MITOCHONDRIAL "HIGH-AFFINITY" BINDING SITES FOR Ca^{2+} - FACT OR ARTEFACT?

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SUMMARY

No "high-affinity" binding sites for Ca^{2+} were found in rat liver mitochondria when care was taken to exclude energy-dependent uptake of Ca^{2+}. When respiration was not completely blocked or when preincubation was too short to drain endogenous energy stores Scatchard plots showed the presence of both low-affinity and apparent high-affinity sites. It is suggested that the latter do not represent true binding sites but result from energy-dependent accumulation of Ca^{2+}.

Mitochondria have been reported to bind Ca^{2+} in an metabolism-independent way to both high and low-affinity sites (1,2). The former are of special interest since they may represent the carrier that may be involved in energy-dependent transport of Ca^{2+}. Proteins with high-affinity binding capacity have indeed been extracted from mitochondria (3,4). High-affinity binding sites in mitochondria are, however, not seen in the presence of uncoupling agents (1,2). This may be due to an alteration of mitochondrial structure in the uncoupled state. On the other hand it is possible that high-affinity sites have been studied under conditions in which energy has been available for accumulation of Ca^{2+}. We have studied the binding of Ca^{2+} under conditions, where the availability of energy for residual Ca^{2+} transport has been varied. We have also varied the length of the preincubation period and have tested the effect of using a divalent cation ionophore in order to equilibrate the inside and outside concentrations. We failed to see any high-affinity binding in the presence of sufficient doses of respiratory inhibitors, when the mitochondria were incubated long enough to relax an "energized" state or

in the presence of an ionophore for divalent cations.

Material and Methods

Rat liver mitochondria were prepared from young male rats (Sprague-Dawley) by a conventional procedure using 250 mM sucrose medium buffered to pH 6.8 with Tris. No EDTA was used. A stock solution of 200 μM $CaCl_2$ was prepared in 250 mM sucrose buffered to pH 7.4 with Tris. On dilution it was labelled with ^{45}Ca to give a counting rate of 5 000 - 100 000 in 10 min. HOQNO and antimycin (Sigma) were used from 6 mM and 11,6 mM stock solutions in ethanol. X537A was a gift from F. Hoffmann - La Roche & Co.

Mitochondria were preincubated 25° C in 250 mM sucrose buffered to pH 7.4 with Tris in the presence of the inhibitors. Ca^{2+} was added and after 1 min followed a centrifugation for 2 min at 14 000 g. Aliquots of the supernatant were used for measurements of radioactivity. The rest of the supernatant was aspirated, excess fluid carefully removed and the mitochondrial pellet suspended in 1.0 ml 1 M formic acid. After standing for at least 1 h the contents were mixed, centrifuged and an aliquot used for determination of radioactivity. The aliquots were mixed with Instagel (Packard) and counted in a Packard Tricarb liquid scintillator.

Results

Using the conditions employed by Reynafarje and Lehninger (1), e.g. 250 nM rotenone and 225 nM antimycin, we could confirm the finding of two classes of binding sites for Ca^{2+}. However, increasing the concentration of antimycin abolished the "high-affinity" binding sites. Since excess antimycin might be uncoupling, we preferred to use HOQNO, which like antimycin inhibits electron flow between cytochromes b and c but which does not show any uncoupling effects in the concentrations used (5). It is also more convenient to use it for studying the effect of

Abbreviation: HOQNO = 2-Heptyl-4-hydroxyquinoline N-oxide

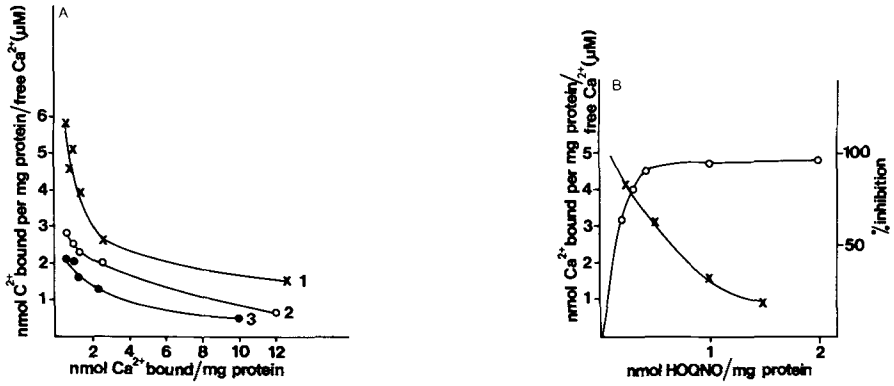

Fig. 1. Effect of varying HOQNO concentration on binding of Ca^{2+}. A. Scathard plots. After preincubation for 1 min in the presence of 0.25 μM rotenone and HOQNO 0.7 - 14 μmol $CaCl_2$ was added per mg protein (4 mg/ml). 1. 0.75 μM HOQNO, 2. 1.5 μM HOQNO, 3. 3.0 μM HOQNO.
B. Comparison of inhibition of respiration on succinate and suppression of binding of Ca to high-affinity sites (at 0.7 μM Ca added/mg protein). o——o Per cent inhibition of respiration. x——x Ratio of bound to free Ca^{2+}.

residual respiration since the degree of inhibition is hyperbolically and not sigmoidally related to concentration of inhibitor (5). Fig. 1A shows that increasing concentrations of HOQNO abolish the "high-affinity" binding sites. This is seen as a lowering of the left-hand rising leg of the Scatchard curves. Fig. 1B shows that the "high-affinity" binding sites have virtually disappeared when respiration is more than 95 % inhibited.

