Journal of Bioenergetics and Biomembranes (1977) 9, 141–149

Effect of Cations on the Temperature Sensitivity of Ca^{2+} Transport in Rat-Liver Mitochondria and Safranine Uptake by Liposomes

Karl E. O. Åkerman

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10, SF–00170 Helsinki 17, Finland

Received 22 April 1976

Abstract

The activation energy of mitochondrial Ca^{2+} transport has been studied in various conditions by Arrhenius plots in the temperature range 6–20°C. In the presence of Mg^{2+} the activation energy is decreased to 18 kJ/mole from that of 40 kJ/mole found in a sucrose medium. In the presence of the polyamine spermine the activation energy is practically 0 kJ/mole. A lanthanide Eu^{3+}, which is a potent inhibitor of Ca^{2+} transport, has no significant effect on the activation energy. In a KCl medium the activation energy is increased to 70 kJ/mole. When both K^+ and Mg^+ are present the activation energy is nonlinear between 11 and 18°C. In the presence of K^+ and spermine it is about 0 kJ/mole between 6 and 13°C and at higher temperatures 68 kJ/mole. Neither Mg^{2+} nor spermine affect the slope of the Arrhenius plot for state 4 respiration. Spermine decreases slightly the activation energy of Ca^{2+}-stimulated respiration. Spermine also decreases the activation energy of valinomycin- or gramicidin-induced safranine uptake by liposomes from 68 to almost 0 kJ/mole between 17 and 30°C. The results indicate that Ca^{2+} binding to the polar head groups of the phospholipids at the membrane surface is the rate-limiting step of mitochondrial Ca^{2+} transport, because agents that inhibit Ca^{2+} binding to these sites (Mg^{2+}, spermine, K^+) have the most marked effect, whereas Eu^{3+}, which, because of the small concentration used, ought to interact mainly with the mitochondrial Ca^{2+} transport system, has no significant effect on the temperature sensitivity of mitochondrial Ca^{2+} transport.

Introduction

The temperature sensitivity of the kinetics of various membrane-linked enzymatic reactions in mitochondria has been studied extensively [1–4]. Arrhenius plots for these enzymes show discontinuities at specific temperatures. These discontinuities have been interpretated to be due to phase changes in the phospholipids of the mitochondrial membrane, because detergent treatment, but not mechanical disruption, abolishes the discontinuities [1]. An increase in the content of unsaturated lipids transfers the points at which the discontinuities occur to lower temperatures [4]. The discontinuities in the Arrhenius plots for mitochondrial ATPase occur at different temperatures compared to the other membrane enzymes [3, 5], probably owing to different phospholipids in vicinity of the enzyme molecules [5]. In other systems good correlation has been obtained between the discontinuities in Arrhenius plots and the phase transitions of the isolated lipids of the appropriate membranes [6–9]. Arrhenius plots for mitochondrial Ca^{2+} transport also shows discontinuities at about the same temperatures as many other membrane-linked functions [3]. It is known that Ca^{2+} is bound to the mitochondrial phospholipds to so-called low affinity sites [10–12]. This binding is competitively inhibited by $Mg^{2+} > K^+ > H^+$ [13–15] and has been suggested to be the first step in the mitochondrial Ca^{2+} transport [14]. Mitochondrial Ca^{2+} transport is competitively inhibited by various lanthanides [16, 17]. Because of the very low K_i for these compounds (about 0.02 μM, [17]) it has been suggested that they interact specifically with a translocase which catalyses the transfer of Ca^{2+} through the mitochondrial membrane [17]. Organic cations like safranine and acridine orange are also taken up by energized mitochondria and induce respiratory responses similar to those of Ca^{2+}, i.e., stimulation of respiration and release of protons [18]. This interaction is competitively inhibited by Ca^{2+}. Safranine but not Ca^{2+} is also taken up by liposomes on induction of a K^+ diffusion potential by valinomycin [19]. Since Ca^{2+} inhibits also this transport competitively it seems highly probable that the inhibition takes place at the outer surface of the liposomal or mitochondrial membrane.

The aim of this work was to study the effect of cations that react mainly, either with the phospholipids (Mg^{2+}, K^+, spermine) or the Ca^{2+} transport system (Eu^{3+}), in the mitochondrial membrane in order to be able to distinguish which step is temperature sensitive and rate limiting. The results are compared to those obtained with safranine uptake in liposomes.

