high anion concentrations, a form which might possibly be crystallized from solution, would retain six zinc ions, and would have a totally symmetric structure.

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Kinetics of Ca^{2+} Carrier in Rat Liver Mitochondria†

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ABSTRACT: The rate of aerobic Ca^{2+} transport is limited by the rate of the H^+ pump rather than by the Ca^{2+} carrier. The kinetics of the Ca^{2+} carrier has therefore been studied by using the K^+ diffusion potential as the driving force. The apparent V_{max} of the Ca^{2+} carrier is, at 20 °C, about 900 nmol (mg of protein)$^{-1}$ min^{-1}, more than twice the rate of the H^+ pump. The apparent V_{max} is depressed by Mg^{2+} and Li^+. This supports the view that the electrolytes act as noncompetitive inhibitors of the Ca^{2+} carrier. The degree of sigmoidicity of the kinetics of Ca^{2+} transport increases with the lowering of the temperature and proportionally with the concentration of impermeant electrolytes such as Mg^{2+} and Li^+ but not choline. The effects of temperature and of electrolyte do not support the view that the sigmoidicity is due to modifications of the surface potential. Rather, they suggest that Ca^{2+} transport occurs through a multisubunit carrier, where cooperative phenomena are the result of ligand-induced conformational changes due to the interaction of several allosteric effectors with the carrier subunits. In contrast with La^{3+} which acts as a competitive inhibitor, Ruthenium Red affects the kinetics by inducing phenomena both of positive and of negative cooperativity. The Ruthenium Red induced kinetics has been reproduced through curve-fitting procedures by applying the Koshland sequential interaction hypothesis to a four-subunit Ca^{2+} carrier model.

Mammalian cells possess two main systems to control Ca^{2+} concentration in the cytosol: one, the Ca^{2+}–ATPases of the plasma membrane and endoplasmic reticulum; two, the H^+ pump of mitochondria driving an electrical Ca^{2+} carrier. However, the precise mechanism by which these two transporting systems regulate the Ca^{2+} concentration in the living cell is not understood. In heart Affolter et al. (1976) and Carafoli (1975) have proposed a role of mitochondria in regulating myocardial contraction. In the axoplasm, Brinley et al. (1977) have reported that mitochondria buffer the Ca^{2+}

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concentration around 10^{-6} M. In the liver cell Meli & Bygrave (1972) have shown that Ca^{2+} transport via mitochondria could in principle regulate cytoplasmic Ca^{2+}-sensitive enzymes.

Studies on liver mitochondria in vitro have shown that the distribution of free Ca^{2+} across the inner membrane in steady state is kinetic rather than thermodynamic in nature (Puskin et al., 1976; Pozzan et al., 1977; Azzone et al., 1977; Nicholls, 1978). Mitochondria appear to possess two pathways, or mechanisms, for influx and efflux. In steady state Ca^{2+} does not reach thermodynamic equilibrium, but its distribution is determined by the relative rates of the pathways or Ca^{2+} influx and efflux.

To understand more precisely the parameters determining the Ca^{2+} distribution, we found that the measurement of the molecular kinetics of the Ca^{2+} carrier becomes crucial. However, information on this question is incomplete and contradictory. Bygrave et al. (1971) and Reed & Bygrave (1975b) found a K_m around 10^{-6} M and observed that the kinetics of the H^+ pump driven Ca^{2+} uptake is sigmoid rather than hyperbolic in nature. Vinogradov & Scarpa (1973) and Scarpa & Graziotti (1973) found sigmoidal kinetics but much higher K_m. Sigmoidal kinetics were also reported by Sordhal (1975) and by Noak & Heinen (1977). Much lower K_m and almost hyperbolic kinetics were obtained by Carafoli & Azzi (1972) and by Carafoli (1975). Ackerman et al. (1977) found that impermeant cations increase the sigmoidicity of the kinetics of Ca^{2+} uptake and interpreted the effects as due to changes of surface potentials. Hutson (1977) found, measuring the kinetics of Ca^{2+} uptake with the Ca^{2+} ionophore A23187, that the n value calculated from Hill plots was higher than 1 also in sucrose.

Comparison between H^+ pump and K^+ diffusion driven Ca^{2+} transport shows that the rate of aerobic Ca^{2+} transport is limited by the rate of the H^+ pump, while the extent of aerobic Ca^{2+} transport depends on the amount of H^+ in the matrix. In the present study we have made use of the K^+ diffusion potential as the driving force for Ca^{2+} uptake.

A major conclusion emerges from this analysis. The sigmoidicity of the kinetics of Ca^{2+} transport does not appear to be an extrinsic phenomenon induced by nonpermeant electrolytes due to surface potential changes. Rather, several electrolytes may be considered as allosteric effectors inducing conformational changes in a multisubunit carrier. The interactions of these effectors may give rise to both positive and negative cooperativity phenomena. The complex pattern of the Ca^{2+} kinetics is best analyzed by using the Koshland sequential interaction model.

Materials and Methods

Mitochondria were prepared in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, pH 7.4, as described by Massari et al. (1972a). Mitochondria were then washed in a sucrose medium devoid of EDTA and supplemented with 0.05% bovine serum albumin.

