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RESEARCH COMMUNICATION

Coenzyme A is a potent inhibitor of acetyl-CoA carboxylase from rat epididymal fat-pads

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Rat epididymal fat-pad extracts have previously been shown to contain an insulin-stimulated acetyl-CoA carboxylase kinase, which is co-eluted from Mono Q ion-exchange chromatography with a potent inhibitor of acetyl-CoA carboxylase [Borthwick, Edgell & Denton (1990) Biochem. J. **270**, 795–801]. A variety of tests, including reactivity with thiol reagents, identify this inhibitor as CoA. Inhbition requires the presence of MgATP, but is independent of any phosphorylation of the enzyme. The effect is complete in about 5 min and is associated with depolymerization of acetyl-CoA carboxylase. Half-maximal inhibition is observed at about 40 nM-CoA. The inhibitory effects of CoA can be partially reversed by incubation with citrate and more fully overcome by treatment of the enzyme with the insulin-stimulated acetyl-CoA carboxylase kinase.

INTRODUCTION

The regulation of acetyl-CoA carboxylase (acetyl-CoA:CO_2 ligase, EC 6.4.1.2) represents an important element in the control of the overall rate of synthesis *de novo* of long-chain fatty acids [1]. In the short term acetyl-CoA carboxylase activity is affected by allosteric effectors and by phosphorylation at a number of different sites on the enzyme [1,2], with both mechanisms seemingly associated with changes in the polymeric state of the enzyme [1,3]. Phosphorylation by AMP-activated protein kinase and cyclic-AMP-dependent protein kinase produces a decrease in activity [1,2,4], whereas phosphorylation by a less well characterized insulin-stimulated protein kinase leads to an increase in the activity of the enzyme [5]. This insulin-stimulated kinase can be separated by Mono-Q anion-exchange chromatography, and has been found to be co-eluted with a potent low-M_r inhibitor of acetyl-CoA carboxylase [5]. Co-elution of the kinase and the inhibitor actually facilitated the detection of kinase activity, as both phosphorylation and activation of acetyl-CoA carboxylase were enhanced in the presence of the inhibitor. The studies described in this paper were initiated in order to identify this inhibitory component.

EXPERIMENTAL

Materials were as given previously [5], with the addition of bacterial luciferase (from *Vibrio fischeri*) which was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). 2-Oxoglutarate dehydrogenase was prepared from pig heart [6] and was kindly given by Michael Leake of this Department.

Highly purified acetyl-CoA carboxylase was prepared from rat epididymal fat-pads by Mono Q ion-exchange and Superose 6 gel-filtration chromatography [5]. This preparation was judged to be over 90% pure on SDS/polyacrylamide gels. Fractions eluted at 0.175 M-NaCl from the first Mono Q step, which contained the carboxylase inhibitor and kinase (fraction 1), were pooled, and fraction 1A (low M_r, containing inhibitor component) and fraction 1B (high M_r, containing the activating kinase) were prepared by using Centricon-10 micro-concentrators (10000-M_r cut-off; Amicon) as in [5]. The effects of fraction 1A or CoA on purified acetyl-CoA carboxylase were determined by preincubating the enzyme (0.1 mg/ml) in basic incubation buffer [potassium phosphate (5 mM, pH 7.4) containing citrate (0.6 mM), EDTA (0.5 mM), NaCl (86 mM), $MgCl_2$ (5 mM), dithiothreitol (0.5 mM)] plus further additions as stated for 15 min at 30 °C in a total volume of 50 μl. Acetyl-CoA carboxylase activity was then assayed [5]. The maximum (total) activity of the enzyme was determined after preincubation of samples of the purified enzyme for 20 min with 20 mM-citrate [5]. One unit of enzyme activity is defined as the amount catalysing the formation of 1 μM-malonyl-CoA/min at 30 °C.