Materials and Methods

Rat-liver mitochondria were prepared from young male Sprague Dawley

rats by a conventional method [20]. Liposomes were prepared according to Saha and co-workers [22].

The initial rate of mitochondrial Ca^{2+} transport was measured with the murexide technique [21] in an Aminco DW2 spectrophotometer using the wavelength pair 540–507 nm. Oxygen consumption was measured polarografically. The kinetics of safranine stacking was measured using the wavelength pair 524–554 [19].

Reaction media: 0.25 M sucrose, 20 mM Tris-Clm pH 7.5 (sucrose-medium) and 130 mM KCl, 20 mM tris-Cl, pH 7.5 (KCl-medium).

Reagents: spermine (Fluka AG Buchs SG, Switzerland), $EuCl_3$ (Koch-Light Laboratories Ltd., Colnbrook Buchs, England), valinomycin (Sigma Chemical Co., St. Louis, Missouri), cardiolipin (The Sylvana Company, Millburn, New Jersey 07041), Soy-lecithin (NB Co., Cleveland, Ohio 44128), safranine and murexide (Merck AG, Darmstadt, GFR).

Results

Effect of Mg^{2+}, Spermine, and Eu^{3+} on the Temperature Sensitivity of Mitochondrial Ca^{2+} Transport

In the sucrose-medium the activation energy as calculated from the Arrhenius plots in Fig. 1 is about 40 kJ/mole between 6 and 20°C. In the presence of Mg^{2+} it decreases to 18 kJ/mole and spermine to almost 0 kJ/mole in the temperature range studied. Figure 1 also shows that the

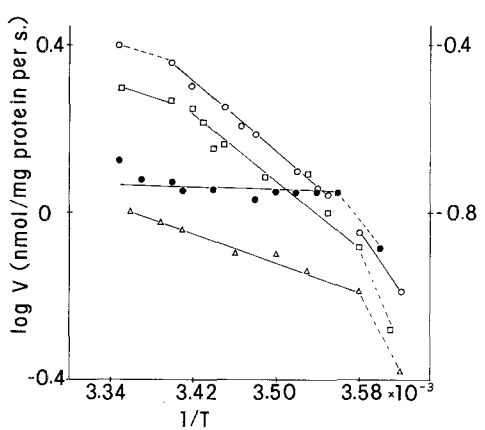

Figure 1. Arrhenius plots for mitochondrial Ca^{2+} transport in the presence of Mg^{2+}, spermine, and Eu^{3+}. Incubations were made in the sucrose medium containing 8 mM succinate and 5 μM rotenone (○–○), 2 mM $MgCl_2$ (△–△), 400 μM spermine (●–●), or 0.02 μM Eu^{3+} (□–□, scale on the right). Mitochondrial protein was 1 mg/ml, the volume 2,5 ml, Ca^{2+} concentration 40 μM and murexide concentration 8 μM.

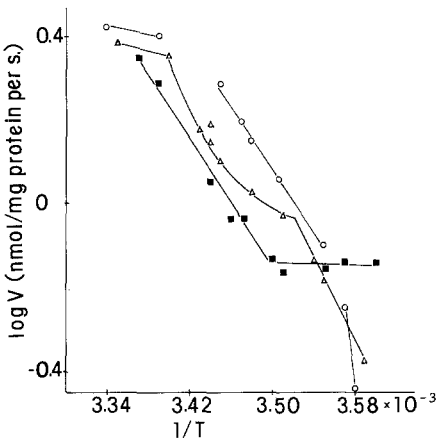

Figure 2. Arrhenius plots for mitochondrial Ca^{2+} transport in the presence of Mg^{2+} and spermine. Conditions as in Fig. 1 except that incubations were made in the KCl medium (O–O, 2 mM $MgCl_2$ (Δ–Δ) and 200 μM spermine (■–■).

lanthanide Eu^{3+} has no significant effect on the slope of the Arrhenius plot even at a concentration (0.02 μM) that inhibits mitochondrial Ca^{2+} transport by 60%. The discontinuities of the Arrhenius plots in Fig. 1 are difficult to demonstrate in the present conditions. They appear to occur close to 6 and 10°C. However the effects of the agents studied on the slope of the Arrhenius plot between 6 and 20°C are highly reproducible.

