The Initial Velocities of Calcium Uptake by Rat Liver Mitochondria*

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SUMMARY

Initial velocities of energy-dependent Ca++ uptake were measured in rat liver mitochondria by stopped flow techniques and dual wavelength spectroscopy in the presence of the Ca++ indicator murexide.

The initial rate of energy-dependent Ca++ uptake by mitochondria was 0.1 to 0.3 nmole per s per mg of protein at 5 to 15 μM Ca++, and increased to 8.2 nmoles of Ca++ per s per mg at 200 μM Ca++. When initial rates of Ca++ uptake by mitochondria were plotted against the Ca++ concentration in the medium, a sigmoidal curve was obtained in which half-maximal rate of Ca++ transport occurred at Ca++ concentration of 55 to 70 μM. A Hill plot of the data yields a straight line with slope, n, of 1.63. Comparable values of Ca++ transport were obtained when Ca++ was added to mitochondria oxidizing either succinate or glutamate and malate. Similar sigmoidicity was shown with Mn++ uptake; however the maximal rate of accumulation was 3.8 nmole of Mn++ per s per mg of protein and the half-maximal rate was reached at 170 μM Mn++. A model, which takes into account previous and present data, is presented for the uptake of Ca++ and Mn++ in liver mitochondria. The results obtained and the proposed mechanism are discussed in terms of the physiological regulation of cellular [Ca++] homeostasis in liver cells.

Energy-dependent Ca++ uptake by mitochondria has attracted much attention by several investigators. Extensive studies by the group of Chance (1, 2) and Lehninger (3, 4) have clearly indicated an obligatory relationship between Ca++ accumulation and energy utilization at the level of the respiratory chain. The recent findings of Mela (5) that Ca++ uptake by mitochondria is inhibited by lanthanides have suggested that the process of Ca++ uptake is mediated by a carrier localized in the inner membrane compartment. Similar conclusions have been proposed by Reynafarje and Lehninger (6) who equate the so-called "high affinity" binding sites for Ca++ in respiration-inhibited mitochondria with the carrier sites. Although the occurrence of a carrier for Ca++ transport in mitochondria is widely accepted (2, 3, 7) there are discrepancies concerning the number of carrier sites and their affinity for Ca++ (8). Furthermore, detailed studies on the binding of Ca++ to the hypothetical carrier have not been made.

The affinity of the transport system for Ca++ has been studied thus far by means of either 4Ca++ uptake (9, 10) or measurements of events occurring simultaneously with Ca++ uptake, such as increase in rate of oxygen consumption (1) or shift of oxidation-reduction state of cytochrome b or flavoprotein (11-13). All of these methods are indirect or obtained under ill-defined kinetic conditions and the value obtained for the Km of the Ca++ transport ranges between 1 and 50 μM (9-11, 13).

In this paper, we present data on the initial velocity of Ca++ uptake by isolated rat liver mitochondria, measured by stopped flow techniques using suitable calcium indicators. This approach provides a direct measurement of the initial velocity of Ca++ uptake and a more reliable value of the Ca++ at which half-maximal activation of the transport system occurs. Furthermore, the kinetic data obtained give insight on the interaction of Ca++ with the transport system and on the over-all process of Ca++ translocation in mitochondria. A preliminary report of these data has been presented (14).

EXPERIMENTAL PROCEDURE

Mitochondria were prepared from rat liver homogenates as described previously (15) in a medium containing 0.25 M sucrose and 1 mM EDTA (pH 7.2). The same medium was used for the first two washings and a 0.25 M sucrose solution, which was denaturated and contained less than 0.2 μM Ca++, was used for the last washing and final suspension of mitochondria. The content of Ca++ in such mitochondrial preparations was measured by atomic absorption spectrophotometry and varied from 10 to 15 nmole of Ca++ per mg of protein. Protein was determined by the biuret method (10) with crystalline bovine serum albumin used as a standard.

