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Oxidation of acetate, acetyl CoA and acetylcarnitine by pea mitochondria

David R. Thomas* and Clifford Wood
Department of Plant Biology, Newcastle University, Newcastle upon Tyne NE1 7RU, U.K.

Abstract. Acetylcarnitine was rapidly oxidised by pea mitochondria. (−)-carnitine was an essential addition for the oxidation of acetate or acetyl CoA. When acetate was sole substrate, ATP and Mg^{2+} were also essential additives for maximum oxidation. CoASH additions inhibited the oxidation of acetate, acetyl CoA and acetylcarnitine. It was shown that CoASH was acting as a competitive inhibitor of the carnitine stimulated O_2 uptake. It is suggested that acetylcarnitine and carnitine passed through the mitochondrial membrane barrier with ease but acetyl CoA and CoA did not. Carnitine may also buffer the extra- and intra-mitochondrial pools of CoA. The presence of carnitine acetyltransferase (EC 2.3.1.7) on the pea mitochondria is inferred.

Key words: Acetyl-group oxidation – Carnitine – Mitochondria – *Pisum*.

Introduction

Slices of avocado mesocarp, which contains (−)-carnitine (Panter and Mudd 1969) oxidised palmitate. Exogenously supplied (−)-carnitine stimulated and (+)-carnitine inhibited this oxidation (Panter and Mudd 1973). CoASH, Mg^{2+}, ATP and (−)-carnitine were necessary additions to the incubation media for pea mitochondria to oxidise palmitate at a maximum rate (McNeil and Thomas 1976). Investigation of the carnitine stimulated oxidation of fatty acids of different chain lengths showed two maxima, one for short chain acids (C4:0) and one for long chain acids (C16:0) (Thomas and McNeil 1976). Thomas and McNeil (1976) suggested that there were at least two carnitine acyltransferases present in their pea mitochondrial preparations, a long-chain and a short-chain transferase. These enzymes transfer acyl groups from acyl CoA to carnitine (Fritz 1963). It was postulated that carnitine acted, as proposed by Fritz (1963) for mammalian mitochondria, by facilitating the transport of acyl groups across the mitochondrial membranes to intramitochondrial β-oxidation sites (McNeil and Thomas 1976; Thomas and McNeil 1976). (−)-carnitine is known to be present in pea cotyledons (McNeil and Thomas 1975). In this report the possible functions of a carnitine short-chain acyltransferase (carnitine acetyltransferase, EC 2.3.1.7) in pea mitochondria is examined by studying the oxidation of acetate, acetyl CoA and acetylcarnitine by the mitochondria.

* To whom correspondence should be addressed

Materials and methods

Pea seeds (*Pisum sativum* L c.v. Alaska) were germinated in the dark at 25° C for 48 h as described previously (Thomas and McNeil 1976). The mitochondria were isolated and O_2 uptake measured by the methods used by McNeil and Thomas (1976). Bovine serum albumin (Fraction V purchased from Sigma, London Chemical Co., Poole, Dorset, U.K.) was purified by the method of Thomas et al. (1982). Details of incubation mixtures are given in the legends to the Tables and Figures in the text. The terminology of Chance and Williams (1956) and Chance (1959) as defined for steady state conditions of plant mitochondria by Bonner (1973) is followed. Protein was determined by the method of Lowry et al. (1951).

Results and discussion

Thomas and his co-workers (1981) demonstrated that carnitine supplied to barley leaves was not utilized as a respiratory substrate, it remained as carnitine or was converted to an acylcarnitine. Carnitine did not increase O_2 uptake when supplied to pea mitochondria in the presence of "sparker" quantities of malate (Table 1). Any alteration in O_2 uptake reported in this paper therefore cannot be attributed to carnitine's utilization per se as a respiratory substrate by the mitochondria.

