ACTIVATION ENERGIES AND ENTHALPIES DURING Ca^{2+} TRANSPORT IN RAT LIVER MITOCHONDRIA

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Received 4 June 1979

1. Introduction

Although there is general agreement that Ca^{2+} transport depends on the operation of a specific divalent cation carrier, very little is known about the molecular properties of the carrier. Hutson [1] has tried to distinguish between a mobile carrier and a pore mechanism by studying the effect of temperature on Ca^{2+} transport. A break in the Arrhenius plot has been observed.

In membrane vesicles from *E. coli*, the Arrhenius plots for active transport display, below the temperature at which lipids undergo transition from the disordered to the ordered state, a slope which is steeper with respect to the temperature above the transition [2–4]. Arrhenius plots have also been reported to be triphasic with the slope becoming again less steep at the lower temperature [4].

In interpreting the effect of temperature on active transport a crucial question concerns the identification of the rate limiting reaction. If the Ca^{2+} carrier operates at a rate faster than that of the H^+ pump the question arises as to whether the Arrhenius plots reflect properties of the Ca^{2+} carrier or rather of the H^+ pump.

This uncertainty has been eliminated in the present study by replacing H^+ extrusion via the aerobic H^+ pump with K^+ diffusion via valinomycin as the driving force for Ca^{2+} influx. Since the rate of K^+ diffusion depends on the amount of valinomycin, the rate of Ca^{2+} transport may be rendered rate limiting by using an excess of valinomycin. The Arrhenius plots for Ca^{2+} transport have been compared with those of other ion transport reactions utilizing either pore mechanisms such as gramicidin, or mobile carriers such as uncouplers, valinomycin or nigericin. The absence of breaks in the Arrhenius plots, the low activation enthalpy and the high turnover number suggest that Ca^{2+} transport in liver mitochondria occurs through a pore.

2. Experimental

Mitochondria were prepared according to standard procedures. The rate of Ca^{2+} influx was measured by using Antipyrilazo III as a free Ca^{2+} indicator. The experiments have been performed in a DWR Aminco spectrophotometer equipped with magnetic stirring. The rate of K^+ efflux was measured with a selective K^+ electrode kept in a thermostated glass vessel with magnetic stirring. The recording apparatus was a Radiometer pH meter connected to a Texas recorder. Both in the spectrophotometric and electrometric experiments the mixing time was below 0.5 sec. In all cases rotenone-treated mitochondria were preincubated for 3 min and the reaction initiated by the addition of an ionophore inducing K^+ transport. For each cation transport analyzed, the rate-limiting step of the reaction was established by suitable titrations with the various ionophores.

3. Results

Figure 1 shows Arrhenius plots obtained under conditions where the rate-limiting reaction was the transport of ions utilizing a mobile carrier mechanism.