The rate of Ca^{2+} influx into rotenone-treated mitochondria has been measured by recording with a K^+ electrode the rate of K^+ efflux following the addition of valinomycin (Azzone & Azzi, 1966; Rossi et al., 1967; Scarpa & Azzone, 1970). The rate of K^+ efflux was then calculated by taking into account the logarithmic response of the electrode. The K^+ electrode used in the present study had a diameter of 12 mm (Schott, Mainz). The response time of the electrode was below 0.2 s. The recording apparatus was constituted by a Radiometer pH meter connected with a Texas recorder. The response time of the recorder was 0.5 s. The chart speed during K^+ efflux was 20 cm/min. The experiments were carried out in a glass cuvette with magnetic stirring. Thermoequilibration was obtained by water circulation employing a Colora thermostat. The mixing time of the apparatus was below 0.5 s as indicated by the time required from full equilibration of known amounts of K^+. The sensitivity of the method was dependent on the amount of K^+ added to the medium. At low K^+ concentrations, the recorder pen deflection was 0.4 cm/μM K^+.

Although K^+ efflux leads to an increase of the K^+ concentration in the medium and, thus, a gradual decrease of the driving force for Ca^{2+} uptake, i.e., the K^+ concentration gradient, the rate of K^+ efflux was usually linear for a period of time longer than 5 s at concentrations of free Ca^{2+} above 20 μM. The linearity of the rate of Ca^{2+} efflux is probably due to the relative constancy of the concentration of free K^+ in the matrix; Ca^{2+} entering the matrix becomes bound to phospholipids; this results in a decrease of osmotically active species in the matrix and in mitochondrial shrinkage (Massari et al., 1972b). In the range between 1 and 10 μM free Ca^{2+}, the extent of linearity was reduced although the concentration of free $[Ca^{2+}]_0$ was kept constant by the Ca^{2+} buffer. The Ca^{2+} influx was always calculated on the initial rate of K^+ efflux following the addition of valinomycin. In all experiments it was ascertained that the rate-limiting reaction was constituted by Ca^{2+} and not by K^+ transport. This was done by recording the rate of K^+ efflux at increasing valinomycin concentrations and then selecting a valinomycin concentration at which the rate of K^+ efflux remained constant. That under these conditions K^+ transport was faster than Ca^{2+} transport was further ascertained by adding an H^+ ionophore, such as FCCP, in excess. This resulted in a further increase of the rate of K^+ efflux.

The kinetics at concentrations of free Ca^{2+} between 1 and 10 μM were carried out by using the nitrilotriacetic Ca^{2+} buffers as suggested by Reed & Bygrave (1979b). The values for the concentration of free Ca^{2+} in the presence of nitrilotriacetic acid were taken from Reed & Bygrave (1975a). The use of the Ca^{2+} buffers has three advantages: (a) it provides a constant and known Ca^{2+} concentration independently of the amounts of endogenous Ca^{2+} or of residual EDTA, (b) it maintains a constant concentration of free Ca^{2+} in the medium during the whole process of Ca^{2+} uptake, and (c) it eliminates interference due to passive binding of Ca^{2+} at the outer mitochondrial surface. Nitrilotriacetic acid was preferred to EGTA because its buffering power for Ca^{2+} lies in the region 10^{-5}–10^{-6} M free Ca^{2+}, which is crucial for the kinetics of mitochondrial Ca^{2+} transport. Above 10 μM $[Ca^{2+}]_0$, nitrilotriacetic acid was omitted and the concentration of free Ca^{2+} was calculated by also taking into account the amount of endogenous Ca^{2+} measured by atomic absorption spectrophotometry. The values in the figures refer to the initial rates after correction for the rate of K^+ efflux occurring in the absence of Ca^{2+}. When $[Ca^{2+}]_0$ was above 10 μM, the rate of K^+ leak, either in sucrose or in media containing impermeant cations, was negligible with respect to the rate of the Ca^{2+}-stimulated K^+ efflux. At $[Ca^{2+}]_0$ between 1 and 10 μM, the rate of K^+ leak constituted a more significant portion (up 10–20%) with respect to the Ca^{2+}-stimulated K^+ efflux.

When Mg^{2+} was also added to the medium, it was necessary to recalculate the concentrations of free Ca^{2+} by taking into account the dissociation constants for the Mg^{2+}–nitrilotriacetic acid complex (Sillen & Martell, 1964).

Figure 1 shows a plot for the concentration of free Ca^{2+} in nitrilotriacetic acid in the presence of Mg^{2+}. Since the dissociation constants for Ca^{2+} in nitrilotriacetic acid buffers are

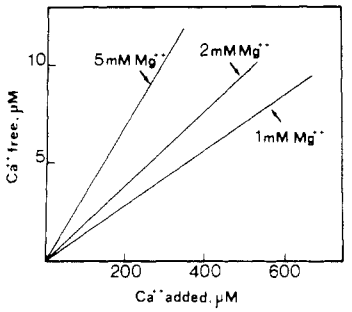

FIGURE 1: Amount of free $[Ca^{2+}]$ in nitrilotriacetic acid buffers in the presence of Mg^{2+}. The concentration of nitrilotriacetic acid was 20 mM at pH 7.4. The concentrations of total Mg^{2+} were 8 and 20.5 mM, yielding a concentration of free Mg^{2+} of 1, 2, and 5 mM, respectively. Calculations are made on the constants reported by Reed & Bygrave (1975a) for the dissociation of nitriloacetic acid and the complexation of Ca^{2+}. For the complexation of Mg^{2+}, it has been assumed that the constant is $10^{5.35}$