In the KCl medium the activation energy increases to 70 kJ/mole (Fig. 2). In the presence of Mg^{2+} the Arrhenius plot is nonlinear between 11 and 18°C. Spermine decreases the activation energy to almost 0 kJ/mole between 6 and 13°C. At higher temperatures the activation energy is about 68 kJ/mole. Figure 2 also shows that the discontinuities are more clearly seen in the KCl medium than in sucrose (see Fig. 1). In the presence of

TABLE I. Effect of temperature on the initial rate of mitochondrial Ca^{2+} transport (Conditions as in Fig. 1)

	Initial rate of Ca^{2+} uptake (nmol/mg protein per sec)					
	Sucrose medium			KCl		
Temperature (°C)	Control	Mg^{2+} (2 mM)	Spermine (400 μM)	Control	Mg^{2+} (2 mM)	Spermine (400 μM)
6	0.66	0.25	0.5	0.46	0.72	1.1
13	1.4	0.6	1.3	1.52	1.0	1.1
18	1.8	0.7	1.3	2.4	1.6	2.0

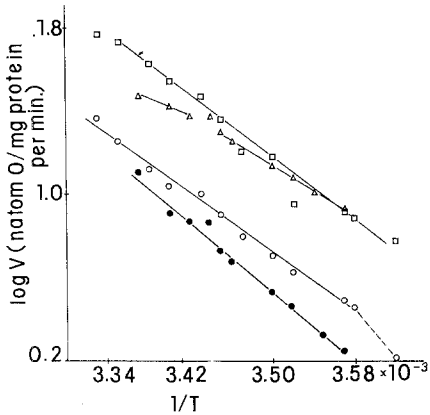

Figure 3. Arrhenius plots for mitochondrial state 4 and Ca^{2+}-stimulated respiration in the presence of spermine. Mitochondrial oxygen consumption was measured in the sucrose medium containing 1 mM KH_2PO_4, 8 mM succinate, and 5 μM rotenone. State 4 respiration: control (O–O) and 500 μM spermine (●–●). Ca^{2+}-stimulated respiration: control (□–□) and 500 μM spermine (△–△). Mitochondrial protein was 1.8 mg/ml, the volume 1 ml, and the Ca^{2+} concentration 100 μM.

Mg^{2+} there is a shift of the point at which the lower discontinuity occurs from 6 to 11°C.

Table I shows for comparison the initial rates of mitochondrial Ca^{2+} transport at three temperatures in the different conditions used.

Effect of Spermine on the Temperature Sensitivity of State 4 and Ca^{2+}-Stimulated Respiration

Mitochondrial respiration is due to the activity of the intrinsic proteins of the membrane. Spermine is thought to interact mainly with the polar head groups of phospholipids [23]. Thus no effect of spermine on the state of the interior of the membrane is expected. Figure 3 shows that spermine indeed does not affect the slope of the Arrhenius plot for state 4 respiration significantly. It only causes a slight reduction in the respiratory rate. Figure 3 also shows that spermine causes a slight decrease in the slope of the Arrhenius plot for Ca^{2+}-stimulated respiration.

Effect of Spermine on the Temperature Sensitivity of Safranine Uptake by Liposomes

Figure 4 shows Arrhenius plots for safranine uptake induced either with valinomycin or gramicidin. The activation energy calculated from the plots is 68 kJ/mole. It seems clear that the temperature sensitivity of the process

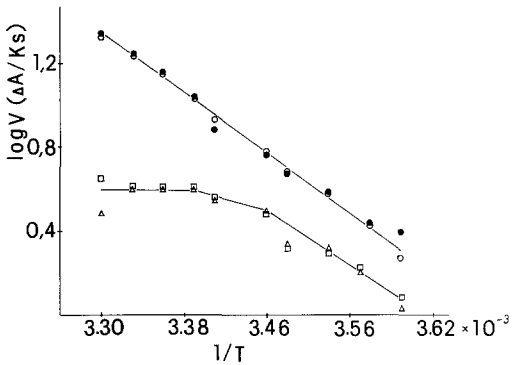

Figure 4. Arrhenius plots for valinomycin- and gramicidin-induced safranine uptake in the absence and presence of spermine. Liposomes were prepared in the KCl medium and suspended in a medium containing 60 mM choline–chloride, 140 mM sucrose, and 20 mM tris-Cl (pH 7.5), 40 ng/ml valinomycin (○–○), 400 nM gramicidin (●–●), 40 ng/ml valinomycin and 400 μM spermine (□–□), and 400 nM gramicidin and 400 μM spermine (△–△). Liposomal phosphate concentration was 0.2 mM, the volume 2,5 ml, and the safranine concentration 20 μM.

is due to the transport of safranine and not to the response of the ionophores because the Arrhenius plot for the uptake induced by valinomycin is similar to that for gramicidin. The response of valinomycin is highly sensitive to changes in temperature whereas that of gramicidin is not [24]. Figure 4 shows that spermine decreases the activation energy to almost 0 kJ/mole between 17 and 30°C. At lower temperatures it is about 68 kJ/mole.

