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CATION BINDING TO SUBMITOCHONDRIAL PARTICLES

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SUMMARY

1. Cation binding to submitochondrial particles has been measured in the absence of metabolism.

2. Both monovalent and divalent cations can bind to the same sites and they compete with each other for the binding.

3. The maximal binding capacity is 25 nmoles/mg protein and 50 nmoles/mg protein for Ca^{2+} and Rb^{+}, respectively.

4. Local anesthetics are competitive inhibitors of the binding.

5. Phospholipids have been found to be the anionic sites to which cations are bound.

6. The relationship of cation binding to energy-linked ion translocation is discussed.

INTRODUCTION

Mitochondria from various sources bind cations in the absence of metabolism. The first observations of this phenomenon were made by SLATER AND CLELAND[1], SARIS[2] and CHAPPELL, COHN AND GREVILLE[3], and quantitative studies were begun by ROSSI, AZZI AND AZZONE[4]. Ca^{2+} has been most frequently employed[1-5], but Mn^{2+} (ref. 3) and Rb^{+} (ref. 6) have also been shown to be bound to mitochondria in the absence of metabolism.

Valuable as these studies have been, they have left open some questions concerning the site to which cations are bound, the type of binding and the reciprocal influences of different cations on the binding.

In this paper studies on the binding of Rb^{+} and Ca^{2+} to sonicated particles of rat liver mitochondria are presented. It will be shown that monovalent and divalent cations are bound to the same site and that they compete with each other. Moreover, the localization of the binding sites in the mitochondrial membranes and their characteristics will be discussed in relation to the energy-linked ion translocation.

Abbreviation: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

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METHODS

Mitochondria were prepared from rat liver homogenized in 0.25 M sucrose, 0.005 M Tris–HCl (pH 7.5), $5 \cdot 10^{-4}$ M ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA). EGTA was omitted in the final mitochondrial suspension.

Submitochondrial particles were prepared as follows: 5–7 mg of mitochondrial protein, suspended in 5 ml of buffered sucrose, were sonicated at 0° for 90 sec at 3 A with a Branson sonifier. After a centrifugation at 9000 × *g* for 5 min in order to remove larger particles, the suspension was spun at 130000 × *g* for 45 min in an MSE ultraspeed centrifuge. The pellet protein, resuspended in sucrose, represented 25 % of the original protein.

Phospholipid-depleted mitochondria were prepared by the method of FLEISCHER, FLEISCHER AND STOECKENIUS[7] using a mixture of acetone, 10 % vol. water and NH_3. Phospholipid was restored to phospholipid-depleted mitochondria by addition of commercial phospholipid preparations (Sigma) suspended in water by sonication.

Total phosphorus was determined after ashing the samples with concentrated sulfuric acid by isobutanol–benzene extraction according to the method of LINDBERG AND ERNSTER[8].

All the experiments were carried out in 2-ml samples of medium at 0° and the added particles were preincubated for 5 min in the presence of an excess of rotenone and antimycin. Protein was always in the range of 1 mg/ml. After an incubation time of 10 min in the presence of radioactive ion (specific activities: $^{45}Ca^{2+}$, 30000 counts per min per μmole; $^{86}Rb^{+}$, 40000 counts/min per μmole), particles were sedimented at 130000 × *g* for 45 min; mitochondria, phospholipid-depleted mitochondria and phospholipid-treated mitochondria were sedimented by centrifugation at 20000 × *g* for 10 min. The pellets were washed twice in cold sucrose, dried and solubilized in 1 M formic acid. Aliquots of the formic acid solution were analyzed for radioactivity. The amount of radioactivity found in the pellet was corrected for the free radioactivity present in the total water content of the pellet. Water was calculated from the difference between wet and dry weights and the water content of the pellets did not vary under the different experimental conditions.

Protein was measured by the biuret method[9].

RESULTS

Cation binding to submitochondrial particles

In order to have quantitative data on cation binding in the absence of metabolism, sonicated particles have been used instead of intact mitochondria, where permeability barriers to monovalent ions, protons and Mg^{2+} are maximally decreased[10,11].

The data reported in Fig. 1 allow one to compare Ca^{2+} binding to intact mitochondria and to submitochondrial particles, both treated with respiratory inhibitors. Both mitochondria and particles were kept in contact with $^{45}Ca^{2+}$ for different times and then centrifuged; the $^{45}Ca^{2+}$ bound was then plotted against the incubation time. Even after a long incubation period, a considerable difference existed between the $^{45}Ca^{2+}$ bound per mg protein to intact mitochondria and the $^{45}Ca^{2+}$ bound per mg

protein to submitochondrial particles: particles bound about twice as much Ca^{2+} as intact mitochondria. The increase in the specific activity of Ca^{2+} bound per mg protein of mitochondrial particles suggests that proteins (like matrix) which are lost during the sonic treatment, do not significantly contribute to the binding.

