THE MITOCHONDRIAL HANDLING OF D,L-THIOCARNITINE AND ITS *S*-ACETYL DERIVATIVE

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Received 16 September

1. Introduction

The sulphur derivatives of carnitine have not been studied since the brief statement in 1970 [1] that D,L-thiocarnitine stimulates the mitochondrial oxidation of palmityl coenzyme A, a characteristic property of carnitine itself, while its acetyl derivative, D,L-ATC is a substrate for the exchange enzyme, CAT. We have therefore studied further the properties of these two substances and now report on their behaviour as carnitine analogues and the effect of adding them to rat heart mitochondria.

2. Materials and methods

Rat heart mitochondria were prepared as in [2] and used within 1 h of preparation. Proteins were assayed by a biuret method [3]. Other assays are as shown in the figures and table. CAT and other enzymes necessary were purchased from Boehringer, Mannheim. D,L-Thiocarnitine and D,L-ATC were donated by Sigma Tau Ltd., Rome. The buffer used (except where otherwise mentioned) was 0.125 M KCl containing 20 mM Tris–HCl (pH 7.4) and 0.1 mM EDTA.

Abbreviations: D,L-ATC,D,L-*S*-acetylthiocarnitine; CAT, carnitine acetyl transferase (EC 2.3.1.7); DTNB, 5,5'-dithiobis(-2-nitrobenzoic acid); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone

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3. Results

The two sulphur analogues of carnitine and acetylcarnitine were examined as substrates in the reaction catalysed by CAT [4]:

Acetylcarnitine + Coenzyme A ⇌

Acetylcoenzyme A + Carnitine

The results (fig.1) show that ATC substitutes for L-acetylcarnitine in the forward direction and gives a similar rate and equilibrium constant, whereas D,L-thiocarnitine, when used in place of carnitine in the backward reaction, is a far less effective substrate. CAT is known [6] to be inhibited by both D-carnitine and D-acetylcarnitine and one explanation for our results may be that D-thiocarnitine, when present at high initial concentration, is also an inhibitor whereas D-ATC is not.

These findings are of special interest when considering the behaviour of the two analogues upon addition to rat heart mitochondria. D,L-Thiocarnitine added at 20°C in the presence of FCCP, pyruvate and malonate gives no stimulation of oxygen uptake. Moreover the stimulation normally obtained with carnitine under these conditions [7] is inhibited somewhat by D,L-thiocarnitine, whether added before or after carnitine (fig.2). In contrast, D,L-ATC, when added as an oxidisable substrate to rat heart mitochondria, together with FCCP and malate, induces an oxygen consumption which is hardly less than that obtained with L-acetylcarnitine itself [8]. Thus, in two assays at 20°C and using an initial concentration of 5 mM, oxygen consumptions were from L-acetylcarnitine and D,L-ATC, respectively

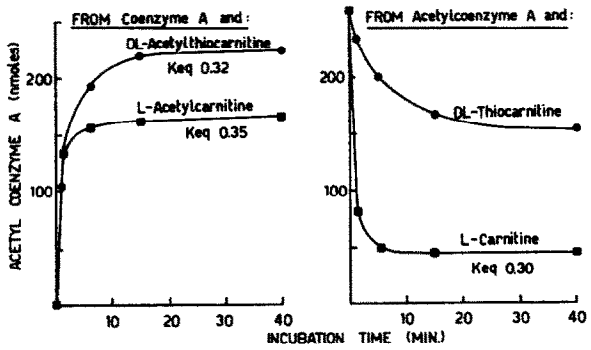

Fig.1. Comparison of the behaviour of carnitine substrates with their sulphur derivatives in the reaction catalysed by CAT. CAT (2 IU/ml medium) was added to deaerated 0.1 M phosphate buffer (pH 7) containing EDTA (0.5 mM) and: either coenzyme A (0.5 mM) with D,L-ATC (1 mM) or L-acetylcarnitine (0.5 mM); or acetylcoenzyme A (0.25 mM) with D,L-thiocarnitine or L-carnitine (0.5 mM). After incubation at 30°C for the times indicated, samples (2 ml) were acidified to 0.8% (w/v) in perchloric acid. After subsequent neutralisation with KOH, acetylcoenzyme A concentrations were assayed [5]. Coenzyme A solutions were standardized immediately before incubation with DTNB [9] and acetylcoenzyme A as above. Small gains or losses in the latter after incubation were subtracted. Equilibrium constants are calculated in the direction shown in the text assuming that the D-forms are inactive. A value for K_{eq} with acetylcoenzyme A and carnitine of 0.6 has been reported [6].