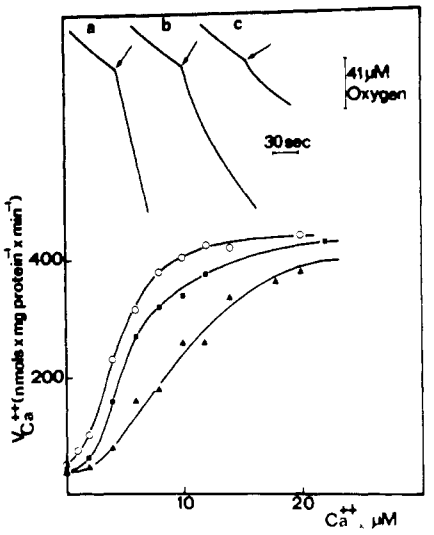

FIGURE 2: Dependence of rate and extent of Ca^{2+} uptake on the availability of H^+ in the matrix. The medium contained 0.2 M sucrose, 20 mM Tris-HCl, pH 7.4, 2 μM rotenone, 2 mM succinate, and 1 mg/mL mitochondrial protein. Temperature was 20 °C. In the upper part the oxygraph traces indicate the respiratory rate following the addition of 10 mM nitrilotriacetic acid and 1 mM $CaCl_2$ under three experimental conditions: (a) with 2 mM P_i; (b) with 10 mM acetate; (c) with 100 μM *N*-ethylmaleimide. In the lower part are the respiratory rates in the presence of (○) 1 nmol of A23187 (mg of protein)$^{-1}$, (■) 2 mM P_i, and (▲) 10 mM acetate.

very sensitive to pH, it was ascertained that, both in the absence and in the presence of Mg^{2+}, the pH remained constant.

Aerobic Ca^{2+} transport was measured with a Clark electrode in a thermoequilibrated cuvette. The rate of O_2 consumption was transformed into the rate of Ca^{2+} transport by assuming a stoichiometry of 4 Ca^{2+}/oxygen with succinate as a substrate.

A23187 was kindly provided by Dr. R. J. Hosley, Lilli Research Laboratories. All chemicals were of analytical grade.

Results

Figure 2 shows the respiratory rates during operation of the H^+ pump driven Ca^{2+} transport. In trace a, addition of Ca^{2+} to mitochondria supplemented with 2 mM P_i resulted in a respiratory rate of about 100 natoms of oxygen (mg of protein)$^{-1}$ min^{-1} which remained linear. A linear respiratory rate was also obtained when Ca^{2+} was added together with A23187 (Reed & Lardy, 1972). In trace b, addition of Ca^{2+} supplemented with 10 mM acetate resulted in a respiratory rate which was initially as rapid as that occurring with P_i and then

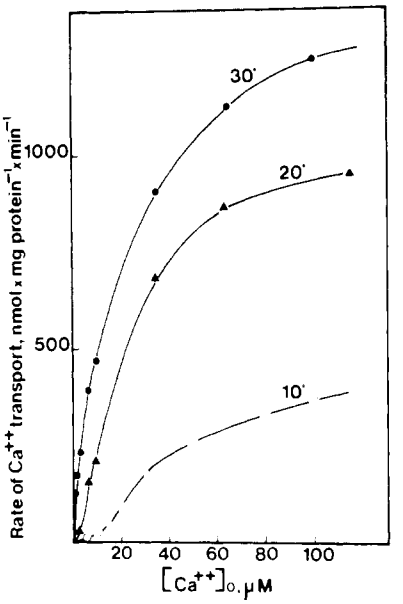

FIGURE 3: Rate of K^+ diffusion driven Ca^{2+} transport in sucrose at various temperatures. The medium contained 0.2 M sucrose, 20 mM Tris-HCl, pH 7.4, 2 mM P_i, 100 μM KCl, 2 μM rotenone, and 1.8 nmol of valinomycin (mg of protein)$^{-1}$. The values below 10 μM $[Ca^{2+}]_0$ were obtained in the presence of 10 mM nitrilotriacetic acid. The amount of mitochondrial protein was 0.5 or 1 mg/mL.

tended to level off. In trace c, addition of Ca^{2+} to mitochondria supplemented with 100 μM *N*-ethylmaleimide resulted in a short stimulation of the respiration which rapidly leveled off. The leveling off of the respiratory rate was less sharp than that observed after addition of a fixed amount of Ca^{2+}, say, 100–200 μM. This is due to our using the nitrilotriacetic acid buffer, which maintains a constant $[Ca^{2+}]_0$. Figure 2 shows the respiratory rates as a function of the Ca^{2+} concentrations in the absence and presence of A23187 or P_i or acetate. The plot was sigmoid in accord with previous reports (Reed & Bygrave, 1975b; Vinogradov & Scarpa, 1973). Furthermore, the respiratory rate increased in the order acetate < P_i < A23187. Since acetate, P_i, and A23187 act by transferring H^+ into the matrix, the results of Figure 2 indicate that the rate of aerobic Ca^{2+} transport is dependent on the rate at which H^+ is translocated in the mitochondrial matrix. This is in accord with a dependence of the rate of the H^+ pump on the H^+ concentration in the matrix. In Figure 2 the respiratory rates, in the presence of *N*-ethylmaleimide, have not been reported, since the extent of respiratory stimulation was too limited to permit accurate measurements (Harris & Zaba, 1977).