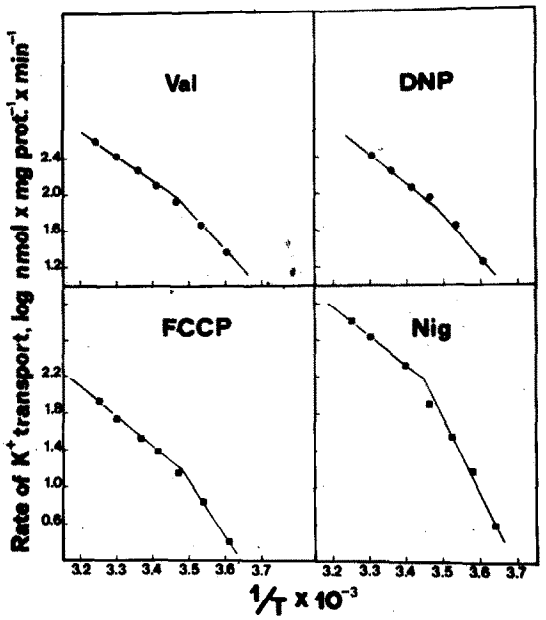

Fig.1. Arrhenius plots for transport catalyzed by valinomycin, dinitrophenol, FCCP and nigericin. The plots indicate four conditions for which the rate-limiting step for transport was that catalyzed by valinomycin, dinitrophenol, FCCP and nigericin, respectively. The medium contained in all cases 0.2 M sucrose, 20 mM Tris–HCl (pH 7.4), 2 μM rotenone and 200 μM KCl. Other additions were as follows. For the valinomycin catalyzed transport 1 nmol FCCP/mg prot and 12 pmol valinomycin/mg protein. For the dinitrophenol catalyzed transport, 100 μM EGTA, 10 μM dinitrophenol and 1 nmol valinomycin/mg protein. For the FCCP catalyzed transport, 100 μM EGTA, 68 pmol FCCP/mg protein and 2 nmol valinomycin/mg protein. For the nigericin catalyzed transport 100 μM EGTA and 120 pmol nigericin/mg protein.

The following types of ion transport were analyzed: H^+ via FCCP; H^+ via dinitrophenol; K^+ via valinomycin; H^+ and K^+ via nigericin. H^+ transport via FCCP or dinitrophenol was rendered rate-limiting by measuring the rate of K^+ efflux in exchange with H^+ in presence of an excess of valinomycin and of limiting amounts of FCCP or dinitrophenol. K^+ transport was rendered rate-limiting by measuring the rate of K^+ efflux in exchange with H^+ in presence of an excess of dinitrophenol and of limiting amounts of valinomycin. In the case of nigericin both K^+ efflux and H^+ influx go via nigericin and the rate of exchange is a function of the amount of nigericin. Figure 1 shows that in all cases there was a break in the Arrhenius plot at about 14°C with a steeper slope in the low temperature region. The activation energy was 20.1 ± 1 kcal/mol below 14°C and 14.2 ± 1 kcal/mol above 14°C, respectively. Läuger [5] has shown that the transport via a mobile carrier involves 3 steps: (a) formation of the ion–ionophore complex at the interface; (b) translocation of the complex through the hydrophobic core; and (c) return of the ionophore. If the translocation of the charged complex is rate-limiting, the plot of rate of transport versus $\Delta\psi$, should be exponential. Figure 2 shows the rates of ion transport versus $\Delta\psi$ as measured at various temperatures with the various carriers. In all cases it is seen that the relation between rate of transport and $\Delta\psi$ is exponential both below and above the transition temperature. The experiment of fig.2 thus indicates that the transition of the charged complex under the electrical field is always rate-limiting both below and above the transition temperature and thus excludes that the breaks observed in the plot of fig.1 are due to the fact that other steps become rate-limiting, for example formation of the complex (which should yield a saturation plot) or migration of the neutral ionophore (which should yield a $\Delta\psi$ independent relation).

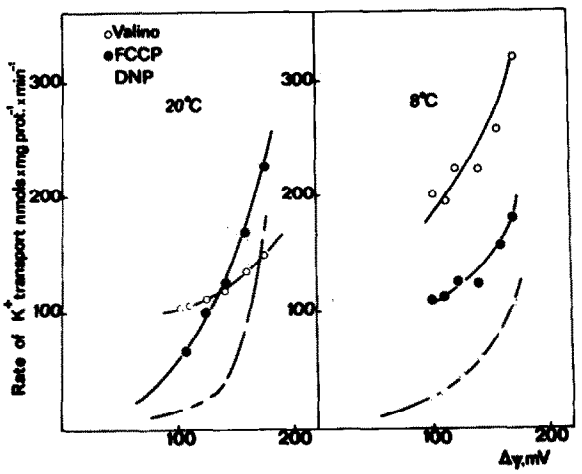

Fig.2. Dependence of the rate of mobile carrier induced transport on membrane potential. The medium contained 0.2 M sucrose, 20 mM Tris–HCl (pH 7.4), 3 μM rotenone, 50 μM EGTA, 2 mM phosphate and variable amounts of KCl in order to vary $\Delta\psi$. Mitochondria 1 mg/ml. 12 or 20 pmol valino-/mycin/mg protein and 2 nmol FCCP/mg protein; 80 pmol FCCP/mg protein and 1 nmol valinomycin/mg protein; 1 nmol dinitrophenol/mg protein and 1 nmol valinomycin/mg protein. The figures on the right and on the left refer to experiments carried out at 8°C and 20°C, respectively.