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Table 1. Oxidation of acetate, acetyl CoA, acetylcarnitine and (−)-malate by pea mitochondria. The basic medium contained: sucrose, 200 mM; K_2HPO_4, 4 mM; NAD+, 0.23 mM; TPP, 0.17 mM; 0.5% (w/v), bovine serum albumin; Tris buffer, 20 mM pH 7.0 and 2.5–3.5 mg mitochondrial protein, in a total volume of 3 ml. Additions were as follows: CH_3COOK, 10 mM; $MgSO_4$, 1 mM; CoASH, 0.1 mM; (−)-carnitine, 1 mM; (−)-malate 0.1 mM; ATP, 1 mM; acetyl CoA, 3 mM; acetylcarnitine, 1 mM. State 3 rates were measured after the addition of 0.6 μmol ADP. The results are typical of those obtained with 6 different mitochondrial preparations (RCR = respiratory control ratio)

Substrate and cofactors added to basic medium	O_2 uptake (nmol min^{-1} mg^{-1} protein)		RCR
	State 3	State 4	
Acetate, CoASH, Mg^{2+}, ATP	7.6	6.8	1.12
Acetate, CoASH, Mg^{2+}, ATP, (−)-carnitine	19.1	13.3	1.44
Acetyl CoA	6.1	5.4	1.13
Acetyl CoA, (−)-carnitine	18.7	11.6	1.61
Acetylcarnitine	20.0	13.0	1.54
(−)-malate, (−)-carnitine	5.7	5.5	1.04
(−)-malate	5.7	5.6	1.02

Table 2. Cofactor requirements for pea mitochondria oxidising acetate. The basic medium contained: sucrose, 200 mM; K_2HPO_4, 4 mM; CH_3COOK, 10 mM (−)-malate, 0.1 mM; NAD+, 0.23 mM; TPP, 0.17 mM; 0.5% (w/v) bovine serum albumin; Tris buffer, 20 mM pH 7.0 and 2.5–3.5 mg protein in a total volume of 3 ml. Additions were as follows: CoASH, 0.1 mM; ATP, 1 mM; $MgSO_4$, 1 mM and (−)-carnitine, 1 mM. State 3 rates were measured after the addition of 0.6 μmol ADP. The results are typical of those obtained in five experiments with different mitochondrial preparations

Cofactors added to basic medium	Oxygen uptake (nmol min^{-1} mg^{-1} protein)		RCR
	State 3	State 4	
CoASH, Mg^{2+}, ATP, (−)-carnitine	21.2	12.7	1.67
CoASH, Mg^{2+}, ATP	12.2	10.8	1.13
CoASH, Mg^{2+}, (−)-carnitine	10.0	10.0	1.00
CoASH, ATP, (−)-carnitine	12.3	9.7	1.27

The data of Table 1 shows that (−)-carnitine was required to obtain the maximum rates of O_2 uptake and the highest RCR (respiratory control ratio) values when acetate was substrate. In the absence of (−)-carnitine very little difference between state 3 and state 4 O_2 uptake was observed which accounted for the low RCR values. When acetyl CoA was substrate, O_2 uptake was low and similar to that observed for acetate without (−)-carnitine for "sparker" malate alone and for malate in the presence of (−)-carnitine. (−)-carnitine greatly stimulated the O_2 uptake of mitochondria supplied acetyl CoA. This result was in contrast to that obtained by Edwards et al. (1974) with uncoupled ox liver mitochondria. These workers found that although acetylcarnitine was oxidised competently, acetyl CoA oxidation was hardly affected by (−)-carnitine. The results obtained here with pea mitochondria (Table 1) demonstrate that acetyl groups from exogenously supplied acetyl CoA were not accessible to the tricarboxylic acid cycle unless (−)-carnitine was present. Hence acetyl CoA did not pass through the mitochondrial membrane barrier. Acetylcarnitine as sole substrate resulted in high O_2 uptake and high RCR values. It is suggested that acetylcarnitine was able to pass through the mitochondrial membrane barrier and the acetyl group then entered the tricarboxylic cycle. As stated, acetyl CoA did not enter the mitochondria but it is suggested that in the presence of (−)-carnitine the reaction catalysed by carnitine acetyltransferase, an enzyme known to be present in animal mitochondria (see Frenkel and McGarry 1980), would occur and form acetylcarnitine. The acetylcarnitine would then penetrate the membrane barrier to the matrix of the mitochondria.