Figure 3 shows the rate of Ca^{2+} transport, driven by the K^+ diffusion potential, as a function of the Ca^{2+} concentration. As in the case of the aerobic experiments, the kinetics of Ca^{2+} uptake driven by K^+ diffusion was sigmoid in nature. The sigmoidicity is apparent in either the regular or double-reciprocal plots (not shown). In this latter case the sigmoidicity appears as a concavity directed upward. The extent of sigmoidicity, however, was not constant but increased with the lowering of the temperature. At 30 °C the kinetics was essentially hyperbolical whereas a marked sigmoidicity was observed at 10 °C. At values of temperatures intermediate between those reported in the plot, intermediate degrees of sigmoidicity were observed (not shown). Since all the kinetics of Figure 3 were obtained in sucrose media, the experiment does not support the conclusion of Ackerman et al. (1977) that the sigmoidicity of the kinetics of Ca^{2+} uptake is induced by the presence of nonpermeant electrolytes. Figure 3 shows also

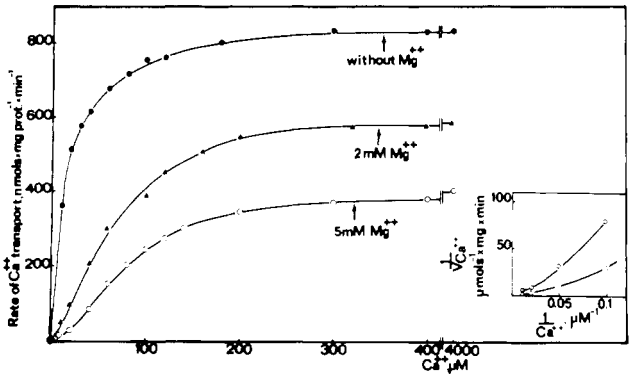

FIGURE 4: Rate of K^+ diffusion driven Ca^{2+} transport in the presence of Mg^{2+}. Experimental conditions are as described in Figure 3. Temperature was 20 °C. Amounts of free Mg^{2+} were as indicated in the figure (2 and 5 mM). The calculation of free Mg^{2+} in the presence of nitrilotriacetic acid was done on the basis of the data of Figure 2. In the insert is a double-reciprocal plot constructed with few significative kinetic values.

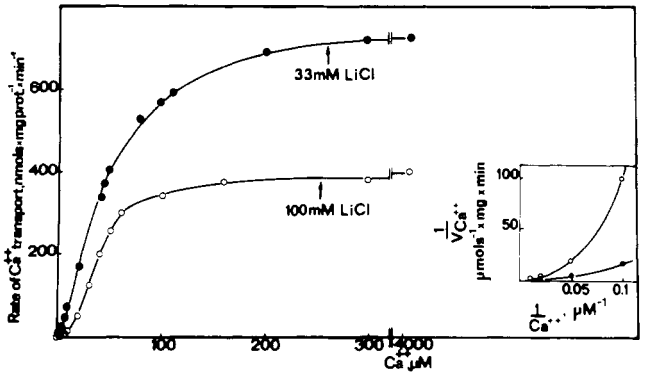

FIGURE 5: Rates of K^+ diffusion driven Ca^{2+} transport in the presence of LiCl. Experimental conditions are as described in Figure 3 except that sucrose was replaced by LiCl in the concentrations indicated in the figure. Temperature was 20 °C. Concentrations of LiCl intermediate between those indicated gave intermediate degrees of sigmoidicity. In the insert is shown a double-reciprocal plot constructed with few significative kinetic values.

that the maximal rates of Ca^{2+} uptake were 1400, 900, and 400 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1} at 30, 20, and 10 °C, respectively. These values are more than twice higher than those observed when Ca^{2+} uptake is driven by the respiratory chain coupled proton pump. This indicates that, when Ca^{2+} uptake is coupled with aerobic H^+ extrusion, the rate-limiting reaction is at the level of H^+ extrusion and not of Ca^{2+} transport.

Since mitochondria under physiological conditions operate in the presence 0.15 M K^+ and about 2 mM Mg^{2+} and since electrolytes have been reported to induce a sigmoid kinetics, the kinetics of Ca^{2+} uptake in the presence of nonpermeant univalent and divalent cations was analyzed. Figure 4 shows the rates of Ca^{2+} uptake as a function of the Ca^{2+} concentration in the absence of Mg^{2+} and in the presence of 2.0 and 5.0 mM Mg^{2+} at 20 °C. In the absence of Mg^{2+} the kinetics was hyperbolical. Addition of Mg^{2+} induced sigmoidicity in the kinetics. The degree of sigmoidicity increased with the increase of Mg^{2+}. The increase of sigmoidicity is indicated in the double-reciprocal plots by an increase of upward concavity. Figure 4 shows also that addition of Mg^{2+} caused a decrease of the V_{max} in the double-reciprocal plots. Vinogradov & Scarpa (1973) have shown that when, in the double-reciprocal plot, $1/[Ca^{2+}]$ is replaced by $1/[Ca^{2+}]^2$, a linear relation is obtained. We have applied this procedure also in the case of data reported in Figures 3 and 4. To obtain, however, a linearization of the double-reciprocal plots, it is necessary to vary the exponent on the abscissa according to the degree of sigmoidicity: i.e., an increased sigmoidicity requires an increase of the exponent on the Ca concentration. Linearization of the double-reciprocal plots allows a more precise determination of the V_{max} and of the apparent K_m for Ca^{2+} transport. The effect of Mg^{2+} is that of decreasing the V_{max} and of increasing the K_m. These effects characterize Mg^{2+} as a noncompetitive inhibitor.

