Cooperative Interactions in Energy-dependent Accumulation of Ca^{2+} by Isolated Rat Liver Mitochondria

THE energy-dependent accumulation of Ca^{2+} by isolated rat liver mitochondria is intimately associated with oxidative phosphorylation[1]. Available evidence supports the idea that, like the permeases postulated for some mitochondrial metabolites[2], this active accumulation of Ca^{2+} may involve a "carrier" in the mitochondrial membrane specific for Ca^{2+} (ref. 3). Several studies have shown that the energy-independent "binding" of Ca^{2+} to sites on the (inner membrane of), intact mitochondria and of submitochondrial particles exhibits hyperbolic saturation curves as a function of Ca^{2+} concentration[4,5].

We now present evidence that the energy-dependent accumulation of Ca^{2+}, in contrast to its binding by rat liver mitochondria, exhibits sigmoid kinetics with respect to Ca^{2+} concentrations. This is suggestive of cooperative behaviour[6].

Representative data (Fig. 1) show the influence of increasing concentrations of total added Ca^{2+} on the respiration-supported accumulation of Ca^{2+}. The curve relating these two parameters deviates from the Langmuir isotherm and is strongly sigmoidal; this is further indicated by the nonlinearity of the Lineweaver–Burk plot. The reaction medium contained 6 mM ATP enzyme hydrolysis of which was blocked by the addition of oligomycin. Using a value[7] of 32,000 at *p*H 6.97 for the stability constant of $CaATP^{2-}$, we determined the concentration of "added" and of "free" Ca^{2+}, assuming $CaATP^{2-}$ to be the predominant species of chelated Ca^{2+}. As Fig. 2 shows, the ATP bound about 99% of the total added Ca^{2+}. Moreover, the displacement of the concentration curve, resulting from chelation of Ca^{2+} by ATP, is equivalent for all concentrations of "free" Ca^{2+}; the plot relating "added" Ca^{2+} to "free" Ca^{2+} is linear. Thus the data obtained and expressed as a function of total added Ca^{2+} reflect a situation which occurred at very much lower concentrations of "free" Ca^{2+}. Hill plots of the data shown in Fig. 2 give parallel lines with a value for the Hill coefficient of 1.8 and values for half-maximal saturation ("K_s") of 140 μM and 2 μM for "total" and "free" Ca^{2+}, respectively. Because the amount of Ca^{2+} accumulated is much greater than the amount of "free" Ca^{2+} present in the lower concentration range, these values are not necessarily kinetic parameters and therefore do not necessarily represent Michaelis constants. On the other hand, we could have been measuring the initial linear rate of accumulation,

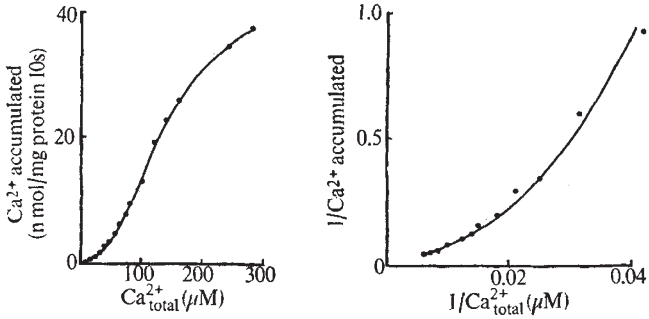

Fig. 1 Ca^{2+} accumulation as a function of increasing concentration of added Ca^{2+} was measured in a medium containing 200 mM sucrose, 15 mM Hepes buffer (*p*H 7.4), 2 mM succinate, 6 mM ATP, 1 μg of oligomycin, 1.2 mg of rat liver mitochondria protein and increasing amounts of Ca^{2+} (containing 0.1 μCi of $^{45}Ca^{2+}$) as indicated. The final volume was 0.5 ml. and the temperature was 25° C. All components except Ca^{2+} were preincubated for 1 min. The reaction was initiated by the addition of Ca^{2+} and after 10 s 0.10 ml. of the suspension was removed rapidly and the mitochondria were removed by 'Millipore' filtration. The filters were rapidly washed, then dried and counted for radioactivity by liquid scintillation. Control experiments[12] have shown that the use of $^{45}Ca^{2+}$ reflects net movement of Ca^{2+} and not an isotopic exchange.