When submitochondrial particles were incubated in the presence of different concentrations of $^{45}Ca^{2+}$ and were centrifuged after 10 min at 0°, they retained different amounts of $^{45}Ca^{2+}$ at different concentrations of $^{45}Ca^{2+}$ in the incubation medium. The values of $^{45}Ca^{2+}$ bound per mg protein, plotted as a function of the concen-

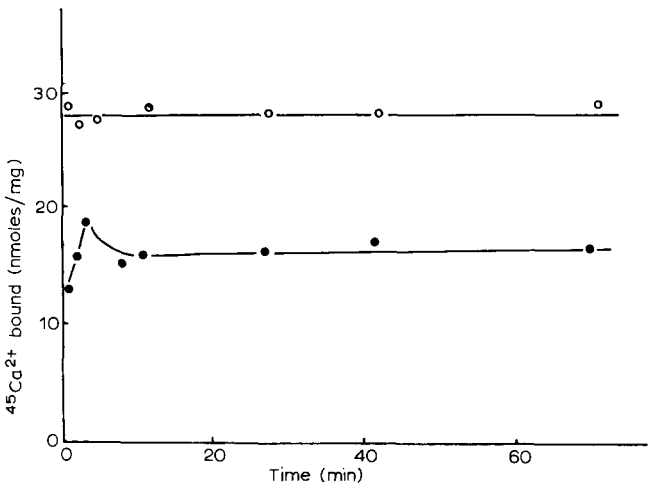

Fig. 1. Ca^{2+} binding by mitochondria (●—●) and submitochondrial particles (○—○). Experimental conditions: the sample contained 0.25 M sucrose, 0.005 M Tris–HCl (pH 7.5), 300 μM $^{45}CaCl_2$, 3.2 mg mitochondrial protein or 2.9 mg sonicated particles in a final vol. of 2 ml. Mitochondria and particles were preincubated for 5 min in the presence of 5 μM rotenone and 10 μg antimycin A. Temp., 0°. After incubation for various lengths of time, the samples were centrifuged at 130000 × g for 30 min. Ions were measured as described in METHODS.

tration of $^{45}Ca^{2+}$ added, gave a hyperbolic curve (Fig. 2), indicating a progressive saturation of a fixed number of binding sites.

A double-reciprocal plot of the same data resulted in a straight line, the intercept on the ordinate being the maximal binding capacity of the mitochondrial particles. By extending the plot to the intersection on the abscissa, the $-1/K_m$ was obtained, which is a measure of the affinity of the cation for its binding site. Under the conditions reported in Fig. 2 the maximal binding capacity for Ca^{2+} of sonicated particles at pH 7.5 was 25 μmoles/g protein, and the K_m for Ca^{2+} was 100 μM. If instead of $^{45}Ca^{2+}$, $^{86}Rb^{+}$ was used as a cation, similar results were obtained (Fig. 3), the only difference being that the maximal binding capacity for Rb^{+} was 50 μmoles/g protein and the K_m 5 mM.

Interaction of different cations at the level of the binding

As both Ca^{2+} and Rb^{+} have been shown to be bound to the mitochondrial structures, an investigation was undertaken to ascertain whether inorganic cations competed for the same binding sites. The binding of $^{45}Ca^{2+}$ or $^{86}Rb^{+}$ was thus measured in the presence of different amounts of another cation, and the reciprocal of the nmoles

of $^{45}Ca^{2+}$ bound per mg protein was plotted against the concentration of the inhibiting cation (see ref. 10 and DISCUSSION). Such a plot gives straight lines at different cation concentrations, the intersection of these straight lines occurring at the ordinate $-K_i$.

In Fig. 4A the effect of KCl concentration on $^{45}Ca^{2+}$ binding to submitochondrial particles is reported. Fig. 4 indicates that K^+ acts as a competitive inhibitor of Ca^{2+} binding. The K_i of K^+ was 7 mM.

In Fig. 4B the effect of Rb^+ on $^{45}Ca^{2+}$ binding was studied and the results presented in a double-reciprocal plot. In the case of Rb^+ the inhibition is also of a competitive type.