Figure 5 shows the kinetics of Ca^{2+} uptake in the presence of either 30 or 100 mM LiCl. The increase of upward concavity in the double-reciprocal plots indicates that the degree of sigmoidicity increases with the Li^+ concentration. Furthermore, and similarly to the case of Mg^{2+}, there was a decrease of the V_{max} proportional to the increase of Li^+ concentration. These effects also characterize Li^+ as a noncompetitive inhibitor.

To determine more quantitatively the effects of temperature and of impermeant cations on the sigmoidicity, we have inserted the data in Hill plots. Figures 6 and 7 show the effect

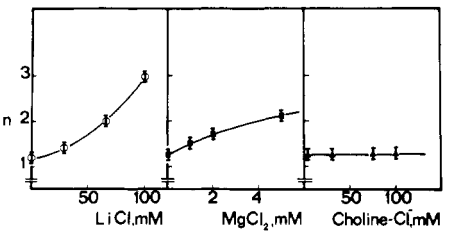

FIGURE 6: Effects of Li^+, Mg^{2+}, and choline$^+$ on the Hill n value. The kinetic values were taken from experiments carried out under the conditions reported in Figures 3–5. The concentration of choline was varied in the same manner as in the case of Li^+. The values for the V_{max} in the Hill plot were calculated from double-reciprocal plots after linearization obtained by inserting an exponent in the Ca^{2+} concentration. The magnitude of the exponent was changed according to the degree of sigmodicity. Each point is the average of at least three to four independent experiments. Temperature was 20 °C.

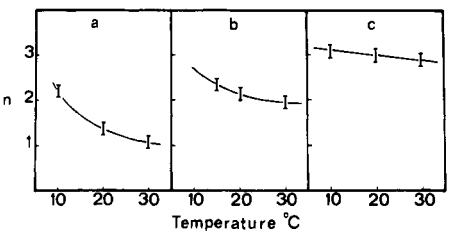

FIGURE 7: Effects of temperature on the Hill n value in the absence and presence of Mg^{2+} and Li^+. The kinetic values were taken from experiments carried out under the conditions reported in Figures 3–5. The values for the V_{max} were calculated as described in Figure 6. Each point is the average of at least three to four experiments. (a) No addition; (b) 2 mM Mg^{2+}; (c) sucrose replaced with 100 mM Li^+.

of temperature and of impermeant cations on the n values as calculated from Hill plots. As seen in Figure 6, whether in the absence or in the presence of impermeant cations, the increase of temperature resulted in a decrease of n from values greater than 2 to values of 1. The effect of temperature on the sigmoidicity was more marked in sucrose. In the presence of impermeant cations, even at 30 °C, the n index was considerably greater than 1. The increase of concentration and Li^+ and Mg^{2+} resulted in an increase of n. At high concentrations of Li^+ and Mg^{2+}, values considerably greater than 2 were obtained. The variability of n as a function of temperature and impermeant cation concentration and the fact that n may by higher than 2 indicate that n should be taken rather as an expression of the minimal number of subunits in the Ca^{2+} carrier. Figure 7c shows that choline had little capacity in inducing an increase of n. Since choline has an effect close

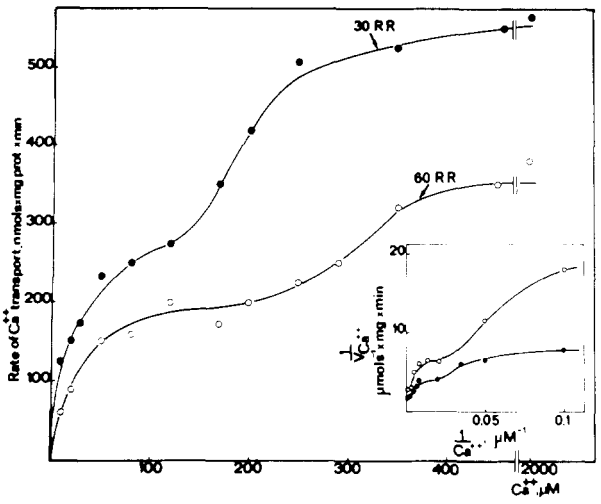

FIGURE 8: Rate of K^+ diffusion driven Ca^{2+} transport in the presence of Ruthenium Red. Experimental conditions are as described in Figure 3. Amounts of Ruthenium Red are 35 and 70 pmol (mg of protein)$^{-1}$; 0.5 or 1 mg of protein per mL. Temperature was 20 °C. In the insert is shown a double-reciprocal plot containing all kinetic values.

to that of Li^+ on the surface potential, this does not support the view of a correlation between sigmoidicity and surface potential (Ackerman et al., 1977).

Figure 8 shows the rates of Ca^{2+} uptake vs. Ca concentrations in the presence of 30 and 70 pmol of Ruthenium Red (mg of protein)$^{-1}$. Ruthenium Red caused a decrease of the V_{max} (Reed & Bygrave, 1974). This also characterizes Ruthenium Red as a noncompetitive inhibitor. Figure 8 further shows that in the plot appear two inflections in the saturation curves. The existence of such inflections, also denoted as "bumps", has already been observed previously in the case of the binding of NAD to glyceraldehyde-3-phosphate dehydrogenase (Conway & Koshland, 1968) and of the kinetics of CTP synthetase (Levitzki & Koshland, 1969) and taken as an evidence for the simultaneous existence of positive and negative cooperativity between the enzyme subunits.

