Physiological mechanism by which acetyl CoA carboxylase is regulated

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Summary. Compared with rats fed a normal diet, the activity of the protein inhibitor of acetyl CoA carboxylase in rat liver doubled after 48 h of fasting. Conversely, acetyl CoA carboxylase activity was diminished by nearly one half. In animals fasted and then subsequently refed a fat free diet, acetyl CoA carboxylase activity was elevated by nearly 9-fold, with a concomitant decrease in the activity of the protein inhibitor by about 9-fold as compared to fasted rats. Hence it appears that the regulatory protein inhibitor for acetyl CoA carboxylase is of physiological significance for fatty acid biosynthesis.

The important role of acetyl CoA carboxylase (E.C. 6.4.1.2) as the rate-limiting enzyme in catalyzing the first step in a series of reactions leading to the biosynthesis of long chain fatty acid from acetyl CoA[3,4], has prompted many investigations on the mechanism(s) by which this enzyme is regulated. It has been reported that acetyl CoA carboxylase (ACX) is regulated by a phosphorylation (inactive) – dephosphorylation (active enzyme) mechanism[5]. Others[6,7] have argued against such a mechanism, since pure rat liver acetyl CoA carboxylase contains 2.1 phosphate groups per 215,000 daltons of peptide[8] and it was found that fully phosphorylated chicken liver ACX maintained full activity[9]. It has also been reported that the activity of ACX is depressed after incubation of isolated rat liver cells with glucagon[10-12], but contrast to these findings, other studies[13-15] indicated that ACX activity after glucagon treatment was not diminished.

In this communication, we report that the regulatory protein inhibitor for ACX has physiological significance in fatty acid biosynthesis.

Materials and methods. Preparation of acetyl CoA carboxylase: Male Holtzman rats weighing 180–200 g were housed in a room under a 12-h light-dark cycle and maintained for at least 2 weeks on normal purina chow diet prior to experimentation. The rats were divided into 5 groups as follows: a) fed ad libitum, b) fasted for 48 h, c) fasted for 48 h and then fed a fat-free diet for 48 h, d) fasted for 48 h and then fed a fat-free diet for 24 h and e) same as d) but the rats were injected i.p. with glucagon (200 µg/100 g b.wt) 1 h prior to killing. All rats were killed by decapitation and their livers were removed, weighed and placed in ice-cold homogenization medium. The livers were homogenized in a Waring blendor, followed by 3 strokes of a motor-driven Teflon pestle in a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 100,000 × g for 75 min. The supernatant solution was adjusted to 30% saturation with ammonium sulfate. The precipitate, which contained acetyl CoA carboxylase, was collected by centrifugation, while the supernatant solution, which contained the protein inhibitor, was further adjusted to 40% saturation with ammonium sulfate. Complete details of the isolation of the enzyme and the protein inhibitor has been reported[16].

Perfusion of rat liver. Hepatocytes were obtained from male Holtzman rats which were fasted for 48 h and followed by feeding a fat free diet for 48 h prior to liver perfusion. Perfusion of the liver was done in situ via the portal vein with Ca^{++} and glucose free Krebs-Hanslet medium as described by Berry and Friend[17].

The hepatocytes were incubated with and without glucagon (10^{-6} M) between 0 and 90 min in sealed Erlenmeyer flasks under 95% O_2 and 5% CO_2 at 37 °C in a Dubnoff metabolic incubator (90 oscillations/min). The reaction was terminated by aspirating the medium from the flasks and the cells were washed 3 times with ice-cold 0.15 M potassium chloride and suspended in phosphate buffer. Disruption of the cells was carried out by freezing and thawing followed by pulsed sonication in a Sonifer cell Disrupt W-350 for 1 min at 4 °C. Acetyl CoA carboxylase and the protein inhibitor from the cell suspension were obtained as outlined above.

Enzyme assay. Acetyl CoA carboxylase activity in the absence and in the presence of the protein inhibitor was measured by the radiochemical assay in which $^{14}CO_2$ was incorporated into malonyl CoA as has been described[16].

Protein determination. The concentration of protein in ACX and the protein inhibitor was measured by the method of Lowry et al.[18] using crystalline bovine albumin as standard.

Results and discussion. When rats were fasted for 48 h, ACX levels fell by more than 60% while protein inhibitor activity doubled as compared to rats fed ad libitum (table). On the other hand, in rats fasted for 48 h followed by feeding a fat-free diet for 48 h, ACX activity was increased more than 3-fold above normal and, conversely, the protein inhibitor decreased by more than 4-fold.

When rats were fasted and fed a fat-free diet for 24 h and injected with glucagon 1 h prior to killing, there was a 30% decrease in ACX activity with a concomitant increase in inhibitor activity by 37% as compared to similarly treated rats without glucagon (table).

Acetyl CoA carboxylase and protein inhibitor activities determined from hepatocytes incubated with and without glucagon at different intervals of time revealed that glucagon neither affects ACX activity nor protein inhibitor activity. These results are in agreement with others[13-15].

