# Distinct type-2A protein phosphatases activate HMGCoA reductase and acetyl-CoA carboxylase in liver

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**Abstract Acetyl-CoA carboxylase and HMGCoA reductase are inactivated by the same AMP-activated protein kinase and are activated by type-2A protein phosphatase. To determine whether the same species of protein phosphatase-2A were involved, we studied the interconversion of acetyl-CoA carboxylase and HMGCoA reductase in isolated rat hepatocytes. We show that (i) these enzymes are differently regulated in hepatocytes and (ii) the species of type-2A protein phosphatase involved in their activation are different and can be separated by anion-exchange chromatography.**



*Key words:* Acetyl-CoA carboxylase; AMP-activated protein kinase; Glutamine; HMGCoA reductase; Protein phosphatase-2A

## 1. Introduction

Acetyl-CoA carboxylase (ACC), a regulatory enzyme of lipogenesis, is controlled by phosphorylation/dephosphorylation of several Ser residues [1]. In hepatocytes, inactivation of ACC occurs in response to glucagon and results from the phosphorylation of $Ser^{79}$ by the AMP-activated protein kinase (AMPK) [2]. In adipocytes, activation of ACC follows insulin treatment and was reported to be mediated by the phosphorylation of an as yet unidentified site located within a tryptic peptide called the 'I-peptide' [3]. Activation of ACC was also observed in hepatocytes treated with $Na^+$-cotransported amino acids, such as glutamine and proline [4]. The activation of ACC in glutamine-treated hepatocytes was proposed to be mediated by glutamate [5], which accumulates in these cells [6,7], and stimulates a protein phosphatase that was recently identified as a new species of hepatic type-2A protein phosphatase (PP-2A) [8]. This glutamate-activated protein phosphatase (GAPP) reverses the in vitro inactivation of ACC by AMPK [8].

Another well-known substrate of AMPK is hydroxymethylglutaryl-CoA (HMGCoA) reductase [1], a regulatory enzyme in cholesterol synthesis that is anchored in the endoplasmic reticulum membrane. Its C-terminal domain contains the catalytic site and can be released as a soluble, active fragment of 53 kDa by proteolysis [9]. Phosphorylation by AMPK of $Ser^{871}$ in the C-terminal domain inactivates HMGCoA reductase [10]. Incubation of hepatocytes with okadaic acid, a potent inhibitor of type-1 protein phosphatases (PP-1) and PP-2A [11], completely inactivated HMGCoA reductase within a few minutes [12]. PP-2A contributes the major proportion of HMGCoA reductase phosphatase in intact cells, because PP-1 accounts for only a few percent of HMGCoA reductase phosphatase in liver extracts [13].

Therefore, liver HMGCoA reductase and ACC appear to be covalently regulated in a similar way. Both of them are inactivated by AMPK and activated by type-2A phosphatases. In addition, both activities follow a diurnal rhythm with a peak of activity in the middle of the dark period, when rats are eating [14,15]. For ACC, the diurnal variation results from modification in the phosphorylation state rather than from changes in enzyme content [14], whereas for HMGCoA reductase, changes in both the amount and the phosphorylation state are involved [15]. The variations in the phosphorylation state of both enzymes during a 24-h period do not result from changes in AMPK activity and could therefore be mediated by variations in the activity of PP-2A [14]. Glutamine could stimulate PP-2A when the rats start eating and, thereby, activate HMGCoA reductase and ACC. In this work, we tested whether incubation of hepatocytes with glutamine could activate HMGCoA reductase, as well as ACC.

## 2. Materials and methods

### 2.1. Material

Phosphorylase *b* and phosphorylase kinase were from Sigma and radiochemicals from Amersham. The synthetic SAMS-peptide (HMRSAMSGLHLVKRR), a substrate for AMPK [16], was synthetized by V. Feytons (Leuven, Belgium) [8]. ACC was purified to homogeneity from livers of re-fed rats [5]. AMPK was purified as far as the Blue Sepharose step [17]. The purified C-terminal catalytic domain of HMGCoA reductase was purified on $Ni^{2+}$-agarose [18] and was phosphorylated by [$\gamma$-$^{32}$P]ATP in the presence of AMPK as described [19]. Phosphorylase *b* was phosphorylated by [$\gamma$-$^{32}$P]ATP in the presence of phosphorylase kinase [20].

### 2.2. Cell isolation and incubation

Male Wistar rats (200–300 g) were maintained on a regime of room light from 22:00 to 10:00 h and darkness from 10:00 h to 22:00 h. Hepatocytes [21] were prepared at 10 h, i.e. at the beginning of the dark period when the animals start eating. This time was chosen because HMGCoA reductase should be phosphorylated and thus a better substrate for protein phosphatases [15]. Cells were incubated at

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*Abbreviations:* ACC, acetyl-CoA carboxylase; AICAriboside, 5-amino 4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; GAPP, hepatic glutamate-activated protein phosphatase; HMGCoA, hydroxymethylglutaryl coenzyme A; PP, protein phosphatase


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30 mg wet weight/ml of incubation medium in Krebs-Henseleit bicarbonate buffer [22] in equilibrium with a gas phase containing $O_2/CO_2$ (95:5) and supplemented with 10 mM glucose.