Discussion

H^+ Pump and K^+ Diffusion Driven Ca^{2+} Transport. Large variations are found among various reports on the rate of aerobic Ca^{2+} transport. Heaton & Nicholls (1976) calculated a membrane conductance for Ca^{2+} of 1 nmol (mg of protein)$^{-1}$ min^{-1} mV^{-1} from the steady rate aerobic Ca^{2+} transport in the presence of A23187 but considered the possibility that the rate of Ca^{2+} transport may be limited by the rate of H^+ extrusion rather than by the Ca^{2+} carrier. A similar view was advanced by Hutson (1977) on the basis of the dependence of the aerobic V_{max} on the type of substrate. On the other hand, Reed & Bygrave (1975b) proposed that the rate-limiting step for Ca^{2+} transport be the release of Ca^{2+} bound to the carrier in exchange with matrix H^+.

K^+ gradient has been used to drive energy-requiring reactions in several instances: (a) Ca^{2+} uptake (Azzone & Azzi, 1966; Rossi et al., 1967; Scarpa & Azzone, 1970); (b) ATP synthesis (Cockrell et al., 1967; Rossi & Azzone, 1970; Azzone & Massari, 1971); (c) reversal of electron transport (Cockrell et al., 1967; Conover, 1971). Ca^{2+} translocation in mitochondria is generally accepted to take place via a native carrier. Interaction of Ca^{2+} with the carrier originates a charged complex which translocates Ca^{2+} across the membrane, the driving force being an electrical field. The Ca^{2+} carrier is a membrane constituent independent of electron transport and ATPase complexes. It is therefore immaterial whether the electrical field driving Ca^{2+} influx is generated by H^+ extrusion coupled to electron transport or by K^+ diffusion down a K^+ gradient. That valinomycin-catalyzed K^+ diffusion and respiration-catalyzed H^+ extrusion drive Ca^{2+} transport through a similar molecular mechanism is supported by the observation that they are both inhibited by the specific Ca^{2+} carrier inhibitors, Ruthenium Red and La^{3+} (Scarpa & Azzone, 1970). Further support is provided by the observation that the charge stoichiometry is 2 K^+/Ca^{2+} in the case of the K^+ diffusion driven process and 2 H^+/Ca^{2+} for the H^+ pump driven process (Azzone & Azzi, 1966; Rossi et al., 1967; Azzone et al., 1977b; Reynafarje & Lehninger, 1977). K^+ diffusion driven Ca^{2+} transport possesses some limitation with respect to H^+ pump driven transport: (a) only initial kinetics and not steady-state kinetics can be analyzed; (b) the effect of high $[K^+]_0$ cannot be analyzed; and (c) the dimension of the K^+ gradient decreases with aging since mitochondrial storage is accompanied by K^+ leakage.

The V_{max} of K^+ diffusion Ca^{2+} transport, at 20 °C in sucrose, is over 900 nmol (mg of protein)$^{-1}$ min^{-1}. The Ca^{2+}-stimulated respiration is, under the same conditions, 100 natoms (mg of protein)$^{-1}$ min^{-1}, corresponding to a rate of Ca^{2+} transport of 400 nmol (mg of protein)$^{-1}$ min^{-1}, at a ratio of 4 Ca^{2+}/oxygen with succinate as substrate. Thus, during H^+ pump driven Ca^{2+} transport, the Ca^{2+} carrier operates at less than half its maximal rate. This indicates that during H^+ pump driven Ca^{2+} transport the rate of exchange of Ca^{2+} with matrix H^+ is not rate limiting (Reed & Bygrave, 1975b) and that the Ca^{2+} conductance is underestimated by at least a factor of 2 (Heaton & Nicholls, 1976). Other parameters are also likely to exert their effects on the H^+ pump rather than on Ca^{2+} transport. Also, the extent of Ca^{2+} influx depends, under aerobic conditions, on the amount of H^+ in the matrix. This explains why the respiratory stimulation due to Ca^{2+} is linear only when there is a continuous influx of H^+ into the matrix as induced by weak acids or by A23187. The difference between P_i and acetate is that, in the presence of P_i, Ca^{2+} is accumulated as $(Ca)_3(PO_4)_2$ precipitate, while in the presence of acetate Ca^{2+} is accumulated as osmotically active $Ca(OAc)_2$. Only a minuscule amount of Ca^{2+} can be transported without regeneration of H^+ in the matrix (Harris & Zaba, 1977). The previous conclusion, namely, that mitochondria are capable of an aerobic Ca^{2+} uptake of 50–70 nmol (mg of protein)$^{-1}$, in the absence of permeant anions, is presumably due to the absence of SH inhibitors required to abolish the reuptake of the endogenous-released P_i.

Kinetics of the Ca^{2+} Carrier. Information on the kinetics of the Ca^{2+} carrier is still preliminary. Vinogradov & Scarpa (1973) find Mg^{2+} to be a competitive inhibitor of Ca^{2+} transport. Other electrolytes affect the extent of sigmoidicity of the kinetics more than the V_{max}. Hutson (1977) also observes a decrease of the apparent K_m and a constant V_{max} with 1–2 mM Mg^{2+} and concludes a competitive inhibition by Mg^{2+}. Ackerman et al. (1977) find competive inhibition by Mg^{2+}, K^+, and La^{3+}. As to the sigmoidicity, Vinogradov & Scarpa (1973) find a value of n, from Hill plots, of 1.63 in the presence of K^+ and Mg^{2+} and suggest the presence of at least two subunits in the Ca^{2+} carrier. Reed & Bygrave (1975b) find a Hill coefficient of 1.7 in sucrose at 0 °C; the Hill coefficient undergoes a slight increase in the presence of acetate, but it is not changed by replacing sucrose with KCl. On the other hand, Ackerman et al. (1977) find sigmoidicity in the presence of nonpermeant electrolytes but not in sucrose at 5 °C.

