DIFFERENCE BETWEEN ATRACTYLOSIDE AND CARBOXYATRACTYLOSIDE ON THE BINDING TO THE MITOCHONDRIAL MEMBRANE

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1. Introduction

It has been shown by a number of investigations that the inhibition of oxidative phosphorylation induced by atractyloside or carboxyatractyloside has to be ascribed to a primary inhibition of adeninenucleotide translocation across the mitochondrial membrane [1,2].

Carboxyatractyloside, like atractyloside [3] is in fact inactive on ATPase activity of submitochondrial particles [2] a result recently confirmed by Vignais et al [4].

However it has also been observed that the kinetics of the inhibition of oxidative phosphorylation is different; atractyloside being a competitive inhibitor toward adeninenucleotides [5,6] and carboxyatractylosine a non competitive inhibitor [2,4]. The conclusion has been drawn from these investigations that carboxyatractyloside is more tightly bound to the mitochondrial membrane than atractyloside [2].

The molecular nature of the binding between the inhibitors and the mitochondria is still unknown and a possible approach to the problem is that of analyzing some parameters affecting the binding itself.

The influence of pH on the binding of atractyloside or carboxyatractyloside to the mitochondrial membrane has been studied in this paper. A pH dependence has been found for the effect of carboxyatractyloside in beef heart mitochondria [7].

2. Materials and methods

Rat liver mitochondria were isolated by conventional techniques in a medium of 0.25 M sucrose, 4 mM Tris–HCl pH 7.4.

Beef heart mitochondria were isolated as described by Scherer and Klingenberg [8] with minor changes. The mitochondrial swelling shrinkage was followed by measuring absorbance changes at 546 nm in an Eppendorf photometer equipped with a recorder. In order to measure the small changes in the absorbance the light path in the cuvette was 5 mm.

The binding of carboxyatractyloside to rat liver mitochondria has been followed by measuring the concentration of the drug on the supernatant after rapid centrifugation. The amount of carboxyatractyloside was estimated by following the inhibition of oxygen uptake (oxygen electrode) in tightly coupled mitochondria and using a calibration curve obtained with known amount of the drug. This method [9] has a sensitivity comparable to those using labelled compounds [10] and is devoid of contamination of foreign labelled material without biological activity see [11].

Atractyloside and carboxyatractyloside [12] were obtained from Inverni and Della Beffa (Milano). Bonkrekic acid was a generous gift from Professor W. Berends (Delft), all other reagents were analytical grade.

3. Results

3.1. *Kinetics of the inhibition by atractyloside and carboxyatractyloside of adeninenucleotide translocation.*

The competition between atractyloside and adeninenucleotides has been demonstrated on ADP-stimulated respiration, ATPase activity DNP-stimulated and ATP–P_i exchange [6] whereas no 'pure' competition has been observed when the adeninenucleotides translocation step has been analyzed [11] even if it is evident the

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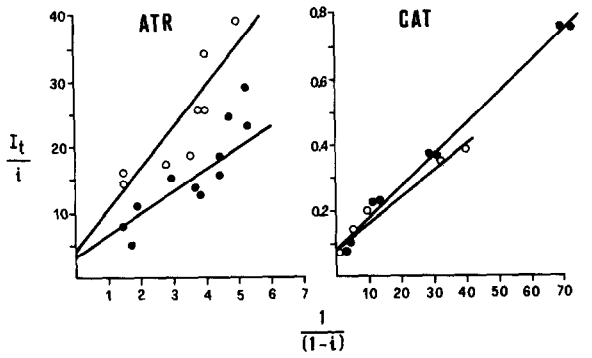

Fig.1. Inhibition by atractyloside (ATR) and carboxyatractyloside (CAT) of adeninenucleotides exchange in rat liver mitochondria. Measurement of 'back-exchange' [18] by the addition (10 sec) of 0.1 mM ADP (•) or 1 mM ADP (○) to mitochondria where endogenous adeninenucleotides are labelled (^{14}C) by preincubation with [^{14}C] ADP. Incubation in 0.25 M sucrose, 1 mM EDTA, 20 mM TRA medium pH 7.2, 10°. I_t = total inhibitor concentration (μmoles/g protein); i = degree of inhibition evaluted from absolute translocation rates.

possibility of partially overcoming the inhibition by increasing the concentration of ADP. As shown in fig.1 atractyloside behaves as a partially competitive inhibitor of the translocation of adeninenucleotides observed by the technique of back-exchange whereas the inhibition by carboxyatractyloside is unaffected by the presence of adeninenucleotides over a wide range of concentrations.