Discussion

The results of this work show that agents known to interact mainly with the polar head groups of phospholipids have the most marked effects on the temperature sensitivity of mitochondrial Ca^{2+} transport. Mg^{2+} and K^+ are known inhibitors of Ca^{2+} binding to the phospholipids at the outer surface of the mitochondrial membrane [13–15]. Neither one is translocated by mitochondria in the conditions used. Also spermine inhibits Ca^{2+} binding to these sites [36]. Mg^{2+} [25, 26] and spermine [23, 27, 28] are known stabilizers of various membrane systems. The main stabilizing effects are probably due to the fact that Mg^{2+} has two charges and spermine has four. Thus they could be able to link the polar head groups of different phospholipid molecules to each other and restrict their mobility. Divalent cations are known to compress and increase the surface potential of

monolayers [28] and to increase the transition temperature of bilayers [8, 30]. Spermine decreases the anodic electrophoretic mobility of mitochondria [23]. It thus appears that the mobility of the polar head groups of phospholipids is responsible for the temperature sensitivity of mitochondrial Ca^{2+} transport. The role of the surface properties of membranes in the transport of ions has been discussed by Haydon and Hladky [31].

It is noteworthy that Mg^{2+} causes a decrease in the activation energy of mitochondrial Ca^{2+} transport. Spermine has an even more dramatic effect. It decreases it to almost 0 kJ/mole. The fact that the activation energy when both K^+ and spermine are present increases at higher temperatures is probably due to a more effective competition for the binding sites by K^+ when the membrane expands at higher temperatures. The Arrhenius plot is nonlinear when K^+ and Mg^{2+} are present. A similar result has been described for ATP-driven Ca^{2+} transport measured in the presence of NaCl and MCl_2 [3]. The workers interpreted the nonlinearity to be due to the use of ATP as energy source instead of respiration. However, the results presented in this work seem to indicate that Mg^{2+} is responsible for the nonlinearity.

The significant decrease in activation energy in the presence of spermine is compatible with a pore or channel mechanism of Ca^{2+} transport. Thus the conductance of bilayers in the presence if ionophores, which have been suggested to form pores, is also insensitive to temperature, while this is not the case in the presence of ionophores of the mobile carrier type [24].

It is also noteworthy that Eu^{3+}, a lanthanide, has no significant effect on the temperature sensitivity of mitochondrial Ca^{2+} transport. Eu^{3+} should not affect Ca^{2+} binding to the phospholipids because of the very low concentration used (0.02 μM).

Monovalent cations are known to fluidize bilayers [30] and monolayers [32]. Thus K^+ is expected to have an opposite effect on the temperature sensitivity of mitochondrial Ca^{2+} transport to that of Mg^{2+}, and K^+ indeed increases the slope of the Arrhenius plot.

The fact that spermine has no significant effect on the temperature sensitivity of state-4 respiration, which measures the function of the intrinsic proteins in the mitochondrial membrane, further supports the proposal that the temperature sensitivity is due to the state of the surface and not the interior of the membrane.

Spermine has similar effects on liposomal safranine uptake as on mitochondrial Ca^{2+} transport. It thus seems probable that the temperature sensitivity of both responses are due to a similar phenomena. This effect of spermine on liposomal safranine uptake gives further indirect evidence about the role of phospholipids in mitochondrial Ca^{2+} transport.

It is suggested that mitochondrial Ca^{2+} transport occurs in at least two steps. The first step is the association of Ca^{2+} with the polar head groups of the phospholipids at the outer surface of the membrane. This step is influenced by the mobility of the groups and thus temperature sensitive. It is also influenced by other cations. In the second step Ca^{2+} is transported through the hydrophobic core of the mitochondrial membrane presumably by a pore-typed mechanism. This step is electrogenic [33, 34], probably driven by the mitochondrial membrane potential [35], and is inhibited by lanthanides and ruthenium red [16, 17].

Acknowledgments

This study was aided by a grant by the Sigrid Jusélius Foundation. The author wishes to thank Professor N.-E. Saris for helpful discussion and criticism.