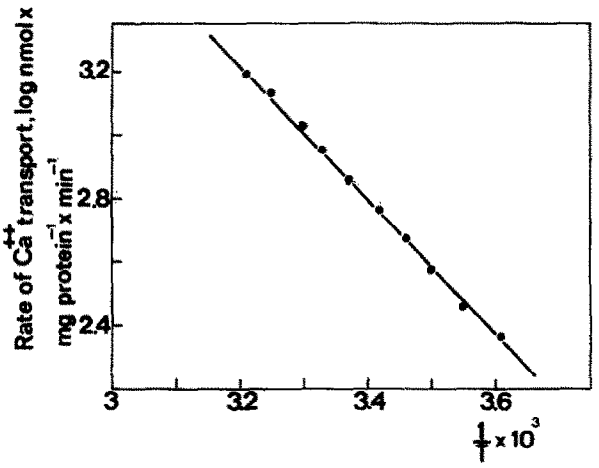

Fig.3. Arrhenius plots for K^+ diffusion driven Ca^{2+} transport. The medium contained 0.2 M sucrose, 20 mM Tris–HCl (pH 7.4), 2 mM P_i, 2 μM rotenone, 200 μM KCl and 0.7 mg/ml mitochondrial protein.

Figure 3 shows the Arrhenius plot for Ca^{2+} transport. Ca^{2+} transport was rendered rate-limiting by using an excess of valinomycin. Plots like those of fig.3 were independent of the Ca^{2+} concentration provided that a suitable excess of valinomycin was used. This was tested by running control experiments in the presence of amounts of FCCP providing rates of K^+ efflux higher than those observed in the presence of Ca^{2+}. Figure 3 indicates that there is no break in the Arrhenius plot and that the activation energy is 9.8 ± 1 kcal/mol. Similar results were obtained by replacing Ca^{2+} with Mn^{2+}.

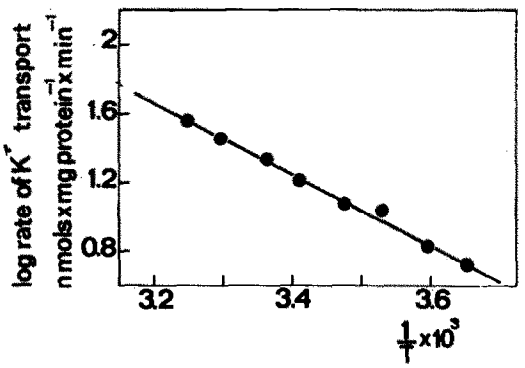

Fig.4. Arrhenius plot for gramicidin catalyzed transport. The medium contained 0.2 M sucrose, 20 mM Tris–HCl (pH 7.4), 2 μM rotenone, 2 mM P_i, 200 μM KCl. Gramicidin was 1 nmol/mg protein.

In order to validate the concept that the difference between the Arrhenius plots of figs.1 and 3 reflect a different nature of carrier mechanism the behaviour of gramicidin was investigated. Ion transport through gramicidin, which is a pore-forming ionophore, occurs after a lag phase, presumably the time required to form the pore. The rate of ion transport in the experiment of fig.4 was calculated after completion of the lag phase. Figure 4 shows that the Arrhenius plot for gramicidin catalyzed transport was also without breaks. Furthermore the activation energy was low, i.e., 9.6 ± 1 kcal/mol, about the same magnitude as that found in fig.3 when Ca^{2+} transport was rate-limiting.

4. Discussion

Vinogradov and Scarpa have favoured a mobile carrier nature of Ca^{2+} transport on the basis of saturation kinetics [6]. Reed and Bygrave [7] have proposed a mobile carrier model where the distribution of the carrier between the two aqueous phases and the rate of translocation are pH dependent. Hutson [1] has suggested that the break in the Arrhenius plots at 22–23°C may reflect dependence of the respiratory chain on the lipid fluidity.

