^{2+} binding. For each point — 5 mg mitochondrial protein were suspended in 2.0 ml incubation medium containing 250 nM rotenone, 225 nM antimycin A, and 2.25 μM FCCP. The mitochondria were preincubated for 1 min in the above medium. The lanthanide (75 nmoles) was added and incubated for 15 sec, followed by 250 nmoles $^{45}CaCl_2$ for 15 sec. Mitochondria were centrifuged down and the Ca^{2+} removed from supernatant measured.

The ionic radii of the lanthanides and Ca^{2+} were obtained from CRC Handbook of Chemistry and Physics (1966), R. C. Weast, Ed., The Chemical Rubber Company, Ohio and Templeton, D. H. and Dauben, C. H. (1954) J. Amer. Chem. Soc., 76, 5237-5239.

Fig. 2 Lanthanide inhibition of respiration-dependent Ca^{2+} binding. For each point — 5 mg mitochondrial protein were incubated in 2.0 ml medium containing 250 nM rotenone and 4 mM succinate. After 1 min 2.5 nmoles of lanthanide were added and incubated for 15 sec, followed by 250 nmoles $^{45}CaCl_2$ for 15 sec. Incubation was quenched with 500 μM EGTA and centrifuged down. The $^{45}Ca^{2+}$ lost from the medium was determined.

transport of the rare-earth cations were minimized by very short incubation times.* Although the results from Figure 1 are easily interpreted as weak field interactions, the respiration-dependent lanthanide binding selectivity (Fig. 2) cannot be regarded as representative of strong site binding, which would require that the smaller members of the lanthanide series should be the best inhibitors. It is also difficult to explain this pattern solely on inter-

*Reed and Bygrave (1) have shown that the lanthanide ions can be actively transported by rat liver mitochondria, although at a rate much slower than Ca^{2+}. Incubation times here are sufficiently short to ensure no significant movement of any of the rare-earth ions into the matrix space. Control experiments with $^{151}Sm^{3+}$ show that with 30 sec incubation less than 1% of the added Sm^{3+} is inaccessible to EGTA. With the concentrations of Ca^{2+} and inhibitors used and short incubation times, the rate of Ca^{2+} uptake is very slow. The membrane binding sites would be saturated, thus the inhibition approaches an equilibrium steady state.

mediate field strength.

However, by considering the contribution to the intrinsic site field strength that can be provided if the site contains more than one functional group to bind the cation, this sequence can be explained. If the binding site is specific for Ca^{2+}, the conformation and coordination with another cation will contribute significantly to the free energy balance of the cation:site interaction. Those cations which are closest to Ca^{2+} in ionic size will achieve stronger coordination with least deformation of the binding site. Therefore, those lanthanide ions which more closely approach the ionic size of Ca^{2+} will be the best inhibitors of Ca^{2+} uptake by virtue of greater ion:site interaction. In Fig. 2, Sm^{3+}, whose ionic radius (0.964 Å) is closest to that of Ca^{2+} (0.979 Å), is the best inhibitor. Inhibitory potency falls off as the lanthanide ions become larger or smaller than Ca^{2+}. In the latter cases, maximum strength of coordination may not be achieved due to steric resistance of the site to conformational change.

The lanthanide inhibition patterns for the inhibition of Ca^{2+} binding and/or transport in respiring and non-respiring mitochondria clearly show that rat liver mitochondria have two distinct types of Ca^{2+}:site interactions, one which appears to be Ca^{2+} specific (Fig. 2) and one which is relatively non-specific (Fig. 1).

Another type of conclusion may be drawn, regarding the relationship of lanthanide binding to energy-dependent Ca^{2+} binding and/or transport. Two alternative views are possible:

1. Two classes of Ca^{2+} binding sites, specific and non-specific, may exist in the inner membrane, but without respiration to facilitate Ca^{2+} transport into the matrix, the Ca^{2+}-specific sites may not be observable with existing methods if their number is very much less than the number of non-specific sites.

2. The Ca^{2+}-specific sites may be dependent upon respiration not only for the transport function but also for their existence. Energization of the membrane may induce a conformational change in membrane substituents which maximizes the Ca^{2+}:site interaction, thus providing Ca^{2+} selectivity.

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