To assess polymerization, purified acetyl-CoA carboxylase was preincubated as above for 20 min in the presence of the appropriate effectors and then centrifuged in an Airfuge (Beckman) for 15 min at 130000 g. Samples of the supernatants and the enzyme incubation mixtures before centrifugation were incubated for a further 15 min with 20 mM-citrate and then assayed for carboxylase activity. The proportion of enzyme activity remaining in the supernatant after centrifugation was taken as a measure of the amount of acetyl-CoA carboxylase in the depolymerized form.

CoA was assayed as NADH formed by 2-oxoglutarate dehydrogenase with NADH-utilizing bacterial luciferase [7].

RESULTS

Preliminary studies on the inhibitor present in fat-pad extracts

Initial studies confirmed that the low-M_r inhibitor in fraction 1A only acted in the presence of MgATP, although inhibition did not involve any change in the phosphorylation state of the enzyme [5]. Further studies indicated that the inhibitor was

Abbreviations used: NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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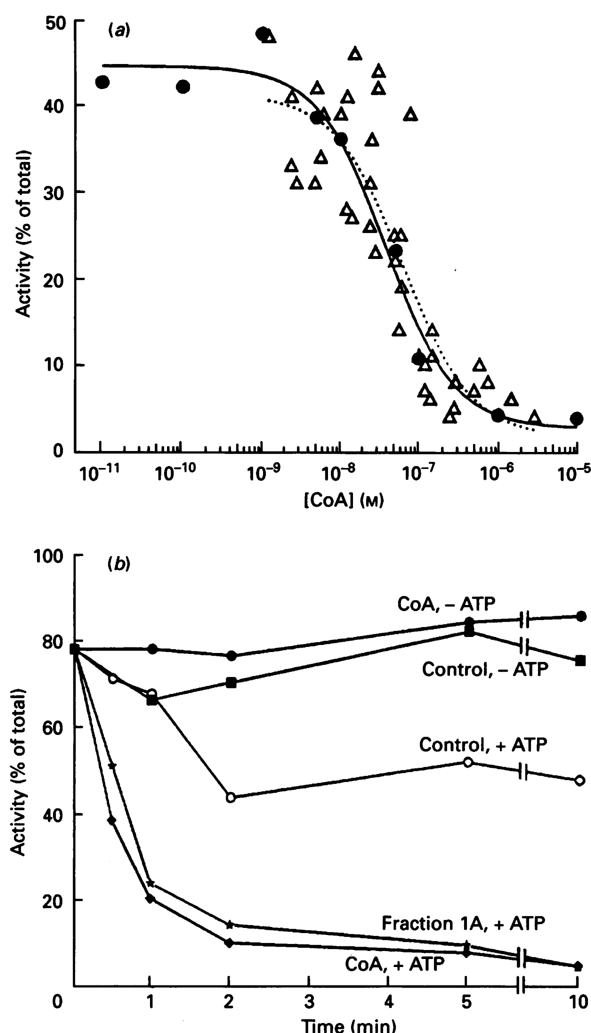

Fig. 1. Effect of CoA on adipose-tissue acetyl-CoA carboxylase activity

(*a*) Acetyl-CoA carboxylase was incubated for 15 min in the presence of 50 μM-ATP (see the Experimental section) with the appropriate concentration of CoA (●, continuous line). Also shown are the effects observed with samples (0.1–5 μl) of six separate preparations of fraction 1A plotted in terms of their measured CoA contents (△, dotted line). Both lines are fitted to the equation

$$v = \frac{V_{max.}}{1+(I/K_i)} + V_0.$$

(*b*) Acetyl-CoA carboxylase was incubated in the presence of 50 μM-ATP (see the Experimental section) with no additions (○), 1.6 μM-CoA (◆) or fraction 1A (equivalent to 1.6 μM-CoA) (★) for the times indicated. Enzyme activity was also determined after incubation without (■) or with (●) CoA (1.6 μM) in the absence of ATP. Similar results were obtained with two further preparations of acetyl-CoA carboxylase.

unaffected by boiling for 10 min at pH 7.0, stable from pH 1 to pH 11 at 0 °C and resistant to incubation with trypsin, chymotrypsin, V8 protease and alkaline phosphatase. However, inhibitory activity was removed by treatment of fractions with activated charcoal, was eluted from Mono Q columns after ATP and had an apparent M_r of less than 3000 on gel-permeation chromatography (Sephadex G-25). Taken together, these observations suggested the inhibitor may be nucleotide-based. We then observed that treatment of fraction 1A with thiol reageants [*N*-ethylmaleimide (NEM) or 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)] was found to abolish inhibitory activity (see below), and this indicated that the inhibitor might be CoA.