In Fig. 4C a competition is reported between Ca^{2+} and Mg^{2+}. No effect of Mg^{2+} was seen in intact mitochondria, probably because of the impermeability of the mitochondrial membrane to Mg^{2+}.

From these results and similar data on the inhibition of Rb^+ binding by several cations, the conclusion can be drawn that all the cations mentioned have the same binding site in mitochondria, and that they compete with each other, exhibiting the following order of affinity: $Mg^{2+} > Ca^{2+} > Rb^+ > K^+$.

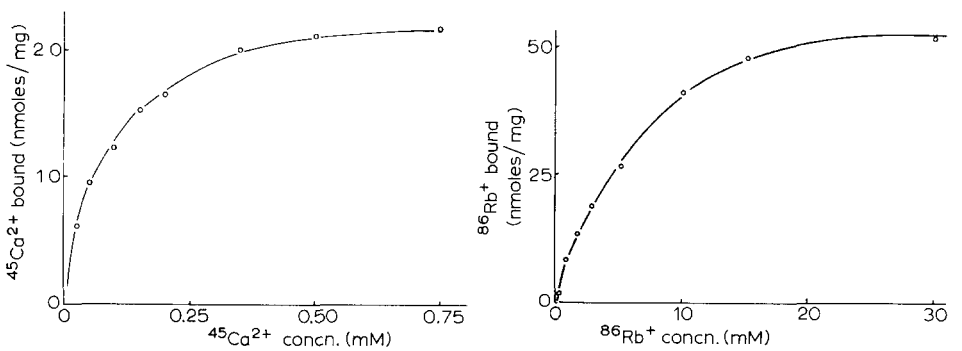

Fig. 2. Dependence of Ca^{2+} binding to submitochondrial particles on Ca^{2+} concentration. Experimental conditions as in Fig. 1, except that $^{45}Ca^{2+}$ concentrations are indicated in the figures and protein was 3.1 mg.

Fig. 3. Dependence of Rb^+ binding to submitochondrial particles on Rb^+ concentration. Experimental conditions as in Fig. 1, except that $^{45}Ca^{2+}$ was not added and 2.3 mg protein were present. $^{86}Rb^+$ concentrations are indicated in the figure.

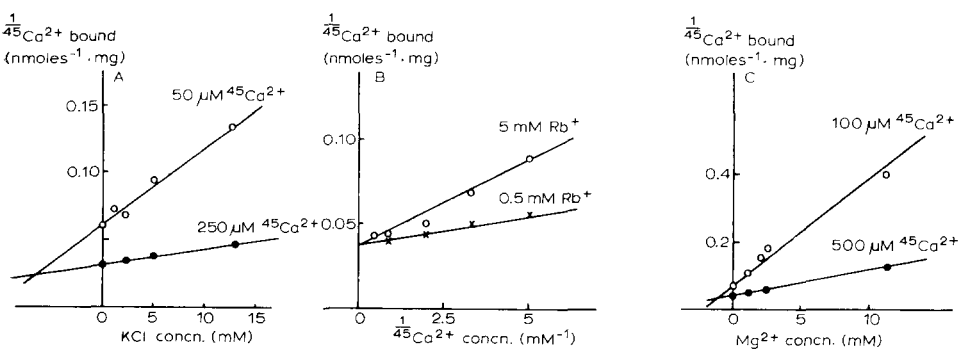

Fig. 4. Effect of K^+, Rb^+ and Mg^{2+} on Ca^{2+} binding. Experimental conditions as in Fig. 1. Ion concentrations are indicated. In A, 1.8 mg protein were present in B, 3.5 mg, in C, 2.2 mg.

In Figs. 5A and 5B the influence of Na^+ on the binding of $^{86}Rb^+$ or $^{45}Ca^{2+}$ to submitochondrial particles is reported. Fig. 5 indicates that the inhibition of the binding by Na^+ is of the uncompetitive type. The interpretation of this result is not clear at the present moment and further investigations are required in order to understand the mechanism of action of Na^+.

