HIGH AFFINITY BINDING OF Ca^{++} IN MITOCHONDRIA: A REAPPRAISAL

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SUMMARY: It has been postulated and generally accepted that mitochondria contain two sets of binding sites for calcium, high affinity and low affinity sites. The high affinity binding of calcium is insensitive to low levels of respiratory inhibitors (antimycin plus rotenone) but completely suppressed by uncouplers. We have found that in beef heart mitochondria high affinity binding is completely suppressed by relatively high levels of respiratory inhibitors for the first two coupling sites, and that these levels of inhibitors have no significant effect on oxidative phosphorylation or energy-linked calcium uptake driven by electron flow at the third coupling site. Similar results have also been obtained in rat liver mitochondria. It is concluded that the so-called high affinity binding of calcium observed in the presence of low levels of inhibitors of respiration is actually the result of energy-linked uptake of calcium due to residual electron transfer remaining in the presence of low levels of inhibitors.

A mechanism for the active transport of calcium in mitochondria has been proposed by Lehninger (1) which involves membrane proteins as the molecular vehicle for transport. According to this mechanism, the initial event in active transport of calcium is the respiration-independent binding of calcium to specific sites apparently located on the inner mitochondrial membrane. In a series of papers from Lehninger's laboratory (2-5), evidence has been presented that vertebrate mitochondria show two types of binding for Ca^{++}-- high affinity binding (Km = 0.1-1.0 μmolar, 1-10 nmoles per mg protein) and low affinity binding (Km = 100 μmolar, 40-60 nmoles per mg protein). It was concluded that respiratory inhibitors have no effect on high affinity binding but that uncouplers completely suppress high affinity binding. In fact, high affinity binding is defined as the binding which is unaffected by respiratory inhibitors and eliminated by uncouplers. The high affinity binding sites are considered by Lehninger to be involved in the mechanism of active transport of calcium, although Mela and Chance (6), on the basis of lanthanium inhibition studies, have suggested

that the high affinity Ca^{++} binding sites may be different than the "functionally active divalent cation carrier."

In the present communication, we shall be presenting evidence that the so-called high affinity binding of Ca^{++} can be completely suppressed by respiratory inhibitors as well as by uncouplers and that the phenomenon apparently involves nothing more than the active transport of Ca^{++} in the micromolar range of concentration.

MATERIALS AND METHODS

Heavy beef heart mitochondria were isolated in a medium 0.25 M in sucrose, 10 mM in Tris-Cl (pH 7.8) as previously described (7). Rat liver mitochondria (Holtzman strain, 200-275) were isolated by the method of Schneider as described by Umbreit, Burris and Stauffer (8) in the same medium as for heart muscle except for a lower pH (7.4). The livers were minced finely with scissors and homogenized in a Potter-Elvehjem glass homogenizer (Thomas, Type C). Protein was determined by the biuret method of Gornall et al. (9). Calcium binding was measured by the method described by Reynafarje and Lehninger (2). Various levels of calcium chloride (labelled with isotopic $^{45}Ca^{++}$) were added to a mitochondrial suspension (2.5 mg protein per ml) one minute after the addition of the appropriate level of inhibitor. One minute later, mitochondria were sedimented by centrifugation (1 to 1.5 min. at maximum speed in a Misco high-speed centrifuge, Model 5500). The amount of $^{45}Ca^{++}$ remaining in the clear supernatant fluid was determined using a Beckman Tri-Carb liquid scintillation counter. Further details are described in the legend to the figure and tables. The energy-linked uptake of $^{45}Ca^{++}$ was determined by incubating for one minute mitochondria (2.5 mg/ml) in a solution containing sucrose, 0.25 M, KH_2PO_4, 10 mM, pH 7.0, Tris-ascorbate, 5 mM and the appropriate level of inhibitors. Calcium chloride ($^{45}Ca^{++}$) (200 nmoles per mg protein) was added followed by $N_1N_1N_1N^1$--tetramethyl-p-phenylenediamine (TMPD), 0.25 mM. Thirty seconds later, the mitochondria were sedimented from the supernatant fluid (Misco) and the radioactivity remaining in the supernatant fraction determined as above.

ATP synthesis was determined by the method described by Southard and Nitesewojo (10). Mitochondria (1-2 mg/ml) were incubated for one minute in a medium containing sucrose, 0.25 M; Tris-Cl, 10 mM, pH 7.4; $MgCl_2$, 10 mM; glucose, 5 mM; hexokinase, 5 units/ml; and the appropriate level of inhibitor. After one minute, ADP, 2 mM and KH_2PO_4 ($^{32}P_i$), 10 mM were added followed by Tris-ascorbate, 5 mM and TMPD, 0.25 mM. The reaction was allowed to continue until the solution was anaerobic (approximately 3 min.) as measured with an oxygen-sensitive electrode. All experiments were performed at 30°. Inhibitors and uncouplers were added as their ethanolic solutions. Controls contained the same amount of ethanol minus the inhibitors.