Ca++ uptake by mitochondria was measured spectrophotometrically in reaction mixtures containing 0.15 M sucrose, 0.075 M KCl, 5 mM K2HPO4 (pH 7.3), 2 mM MgCl2, 7 mM sodium succinate, 3 μM rotenone, 35 μM murexide, and 2 to 3 mg of mitochondrial protein per ml (17). The same medium was used...
by mitochondria were measured after addition of different amounts of Ca++. The result of such experiments is shown in Fig. 2. The initial rate of Ca++ uptake by mitochondria, when plotted against Ca++ concentration in the medium, results in a slope of sigmoidal shape and a nonlinear Lineweaver-Burk plot. However, by plotting the square of Ca++ ion concentration on the abscissa instead of Ca++ concentration (Fig. 2B), the same data produce an hyperbolic curve and a linear double reciprocal plot. In different preparations of rat liver mitochondria, half-maximal rate of Ca++ transport was obtained at concentration of $60 \pm 10 \mu M$ and the maximal velocity of the Ca++ transport was $8 \times 13$ nmoles of Ca++ per s per mg of protein. Similar values were also obtained when Ca++ was added to mitochondria oxidizing glutamate and malate instead of succinate. Two suggestions can be drawn from this result. (a) The saturation of the initial rate of Ca++ transport, which occurs at about $200 \mu M$ Ca++, suggests the existence of carrier-mediated transport.

(b) The sigmoidicity of the curve indicates cooperativity in Ca++ transport, namely the existence of more than one binding site for the Ca++ on the carrier. By plotting the initial velocities of Ca++ uptake against the Ca++ concentration in the form of the Hill plot, a good straight line with a slope, $n$, of 1.63 was obtained (not shown).

Since Ca++ can be bound to the surface of rat liver mitochondria in the absence of an energy source (23), the above experiments were carried out in a medium containing Mg++ and K++. We have previously reported that the presence of 2 mM MgCl$_2$ and 50 mM KCl decreases by more than 90% the amount of Ca++ bound to energy-independent sites of mitochondria, without interfering significantly with the energy-linked Ca++ transport (23, 24). Fig. 3 shows that in our conditions the Ca++ bound to energy-independent binding sites is negligible. Identical deflections and linearity were obtained by adding micromolar amounts of Ca++ either to the medium without mitochondria or to the medium supplemented with mitochondria and inhibitors of respiration (antimycin A) and uncouplers of respiration (FCCP), under conditions in which the energy-dependent accumulation of Ca++ was abolished.

Since mitochondria are able to accumulate other divalent cations in a process which requires energy (1-4, 25), we investigated whether the uptake of other divalent cations shows a cooperativity similar to that described for Ca++. Fig. 4 shows the initial rates of the energy-dependent accumulation of Mn++ as a function of Mn++ concentration in the medium. The curve obtained has a sigmoidicity similar to that described for Ca++ and an hyperbolic curve was obtained by plotting Mn++ uptake versus the square of Mn++ concentrations. In this case, the half-maximal rate was reached when Mn++ concentration was $170 \mu M$ and maximal rate of accumulation was $4 \times 5$ nmoles per s per mg of protein. Because qualitatively similar results were obtained with Sr++, we suggest that a similar mechanism is operative for the transport of Ca++, Mn++, and Sr++ in rat liver mitochondria.

If a similar transport system is present for Mn++ and Ca++, one should expect enhanced transport of one cation in the presence of the other cation, since cooperativity is observed in the transport of both ions. Fig. 5 shows the result of an experiment in which the titration of the initial rates of Mn++ accumulation at different Mn++ concentrations were compared with that obtained with mitochondria preloaded with small amounts of Ca++. Preloading of mitochondria with Ca++ not only increases the initial rate of Mn++ accumulation, but also changes the apparent shape of the titration curve. The sigmoidal shape was substituted by a hyperbole and, for any Mn++ concentrations, the maximal rate of Mn++ transport was significantly higher than in the control experiment. This result provides further support for the operation of a single divalent cation