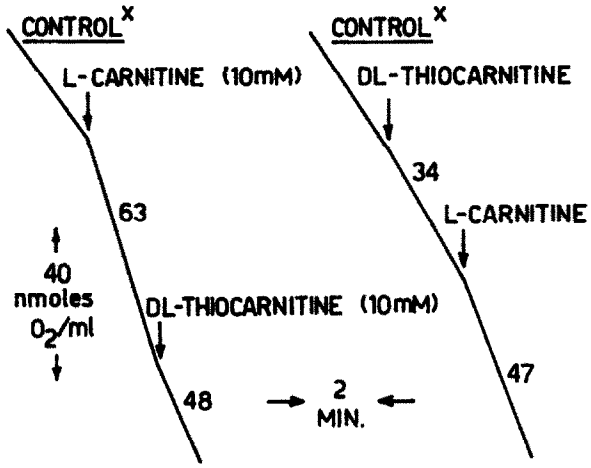

Fig.2. Effect of thiocarnitine and carnitine on oxygen uptake by rat heart mitochondria. Mitochondria (2.5 mg protein/ml medium) in buffer containing FCCP (2 nM), pyruvate (1 mM) and malonate (1 mM) were incubated with stirring at 20°C in the chamber of a Clark-type oxygen electrode with the addition of carnitine or thiocarnitine as indicated.

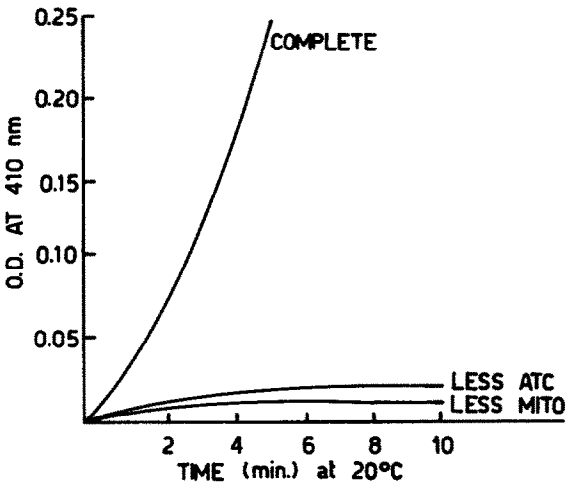

Fig.3. Assay of thiol formation from D,L-ATC. To 1 ml buffer containing D,L-ATC (1.25 mM), DTNB (0.2 mM) and FCCP (2 nM) was added 2 mg mitochondrial protein. The extinction increase at 412 nm was read against a blank containing the mitochondria in buffer with FCCP.

(nmol . min^{-1} . mg $protein^{-1}$), 13 ± 2.25 and 10.5 ± 1.25. The finding that ATC is readily oxidised implies that free thiocarnitine is released. Formation of this substance is easily followed by assaying the rate of production of new thiol groups using the DTNB reagent [9]. This formation has been observed continuously by incubating mitochondria with ATC in the presence of DTNB (fig.3). It can also be followed discontinuously by precipitating the mitochondrial proteins before adding the reagent.

The release of thiol from ATC by rat heart mitochondria requires the availability of free mitochondrial coenzyme A. This is shown by preincubating mitochondria with acetoacetate and 2-oxoglutarate under conditions shown [10] to trap the coenzyme as its acetoacetyl derivative. Thiol formation on the subsequent addition of ATC is then inhibited by >90%. The effect of L-carnitine and L-acetylcarnitine, respectively, on the release of thiol from ATC has also been determined and Dixon plots (fig.4) show that both substances are competitive inhibitors of the reaction. However a much more potent inhibitor is L-palmitoylcarnitine which exhibits non-competitive kinetics (not shown) with K_i 0.3 mM.

When D,L-ATC is added to rat liver mitochondria instead of those from rat heart, there is little release of thiol nor is there any significant oxygen uptake:

similar results have been reported for L-acetylcarnitine and attributed to the fact that rat liver mitochondria have only 1.1% of the activity of rat heart mitochondria [11].