The key reagents were obtained from the following sources: antimycin A, rotenone, oligomycin and 2,4-dinitrophenol (DNP) from Sigma Chemical Company, calcium chloride ($^{45}Ca^{++}$) from New England Nuclear, and sucrose from Schwarz/Mann (ultrapure, density gradient grade).

RESULTS

Figure 1 shows that the uptake of isotopic calcium in the range 2-20 nmoles Ca^{++} per mg protein is progressively reduced by increasing concentrations of antimycin plus rotenone to the level of uptake obtaining in the presence of DNP. Since ATP can energize active transport of Ca^{++}, we have routinely used oligomycin to suppress hydrolysis of endogenous ATP in addition to the inhibitors for electron transport. Oligomycin alone does not suppress "high affinity Ca^{++} binding" (Table 1), a token that electron transfer is the principle mode of energization in beef heart mitochondria under these special conditions. Table 1 also shows the effect of the inhibitors of electron transfer individually and in combination on the uptake of 2 nmoles Ca^{++} per mg protein. Antimycin is the most effective inhibitor of Ca^{++} uptake but the combination of antimycin, rotenone and oligomycin is required to reduce the level of calcium uptake to that observed in the presence of the uncoupler, DNP.

Since it could be argued that the levels of respiratory inhibitors required for complete suppression of high affinity Ca^{++} binding are overlapping with levels

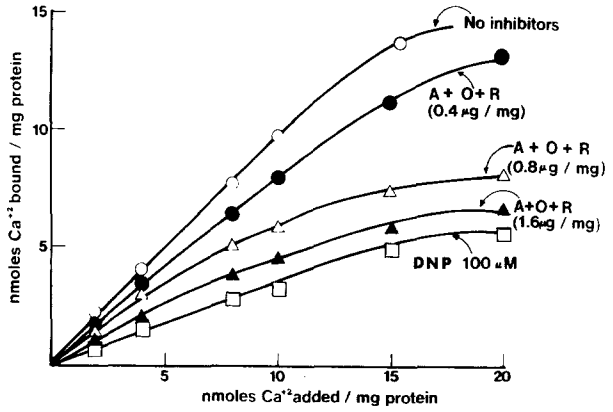

Figure 1. Effect of respiratory inhibitors and uncouplers on high affinity binding of Ca++ in beef heart mitochondria. Mitochondria (2.5 mg/ml) were incubated for one minute at 30° in the presence of the indicated amount of respiratory inhibitor (A = antimycin, O = oligomycin, R = rotenone, and at the concentration indicated in the figure) or 2,4-dinitrophenol (DNP) in a solution containing sucrose, 0.25 M and Tris-Cl, 2 mM, pH 7.4. After the appropriate amount of $CaCl_2$ ($^{45}Ca^{++}$) was added, the mitochondria were incubated for one minute and then centrifuged for one minute (Misco). Radioactivity remaining in the clear supernatant fluid was determined as described in Materials and Methods.

at which these inhibitors have uncoupling action, we tested the action of these inhibitors on active transport of Ca^{++}, energized by ascorbate plus TMPD and on oxidative phosphorylation. As shown in Table 2, levels of antimycin and rotenone sufficient to completely abolish high affinity Ca^{++} binding have almost no effect on the capacity of the mitochondrion to accumulate calcium, and have a marginal effect on oxidative phosphorylation. Ruthenium red abolished about 85% of the high affinity Ca^{++} binding capability, and completely suppressed energy-linked uptake of Ca^{++}.

The effect of respiratory inhibitors shown above with beef heart mitochondria has been duplicated in rat liver mitochondria (Table 3), i.e., with the same mitochondrial preparation which provided the experimental foundations for high affinity Ca^{++} binding (2). As in beef heart mitochondria, rat liver mitochondrial high affinity Ca^{++} binding is completely suppressed by antimycin plus rotenone at levels that have practically no effect on energy-linked uptake of

TABLE 1. Effect of Inhibitors of Respiration, Oligomycin and Uncouplers on High Affinity Ca++ Binding in Beef Heart Mitochondria

	nmoles Ca^{++} Bound/mg Protein				
		μg inhibitor/mg protein			
No Inhibitor	1.94	0.4	0.8	1.6	2.4
Rotenone	----	1.89	1.92	1.86	1.77
Oligomycin	----	1.91	1.92	1.90	1.92
Antimycin	----	1.89	1.84	1.43	1.17
Antimycin + Rotenone	----	1.80	1.36	0.85	0.87
Antimycin + Oligomycin + Rotenone	----	1.60	1.17	0.77	0.73
		Concentration of KCN			
Dinitrophenol (100 μM)	0.81	1 mM		4 mM	10 mM
KCN	----	1.27		1.00	0.68

Conditions are the same as described in the legend for Figure 1. Two nmoles $CaCl_2$ ($^{45}Ca^{++}$) were added per mg protein.

calcium energized by ascorbate plus TMPD, and only a marginal effect on oxidative phosphorylation.