Acetate was not oxidised rapidly except in the presence of (−)-carnitine (Tables 1 and 2). Also ATP and Mg^{2+} were essential additives to the medium in order to obtain high O_2 uptake and high RCR values (Table 2). The omission of ATP reduced state 3 O_2 uptake rates to the low value of the state 4 rates giving RCR values of unity. Omission of Mg^{2+} also substantially reduced state 3 O_2 uptake rates thus reducing the RCR. Presumably, some Mg^{2+} remained in the mitochondria preparations to account for the low but observed state 3 rate of O_2 uptake in the presence of (−)-carnitine (Table 2).

Interestingly the data in Table 3 shows that if CoASH was omitted when Mg^{2+}, ATP and (−)-carnitine were present in the incubation mixture for mitochondria oxidising acetate, the O_2 uptake in both state 3 and state 4 was the highest achieved in any experiment with a resulting high RCR value. Additions of CoASH reduced the oxygen uptake for both state 3 and state 4 oxidation of acetate by the mitochondria. The addition of CoASH also severely inhibited state 3 and state 4 O_2 uptake both when acetylcarnitine was sole substrate and when acetyl CoA was substrate in the presence of (−)-carnitine (Table 3). Lineweaver-Burk plots demonstrate that CoASH was acting as a competitive inhibitor of carnitine action (Fig. 1).

Table 3. Effect of CoASH on the oxidation of acetate, acetyl CoA and acetylcarnitine by pea mitochondria. The basic medium contained: sucrose, 200 mM; K_2HPO_4, 4 mM; (−)-malate, 0.1 mM; NAD+, 0.23 mM; TPP, 0.17 mM; (−)-carnitine, 1 mM; 0.5% (w/v) bovine serum albumin; Tris buffer, 20 mM pH 7.0 and 2.5–3.5 mg mitochondrial protein in a total volume of 3 ml (−)-carnitine was omitted from this basic medium when acetylcarnitine was substrate. Additions were as follows: CH_3COOK, 10 mM; acetyl CoA, 3 mM; acetylcarnitine, 1 mM; $MgSO_4$, 1 mM; ATP, 1 mM. CoASH additions are stated in the table. State 3 rates were measured after the addition of 0.6 µmol ADP. The results are typical of those obtained in five experiments with different mitochondrial preparations

Substrate and cofactors added to basic medium	O_2 uptake (nmol min^{-1} mg^{-1} protein)		
Acetate, Mg^{2+}, ATP, (−)-carnitine	27.7	14.1	1.96
Acetate, Mg^{2+}, ATP, (−)-carnitine plus 0.1 mM CoASH	21.2	12.7	1.67
Acetate, Mg^{2+}, ATP, (−)-carnitine plus 0.2 mM CoASH	15.3	12.6	1.21
Acetyl CoA, (−)-carnitine	18.7	11.6	1.61
Acetyl CoA, (−)-carnitine plus 0.1 mM CoASH	9.0	8.5	1.06
Acetylcarnitine	20.0	13.0	1.54
Acetylcarnitine plus 0.1 mM CoASH	7.8	6.0	1.30

