ENERGY-LINKED REACTIONS IN MITOCHONDRIA : THE EFFECTS OF SOME INHIBITORS AND THEIR USE IN THE LOCATION OF THE TRANSHYDROGENASE ENZYME

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Summary

The effects of 2-phenylisatogen, 2-phenylindolone, N-ethylmaleimide and p-hydroxymercuribenzoate on transhydrogenase reactions are compared. 2-Phenylindolone and N-ethylmaleimide inhibited the energy-linked transhydrogenase at lower concentrations than they inhibited the non-energy-linked transhydrogenase, whereas the reactions were equally sensitive to p-hydroxymercuribenzoate. 2-Phenylisatogen inhibited the transhydrogenase reactions when added to the mitochondria prior to sonication, but not when added directly to the assay medium. The results are discussed in terms of the location of the transhydrogenase enzyme.

Introduction

2-Phenylindolone, an inhibitor of phosphate transport (1), has been shown to inhibit the transhydrogenase reaction catalysed by rat liver submitochondrial particles (2). A closely related compound, 2-phenylisatogen, was found to inhibit ADP-stimulated respiration in rat liver mitochondria (3). In an effort to determine the mechanism of action of 2-phenylisatogen, its action on transhydrogenase reactions was compared with the effects of the phosphate transport inhibitors 2-phenylindolone (1), p-hydroxymercuribenzoate (4) and N-ethylmaleimide (5). We believe that the experiments may shed some light on the location of the transhydrogenase enzyme in rat liver mitochondria.

Methods

Rat liver mitochondria were prepared by the method of Chappell and Hansford (6). The mitochondria (20-25mg/ml) were suspended in a medium containing 0.25M-sucrose, 1mM-ATP, 4mM-magnesium chloride, 1mM-sodium succinate and 10mM-tris-HCl, pH 7.4, and sonicated at maximum output from the large probe of an MSE 100W sonicator for 90s in 30s bursts, with 30s between each burst. The probe was precooled in ice and the mitochondria were cooled during sonication by an ice-water mixture. Unbroken mitochon-

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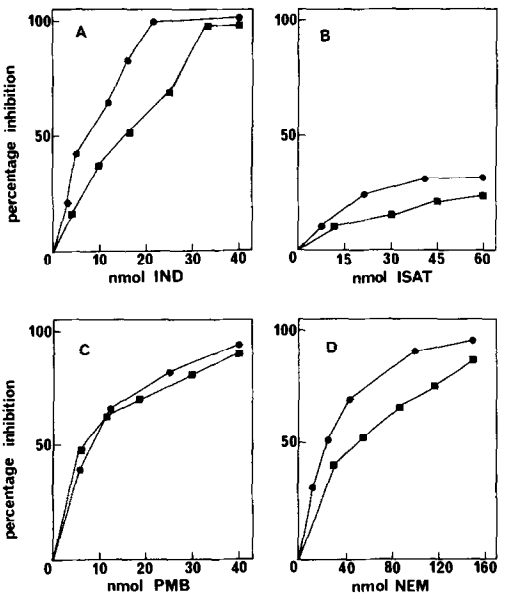

Fig. 1. The effect of inhibitors on the non-energy-linked and ATP-driven transhydrogenases in rat liver submitochondrial particles. a) inhibition by 2-phenylindolone (IND), b) inhibition by 2-phenylisatogen (ISAT), c) inhibition by p hydroxymercuribenzoate (PMB) and d) inhibition by N-ethylmaleimide (NEM). ■ refers to the non-energy-linked transhydrogenase which was measured as follows: 675μmol sucrose, 150μmol tris-HCl, pH 8.0, 5μmol potassium cyanide, 300μg yeast alcohol dehydrogenase, 10μl ethanol and 1.5-2mg rat liver submitochondrial particle protein were added to both blank and experimental cuvettes of 1cm light path. NAD^+ (40nmol) was added to the experimental cuvette, followed 1min later by 1μmol $NADP^+$. The final volume was 3ml. The optical density change at 340nm was monitored using a Pye-Unicam SP 1800 spectrophometer. The reaction temperature was 30° C.