DISCUSSION

The data presented here indicate that high affinity calcium binding may be nothing more than active transport of calcium in mitochondria in which electron transfer has been insufficiently inhibited by low levels of respiratory inhibitors. Increasing levels of antimycin plus rotenone decrease the level of high affinity calcium binding to the level obtained in the presence of uncouplers. In presence of inhibitors at such levels, mitochondria are still capable of coupling electron transfer to the translocation of calcium--an indication that there is no correlation between high affinity binding and calcium translocation

TABLE 2. Effect of Inhibitors on High Affinity Ca^{++} Binding, Active Transport of Ca^{++} and Oxidative Phosphorylation in Beef Heart Mitochondria

Inhibitors	Concentration at which tested	High affinity Ca^{++} binding nmoles/mg	Active Transport Ca^{++} nmoles/mg/30 sec.	P/O
Rotenone	0.5 µg/mg	1.94	195	0.77
Antimycin + Rotenone	1.0 µg/mg	1.52	185	0.62
Antimycin + Rotenone	2.0 µg/mg	1.20	182	0.61
Antimycin + Rotenone	4.0 µg/mg	0.88	169	0.46
DNP	100 µM	0.80	25	0.05
Ruthenium Red	5 nmoles/mg	1.00	30	----

High affinity binding was determined as described in the legend for Figure 1. Active transport and P/O ratios were determined as described in Materials and Methods. In the active transport experiment, the amount of calcium was 200 nmoles per mg protein.

under these conditions. High levels of respiratory inhibitors, particularly antimycin, induce partial uncoupling of oxidative phosphorylation (about 40-50% in rat liver mitochondria and 40% in beef heart mitochondria at the highest level tested) but maximal suppression of high affinity binding.

The low levels of inhibitors normally used to suppress oxygen uptake (measured with oxygen-sensitive electrodes) appear not to prevent completely a limited number of turnovers of the respiratory chain and the generation of an energized state capable of driving limited cation translocation. Let us assume that the active transporting units of the mitochondrion require 10^7 turnovers to translocate a certain relatively large amount of Ca^{++} in 10 minutes at 30°. If the transport units have a turnover of 10^6 per minute without inhibitor and 10^4 per minute with inhibitor (99% inhibition), the inhibition would be suffi-

TABLE 3. Effect of Inhibitors on High Affinity Ca^{++} Binding, Active Transport of Ca^{++} and Oxidative Phosphorylation in Rat Liver Mitochondria

Inhibitor	Concentration at which tested	High affinity Ca^{++} binding nmoles/mg	Active Transport Ca^{++} nmoles/mg/30 sec.	P/O
Rotenone	0.08 µg/mg	1.92	195	0.50 ± .05
Antimycin + Rotenone	0.08 µg/mg	1.48	190	0.49 ± .05
Antimycin + Rotenone	0.16 µg/mg	1.41	193	0.43 ± .05
Antimycin + Rotenone	0.32 µg/mg	1.34	183	0.48 ± .05
Antimycin + Rotenone	0.48 µg/mg	1.27	180	0.36 ± .08
Antimycin + Rotenone	1.20 µg/mg	1.14	172	0.30 ± .06
DNP	50 µM	1.19	68	0.03

Conditions are identical to those described in the legend for Table 2.

cient to prevent effective translocation (less than 1% translocation). However, if the amount of Ca^{++} to be translocated required only 10^1 or 10^2 turnovers of the transporting units, then 99% inhibition of the transporting units by respiratory inhibitors would not be insurance that transport would be suppressed. It might be necessary to achieve 99.9% inhibition or even higher and this would require use of the inhibitor at the upper limit of the permissible range of concentrations.

A number of mechanisms have been proposed to describe active transport of cations in mitochondria in which the translocating system is considered to be a membrane bound protein. By contrast, Blondin, DeCastro, and Senior (11) have postulated an ionophore type molecule as the molecular instrument of cation translocation in mitochondria. The isolation of a Na^+/K^+ specific ionophore (12)

and a divalent metal cation ionophore (13) from beef heart mitochondria has recently been reported. Although the existence of intrinsic ionophores does not eliminate unequivocally the role of high affinity binding in the mechanism of calcium translocation, it does indicate that other alternatives than the postulated high affinity sites invoked by Lehninger (1-4) are available.

Intrinsic mitochondrial ionophores appear to be associated with membrane proteins and not to be freely dispersed in the lipid region of the membrane. Whether one of the ionophore-containing proteins can be identified with the glycoprotein fractions recently isolated from liver mitochondria by Lehninger (14,15) and Sottocasa, et al. (16) remains to be determined.

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