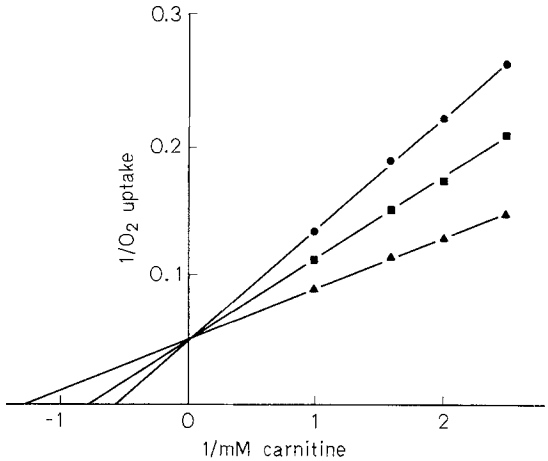

Fig. 1. Lineweaver-Burk plot demonstrating competitive inhibition by CoASH of the (−)-carnitine effect on acetate oxidation. The basic incubation mixture consisted of: sucrose, 200 mM; K_2HPO_4, 4 mM; NAD+, 0.23 mM; TPP, 0.17 mM; CH_3COOK, 10 mM; (−)-malate, 0.1 mM; ATP, 1 mM; $MgSO_4$, 1 mM; 0.5% (w/v) bovine serum albumin; Tris-buffer, 20 mM pH 7.0 and 3.1–3.4 mg mitochondrial protein in a total volume of 3 ml. State 3 rates were measured after the addition of 0.6 µmol ADP. The results were typical of those obtained in four experiments with different mitochondrial preparations. The reciprocal of O_2 uptake was calculated after subtracting the state 3 O_2 uptake (nmol min^{-1} mg^{-1}) protein) with "sparker" malate, 0.1 mM as sole substrate in the basic medium from the state 3 O_2 uptake (nmol min^{-1} mg^{-1} protein) obtained with the basic medium (i.e. CH_3COOK, 10 mM, malate 0.1 mM and cofactors). –●– CoASH, 0.12 mM; –■– CoASH, 0.08 mM; –▲– CoASH, 0.04 mM

It is suggested that the requirement for ATP and Mg^{2+} in acetate oxidation will be for the reaction catalysed by acetyl CoA synthetase (EC 6.2.1.1.).

$$\text{acetate} + \text{ATP} + \text{CoASH} \underset{\rightleftharpoons}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{acetyl CoA} + \text{AMP} + \text{PPi} \quad (1)$$

and that the requirement for (−)-carnitine in the oxidation of acetate and acetyl CoA will be in the reaction (2) catalysed by carnitine acetyltransferase.

$$\text{acetyl CoA} + \text{carnitine} \rightleftharpoons \text{acetylcarnitine} + \text{CoASH}. \quad (2)$$

The effects of CoASH additions to mitochondria oxidising acetate, acetyl CoA or acetylcarnitine were inhibitory. It was shown that CoASH was a competitive inhibitor of (−)-carnitine in this system (Fig. 1). We suggest that enough endogenous CoASH remained outside the mitochondrial membrane barrier to satisfy the requirements of reaction 1. On addition of CoASH, the equilibrium of reaction 2 was displaced to the left leading to the formation of acetyl CoA. This reaction occurring outside the mitochondrial membrane barrier, which as for animal mitochondria is, presumably, the inner membrane (Fritz 1963) would lead to an accumulation of acetyl CoA. As the acetyl CoA so formed is not oxidised (Table 1) and does not permeate the membrane barrier, necessarily a low O_2 uptake was the result of the CoASH additions.

It is postulated that the reactions (1) and (2) occur outside the mitochondrial membrane barrier, that is, on the "c" or cytosol side (Fig. 2) to form acetylcarnitine. The acetylcarnitine in animal mitochondria is known to traverse the inner membrane via an intrinsic translocator mechanism (Pande and Parvin 1980; Tubbs et al. 1980) which we suppose to be present in the pea mitochondria. The acetylcarnitine readily enters the matrix of the pea mitochondria via the translocase (reaction 3, Fig. 2) where it is suggested that an internal carnitine acetyltransferase transfers the acetyl group to CoASH (reaction 4, Fig. 2). The acetyl groups then enter the tricarboxylic acid cycle from acetyl CoA in the reaction catalysed by citrate synthase (EC 4.1.3.7.) (reaction 5, Fig. 2). The (−)-carnitine, once released, may pass back from the mitochondrial matrix ("m" side, Fig. 2) to the cytosol,