The present studies carried out in vivo as well as in isolated hepatocytes to evaluate the role of the protein inhibitor in regulating ACX activity and consequently fatty acid synthesis, strongly suggest the presence of a regulatory protein for

Relationship between acetyl CoA carboxylase and protein inhibitor activities in livers of animals subjected to different nutritional and hormonal conditions

State	Acetyl CoA carboxylase (U/g liver)[a]	Protein inhibitor (U/g liver)[b]
Fed ad libitum	0.050 ± 0.005[c]	0.093 ± 0.007[c]
Fasted	0.019 ± 0.005	0.186 ± 0.016
Fasted 48 h, fed fat-free diet 48 h	0.175 ± 0.018	0.022 ± 0.006
Fasted 48 h, fed fat-free diet 24 h	0.140 ± 0.015	0.110 ± 0.006
Fasted 48 h, fed fat-free diet 24 h and injected with glucagon (10^{-6} M)	0.100 ± 0.013	0.150 ± 0.009

[a] A unit of enzyme activity is defind as the incorporation of 1 µmole of HCO_3^- into malonyl CoA/mg of protein. [b] A unit of inhibitor activity is defined as the amount of inhibitor that is required to reduce acetyl CoA carboxylase activity from 1 µmole of malonyl CoA formed/mg of protein/min to 0.5 µmole. [c] Mean value ± SD expressed for 4 rats; experiments were repeated 3 times. Values are significant in comparison to rats fed ad libitum ($p < 0.01$, Student's t-test).

ACX. Also, it seems that glucagon may play a role in controlling the activity of the protein inhibitor at the in vivo level; however, isolated liver cell studies indicated that neither protein inhibitor nor ACX are under the influence of glucagon. The decrease in ACX activity and the concomitant increase in the inhibitor activity after glucagon injection in rats may be explained by the presence of interfering factor(s).

This study as well as our earlier studies[16,19,20], suggest that ACX is not regulated via covalent phosphorylation or allosteric modulation, but more probably by the presence of other factors such as regulatory protein(s). We therefore believe that more attention should be directed to the investigation of other enzymes, particularly those which are believed to be regulated via phosphorylation where this has been demonstrated in vitro but it is not proven that regulation functions by this mechanism in vivo.

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Cardioactive peptides of the CNS of the pulmonate snail *Lymnaea stagnalis*

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Summary. In the pulmonate snail *Lymnaea stagnalis* the cardioactive effects (tested on isolated auricles) of acetylcholine (ACh), 5-hydroxytryptamine (5-HT), the bivalve tetrapeptide FMRFamide, and of chromatographically separated snail brain substances have been established. Besides ACh and 5-HT, in brain extracts, small FMRFamide-like and large cardioexcitatory peptides were found.

In molluscs the heart rate is accurately controlled[1-4]. In addition to neurotransmitters, e.g. acetylcholine (ACh) and 5-hydroxytryptamine (5-HT), cardioregulatory neuropeptides have been implicated in this control[4]. One of these, the tetrapeptide amide Phe-Met-Arg-Phe-NH_2 of the bivalve *Macrocallista nimbosa*, has recently been purified, identified and synthesized[5]. Cardioactive FMRFamide-like neuropeptides also occur in various gastropods[4,6,8]. Furthermore, from the CNS of *Helix* one or more large cardioactive peptides (LCP; 6000-8000 daltons) have been isolated[6,8]. Immunocytochemical[7] and functional[4,6,8] studies suggest that FMRFamide and related peptides function as neurotransmitters/neuromodulators, whereas LCP has a putative neurohormonal function[6,8]. In the basommatophoran *Lymnaea stagnalis* starvation causes considerable changes in kidney function, water- and ion metabolism, and acid-base balance. Very probably the decrease in heart rate plays a crucial role in these changes[3]. As part of a study of the underlying control mechanisms we plan to investigate the role of cardioactive neuropeptides. In this report, circumstantial biochemical and pharmacological evidence for the existence of FMRFamide-like peptides and LCP in *Lymnaea* CNS will be presented. Further details on the purification and characterization of these peptides will be presented elsewhere. For the in vitro bioassay we used the auricle of the heart of *Lymnaea*, as it is spontaneously active, whereas the ventricle is quiescent.

Materials and methods. The auricle of an adult, laboratory raised[9] *Lymnaea stagnalis* was dissected out, attached to a displacement transducer (designed in the institute), suspended in a 750-µl organ bath, and superfused with aerated (1.72% CO_2 in O_2) snail saline[10] (pH ~ 7.8) maintained at 20 °C, at a rate of 650 µl per min. Test substances were introduced in either of the following ways. 1. As a pulse in the superfusion fluid just before it entered the bath (dose-response curves and fractions of columns). 2. Directly into the bath; in this case superfusion was temporarily arrested (threshold determinations and pretreatments with α-bungarotoxin or methysergide). Contractions were displayed on a recorder. Test drugs were dissolved in distilled water and added to the bath in 5-10-µl doses. All doses were expressed as molar concentrations in the bath. ACh chloride (Sigma), serotonin-creatinine sulphate (Merck), FMRFamide (Serva), α-bungarotoxin (Boehringer) and methysergide (gift from Sandoz) were used. CNS with adhering nerves were excised, collected in a glass homogenizer in solid CO_2, boiled (10 min) in 0.1 M acetic acid (w/v = 1/10) and centrifuged (Janetzki TH 12, 15 min, 12,000 rpm). The pellet was extracted 3 times more. The combined supernatants were lyophilized, redissolved in 0.1 M acetic acid and used for gel filtration on Sephadex G-15 at 4 °C[6]. Incubations with pronase (Merck; final concentration 250 U/ml) were carried out in distilled water at 23 °C for 4 h, and terminated by boiling (15 min). Control incubations were done without pronase.

Results and discussion. Figure 1 depicts a representative