The present study where the kinetics has been analyzed after excluding the H^+ pump as the rate-limiting step reveals the following features of the Ca^{2+} carrier.

(1) The sigmoidicity is temperature dependent, in that the increase of temperature tends to decrease the sigmoidicity. This agrees with Reed & Bygrave (1975b), who find a Hill coefficient of 1.7 in sucrose at 0 °C, but not with Ackerman et al. (1977), who do not find sigmoidicity in sucrose at 5 °C. We attribute the discrepancy to our use of nitrilotriacetic acid–Ca buffers which render more precise the kinetic measurements in the low Ca^{2+} region.

(2) The sigmoidicity is electrolyte dependent in that addition of electrolyte tends to increase the degree of sigmoidicity. This agrees with Ackerman et al. (1977). However, choline does not affect the degree of sigmoidicity, thus excluding an effect on the surface potential and suggesting a more specific effect on the outer surface of the carrier subunits. The decrease of sigmoidicity with the absence of electrolyte and increase of temperature explains the hyperbolic kinetics of Carafoli et al. (1971).

(3) Mg^{2+}, Li^+, and Ruthenium Red do not act as competitive inhibitors. This is indicated by the decrease of the V_{max}. Since this is a point of discrepancy, the question arises as to why previous studies have concluded for a competition by Mg^{2+} and Li^+. We attribute this mostly to the use of the H^+ pump as the driving force for Ca^{2+} transport. Since the respiratory chain is slower than the Ca^{2+} carrier, the V_{max} of Ca^{2+} transport in the absence of inhibitors will reflect the V_{max} of the H^+ pump rather than that of the Ca^{2+} carrier. If the V_{max} of the Ca^{2+} carrier in the presence of inhibitors is still greater than the V_{max} of the H^+ pump, the two V_{max} ± inhibitors may be identical because they reflect the V_{max} of the H^+ pump. The noncompetitive nature of Mg^{2+} inhibition becomes evident under aerobic conditions when Mg^{2+} renders the V_{max} of the Ca^{2+} carrier lower than that of the H^+ pump (unpublished experiments).

(4) The kinetics of the Ca^{2+} carriers shows, in the presence of Ruthenium Red, a peculiar behavior due to the presence of "bumps". It is important to note the fundamental difference between Ruthenium Red and La^{3+}, since La^{3+} appears to act as a purely competitive inhibitor (Scarpa & Azzone, 1970; Reed & Bygrave, 1975b).

The kinetics of Ca^{2+} translocation across the membrane may be described as proposed by Reed & Bygrave (1975b):

$$Ca^{2+}_{out} + C_{out} \xrightarrow{K_b} Ca^{2+}\cdot C_{out} \xrightarrow{K_t} Ca^{2+}\cdot C_{in} \xrightarrow{K_d} Ca^{2+} + C_{in} \quad (1)$$

where C is the carrier and K_b and K_t are the constants for the binding of Ca^{2+} to the carrier and for the rate of translocation of the Ca^{2+} carrier complex across the membrane. The transport eq 1 recalls those used for enzymatic catalysis. We assume that the sigmoidicity reflects cooperative phenomena, that binding of a ligand produces some distortion in a subunit of a multisubunit carrier and that this distortion is transmitted to the neighboring subunit, thus producing cooperativity. The presence of two constants in eq 1, one of binding and another of translocation, K_b and K_t, gives rise to two problems. First, which of the two is the rate-limiting step? Second, what is the role of each step in determining the cooperative kinetics? As to the first problem, as will be shown elsewhere, the relation $V_{Ca^{2+}}$ vs. $\Delta\psi$ is exponential (Laüger, 1972). Due to the analogy of the kinetics of mobile and proteic pore carriers, this indicates that the rate-limiting step in eq 1 is presumably the translocation. As to the second problem, the presence of subunits makes it impossible to distinguish whether allosteric agents affect the cooperativity at the level of the association of Ca^{2+} with the subunits or at the translocation step. The distinction would require a comparison between effects on the binding and on the catalytic steps which is at present out of experimental attack.