By using the sensitive luciferase-linked CoA assay, CoA was found to be present in all the inhibitor fraction-1A preparations tested (average CoA concentration was 3.6 ± 1.4 μM; $n = 10$). Furthermore, CoA was shown to be eluted from Mono Q with 0.175 M-NaCl in fractions which coincided with those containing acetyl-CoA carboxylase inhibitory activity (results not shown).

Effects of CoA on acetyl-CoA carboxylase activity and phosphorylation

CoA was found to be a potent inhibitor of acetyl-CoA carboxylase. Half-maximal inhibition occurred at 37.8 ± 10.2 nM with the preparation of acetyl-CoA carboxylase used in Fig. 1(*a*). Other preparations gave similar values (mean ± S.E.M. for three preparations of enzyme was 38.2 ± 5.6 nM). At maximal inhibitory concentrations, CoA diminished the activity of the carboxylase from approx. 50 % to 5 % of total activity. No inhibition was seen in the absence of MgATP (see Fig. 1*b*). In order to detemine whether the amount of CoA in fraction 1A could account for the observed inhibition of acetyl-CoA carboxylase, a number of different inhibitor preparations were assayed for their CoA content, and then screened for inhibitory potency. As shown in Fig. 1(*a*), the dose–responses for fraction-1A preparations and CoA agree closely, indicating that the CoA in the inhibitor preparations could indeed account for the inhibitory effects of these fractions.

The time course of the effect of CoA on activity (Fig. 1*b*) shows that full inhibition was apparent after incubating the enzyme with CoA for 2–5 min. The similarity between the time course for inhibition by CoA and that of fraction 1A again indicated that the inhibitory component in fraction 1A was CoA. Addition of MgATP alone to different acetyl-CoA carboxylase preparations was found to result in variable amounts of inhibition. With the preparation used in Fig. 1, the loss of activity on addition of MgATP alone was about 30 % of the activity in the absence of MgATP; in other preparations the inhibition by MgATP ranged from less than 10 % to over 80 %. Only preparations exhibiting a loss of less than 35 % of activity on addition of MgATP have been used in the present study. Measurements of the CoA content of preparations showing high amounts of inhibition with MgATP indicate that these preparations contain sufficent CoA to account for much of the observed inhibition.

Previous studies have shown that the inhibition of acetyl-CoA carboxylase by fraction 1A can be abolished by the action of the kinase activity in fraction 1B, and also partially by treatment with citrate (20 mM) [5]. This is confirmed in the results reported in Table 1, and is also found to be the case for CoA. It should be noted that incubation with fraction 1B does not alter the concentration of CoA (S. K. Moule, unpublished work).

Fig. 2 shows the effect of CoA on the phosphorylation of purified acetyl-CoA carboxylase. CoA, like fraction 1A, under conditions causing maximal inhibition had no effect on phosphorylation. However, again like fraction 1A, CoA caused a modest enhancement of approx. 30 % in the incorporation of phosphate into the enzyme by the insulin-stimulated kinase (fraction 1B). The addition of ATP alone to acetyl-CoA carboxylase did not increase the phosphorylation of the enzyme. This was found to be the case even with acetyl-CoA carboxylase preparations showing a high degree of MgATP inhibition, indicating that the phenomenon was not due to phosphorylation of the enzyme.