Effect of pH on cation binding

The effect of pH on cation binding is reported in the double-reciprocal plot of Fig. 6. The maximal binding capacity of submitochondrial particles was the same at different pH's, when extrapolated at infinite Ca^{2+} concentration. This value was 25 μmoles/g protein. On the other hand the affinity increased from 300 μM at pH 6.5, to 70 μM at pH 8.5. Thus the protons acted as competitive inhibitors at the level of

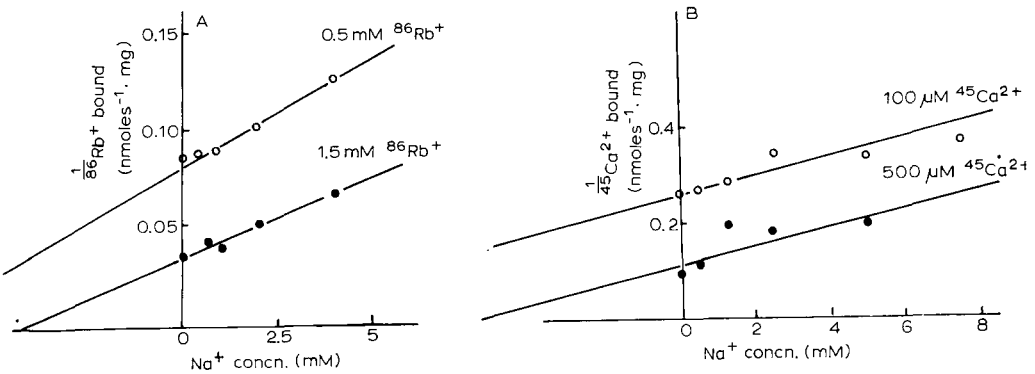

Fig. 5. Effect of Na^+ on Ca^{2+} and Rb^+ binding. Experimental conditions as in Fig. 1, except that ion concentration was: in A, 500 μM or 1.5 mM $^{86}Rb^+$; in B, 100 μM or 500 μM $^{45}Ca^{2+}$. 1.7 mg protein were present.

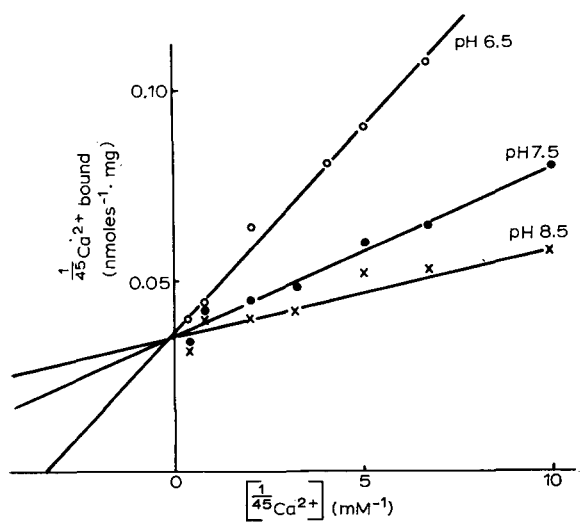

Fig. 6. Effect of pH on Ca^{2+} binding to submitochondrial particles. Experimental conditions: samples contained 0.25 M sucrose, 12.5 mM Tris–HCl at the pH indicated, 5 μM rotenone, 2 μg antimycin A and 1.4 mg protein. Temp., 0°. Other conditions as in Fig. 1.

the binding site of Ca^{2+}. Moreover Rb^+ was found to compete with protons for binding. The amount of Rb^+ bound at infinite concentration was 50 μmoles/g protein, twice the amount of Ca^{2+} bound.

It is important to note here that the affinities of Ca^{2+} and Rb^+ for their binding sites were very close, at high pH, to the affinities for the transport system when ATP or respiration causes an energy-dependent translocation of cations[13,14].

Local anesthetics and cation binding

Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, 2,4-dinitrophenol, valinomycin and gramicidin, even at very high concentrations, did not influence the extent of cation binding to submitochondrial particles. These findings thus excluded the possibility that uncouplers and cation conductors might be bound at the same sites to which cations are bound, and, in addition, excluded the possibility that metabolism might influence the process studied. On the other hand, local anesthetics which have been observed to bind and inhibit cation binding to isolated phospholipid[15–17], have been found to be powerful inhibitors of the binding of cations to submitochondrial particles. In Figs. 7A and 7B, and 7C and 7D, the effects of Nupercaine and Pantocaine on Ca^{2+} binding and Rb^+ binding, respectively, are presented. The inhibition by local anesthetics of cation binding to submitochondrial particles is of the competitive type. The K_i was 0.2 mM for all the anesthetics tested, both with Rb^+ and Ca^{2+}.

Cation binding and phospholipids

CHAPPELL, COHN AND GREVILLE[3] and PEACHEY[18] have suggested phospholipid as the probable binding site for Mn^{2+} and Ca^{2+} during energy-linked translocation.

