INTERACTION OF CALCIUM WITH MITOCHONDRIA DURING CALCIUM FLUX*

C. S. ROSSI, A. ALEXANDRE and C. R. ROSSI

Institute of Biological Chemistry, University of Padova, and Centro per lo Studio della Fisiologia dei Mitocondri del Consiglio Nazionale delle Ricerche, Italy

Received 15 April 1974

1. Introduction

Previous investigations have indicated the existence in the mitochondria of a specific transport (or carrier) system which is involved in the active movements of calcium across the inner mitochondrial membrane [1, 2]. Although these findings favor the general concept of an interaction of the cation with some component of the mitochondrial membrane, no actual detection of calcium bound to the membrane has been so far presented to directly support this conclusion.

Using a procedure developed in our laboratory [3, 4], we report here the detailed kinetic measurements of calcium binding to the mitochondrial structure before the process of translocation has been completed, that is, during the flux (both inward and outward) of the cation across the membrane.

The results clearly suggest that during the flow of calcium a maximum of 0.7 nmoles of calcium can be bound for each of the three energy conserving sites of the respiratory chain (per mg protein). Moreover, the interaction calcium–mitochondrial structure occurs at the level of the same transport system both during the entrance and exit of the ion. Finally, the redox state of the respiratory carriers modulates the rate of ATP hydrolysis, when this reaction is used to drive the active translocation of calcium in mitochondria.

* A portion of this paper has appeared in the abstracts of the IX International Congress of Biochemistry, Stockholm, July 1973.

2. Materials and methods

Rat liver mitochondria were isolated essentially as described by Schneider [5]. The measurements of calcium bound were made by using the mercuric chloride quenching technique described by Alexandre et al. [6], in which the reaction is terminated by addition of 0.2 ml saturated mercuric chloride (at 20°C). The concentration of mercuric chloride is then rapidly lowered to 133 mM, and the complete mixture centrifuged for 90 sec at 15 000 *g* at 0°C. The pellet is washed with 10 ml of cold 6.5 mM mercuric chloride, extracted with 10 ml of Instagel (Packard), and counted for radioactive calcium in a liquid scintillation spectrometer. In parallel experiments the absolute amount of calcium accumulated was estimated as described by Rossi and Lehninger [7]. ATP hydrolysis was followed by measuring the H^+ ejected into the suspending medium with a glass electrode and an expanded scale pH meter linked to a strip chart recorder.

3. Results and discussion

3.1. *Respiration dependent binding of calcium*

In fig. 1 are illustrated the results of a series of experiments in which the amount of calcium 'bound' is measured as a function of the concentration of externally added calcium. It can be seen that the binding reaction follows a typical saturation kinetics, with a maximum binding corresponding to about 2 nmoles of calcium per mg mitochondrial protein. The plateau is clearly not due to an exhaustion of the cation in the assay mixture, since in a number of parallel experi-

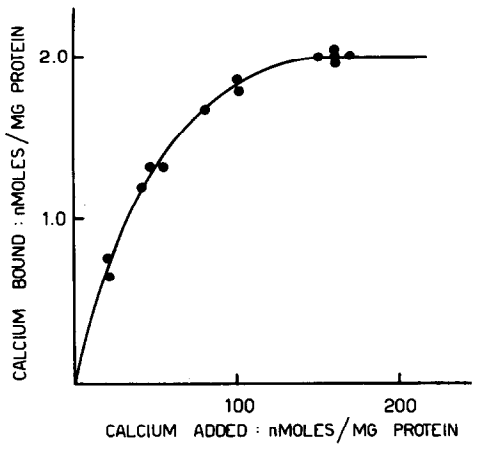

Fig. 1. Bound calcium as a function of added calcium. Experimental conditions as described in table 1, except that β-hydroxybutyrate was the respiratory substrate and the time was 20 sec.

ments the plateau was reached well before the external medium was depleted. Rather, the maximum binding reflects the saturation of some mitochondrial component with calcium. From the data it also appears that the pellet material retains an amount of calcium twenty times as lower as the amount that can be actually accumulated at the storage compartment of the mitochondria (about 40 nmoles per mg protein).

Table 1
Succinate driven calcium binding. Effect of oligomycin respiratory inhibitors, and DNP

Additions	Calcium bound nmoles/mg
1. None	1.33–1.60
2. Oligomycin	1.40
3. DNP	0.15
4. ROT + AA	0.45–0.53
5. ROT + AA + DNP	0.05–0.10

The reaction system contained 10 mM Tris–Cl (pH 7.4), 50 mM NaCl, 50 mM KCl, 10 mM succinate as respiratory substrate, and rat liver mitochondria (30 mg protein in total volume of 2 ml). When present: rotenone was 0.07 $\mu g \times mg^{-1}$ protein, antimycin A 0.14 $\mu g \times mg^{-1}$ protein, oligomycin 1 $\mu g \times mg^{-1}$ protein, and DNP 5×10^{-5} M. $^{45}CaCl_2$ (65 nmoles $\times$ mg^{-1} protein) was added to start the reaction. The reaction was stopped 15 sec later.