● refer to the ATP-driven transhydrogenase which was measured as follows: the non-energy linked reaction in the presence of 20μmol magnesium chloride was followed for about 2min and then 6μmol ATP was added. Corrections were made for the non-energy-linked reaction. The concentrations of the inhibitors refer to nmol/mg submitochondrial particle protein.

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dria were removed at 27,000g for 15 min at 2°C and the supernatant centrifuged at 100,000g for 30 min at 2°C in the 8 x 25 ml rotor of an MSE 40 centrifuge. The pellet was resuspended in 0.25M-sucrose, 4mM-magnesium chloride, 50mM-tris-HCl, pH 7.6, and centrifuged at 100,000g for 30 min at 2°C. The final pellet was suspended in the sucrose-magnesium-tris medium to give a protein concentration of 10-20mg/ml. In some experiments inhibitors were added to the mitochondria immediately before sonication.

Protein was determined by the method of Gornall et al (7) after solubilization with deoxycholate.

The non-energy-linked and ATP-driven transhydrogenases were measured by the method of Griffiths and Roberton (8).

ATP-hydrolysis was measured according to the method of Beechey (9), phosphate release being determined by the method of Fiske and Subbarow (10).

2-Phenylindolone was prepared by the method of Kalb and Bayer (11) and 2-phenylisatogen was synthesized by the method of Bond and Hooper (12). Both compounds were added to the reaction medium as solutions in dimethylformamide (0.5-5µl); controls with this solvent showed that it did not affect the reactions studied. N-Ethylmaleimide and p-hydroxymercuribenzoate were purchased from Sigma Chemical Co. Ltd., London.

Results

The reactions studied in the present work were the non-energy-linked and ATP-driven transhydrogenases catalysed by rat liver submitochondrial particles. Fig. 1 shows that both reactions were completely inhibited by p-hydroxymercuribenzoate, N-ethylmaleimide and 2-phenylindolone. There were, however, some differences in the pattern of inhibition obtained with the three compounds.. First, both reactions were equally sensitive towards p-hydroxymercuribenzoate, but the ATP-driven reaction was consistently more sensitive towards N-ethylmaleimide and 2-phenylindolone than was the non-energy-linked reaction. Second, similar concentrations of p-hydroxymercuri benzoate and 2-phenylindolone were required to inhibit the ATP-driven reaction, but much higher concentrations of N-ethylmaleimide were required for inhibition. We have, however, shown previously that the potency of N-ethylmaleimide can be increased to that of the other two compounds when it is preincubated with the submitochondrial particles for 24h (2). 2-Phenylisatogen was found to be a poor inhibitor of both transhydrogenase reactions (maximum inhibition is in the region of 10-20%). This result was somewhat surprising in view of the close structural similarity between 2-phenylisatogen and 2-phenylindolone.

It was thought that the increased sensitivity of the ATP-driven reaction, as opposed to the non-energy-linked reaction, to N-ethylmaleimide and 2-phenylindolone could be due to an inhibition of ATP-hydrolysis by these compounds. Table 1 shows that N-ethylmaleimide and 2-phenylindolone, as

Table 1

Effect of Inhibitors on ATP hydrolysis in Rat Liver Submitochondrial Particles

INHIBITOR (10nmol/mg SMP protein)	RATE OF ATP HYDROLYSIS (nmol/min/mg SMP protein)
None	125
2-phenylisatogen	158
2-phenylindolone	146
p hydroxymercuribenzoate	128
N-ethylmaleimide	136

125μmol Sucrose, 50μmol tris-HCl, pH8, 3μmol ATP and the inhibitors were added to test tubes in a water bath at 30°C. The reaction was started by the addition of 1mg submitochondrial particle protein to give a final volume of 1ml. After 10min, 3%^{w}/v trichloracetic acid were added to stop the reaction. The tubes were transferred to an ice bucket and then the protein precipitate removed. 0.5ml aliquots of the supernatant were assayed for inorganic phosphate by the method of Fiske and Subbarow (10.)

well as p-hydroxymercuribenzoate and 2-phenylisatogen, had no inhibitory effect on the ATPase catalysed by rat liver submitochondrial particles; in fact all the compounds were found to be capable of stimulating ATP hydrolysis by between 5-20%.