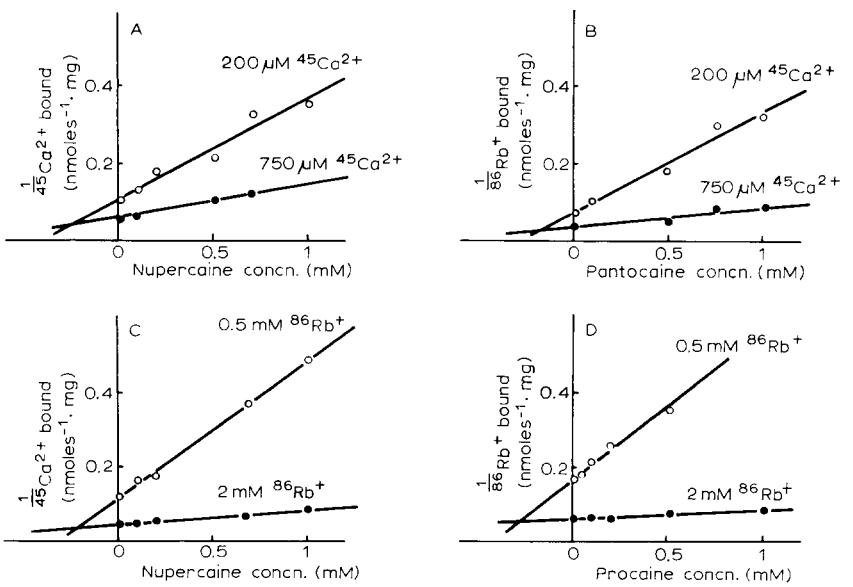

Fig. 7. Effect of local anesthetics on ion binding to submitochondrial particles. Experimental conditions as in Fig. 1 except for the ion concentrations. 1.8 mg protein were present in A and B, 2.5 mg in C and D. In A and B, $^{45}Ca^{2+}$ concentration was 0.2 and 0.75 mM, respectively. In C and D, $^{86}Rb^+$ concentration was 0.5 and 2 mM, respectively.

Protein, on the other hand, has been suggested as being involved in cation binding in the absence of coupled respiration[3].

Nevertheless, measurements of Ca^{2+} binding in phospholipid-deficient mitochondria led to the conclusion that the phospholipid content and cation binding run parallel.

In Table I it is shown that when phospholipids were extracted from mitochondria cation binding was inhibited, and restoration of the phospholipid to mitochondria coincided with restoration of cation binding. The saturation curve of Fig. 8 indicates that the number of binding sites is decreased in lipid-deficient mitochondria to one-third of the value of submitochondrial particles and acetone-extracted mitochondria treated with phospholipids.

TABLE I

EFFECT OF PHOSPHOLIPID CONTENT OF MITOCHONDRIA ON Ca^{2+} BINDING

Experimental conditions: extraction of phospholipids was carried out according to the procedure of FLEISCHER[7]. For the rebinding of phospholipid, a mixture of phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl inositide derived from brain (Sigma) was used. The cation binding was measured in a 0.25 M sucrose medium containing 0.005 M Tris–HCl (pH 7.5), 5 μM rotenone, 2 μg/ml antimycin and 270 μM Ca^{2+}. Protein concentrations were: particles 1 mg/ml, depleted mitochondria 0.6 mg/ml, and reconstituted mitochondria 0.7 mg/ml. Vol., 2 ml; Temp., 0°; incubation time, 10 min.

	Phospholipid P (nmoles/mg protein)	*P (%)*	*Ca^{2+} bound (nmoles/mg protein)*	*Ca^{2+} bound (%)*
Mitochondrial particles	580	100	16	100
Phospholipid-depleted mitochondria	92	16	5.1	32
"Reconstituted" mitochondria	320	55	15.8	99

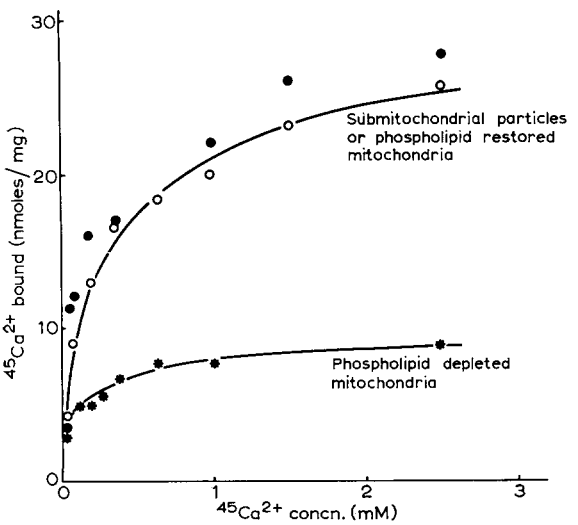

Fig. 8. Ca^{2+} binding and phospholipid in mitochondria. Experimental conditions as in Table I except for Ca^{2+} concentration which is indicated in the figure. Phospholipid content of the three curves is as in Table I. Full circles are phospholipid-restored mitochondria, open circles submitochondrial particles, asterisks phospholipid-depleted mitochondria.