*2.3. Measurement of enzyme activities*

ACC activity was measured in digitonin-permeabilized hepatocytes in the presence of 0.5 mM Mg-citrate (method 2 in [5]). AMPK was measured by phosphorylation of the SAMS peptide in the presence of 0.2 mM AMP in 0–6% polyethylene glycol fractions made from cell homogenates [23]. HMGCoA reductase activity was measured in digitonin-permeabilized hepatocytes [24]. Enzyme activity is expressed as units (U), i.e. μmol of substrate consumed per minute under the assay conditions. The values shown are the means ± SEM for the given number of different preparations of hepatocytes. The statistical significance of differences was calculated by Student's *t* test (paired data).

*2.4. Separation and measurement of GAPP, HMGCoA reductase phosphatase and phosphorylase phosphatase*

A liver cytosolic fraction was fractionated on a DEAE-Sephadex-A50 as described [8]. The ACC phosphatase activity of GAPP was measured by the glutamate-stimulate activation at 37°C of 0.15–0.50 μM purified ACC [8], which was assayed (method 1 in [5]) in the presence of 0.5 mM Mg-citrate. One unit of ACC phosphatase is defined as the amount that generates 1 unit of ACC/min under the specified conditions.

The HMGCoA reductase phosphatase and phosphorylase phosphatase activities were determined at 30°C from the linear rate of dephosphorylation of the $^{32}$P-labelled substrates at a final concentration of 30 μg/ml or 10 μM, respectively [19,20]. The released [$^{32}$P]$P_i$ was measured by liquid scintillation after precipitation by 10% trichloroacetic acid of the remaining substrates together with 3 mg/ml albumin. One unit of HMGCoA reductase phosphatase or phosphorylase phosphatase produces 1 pmol or 1 μmol of phosphate/min under the specified conditions, respectively.

## 3. Results

*3.1. Effect of glutamine on HMGCoA reductase and ACC activity in hepatocytes*

In hepatocytes prepared from rats at the end of the light period, the expressed HMGCoA reductase activity was only about 10% of that in cells prepared from rats taken in the middle of the dark period, as reported previously [15]. Incubation of the cells (prepared at the end of the light period) for 60 min did not result in a significant increase in HMGCoA reductase activity (Fig. 1A). Incubation with 10 mM glutamine did not modify HMGCoA reductase activity (Fig. 1A), whereas it activated (3-fold) ACC in the same experiments (Fig. 1B). Hepatocytes were also incubated in the presence of 1 mM AICAriboside to activate AMPK. AICAriboside is taken up by the cells and converted into ZMP, which stimulates AMPK because its structure is similar to that of AMP [25–27]. As expected, both HMGCoA reductase and ACC were almost completely inactivated within 15 min (Fig. 1), thus confirming that AMPK indeed inactivates both enzymes in intact cells.

*3.2. Effect of glutamine on HMGCoA reductase, AMPK and ACC activity in hepatocytes preincubated without oxygen*

Anoxia is known to deplete ATP and to increase AMP in hepatocytes [28]. We tested whether anoxia could inactivate HMGCoA reductase and allow reactivation by glutamine to take place in a subsequent incubation in the presence of oxygen. After a 30-min incubation without oxygen, AMPK activity was 0.46 ± 0.20 mU/mg of protein (100% of activity in Fig. 2A) and HMGCoA reductase activity was not significantly decreased (7.0 ± 0.9 to 5.5 ± 1.1 mU/g of cells). During the following reoxygenation, AMPK activity decreased by about 60% within 15 min (Fig. 2A) and a concomitant increase in HMGCoA reductase activity was observed (Fig. 2B). Incubation with 10 mM glutamine did not affect AMPK inactivation (Fig. 2A), nor the concomitant activation of HMGCoA reductase (Fig. 2B).

As for HMGCoA reductase, the 30-min incubation without oxygen did not inactivate ACC (10.7 ± 2.1 mU/g of cells with oxygen vs. 11.6 ± 2.2 mU/g of cells without oxygen). This lack of inactivation might be related to the well known anoxia-induced depletion of ATP. By contrast with HMGCoA reductase, ACC activation was not observed upon reoxygenation alone but required the presence of glutamine (Fig. 2C).