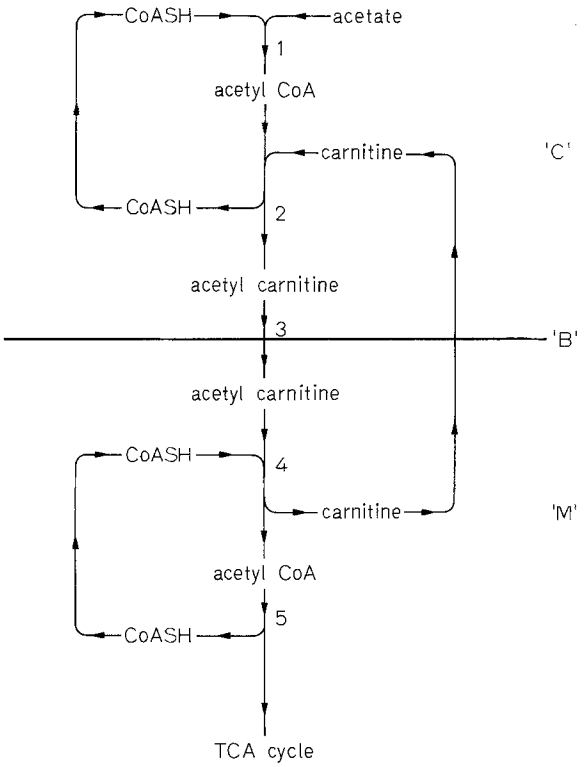

Fig. 2. Postulated reactions occurring on either side of the mitochondrial membrane barrier during the oxidation of acetate in the presence of (−)-carnitine by pea mitochondria. Reaction (1) "c" side acetyl CoA synthetase; (2) "c" side carnitine acetyltransferase; (3) acetylcarnitine/carnitine translocator; (4) "m" side carnitine acetyltransferase; (5) citrate synthase. *C*, cytosol; *B*, mitochondria membrane barrier; *M*, mitochondria matrix

this movement being facilitated by the acylcarnitine/carnitine translocase. It will be necessary in future work to study the kinetics of this transfer but if the process is similar to that in animal mitochondria the transfer will be in a 1:1 ratio of acetylcarnitine entry to carnitine exit (Pande 1975; Pande and Parvin 1976; and Pande and Parvin 1980).

It is apparent from the data of Table 3 and Fig. 1 that the external relative concentrations of (−)-carnitine and CoASH determine the fate of acetyl groups supplied exogenously whether as acetate, acetyl CoA or acetylcarnitine. High concentrations of (−)-carnitine but low of CoASH leads to an increased entry of acetyl groups into the tricarboxylic acid cycle as measured by increased O_2 uptake (Table 3, Fig. 1). Assuming that the (−)-carnitine released in reaction 4 of Fig. 2, leaves the mitochondria to balance the import of acetylcarnitine, the internal carnitine content of those conditions which satisfy acetyl group oxidation would not be so high as to disturb the equilibrium of the reaction catalysed by "m" side carnitine acetyltransferase to the right i.e., (reaction 2 in the text, reaction 4 in Fig. 2) to maintain a high concentration of acetylcarnitine in the matrix. It is envisaged that there may be an increased production of acetyl CoA, e.g. from pyruvate, on the "m" side of the membrane barrier under certain cellular physiological conditions. Hence when the matrix concentration of (−)-carnitine is high, the formation of acetylcarnitine would proceed as the low concentration of CoASH and high concentration of acetyl CoA would displace the equilibrium of the reaction in favour of acetylcarnitine formation. In the model presented in Fig. 2 reactions 1, 2, 3 and 4 are considered to be freely reversible. Therefore excess acetylcarnitine formed internally may well be exported through the membrane barrier to cytosol sites where it may be used for synthetic purposes e.g. fatty acid synthesis.

It is suggested that acetylcarnitine and carnitine freely permetate pea mitochondria membranes whereas CoA and acetyl CoA are rigidly compartmented. Thus the pool of cytosolic carnitine would be available to allow equilibrium between acetyl CoA/CoA and acetylcarnitine/carnitine couples in the mitochondria. This buffering capacity of carnitine may well determine the availability of acetyl group entry into the tricarboxylic acid cycle and may well control its removal to other cellular sites for other biosynthetic purposes. It will be necessary in future work to isolate and study the properties of both the external "c" side, and internal "m" side, carnitine acetyltransferase of pea mitochondria to verify some of the suggestions presented here.

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