As will be discussed later, the present evidence favours a direct binding of cations to phospholipids rather than an indirect effect of phospholipids, such as an increase of the cation affinity of non-phospholipid binding sites.

DISCUSSION

According to the model of acid membranes proposed by MEYER AND SIEVERS[19] and JOHNSON, EYRING AND POLISSAR[20], if a membrane contains attached acid groups, –AH, the following reaction occurs when the membrane is in an aqueous solution of the ion, B^+:

$$\underset{\left(\begin{smallmatrix}\text{in the}\\ \text{membrane}\end{smallmatrix}\right)}{-AH} + \underset{\left(\begin{smallmatrix}\text{in}\\ \text{solution}\end{smallmatrix}\right)}{B^+} \rightleftharpoons \underset{\left(\begin{smallmatrix}\text{in the}\\ \text{membrane}\end{smallmatrix}\right)}{-A^- + B^+} + \underset{\left(\begin{smallmatrix}\text{in}\\ \text{solution}\end{smallmatrix}\right)}{H^-} \qquad (1)$$

The concentration, A, of the ionized groups is

$$A = \frac{K[A^* - A]C_i'}{C_i[H^+]_i'} \qquad (2)$$

where K is the equilibrium constant of reaction (1), A* is the total amount of acid groups, and C_i and C_i' are the concentrations of a given ion in the membrane and in solution, respectively. Eqn. 2 shows that the amount of A varies directly with the concentration of the cation and inversely with the pH. If the pH is sufficiently high, Reaction 1 is complete. This relationship between ion concentration, binding sites and pH has been verified for the preparations of mitochondrial membranes which we used during this work. In other words, when the cation concentration was raised to very high values or at high values of pH, the number of binding sites approached a constant maximum (Fig. 6).

Since the mitochondrial membrane possesses fixed binding sites capable of reversible association with cations, it should be possible to analyze cation binding in terms of the Michaelis–Menten equation[21].

In the presence of cations, the mitochondrial membrane behaves as an acid membrane, the anionic sites being identical for monovalent and divalent cations. By substituting V with $[A^- B^+]$, the ion-binding site, and V_{max} with $[A^-] + [A^- B^+]$, the maximal binding capacity, the Michaelis–Menten equation can be written in the following form:

$$[A^- B^+] = \frac{[A^-] + [A^- B^+]\,[B^+]}{K_m + [B^+]} \qquad (3)$$

from which the reciprocal form of Lineweaver–Burk or the equations for the different types of inhibition could be derived.

In fact the competition found between different cations and the constancy of the saturating positive charges, is strongly in favour of a common site of binding for both univalent and divalent cations. This site of binding is probably on the mitochondrial membrane, the specific activity of Ca^{2+} bound per mg protein in particles being double to that in mitochondria (Fig. 1).

We have discussed the evidence for the presence on the mitochondrial membrane of anionic groups that can bind different cations or protons, depending on their relative concentrations. The evidence that the anionic groups that bind cations are

mainly phospholipids is as follows. First, mitochondria contain large amounts of phospholipids that were shown to bind, when isolated, both monovalent and divalent cations[15–17]. Second, local anesthetics, which have been shown to inhibit ion binding to phospholipids[15,17], do indeed prevent cation binding to mitochondria. Third, extraction of phospholipids from mitochondria causes a decrease of the cation binding capacity of mitochondria that is roughly proportional to the total amount of phospholipid extracted. Fourth, readdition of phospholipids to extracted mitochondria increased both the amount of phospholipids attached to mitochondria and their binding capacity.

As to the relationship of this type of binding and ion translocation in intact mitochondria the following considerations may be made. Unless major changes of the characteristics of the mitochondrial membrane had occurred during the sonic treatment, the above reported data concerning the characteristics of cation binding to sonicated submitochondrial particles can be also applied to intact mitochondria. Moreover, it is possible that the binding analyzed here is the first step in the ion translocation mechanism proposed by CHANCE[13].

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