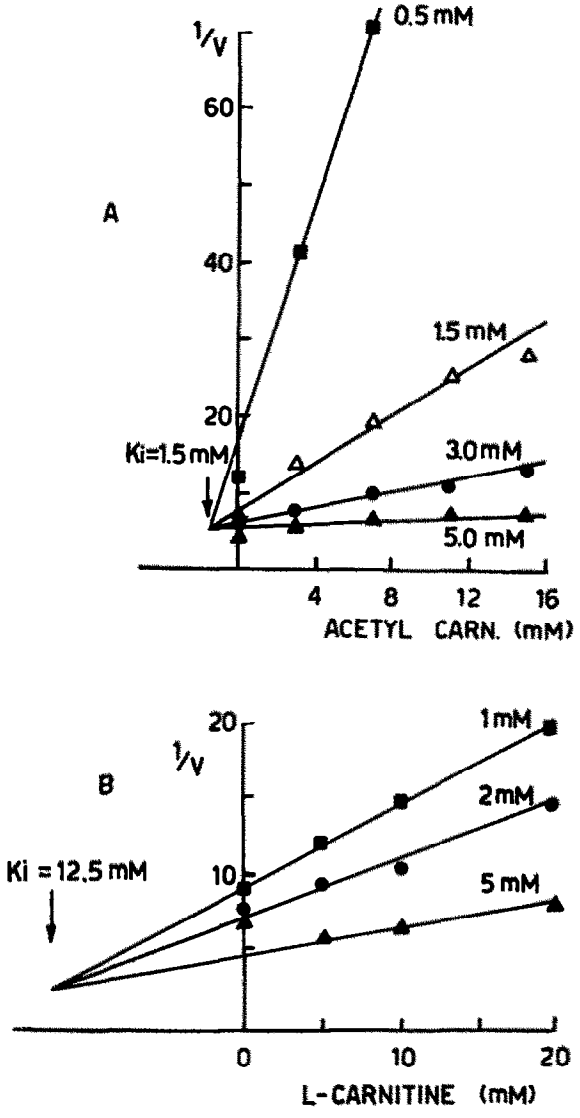

Fig.4. Dixon plots showing the inhibition by L-acetylcarnitine or L-carnitine of thiol formation from D,L-ATC. Mitochondria (2.5 mg protein/ml medium) were added to buffer containing FCCP (2 nM), D,L-ATC (5 mM) and the inhibitor shown. After incubation at 30°C for 4 min 1 ml samples were acidified to 0.6% (w/w) in perchloric acid. Thiol formed was calculated from the increase in extinction at 412 nm [9] after adding 0.5 ml DTNB (1 mM) containing 0.5 M phosphate buffer (pH 7). Values obtained after incubating controls lacking, respectively, mitochondria and ATC were added together and subtracted from the rest.

Table 1
Second order velocity constants for the reaction of D,L-ATC with various acceptors

Acceptor	k ($M^{-1} \cdot s^{-1} \cdot 10^{-3}$)
Coenzyme A	91.3 ± 12
Glycine	4.7
p-Aminobenzoic acid	1.7
Aspartic acid	1.3
Carnitine	Nil
Phosphoric acid	Nil

When coenzyme A was the acceptor, D,L-ATC was added at 20°C in triethanolamine buffer (pH 7.8) with varying initial concentrations of coenzyme A to the system used for assaying the formation of acetylcoenzyme A [5]. Contamination of the assay enzymes with CAT was excluded since no acetylcoenzyme A was formed when L-acetylcarnitine was used in place of ATC. For the other acceptors, the rate of thiol formation was found after adding D,L-ATC together with DTNB (0.5 mM) to a solution at 20°C of the acceptor in Tris–HCl buffer (pH 7.4). Thiol formed without the acceptor present was subtracted

ATC possesses transacetylating activity. Thus it forms acetylcoenzyme A from coenzyme A non-enzymically at a low rate (insignificant under the conditions of fig.1) and, as judged by the release of thiol in the presence of potential acceptors, is also able to acetylate glycine and other amino acids (table 1).

4. Discussion

ATC, determined as an effective substrate for CAT, is metabolised by heart mitochondria. The reaction is conveniently followed by measuring the rate of formation of thiol groups originating from the released thiocarnitine. This release is inhibited by trapping mitochondrial coenzyme A. Both L-acetylcarnitine and L-carnitine are competitive inhibitors. These findings taken together are strong evidence that heart mitochondria can handle ATC in the same way as they handle acetyl carnitine, i.e., by enzymic transacetylation on to endogenous coenzyme A. Although non-enzymic transacetylation has been observed it is difficult to account for the competitive kinetics or the low activity with liver mitochondria if it is other than of minor importance. The powerful inhibition obtained with L-palmitoylcarnitine, which is not a substrate and does not affect the activity of

CAT [12], may be due to its trapping coenzyme A as the palmitoyl derivative; alternatively the entry of ATC into the matrix may require, like L-palmitoyl-carnitine, the operation of the carnitine translocase system [13,14].

The results suggest that D,L-ATC, because of the ease and accuracy of assaying thiol groups, may be a suitable reagent for the further study of acetyl group metabolism in mitochondria.

Acknowledgements

The secretarial help of Mrs Maurizia Cuccia is acknowledged. P. C. J. is grateful to Sigma Tau Ltd., Rome for an award to defray maintenance costs.

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