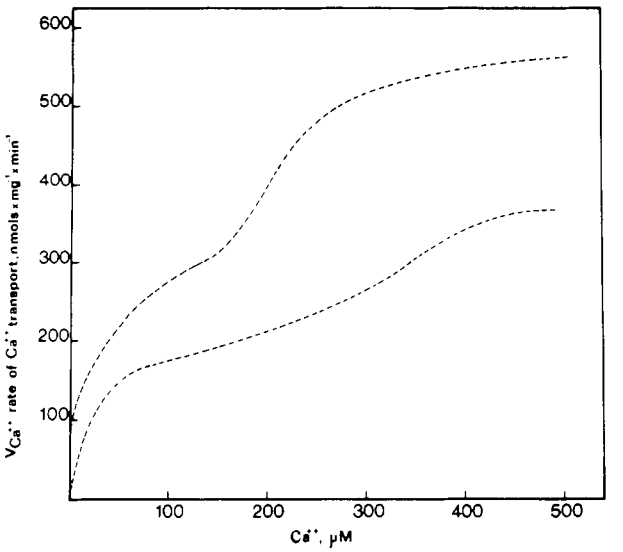

FIGURE 9: Computer-simulated kinetics of Ca^{2+} transport in the presence of Ruthenium Red. The kinetics was obtained by inserting the following values. With 70 pmol of Ruthenium Red (mg of protein)$^{-1}$, V_{max} = 400 nmol (mg of protein)$^{-1}$ min^{-1}, $K_{b_1} = 0.4\ \mu M^{-1}$, $K_{b_2} = 11.7 \times 10^{-3}\ \mu M^{-1}$, $K_{b_3} = 2.5 \times 10^{-4}\ \mu M^{-1}$, and $K_{b_4} = 5 \times 10^{-2}\ \mu M^{-1}$. With 35 pmol of Ruthenium Red (mg of protein)$^{-1}$, V_{max} = 600 nmol (mg of protein)$^{-1}$ min^{-1}, $K_{b_1} = 0.5\ \mu M^{-1}$, $K_{b_2} = 6.25 \times 10^{-3}\ \mu M^{-1}$, $K_{b_3} = 2.10^{-4}\ \mu M^{-1}$, and $K_{b_4} = 0.15\ \mu M^{-1}$.

A cooperative kinetics can be explained on the basis of the "concerted transition" model of Monod et al. (1965), by using appropriate values for the equilibrium constants. The variability of the Hill coefficient, under the effect of temperature and electrolyte, would be due to an effect of temperature and of electrolytes on the equilibrium between the T and R states and on the binding constants for the interaction of Ca^{2+} with the carrier sites. However, the concerted transition model is unable to explain the kinetics with "bumps" of Figure 8, whichever values for equilibrium constants are assumed.

A negative cooperativity and the phenomenon of the "bumps" are open to a more flexible explanation within the sequential interaction model (Koshland, 1970). The model assumes ligand-induced conformational changes determining regulatory and cooperative phenomena. Application of this model to Ca^{2+} transport implies that Mg^{2+} and Li^+ induce a positive cooperativity among the carrier subunits while Ruthenium Red induces a negative cooperativity too. Temperature would enhance interaction among the subunits and thus induce a positive cooperativity. In the model, where the four subunits have free and different interactions among each other, the fractional saturation of the enzyme Y is described by

$$Y = (K_{b_1}[Ca^{2+}] + 2K_{b_1}K_{b_2}[Ca^{2+}]^2 + 3K_{b_1}K_{b_2}K_{b_3}[Ca^{2+}]^3 + 4K_{b_1}K_{b_2}K_{b_3}K_{b_4}[Ca^{2+}]^4)/(1 + K_{b_1}[Ca^{2+}] + K_{b_1}K_{b_2}[Ca^{2+}]^2 + K_{b_1}K_{b_2}K_{b_3}[Ca^{2+}]^3 + K_{b_1}K_{b_2}K_{b_3}K_{b_4}[Ca^{2+}]^4) \quad (2)$$

The kinetics of the Ca^{2+} carrier can be then described by the equation

$$V_{Ca} = E_t K_t Y = V_{max} Y \quad (3)$$

where V_{Ca} is the initial velocity of Ca^{2+} transport, K_t is the translocation constant for $Ca^{2+}\cdot C$ complex, E_t is the total Ca^{2+} carrier concentration, and K_{b_1}, K_{b_2}, K_{b_3}, and K_{b_4} are the equilibrium constants for the interaction of Ca^{2+} with the carrier subunits. The subunits are assumed to be four, and the equilibrium binding constants are assumed to be different

among themselves. Figure 9 shows the kinetics of Ca^{2+} transport in the presence of Ruthenium Red obtained by assuming a translocation constant K_t identical for all carrier subunits and values for the binding constants in the following order: $K_{b_1} > K_{b_2} > K_{b_3} < K_{b_4}$. The kinetics of Figure 9 can be obtained also by assuming that the equilibrium constants for the binding of Ca^{2+} to the four subunits are all identical, $K_{b_1} = K_{b_2} = K_{b_3} = K_{b_4}$, while the translocation constants vary in the order $K_{b_1} > K_{b_2} > K_{b_3} < K_{b_4}$.

In conclusion, the present study suggests that a number of ions, known to affect the kinetics of Ca^{2+} transport, do not act as competitive inhibitors of the Ca^{2+} carrier but rather as allosteric effectors and therefore as ligands, inducing conformational changes on the carrier subunits. As a result, either translocation or binding constants may be modified. This view assumes that the Ca^{2+} carrier (a) has multiple subunits, at least four, and (b) has allosteric sites for the interaction of the various effectors (these sites appear not to overlap with the Ca^{2+} binding sites).

Regulation of Ca^{2+} Concentration in Vitro. The control of mitochondria Ca^{2+} distribution is likely to be crucial in vivo, the dimension of the mitochondria driving force being so large that little Ca^{2+} would be left in the cytosol should Ca^{2+} be allowed to reach thermodynamic equilibrium (Azzone et al., 1977a,b). On the other hand, a kinetic regulation of the Ca^{2+} distribution, where the rate of Ca^{2+} carrier is twice the respiratory rate, can hardly be due to an acceleration of the rate of efflux since this would lead to energy drain rather than to a decrease of the accumulation ratio. The alternative is a regulation of the rate of influx. The effect on cooperativity by impermeant cations, under conditions similar to those existing in vivo, suggests that the cooperativity is playing a major physiological role in permitting mitochondria to buffer the cytosol Ca^{2+} concentration in the 10^{-4}–10^{-5} M region.

Added in Proof

After the paper was submitted for publication, the work of Williams & Barrie (1978) came to our attention, where conclusions similar to ours have been reached.

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