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1. The abbreviation used is: FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.
Fig. 2. Initial velocities of Ca++ uptake at different Ca++ concentrations. The experimental conditions were similar to that of Fig. 1, except for the protein concentration which was 1.9 mg per ml. The reaction was started by discharging in the reaction mixture the amounts of Ca++ indicated in the abscissa. Initial velocity refers to the amount of Ca++ taken up by mitochondria during the first 200 ms. Every point represents the average of three different experiments obtained with the same preparation of mitochondria within a few hours. Due to the low concentration of murexide present and the high K_D of the calcium-murexide complex, under our conditions the chelation of Ca++ with murexide results in negligible pH changes (less than 0.01 pH unit) even at the highest Ca++ concentrations.

Fig. 3. Ca++-induced absorbance changes of murexide in the presence (A) and in the absence (B) of mitochondria. Reaction mixture was as in Fig. 1, except for the murexide which was 60 PM. The medium was also supplemented with 5 μM FCCP and 10 μg of antimycin A per ml and, where indicated, with 2.7 mg per ml of mitochondria.

DISCUSSION

Initial Velocities of Ca++ Uptake—Stopped flow techniques and spectrophotometric detection of the changes in absorbance of murexide provide a valuable tool for the measurement of initial rates of Ca++ binding and Ca++ transport in biological systems (17-19, 22). The intrinsic properties of murexide (higher extinction coefficient (17-19), fast complexation rate with Ca++ (26), extramitochondrial localization (19), and lack of side effects on mitochondrial properties and function (19)) and the availability of sensitive spectrophotometric techniques (20) make possible fast detection of 1 to 2 μM [Ca++] transients in the presence of mitochondrial suspensions. Under our conditions, the amount of Ca++ added for each experiment is close to if not identical with the concentrations of Ca++ free in the medium. This condition was attained: (a) in the presence of a few mM Mg++, which effectively competes with Ca++ for the energy-
Fig. 4. Titration of the initial rate of Mn$^{2+}$ uptake versus [Mn$^{2+}$]. Experimental conditions were as in Fig. 1, except that the reaction was started with Mn$^{2+}$ instead of Ca$^{2+}$ and the mitochondrial protein was 2.2 mg per ml.

Fig. 5. Activation of Mn$^{2+}$ transport by Ca$^{2+}$. Experimental conditions were as in Fig. 1, except that the reaction was started with Mn$^{2+}$. The top curve is an experiment where the mitochondria were incubated with 30 nM Ca$^{2+}$ 45 s prior to the injection of Mn$^{2+}$. Parallel experiments show that at that time all the Ca$^{2+}$ added is accumulated and retained by mitochondria.

Our values for $V_{\text{max}}$ and for initial velocities of Ca$^{2+}$ uptake are higher than previous data obtained with $^{45}$Ca$^{2+}$ and separation of mitochondria through filtration or centrifugation (9, 10). This discrepancy may be ascribed to the fact that initial velocity, and not the average of progressively decreasing velocities, was measured. The Ca$^{2+}$ concentration required for the half-maximal activation of Ca$^{2+}$ uptake was between 50 and 70 nM. This figure is between one and two orders of magnitude greater than that reported recently by Carafoli and Asai (13) and Bygrave et al. (9) and very close to the value reported by Chance and Schoener (11).

A Model for Ca$^{2+}$ Transport in Mitochondria—The linearity of the double reciprocal plot, when Ca$^{2+}$ uptake is plotted versus the square of [Ca$^{2+}$] in the medium, and a Hill coefficient approaching 2 indicates that at least 2 molecules of Ca$^{2+}$ must be bound to the transport system to have Ca$^{2+}$ transported at significant rates. Alternately, our kinetic data could be explained in terms of product inhibition which would be overcome at higher substrate concentrations. Although our kinetic data fail to discriminate between cooperativity and product inhibition, the latter possibility can be excluded by the experiment of Fig. 5.