References

1. J. K. Raison, J. M. Lyons, and W. W. Thompson, *Arch. Biochem. Biophys.*, **142** (1971) 83.
2. P. J. Ainswoorth, R. M. Janki, E. R. Tustanoff, and A. J. S. Ball, *Bioenergetics*, 6 (1974) 135.
3. M. P. Lee and A. R. L. Gear, *J. Biol. Chem.*, **249** (1974) 7541.
4. K. Watson, R. L. Houghton, E. Bertoli, and D. E. Griffiths, *Biochem. J.*, **146** (1975) 409.
5. E. Bertoli, J. B. Finean, and D. E. Griffiths, *FEBS Lett.*, **61** (1976) 163.
6. P. Overath and H. Träuble, *Biochemistry*, **12** (1973) 2625.
7. B. De Kruyff, P. W. M. van Dijck, R. W. Goldbach, R. A. Demel, and L. L. M. van Deenen, *Biochim. Biophys. Acta*, **330** (1973) 269.
8. H. K. Kimelberg and D. Papahadjopoulos, *J. Biol. Chem.*, **246** (1974) 1071.
9. D. Chapman, *Quart. Rev. Biophys.*, **8** (1975) 185.
10. J. B. Chappell, M. Cohn, and G. D. Greville, in: *Energy-Linked Functions of Mitochondria*, B. Chance, Ed., pp. 219–231, Academic Press, New York and London (1963).
11. C. Rossi, A. Azzi, and G. F. Azzone, J. Biol. Chem., **242** (1967) 951.
12. K. C. Reed and F. L. Bygrave, *Biochem. J.*, **142** (1974) 555.
13. A. Scarpa and A. Azzi, *Biochim. Biophys. Acta*, **150** (1968) 473.
14. A. Scarpa and G. F. Azzone, *J. Biol. Chem.*, **243** (1968) 5132.
15. W. E. Jacobus and G. P. Brierley, *J. Biol. Chem.*, **244** (1969) 4995.
16. L. Mela, *Biochemistry*, **8** (1969) 2481.
17. K. C. Reed and F. L. Bygrave, *Biochem. J.*, **140** (1974) 143.
18. R. Colonna, S. Massari, and G. F. Azzone, *Eur. J. Biochem.*, **34** (1973) 577.
19. K. E. Åkerman and N.-E. L. Saris, *Biochim. Biophys. Acta*, **426** (1976) 624.
20. M. K. F. Wikström and N.-E. L. Saris, *Eur. J. Biochem.*, **9** (1969) 160.
21. L. Mela and B. Chance, *Biochemistry*, **7** (1968) 4059.
22. J. Saha, D. Papahadjopoulos, and C. E. Wenner, *Biochim. Biophys. Acta*, **196** (1970) 10.
23. A. Huunan-Seppälä, M.D. thesis (1972) University of Helsinki, Finland, pp. 1–115.

24. S. Krasne, G. Eisenman, and G. Szabo, *Science,* **174** (1971) 412.
25. G. G. Somjen and G. Kato, *Brain Res.,* **9** (1968) 161.
26. L. Packer, K. Utsumi, and G. Mustafa, *Arch. Biochem. Biophys.,* **117** (1966) 381.
27. C. W. Tabor, *Biochem. Biophys. Res. Commun.* **2** (1960) 117.
28. H. Tabor and C. W. Tabor, *Pharmacol. Rev.,* **16** (1964) 245.
29. D. Papahadjopoulos, *Biochim. Biophys. Acta,* **163** (1968) 240.
30. H. Träuble and H. Eibl, *Proc. Natl. Acad. Sci. USA,* **71** (1974) 214.
31. D. A. Haydon and S. B. Hladky, *Quart. Rev. Biophys,* **5** (1972) 187.
32. J. F. Tocanne, P. H. J. Th. Ververgaert, A. J. Verkleij, and L. L. M. van Deenen, *Chem. Phys. Lipids,* **12** (1974) 201.
33. M. J. Selwyn, A. P. Dawson and S. J. Dunnet, *FEBS Lett.,* **10** (1970) 1.
34. H. Rottenberg and S. Scarpa, *Biochemistry,* **13** (1974) 4811.
35. P. Mitchell, *Biol. Rev. (Camb.),* **41** (1966) 445.
36. K. E. O. Åkerman, *J. Bioenerg. Biomembr.,* **9** (1977) 77.