When respiration is almost completely blocked "high-affinity" binding is lost when the time of preincubation is prolonged from the one minute used routinely in earlier studies (1,2), Fig. 2. "High-affinity" binding is also lost in the presence of X537A (Fig. 3) - a ionophore for divalent cations (6). X537A may be uncoupling at higher concentrations by equilibrating not only Ca^{2+} across the membrane but also Mg^{2+}, but no uncoupling was observed in our conditions.

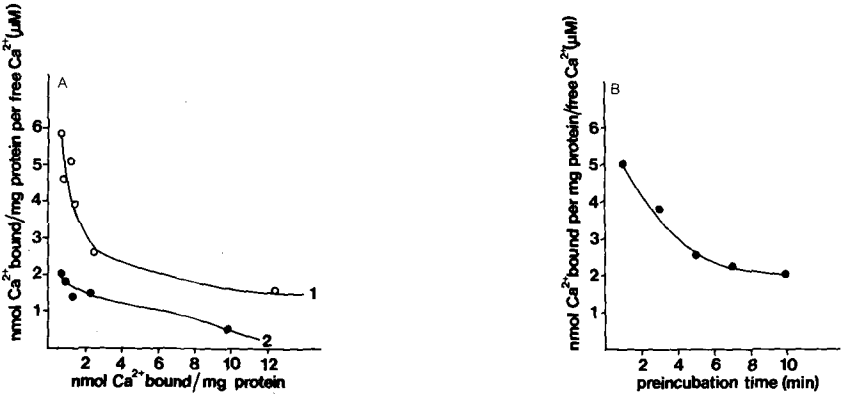

Fig. 2. Effect of time of preincubation of high-affinity binding. A. Scatchard plots in the presence of 0.75 μM HOQNO and 0.25 μM rotenone. 1. 1 min preincubation, 2. 5 min preincubation.
B. Binding at 0.7 μmol Ca/mg protein as function of preincubation time.

Discussion

In this study we have found Scatchard plots indicating "high-affinity" binding sites for Ca^{2+} only when there is still some respiration or when the mitochondria have been preincubated for short periods at room temperature. It is therefore tempting to conclude that a condition for the "high-affinity" binding is the availability of metabolism-derived energy, either directly from respiration or via ion gradients. This is in accordance with the finding (1) that uncoupling agents inhibit the binding. The dependence on a source of energy may be explained in at least two ways:

1. a) The hypothetical carrier is available for binding only when the mitochondria are "energized" or b) it has high affinity for Ca^{2+} only in an "energized" form.
2. The "high-affinity" binding is energy-dependent transport of Ca^{2+} into the inner membrane or the matrix space.

The difference between 1b and 2 is semantic. The former may be regarded as a special case of 2. It is evident that by the second mechanism many features of the "high-affinity" binding can be readily understood. These

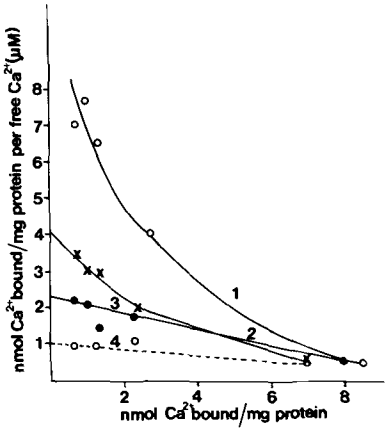

Fig. 3. Effect of Ca^{2+} ionophore and KCN on high-affinity binding. 1. 0.75 μM HOQNO + 5 μM rotenone, 2. in addition 0.56 μg/ml X537A, 3. X537A 2.7 μg/ml, 4. 5mM KCN + 5 μM rotenone.

include the inhibition of "high-affinity" binding by substances that inhibit the transport of Ca^{2+} (1,7) and the presence of these sites only in mitochondria that possess an efficient transport mechanism (2). The "high-affinity" binding vanishes when the mitochondria are aged or water-lysed without a complete loss of the transport function (1). In the last case the critical point is whether the mitochondria are still able to accumulate Ca^{2+} from very low concentration with a low "energy pressure". This would result in Scatchard plots that indicate "high-affinity" binding. The ability of the ionophore X537A to abolish "high-affinity" sites is also in favour of this interpretation.

Our inability to find "high-affinity" sites for Ca^{2+} might be assailed on the grounds that we might have used poorly prepared mitochondria or that the inhibitors were uncoupling. However, the control ratios of our preparations were satisfactory, in excess of four in succinate respiration, and we could see no uncoupling effects, e.g. stimulation of ATPase activities in control experiments. "High-affinity" binding could be inhibited with cyanide, Fig. 3, which certainly is not an uncoupler of oxidative phosphorylation.

It is extremely difficult to rule out energy-dependent transport of Ca^{2+} as the underlying mechanism for "high-affinity" binding. Carrying out incubation at 0^{o} C does not slow metabolism sufficiently to prevent the accumulation of the minute amounts of Ca^{2+} which are added and would furthermore slow down the relaxation of an "energized" state necessitating longer preincubation times. The strongest argument in favour of an energy-independent binding is that hydrogen ions are produced in the transport (8) while little (9) or no (1) production is seen in binding. However, in these studies mainly low-affinity binding was studied (9) or antimycin was added (1). Antimycin probably increases the hydrogen ion permeability of the mitochondria (7). Thus, the minute amounts produced may have been quickly buffered and escaped detection by the comparatively slow glass electrode.

From these data it is evident that experiments on high-affinity binding of Ca^{2+} by mitochondria should be viewed with caution. Furthermore, Scatchard plots are not well suited to study surface binding in a complex, several-compartment system like mitochondria. This does not exclude participation of proteins with high-affinity binding sites in the transport of Ca^{2+}. However, attention should also be given to components with lower affinity which bind Ca^{2+} fast enough to account for the kinetics of transport (7, 10) and the high permeability of mitochondria towards Ca^{2+} (11).

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