The Eason and Stedman treatment see [19] of inhibition kinetics used in fig.1 takes into account the decrease of free inhibitor concentrations in so called 'mutual depletion system'.

This result should be compared with the experiments reported by Vignais et al. [4] – fig.5B of their paper – in which the inhibition of ADP translocation by atractyloside is not clearly competitive, probably, as suggested by the authors, for the tightness of atractyloside binding to the mitochondria and the variation of free inhibitor concentration. In the case of carboxyatractyloside the inhibition is unequivocally 'non-competitive' both in the experiments of Vignais et al. [4] and in those reported in this paper.

3.2. *Influence of pH on the inhibition by carboxyatractyloside of the shrinkage induced by bonkrekic acid in beef heart mitochondria*

The contraction of beef heart mitochondria induced by ADP [13] is one of the most sensitive methods revealing the binding of adeninenucleotides to the mitochandrial membrane. Furthermore a close correspondence between the extent of contraction and the binding of adeninenucleotides has been demonstrated by Scherer and Klingenberg [8] using labeled compounds.

As shown in fig.2 the addition of small amounts of ADP to freshly prepared beef heart mitochondria induce a contraction which is reversed or prevented by the addition of atractyloside or carboxyatractyloside [8].

The difference between atractyloside and carboxyatractyloside van be evidenced when the addition of these compounds is followed by that of bonkrekic acid which induce a contraction in the presence of ADP. In this case the effect of atractyloside is reversible whereas that of carboxyatractyloside is irreversible [8].

However as shown in fig.2 at pH 6.1 also the inhibition by carboxyatractyloside becomes reversible on addition of bonkrekic acid. By plotting the reversal of the effect of carboxyatractyloside induced by bonkrekic acid at different pH ranging from 6.0 to 7.5 a pH-dependence is observed. On the contrary the reversal of atractyloside effect appears unaffected by varying

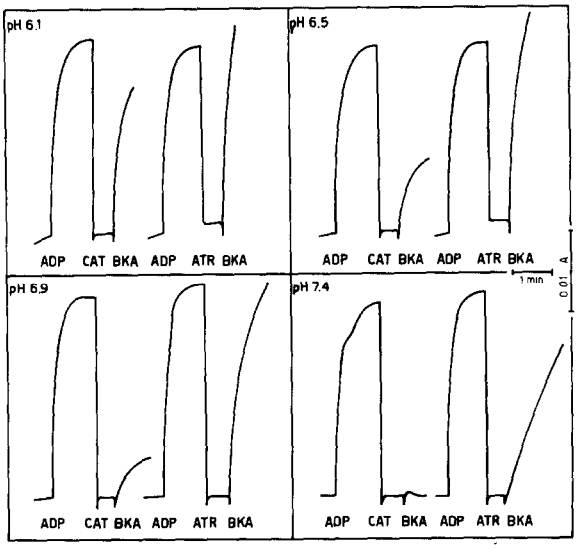

Fig.2. Reversal by bonkrekic acid (BA) of atractyloside (ATR) and carboxyatractyloside (CAT) effect on ADP-induced shrinkage in beef heart mitochondria at different pH. Incubation medium: 0.25 M sucrose, 1 mM EDTA, 10 mM K-PIPES, 0.45 mg of protein in a final volume of 1 ml. Temperature 22°C. ADP 5 μM, ATR 5 μM, CAT 2.5 μM, BKA, 5 μM. Absorbance change was measured as described in Materials and methods.