The lack of effect of 2-phenylisatogen on the transhydrogenase reactions could have been due to one of two reasons. Either the isatogen was inactive or it was active but unable to penetrate to its site of action on the transhydrogenase. In an attempt to distinguish between these alternatives rat liver submitochondrial particles were prepared by sonicating the mitochondria in the presence of 2-phenylisatogen or 2-phenylindolone. Table 2 shows

Table 2

Inhibition of Transhydrogenases in Rat Liver Submitochondrial Particles Prepared in the Presence and Absence of Inhibitors

CONDITIONS	% INHIBITION	
	2-PHENYLISATOGEN	2-PHENYLINDOLONE
1. Non-Energy-Linked Transhydrogenase		
a) Inhibitor added to assay medium	10	38
b) Inhibitor added to sonication medium	50	61
2. ATP-Driven Transhydrogenase		
a) Inhibitor added to assay medium	13	58
b) Inhibitor added to sonication medium	60	69

The Transhydrogenase reactions were measured as described in the legend to Fig 1. Inhibitors were present at a concentration of 10nmol/mg protein. Each figure for %inhibition represents the mean of 7 determinations.

that inclusion of 2-phenylisatogen in the sonicating medium increased the inhibition of both the non-energy linked and ATP-driven transhydrogenases. Under these conditions the inhibition by the isatogen approached that obtained using 2-phenylindolone, the potency of which was also increased to some extent when it was included in the sonicating medium.

Discussion

The results show that p-hydroxymercuribenzoate, N-ethylmaleimide, 2-phenylindolone and 2-phenylisatogen are all capable of inhibiting the transhydrogenase enzyme in rat liver mitochondria. The four compounds all inhibit the non-energy-linked reaction and so must inhibit the transhydrogenase enzyme directly. The transhydrogenase enzyme contains a thiol group (13)

and it is possible that all four compounds are reacting with this group, however, maleimides, indolones and isatogens can react with other nucleophiles.

The inhibition of the transhydrogenase by 2-phenylindolone and by N-ethylmaleimide was increased in the presence of ATP, even though ATP hydrolysis was not inhibited. It has been reported that both the non-energy-linked and energy-linked transhydrogenases are catalysed by the same enzyme (8) and that the presence of energy alters the conformation of the transhydrogenase enzyme (14). It is possible that this energized conformation makes the active site more accessible to the inhibitors.

The problem of accessibility of the transhydrogenase enzyme to inhibitors was illustrated in the experiments using 2-phenylisatogen. The inhibition of the transhydrogenase was greatly increased when 2-phenylisatogen was added to the mitochondria prior to sonication, rather than to the submitochondrial particles. We interpret this to mean that 2-phenylisatogen can reach its site of action in mitochondria, but not in submitochondrial particles. Since 2-phenylisatogen inhibits the transhydrogenase enzyme itself, then these results indicate that the transhydrogenase enzyme in rat liver mitochondria is towards the outside of the inner membrane. Rydstrom et al (14) have interpreted experiments on the inactivation of enzymes using trypsin to indicate that the transhydrogenase enzyme is on the inside of the inner membrane in beef heart mitochondria. It is possible that the position of the transhydrogenase enzyme differs in different mitochondrial preparations. However, we feel that experiments using trypsin are unsatisfactory in two respects. First, little is known of the different susceptibilities of various enzymes to inactivation by trypsin, and selective inactivation may indicate differences in susceptibilities rather than differences in accessibility to trypsin. Second, little is known of the effects of trypsin treatment on the mitochondrial membrane as a whole. Damage of the membrane in one part could possibly affect enzymes in another part of the same membrane, and even on the opposite side of it.

Thus we postulate a transhydrogenase enzyme located towards the outside of the inner membrane of rat liver mitochondria, and which undergoes a conformational change in the presence of a supply of energy. The energized conformation of the enzyme is more accessible to 2-phenylindolone and N-ethylmaleimide.

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