Cooperative interaction has been postulated by Robinson (27) and by Tobin et al. (28) for the binding of Na$^+$ and K$^+$ to the (Na$^+$ + K$^+$)-ATPase and by Bygrave et al. (9) for the ATP-dependent Ca$^{2+}$ in mitochondria. Although our data confirm the concept of cooperativity for Ca$^{2+}$ transport in mitochondria proposed by Bygrave et al. (9), they indicate that the apparent $K_m$ for Ca$^{2+}$ transport is one to two orders of magnitude higher. This discrepancy can be attributable to the different experimental conditions, namely the lack of kinetic parameters and the presence of ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetate-Ca$^{2+}$ buffers in previous experimental conditions.

On the basis of the results presented, we propose that the transport and the binding of Ca$^{2+}$ (and Mn$^{2+}$) in mitochondria follow the scheme presented in Fig. 6. According to this model, the transport system, T, located within the mitochondria inner membrane binds 2 calcium ions and the complex moves across the membrane. By contrast, the mobility of the complex $T$ with 1 calcium ion is very slow or absent (Fig. 6A). In this view, the binding constant of $T$ for Ca$^{2+}$ corresponds to the apparent $K_m$ and the $V_{\text{max}}$ is dictated by the mobility of the complex across the membrane and by the over-all process of Ca$^{2+}$ translocation. As shown in Fig. 6B, a similar mechanism applies to the transport of Mn$^{2+}$; in this case both the affinity of the complex and the over-all translocation rate are lower. The
mobility of the mixed complexes (Fig. 6C) is higher than that of the "pure" Mn++ complex.

The above model accounts for the stoichiometry Ca++-ATP of 2 observed by Rossi and Lehninger (30). Furthermore, the previous observations of Mela and Chance (19) and of Ernster and Nordenbrand (29), that small concentrations of Ca++ enhance and accelerate Mn++ uptake, can be easily explained in terms of the described mobility of T with either Ca++ or Mn++ or both. Although this model accommodates most of the data available in the literature confine cytosolic Ca++ transport when, in response to extra or intracellular events, the cytosolic Ca++ concentration rises.

physiological role with respect to ADP phosphorylation. While the ability of ADP and phosphate to act as chemical high energy intermediates, all of the data available in the literature on Ca++ transport, it fails to determine whether the operation of a transport system for Ca++ in mitochondria results from a chemical high energy intermediate (31, 32) or from electrochemical potential (33).

Cooperativity of Ca++ Uptake and Physiological Significance—The discussion of the proposed mechanism of Ca++ uptake in mitochondria in terms of physiological significance remains speculative. This is mainly due to the present lack of knowledge of (a) the exact concentration of Ca++ within the cytosol of liver cells and its compartmentation, and (b) the physiological role of mitochondrial Ca++ accumulation.

It has been shown that the transport of Ca++ and the phosphorylation of ADP in mitochondria are alternative processes and that the affinity of the energy-conserving sites of the respiratory chain for Ca++ is higher than that of ADP and phosphate (30). The data presented show that Ca++ uptake in isolated mitochondria is slow or absent until the Ca++ concentration reaches 10 to 15 μM, whereupon it increases in a sigmoidal fashion. If the extrapolation of these in vitro data to in vivo conditions is permitted, we propose that, when the levels of cytosolic Ca++ are lower than 10 μM, the energy production by mitochondria will not be affected by Ca++. In contrast, at higher levels of cytosolic [Ca++], Ca++ transport will assume a more significant role with respect to ADP phosphorylation. Although the measurements of Ca++ concentrations within liver cells are few and not supported by firm experimental evidence, all of the data available in the literature confine cytosolic Ca++ levels to values of 0.1 to 10 μM (for a review see Reference 34). This concentration would accommodate both alternative functions of mitochondria within liver cells: ADP phosphorylation at the lower Ca++ levels and Ca++ transport when, in response to extra or intracellular events, the cytosolic Ca++ concentration rises.

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REFERENCES

Fig. 6. A scheme to illustrate the proposed mechanism of energy-dependent Ca++ or Mn++ transport or both by rat liver mitochondria.

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