The thiol reagents DTNB and NEM react with the free thiol group of CoA and, as shown in Table 1 (Expt. 3), this essentially

Table 1. Inhibition of acetyl-CoA carboxylase by CoA under various conditions

Expts. 1 and 2: acetyl-CoA carboxylase was incubated for 15 min (see the Experimental section) in basic incubation buffer with ATP (50 μM) plus appropriate further additions; the concentration of CoA was 100 nM, and the volume of fraction 1A added was adjusted to give the same CoA concentration. Expt. 3: CoA (1 μM), fraction 1A (equivalent to 1 μM) or basic incubation buffer (control) were pretreated with or without 5 mM-DTNB or -NEM for 1 min before 2 μl portions of these samples were added to 43 μl of basic incubation buffer plus 50 μM-ATP (all excess DTNB or NEM was removed by dithiothreitol in the basic incubation buffer). Acetyl-CoA carboxylase (5 μl) was added and activity was assayed after incubation for 15 min (final concn. of CoA in the absence of DTNB or NEM was 40 nM). Results are given as means ± S.E.M.: * $P < 0.05$, ** $P < 0.01$ versus no-treatment control; † $P < 0.05$, †† $P < 0.01$ versus same condition without treatment.

Treatment	Condition...	Acetyl-CoA carboxylase activity (% of total) Control	CoA	Fraction 1A
Expt. 1 ($n = 3$)				
None		49.0±2.5	6.7±1.7**	5.0±1.8**
Fraction 1B (20 μl)		60.6±2.3*	51.0±6.7††	66.7±5.9††
Expt. 2 ($n = 3$)				
None		49.0±5.9	9.3±4.1**	10.3±6.0*
Citrate (20 mM)		89.3±5.0**	44.6±8.8†	49.0±15.9†
Expt. 3 ($n = 4$)				
None		44.8±1.9	18.2±4.0**	15.5±1.6**
DTNB		47.4±2.5	31.8±2.5**†	33.8±1.7**††
NEM		41.2±3.2	45.8±2.0††	40.6±1.1††

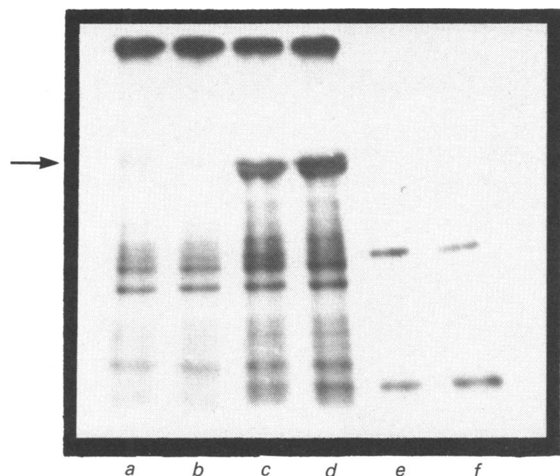

Fig. 2. Effect of CoA on the phosphorylation of acetyl-CoA carboxylase

Acetyl-CoA carboxylase was incubated with [γ-^{32}P]ATP (2340 d.p.m./pmol) for 15 min without further additions (*a*) or in the presence of the following effectors: (*b*) 1 μM-CoA, (*c*) 20 μl of fraction 1B or (*d*) 1 μM-CoA plus 20 μl of fraction 1B. Tracks (*f*) and (*e*) respectively show the phosphorylation of fraction 1B with or without 1 μM-CoA in the absence of acetyl-CoA carboxylase. Phosphorylation was then stopped by addition of SDS/PAGE sample buffer, and samples were separated on SDS/6%-polyacrylamide gels. Autoradiography was carried out with flash-preactivated Kodak X-Omat film [5]. The position of acetyl-CoA carboxylase is indicated by the arrow. Results shown are typical of experiments on at least three separate enzyme preparations.

abolishes the inhibitory effects of CoA. As predicted, these treatments have the same effect on inhibition by fraction 1A. Removal of Mg^{2+} by addition of 10 mM-EDTA, or of ATP by addition of glucose (1.6 mM) and hexokinase (0.6 unit/ml), also led to a rapid reversal of the effects of CoA (results not shown).