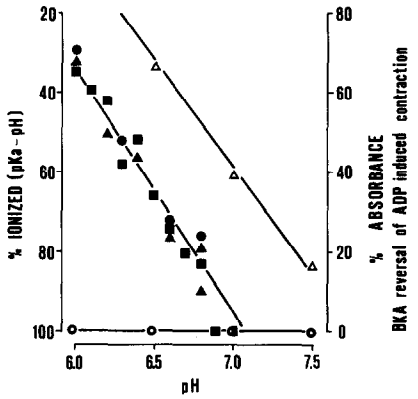

Fig.3. pH dependence of the reversal by bonkrekic acid of carboxyatractyloside effect on ADP induced contraction in beef heart mitochondria. Experimental conditions as in fig.2. Per cent ionization of an anion with a p*K*a of 6.8 (△—-△) and 3.8 (○——○).

the pH of the medium. The pH dependence of carboxyatractyloside effect indicated that the difference in polarity between this compound and atractyloside might play a role in the binding of these drugs to the mitochondrial membrane. The p*K*a of both atractyloside and carboxyatractyloside was therefore estimated by the potentiometric method. A p*K*a of 5.7 was found for atractyloside and a p*K*a′ of 3.8 and a p*K*″ of 6.8 for carboxyatractyloside [14]*. It is shown in fig.3 that the slope of the curve representing the pH-dependence of the bonkrekic acid reversal of caboxyatractyloside effect is similar to that indicating the percentage of ionization of an anion showing a p*K*a of 6.8. This observation might be interpreted as an indication that the carboxylic group of carboxyatractyloside with a p*K*a of 6.8 is regulating the tightness of the binding to the mitochondrial membrane.

3.3. *The binding of carboxyatractyloside to rat liver mitochondria*

It has been shown that the binding of carboxyatractyloside to the mitochondria follows a kinetic suggesting a co-operative behavior [4]. However these results obtained with labelled carboxyatractyloside lend themselves to different interpretations as stressed by Klingenberg et al. [11]. The results reported in fig.4 indicate that the binding of carboxyatractyloside follows a sigmoidal kinetic at low concentrations of mitochondrial protein and a hyperbolic behavior at high concentration. No clear pH-dependence has been found for the binding of carboxyatractyloside to rat liver mitochondria.

* The values of p*K*a are very close to those reported by Vignais et al. [4] and might explain the different behavior of atractyloside and carboxyatractyloside in vivo [15]. Carboxyatractyloside in spite of its higher toxicity is devoid of the nephrotoxic effect of atractyloside, probably because it does not penetrate the tubular membrane and is rapidly eliminated.

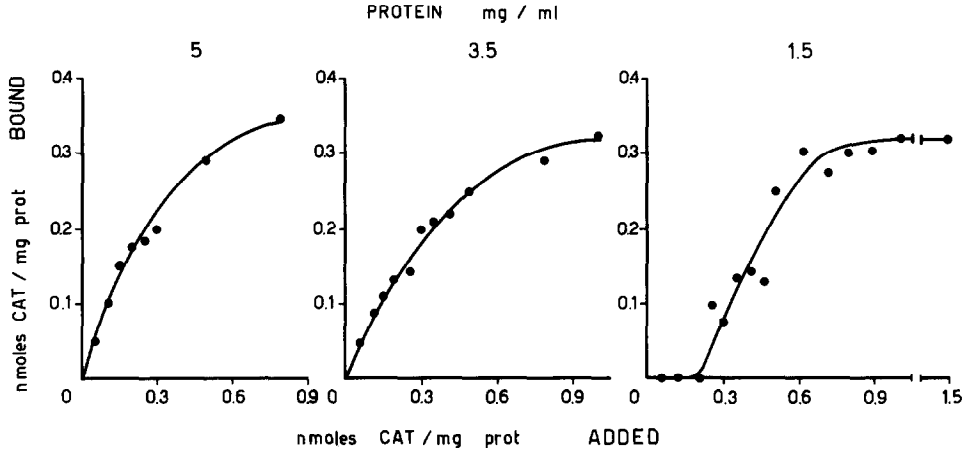

Fig.4. Binding of carboxyatractyloside (CAT) to rat liver mitochondria. Incubation medium was 0.1 M KCl, 20 mM Tris–HCl buffer pH 7.4, 1 mM EDTA. Temperature 0°C. Incubation time : 10 min. After centrifugation the concentration of CAT on the supernatant was estimated as described in Materials and methods. The values are mean values of 5–6 separate experiments.