The binding reflects an energy requiring process, since the reaction is strongly inhibited by respiratory inhibitors, and uncoupling agents (table 1). On the other hand, oligomycin does not affect the binding at concentrations which completely prevent oxidative phosphorylation of ADP.

3.2. *Relationships between the extent of binding and the number of energy conserving sites*

Table 2 shows that the amount of calcium bound differs with the different electron donors assayed. In particular, the amounts of calcium bound are: 2, 1.5, and 0.7 during the oxidation of β-hydroxybutyrate, succinate and TMPD plus ascorbate, respectively. These values, when divided by the number of coupling sites involved in the oxidation of the substrates employed, give the contribution of each energy conserving site: 0.7 nmoles of calcium are bound for each electron couple passing through each energy conserving span of the respiratory chain. One additional point can be made here: all of the calcium added is accumulated in the intramitochondrial compartment during the period of calcium-stimulated respiration [7], so that the net amount of calcium moved is the same for all the substrates tested, despite of the different rates of calcium induced respiration. It follows therefore, that the rate of *net* uptake (as measured by the rate of calcium-evoked oxygen consumption) appears to proceed independently from the extent of the *binding* of the cation reported in this paper. In this light, the following tentative conclusion can be drawn: the translocation of calcium occurs via three

Table 2
Stoichiometry between calcium bound and energy conserving sites of the respiratory chain

Substrate	Ca^{2+} bound/mg protein
β-Hydroxybutyrate	2.10/3 = 0.70
Succinate	1.55/2 = 0.77
Ascorbate + TMPD	0.68/1 = 0.68

Experimental conditions as described in table 1, except that the respiratory substrates were 20 mM β-hydroxybutyrate or 10 mM succinate or 20 mM ascorbate (+ 0.30 mM N,N_1,N',N'-bound are divided by the number of phosphorylation sites in bound are divided by the number of phosphorylation sites in the respiratory chain.

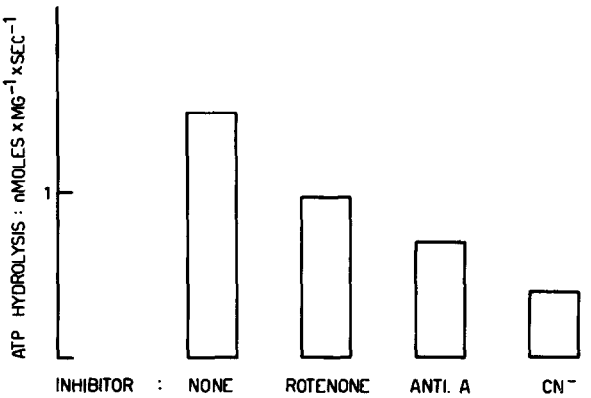

Fig. 2. Calcium induced ATP hydrolysis. Effect of respiratory inhibitors on the initial rate of ATPase activity. The reaction system contained 10 mM Tris–Cl (pH 7.4), 50 mM NaCl, 50 mM KCl, 5 mM ATP, and rat liver mitochondria (5 mg protein) in total volume of 2.0 ml. Incubated at 21°C. The respiratory inhibitors were: rotenone 0.07 $\mu g \times mg^{-1}$ protein, antimycin A 0.14 $\mu g \times mg^{-1}$ protein, and KCN 100 μM. 160 μM $CaCl_2$ was added to start the reaction.

operationally distinguishable 'interaction points' whose binding capacity appears not correlated with the velocity of the ion flux. This conclusion is also supported by the experiments in which the driving force for calcium transport is derived from the hydrolysis of ATP. In the experiments of fig. 2 the initial rate of calcium-activated ATPase has been measured during different redox states of the respiratory carriers. As shown, the initial velocity of ATP hydrolysis progressively diminishes following the reduction of respiratory carriers: from the value of 1.5 nmoles (per sec, per mg protein), the rate of the reaction declines to 1, 0.55, and 0.4 upon addition of rotenone, antimycin A, and cyanide, respectively. It is noteworthy that this effect of respiratory inhibitors is in accordance with earlier findings on ATP-linked calcium accumulation. In fact, Bielawsky and Lehninger [8] reported that the respiratory inhibitors produce some significant differences in the rates of calcium uptake and in the oscillations (H^+ bounces) of proton movements accompanying the translocation.

With these results at hand, since the ATPase reaction is connected with reversible steps in the energy-transducing process in which the respiratory carriers are involved, it would follow that also the reverse process of oxidative phosphorylation occurs through the operation of transducing devices which can be functionally discriminated from each other.