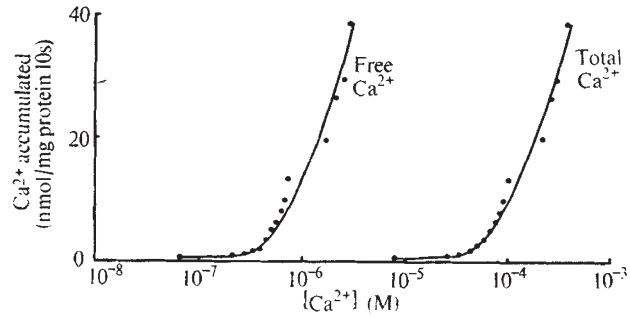

Fig. 2 Ca^{2+} accumulation expressed as a function of total "added" and of "free" Ca^{2+} concentration in the reaction medium described in Fig. 1.

for the concentration of free Ca^{2+} may remain virtually constant over the 10 s incubation period as a result of the $CaATP^{2-}$ complex acting as a "Ca^{2+} sink". In any case, our data provide reliable information about the high affinity of the accumulation process for "free" Ca^{2+}. With these data in mind we examined the accumulation of Ca^{2+} in the system described in Fig. 1 in the absence of added ATP, at 0° C and for a greater range of Ca^{2+} concentration. The curve relating Ca^{2+} accumulation to Ca^{2+} concentration was sigmoidal, and the Hill plot gave a value of 1.73 for the Hill coefficient. Thus the cooperativity we have observed seems to be a function of the active accumulation process and is not simply a function of, for example, chelation of the added Ca^{2+} by ATP. Our affinity data for the accumulation process are in excellent agreement with those of Carafoli[8], who reported that the K_m for Ca^{2+} was of the order of 10^{-6} M (compare ref. 3).

It has been shown[1] that two Ca^{2+} ions are accumulated by rat liver mitochondria for each high-energy "bond" utilized. Our finding that the energy-dependent accumulation of Ca^{2+} is apparently associated with cooperative behaviour in the mitochondrial membrane(s) with values of the Hill coefficient approaching 2 suggests that the interaction of one Ca^{2+} ion with components of the membrane facilitates the interaction of a second Ca^{2+} ion. Recently, evidence has been presented for cooperative interactions between the Na^+ binding sites[9] and between the K^+ binding sites[10] of the $(Na^+ + K^+)$–ATPase. It would be interesting to determine whether other metabolite and ion accumulation/exchange processes in the mitochondrial membrane[2], as well as processes linked with the mechanism of oxidative phosphorylation, also exhibit cooperativity. Finally, these data provide further evidence to support the idea that intercellular movements of metal ions are important in metabolic regulation[1,11].

We thank Dr D. D. Perrin for assistance with some computations.

F. L. BYGRAVE
K. C. REED
T. SPENCER

Department of Biochemistry, Australian National University, Faculty of Science, Canberra

Received June 4, 1970.

1 Lehninger, A. L., Carafoli, E., and Rossi, C. S., *Adv. Enzymol.*, **29**, 259 (1967).
2 Klingenberg, M., *FEBS Lett.*, **6**, 145 (1970).
3 Reynafarje, B., and Lehninger, A. L., *J. Biol. Chem.*, **244**, 584 (1969).
4 Scarpa, A., and Azzone, G. F., *Biochim. Biophys. Acta*, **173**, 78 (1969).
5 Jacobus, W. E., and Brierley, G. P., *J. Biol. Chem.*, **244**, 4995 (1969).
6 Monod, J., Changeux, J. P., and Jacob, F., *J. Mol. Biol.*, **6**, 306 (1963).
7 O'Sullivan, W. J., and Perrin, D. D., *Biochemistry*, **3**, 18 (1964).
8 Carafoli, E., *Biochem. J.*, **116**, 2*P* (1970).
9 Tobin, T., Banerjee, S. P., and Sen, A. K., *Nature*, **225**, 745 (1970).
10 Robinson, J. D., *Nature*, **220**, 1325 (1968).
11 Bygrave, F. L., *Nature*, **214**, 667 (1967).
12 Reed, K. C., and Bygrave, F. L., *Proc. Austral. Biochem. Soc.*, 34 (1970).