4. Conclusions

The following conclusions can be drawn from the results presented in this paper:

(a) Competitive inhibition between atractyloside and adeninenucleotides on oxidative phosphorylation is evident when the overall process is observed whereas is not clearly demonstrable when only the step of translocation is analyzed. This result can be interpreted as indicating that the translocation of adeninenucleotides is not a rate-limiting step on the process of oxidative phosphorylation and/or that in intact mitochondrial translocation and energy transfer are not completely independent. Such interpretation is in agreement with the finding reported by Souverin et al. [16] showing that the energy state of mitochondria affects the translocation of adeninenucleotides most probably through a conformational modification of the translocator. More recently Out and Kemp [17] have reached the conclusion that the transport of adeninenucleotides and phosphorylation operate as single unit. The effect of carboxyatractyloside is independent of the presence of adeninenucleotides over a wide range of concentrations.

(b) In beef heart mitochondria the binding of carboxyatractyloside seems to be affected by the p*K*a of one of the two carboxylic groups of the drug. This indicates that in the binding of carboxyatractyloside to the mitochondrial membrane electrostatic forces are involved.

(c) The binding of carboxyatractyloside to the mitochondria is influenced by the concentration of mitochondrial proteins in the medium. The concentration of mitochondrial is affecting the rate and not the maximal capacity of mitochondria to bind carboxyatractyloside.

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References

[1] Heldt, H. W., Jacobs, H. and Klingenberg, M. (1965) Biochem. Biophys. Res. Commun. 18, 174–179.
[2] Luciani, S., Martini, N. and Santi, R. (1971) Life Sci. 10, 961–968.
[3] Löw, H., Vallin, I. and Alm, B. (1963) in: Energy Linked Functions of Mitochondria (Chance, B., ed.), pp. 5–25, Academic Press, New York.
[4] Vignais, P. V., Vignais, P. M. and Defaje, G. (1973) Biochemistry 12, 1508–1519.
[5] Bruni, A., Contessa, A. R. and Luciani, S. (1962) Biochim. Biophys. Acta 60, 301–311.
[6] Bruni, A., Luciani, S. and Bortignon, C. (1965) Biochim. Biophys. Acta 97, 434–441.
[7] Luciani, S. and Varotto, R. (1974) in: Abstracts 9th FEBS Meeting, Budapest, p. 275.
[8] Scherer, B. and Klingenberg, M. (1974) Biochemistry 13, 161–170.
[9] Bruni, A., Contessa, A. R. and Scalella, P. (1965) Biochim. Biophys. Acta 100, 1–12.
[10] Klingenberg, M., Falkner, G., Herdelt, H. and Grebe, K. (1971) FEBS Lett. 16, 296–300.
[11] Klingenberg, M., Scherer, B., Stengel-Rutkowski, L., Buchholz, M. and Grebe, K. (1973) in: Mechanisms in Bioenergetics (Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds.), pp. 257–284, Academic Press, New York.
[12] Danieli, B., Bombardelli, E., Bonati, A. and Gabetta, B. (1972) Phytochemistry 11, 3501–3504.
[13] Stoner, C. D. and Sirak, H. D. (1973) J. Cell. Biol. 56, 51–64.
[14] Luciani, S., Carpenedo, F., Palatini, P. and Santi, R. (1973) Boll. Soc. It. Biol. Sper. 49, 83.
[15] Carpenedo, F., Luciani, S., Scaravilli, F., Palatini, P. and Santi, R. (1974) Arch. Toxicol. 32, 169–180.
[16] Souverijn, H. J. M., Huisman, L. A., Rosing, J. and Kemp, A. (1973) Biochim. Biophys. Acta 305, 185–198.
[17] Out, T. and Kemp, A. (1974) Biochem. Soc. Trans. 2, 516–517.
[18] Pfaff, E. and Klingenberg, M. (1968) Europ. J. Biochem. 6, 66–79.
[19] Webb, J. L. (1963) Enzyme and Metabolic Inhibitors, Vol. 1, pp. 73–75, Academic Press, New York.