Major criteria to distinguish whether transport goes via mobile carriers or proteic pores are: (i) the symmetric effect of inhibitors; (ii) the dimension of the turnover constant; and (iii) the dimension(s) of the activation enthalpy. According to the first criterion in case of mobile carriers an equivalent degree of inhibition is expected on influx or efflux processes since the effect of specific inhibitors involves binding of the carrier and then removal of the carrier to an equal extent for influx and efflux processes. This is not the case for pore structures where asymmetric inhibition of efflux and influx processes is expected due to the fact that the non permeant inhibitors reach only the external surface of the pore.

According to the second criterion, in case of mobile carriers the turnover constant reflects the ratio between transport V_{max} and number of carriers. Being kinetically indistinguishable the same definition applies for transport via pore. However, studies in model membranes have indicated a turnover constant several orders of magnitude higher for transport via

pore with respect to transport via mobile carriers [8,9].

According to the third criterion mobile carriers and pores have different energy barriers [10,11]. Mobile carriers dissolve the ion in the hydrocarbon phase. However transport across the membrane still requires the passage of the charged complex through a low dielectric medium and thus the overcome of a large energy barrier. On the other hand, a pore mechanism implies the presence of a hydrophilic channel with a high dielectric constant. The activation enthalpy is expected to be much higher in the former with respect to the latter case. Furthermore, if transport via a mobile carrier depends on lipid fluidity it may be expected that the activation enthalpy be different in different temperature ranges.

As to the symmetry criterion, it is generally accepted that Ca^{2+} influx and efflux are not inhibited to the same extent by Ruthenium Red. Pozzan et al. [12] have suggested that this is due to the fact that binding of Ruthenium Red is $\Delta\psi$ dependent, and that Ca^{2+} efflux occurs mostly under conditions where $\Delta\psi$ is collapsed. However, Caroni et al. [13] have reported that addition of FCCP, which induces collapse of $\Delta\psi$, does not cause release of bound Ruthenium Red. Although binding without inhibition would support Ruthenium Red as an asymmetric inhibitor of a proteic pore, it is still unclear why the EGTA induced Ca^{2+} efflux in static head mitochondria is highly inhibited by Ruthenium Red. The pore nature of Ca^{2+} transport in liver mitochondria is then supported by two lines of evidence.

First, the turnover number of Ca^{2+} transport. On the basis of a V_{max} for Ca^{2+} transport of 600 nmol/mg protein/min [14] and an amount of Ruthenium Red sensitive Ca^{2+} carrier of 100 pmol/mg protein the turnover number is 260/sec. However replacement of Ruthenium Red with T_m [18] results in a number of carrier sites of 1 pmol/mg protein and thus in a turnover number $\geq 26 \times 10^3$/sec. A further increase may be expected parallel to the identification of more specific inhibitors of the Ca^{2+} carrier.

A turnover constant of 26×10^3/sec is smaller than what is found in the case of gramicidin. On the other hand, the turnover constants of the other mobile carriers ionophores are even smaller, FCCP, 13/sec; dinitrophenol, 0.33/sec, valinomycin, 600/sec. This latter value compares well with that found by Pressman [9].

The turnover constant K is related to the activation free energy through eq. (1)

$$K = \frac{RT}{Nh} \exp\left(-\frac{\Delta G}{RT}\right) \quad (1)$$

where N is Avogadro's number, h the Planck's constant, R the gas constant, and T the absolute temperature. A turnover constant of 26×10^3/sec corresponds to an activation free energy ΔG of 11.5 kcal/mol. The value may be expected to decrease parallel to the increase of the turnover number.

The dielectric constant in the channel is related to the activation free energy through the Born eq. [16]:

$$\Delta G = \left[\frac{1}{\epsilon_1} - \frac{1}{\epsilon_2}\right] \frac{(zq)^2}{2r} \quad (2)$$

An activation free energy of 11 kcal, where ϵ_1 and ϵ_2 are the dielectric constants of the pore and of the aqueous solution, respectively, and r the radius of the ion, corresponds to a dielectric constant of 24 for the Ca^{2+} channel. Again this value may further increase with the decrease of the activation free energy.