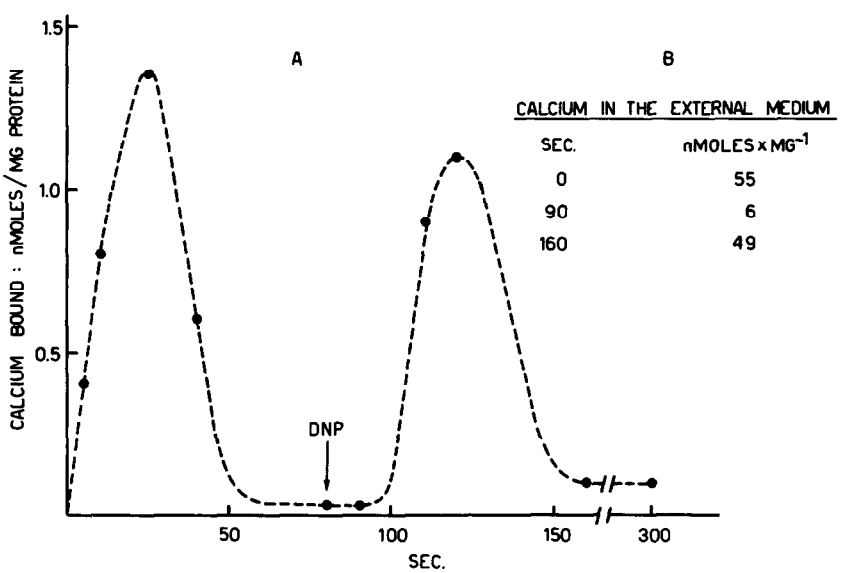

Fig. 3. Time course of calcium binding. Effect of dinitrophenol. Experimental conditions as in table 1. (A) At the point indicated by the arrow 5×10^{-5} M DNP was added to the system. (B) Net calcium accumulation measured in separate vessels by following the disappearance of radioactive ^{45}Ca from the suspending medium.

3.3. *Release of bound calcium*

One of the crucial points in the understanding of the mechanism of calcium movements across the mitochondrial membrane has so far received little attention: the question has never been definitely answered as to whether the flux of calcium follows the same route in either directions: inward and outward. With this respect, Vasington et al. [9] and Rossi et al. [10] have produced some indirect evidence, by using ruthenium red, that both the entrance and the exit of the cation occurs through the same route. In the experiment reported in fig. 3 the time course of respiration driven binding of calcium is shown. As illustrated in the figure, the maximum binding is reached after about 30 sec, and is followed by the release of calcium from its binding sites, with the simultaneous disappearance of the ion from the suspending medium. The addition to the system of DNP after the completion of the net accumulation evokes a prompt rebinding of the cation; subsequently, the binding falls off again and is accompanied by the back release to the external medium of almost all of the previously accumulated calcium. These data give the first direct evidence that the interaction of calcium with mitochondria occurs through the *same* transport (or binding) system, both during the entrance and the exit of calcium.

4. Conclusion

The results discussed in this paper permit the following conclusions. First, the binding of calcium to the mitochondrial structure is detectable during the operation of calcium pump, that is, before the completion of the translocating event. This binding is stoichiometrically related to the number of energy conserving sites; about 0.7 nmoles of calcium are bound (per mg protein) per energy conserving site. The process seems to be mediated by three distinct components of the energy transducing machinery. This last conclusion is also supported by the finding that the rate of calcium-induced ATP hydrolysis is third by third reduced following the corresponding stepwise reduction of the respiratory carriers, as evoked by rotenone, antimycin A, and cyanide.

Second, the interaction of calcium with mitochondria occurs at the level of the same component, both during the entrance and the exit of the ion.

Acknowledgement

The authors wish to thank Mr. Paolo Dalan for his excellent technical assistance.

References

[1] Lehninger, A. L. (1971) Biochem. Biophys. Res. Commun. 42, 312.
[2] Carafoli, E., Gazzotti, P., Vasington, F. D., Sottocasa, G. L., Sandri, G., Panfili, E. and De Bernard, B. (1972) in: Biochemistry and Biophysics of Mitochondrial Membranes. (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E. and Siliprandi, N., eds.) p. 215. Academic Press, New York.
[3] Rossi, C. S., Alexandre, A. and Rossi, C. R. (1973) in: Cytopharmacology of Secretion (Clementi, F., ed.) Raven Press, New York.
[4] Alexandre, A., Rossi, C. S. and Rossi, C. R. in: Calcium binding proteins (Drabikowski, W. and Carafoli, E. eds.) Polish Academy of Sciences, Warsaw, 1974.
[5] Schneider, W. C. (1857) in: Manometric Techniques (eds. Umbreit, W. W., Burris, R. and Sauffer, J. E.) Burgess Publishing Co., p. 188.
[6] Alexandre, A., Rossi, C. R. and Carignani, G. (1973) in: Cytopharmacology of Secretion (ed. Clementi, F.) Raven Press Publishers, New York.
[7] Rossi, C. S. and Lehninger, A. L. (1964) J. Biol. Chem. 239, 3971.
[8] Bielawsky, J. and Lehninger, A. L. (1966) J. Biol. Chem. 241, 4316.
[9] Vasington, F. D., Gazzotti, P., Tiozzo, R. and Carafoli, E. (1972) Biochim. Biophys. Acta, 256, 43.
[10] Rossi, C. S., Vasington, F. D. and Carafoli, E. (1973) Biochem. Biophys. Res. Commun. 50, 846.