The effect of CoA and fraction 1A on the extent of polymerization of acetyl-CoA carboxylase was explored by determining the proportion of enzyme remaining in the supernatant after centrifugation at 130000 *g* [8]. As shown in Table 2, both CoA and fraction 1A greatly increased this proportion, and thus their inhibitory effects are associated with the depolymerization of acetyl-CoA carboxylase. Incubation with a high concentration of citrate decreased the proportion in the supernatant, indicating a greater degree of polymerization, as expected from previous studies [1,2,9,10].

Table 2. Effect of CoA on the polymerization of acetyl-CoA carboxylase

Acetyl-CoA carboxylase was incubated as described in the Experimental section in basic incubation buffer (200 μl) plus 50 μM-ATP and 600 nM-CoA, 20 mM-citrate or fraction 1A (equivalent to 600 nM-CoA) for 20 min at 30 °C; total acetyl-CoA carboxylase activity was then assayed before and after centrifugation at 130000 *g* for 15 min (see the Experimental section). * $P < 0.05$, ** $P < 0.01$ versus control ($n = 3$ separate enzyme preparations).

Condition	Activity in supernatant after centrifugation (%)
Control	38.0±3.7
Citrate	20.7±2.7*
CoA	76.0±12.4*
Fraction 1A	76.4±6.5**

DISCUSSION

The results described in this paper demonstrate that the acetyl-CoA carboxylase inhibitor previously detected in rat epididymal fat-pad extracts [5] is CoA. The inhibitor and CoA have proved indistinguishable in a wide variety of tests.

Palmitoyl-CoA has also been shown to decrease the activity of acetyl-CoA carboxylase ($K_i = 6.5$ nM) [11,12]; however, this effect is not ATP-dependent, and would therefore seem to be quite distinct from the inhibition by CoA. We have found that preparations of both malonyl-CoA and succinyl-CoA can inhibit the enzyme in an ATP-dependent fashion (results not shown), but, since pretreatment with NEM largely abolishes their ability to alter enzyme activity, it seems likely that contamination by CoA may be responsible for inhibition.

Our results are in direct conflict with those of Kim and colleagues on various rat liver acetyl-CoA carboxylase preparations [13–15]. Their studies reported activation by CoA (K_a = 15 μM) occurring in the absence of MgATP, together with a CoA-activated acetyl-CoA carboxylase kinase which apparently inhibited the enzyme. The reason for the discrepancies between the results of Kim and co-workers and those reported here is not entirely clear, but may in part reflect differences in the acetyl-CoA carboxylase preparations used. With the adipose-tissue enzyme we have been unable to detect any activation, even at the much higher CoA concentrations used by Kim and co-workers. Preliminary experiments indicate that acetyl-CoA carboxylase purified by avidin affinity chromatography from rat liver shows a similar CoA inhibition to that displayed by the white-adipose-tissue enzyme in the present study (N. J. Edgell, S. K. Moule & R. W. Brownsey, unpublished work).

As mentioned in the Results section, a number of acetyl-CoA carboxylase preparations were contaminated with sufficient amounts of CoA to give rise to significant inhibition of the enzyme on addition of MgATP. The fact that the binding of CoA to the enzyme is sufficently strong for it to co-purify indicates that it is important to evaluate the contribution of CoA to MgATP-dependent inhibition of acetyl-CoA carboxylase preparations. This may be particularly relevant in studies on protein-kinase-mediated alterations of acetyl-CoA carboxylase activity.

We are not aware of any measurements of the concentration of CoA in rat epididymal fat-cells, but it would seem likely that the concentration is considerably greater than the K_i for acetyl-CoA carboxylase inhibition. If this is the case, changes in CoA concentration may not play a direct role in the physiological regulation of the enzyme. However, it is possible that the inhibitory effects of CoA would have a major influence on both the basal activity and the polymerization of the enzyme, as well as on its response to other regulatory mechanisms, including protein kinases.

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