Second, an activation enthalpy of 9.5 kcal/mol and the absence of breaks in the Arrhenius plots. The activation enthalpy of 9.5 kcal corresponds very well to that found, both in mitochondria and model systems for the classical pore forming antibiotic gramicidin (cf. fig.4 and [11]). On the other hand, this low activation enthalpy clearly distinguishes transport by Ca^{2+} and gramicidin from that mediated by mobile carriers such as valinomycin, nigericin, uncouplers. In these latter cases the activation enthalpy is around 20 below, and around 14 kcal/mol above, the break in the Arrhenius plots.

Unfortunately, the uncertainty in the ΔG do not permit to know, by comparing with the activation enthalpy values, whether ΔS is positive or negative, which would provide further information on the Ca^{2+} channel.

The molecular nature of the breaks in the Arrhenius plot of fig.1 requires further investigation. The conductance for valinomycin and monactin [11] has been shown to undergo a marked reduction below the temperature of membrane-'freezing', which suggests that mobile carriers are immobilized in a 'frozen' layer. Other mitochondrial properties also show breaks in

Arrhenius plots between 14°C and 20°C. The break presumably does not reflect a phase transition since this seems to occur in the mitochondrial lipids around −4°C [17]. Furthermore, there are no reports of a marked temperature dependence of the solubility of the carriers in the membrane phase in model systems. Thus the break although suggesting a dependence of mobile carrier catalyzed transport on the lipid fluidity may reflect a more complex change in the bilayer structure.

References

[1] Hutson, S. M. (1977) J. Biol. Chem. 252, 4539–4545.
[2] Schairer, H. V. and Overath, P. (1969) J. Mol. Biol. 44, 209–214.
[3] Wilson, G., Rose, S. P. and Pox, C. P. (1970) Biochem. Biophys. Res. Commun. 38, 617–623.
[4] Schechter, E., Leteillier, L., Gulik-Krzywicki, T. (1974) Eur. J. Biochem. 49, 61–76.
[5] Läuger, P. (1972) Science 178, 24–30.
[6] Vinogradov, A. and Scarpa, A. (1973) J. Biol. Chem. 248, 5527–5531.
[7] Reed, K. C. and Bygrave, P. L. (1975) Eur. J. Biochem. 55, 497–504.
[8] Schnell, K. F. (1977) J. Membrane Biol. 37, 99–136.
[9] Pressman, B. (1976) Annual Review Biochem. 16, 5618–5624.
[10] Parsegian, A. (1969) Nature 221, 844–846.
[11] Ginzburg, S. and Noble, P. (1974) J. Membrane Biol. 18, 163–176.
[12] Pozzan, T., Bragadin, M. and Azzone, G. F. (1977) Biochemistry 16, 5618–5625.
[13] Caroni, P., Schwerzmann, K. and Carafoli, E. (1978) FEBS Lett. 96, 339–342.
[14] Azzone, G. F., Bernardi, P. and Brogadin, M. (1979) in: The Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E. et al. eds) Dev. Bioenerg. Biomembr. vol. 3, pp. 183–192, Elsevier/North-Holland, Amsterdam, New York.
[15] Ketterer, B., Neumke, B. and Läuger, P. (1971) J. Membrane Biol. 5, 225–245.
[16] Born, M. (1920) Z. Physik 1, 45.
[17] Hackenbrock, C. R., Höcli, M. and Chau, M. (1976) Biochim. Biophys. Acta 455, 466–484.
[18] Crompton, M., Sigel, E. and Carafoli, E. (1979) in: The Function and Molecular Aspects of Biomembrane Transport, (Quagliariello, E. et al. eds) Dev. Bioenerg. Biomembr. vol. 3, pp. 171–174, Elsevier/North-Holland, Amsterdam, New York.