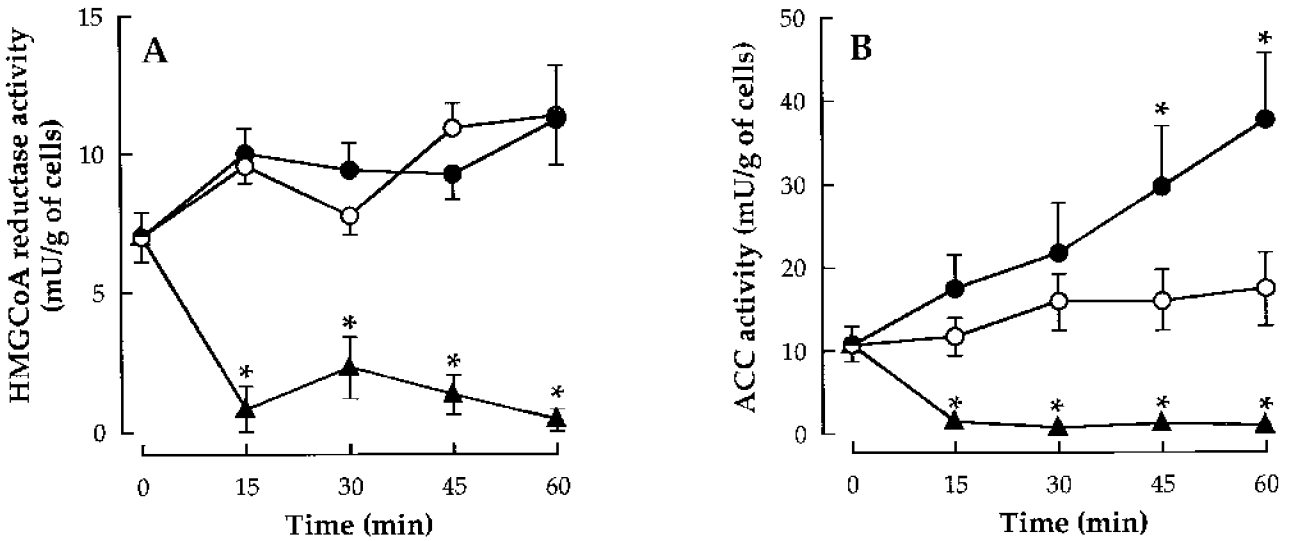


Fig. 1. Time-course of the effect of glutamine or AICAriboside on HMGCoA reductase (A) or ACC (B) activity in hepatocytes. Hepatocytes were incubated for 15 min in the presence of 10 mM glucose, and then in the absence (○) or presence of 10 mM glutamine (●) or 1 mM AICAriboside (■) for the indicated times. The results are the means ± S.E.M. for three (A) or five (B) different cellular preparations. *Significantly different ($P \leq 0.05$) from the respective control value.

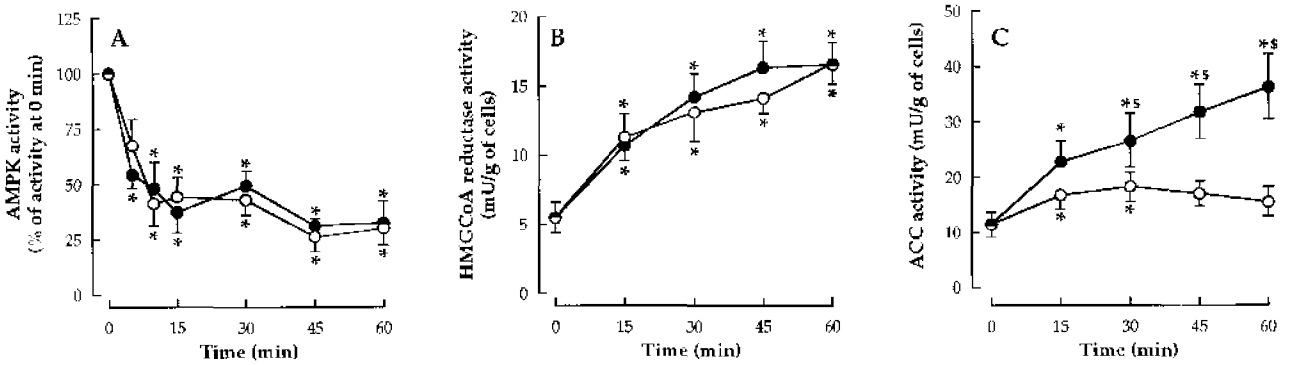


Fig. 2. Time-course of the effect of glutamine on AMPK (A), HMGCoA reductase (B) or ACC (C) activity in hepatocytes during reoxygenation. Hepatocytes were incubated for 30 min in the presence of 10 mM glucose but in the absence of oxygen [28]. Cells were then reoxygenated and further incubated in the absence (○) or presence (●) of 10 mM glutamine for the indicated times. The results are the means ± S.E.M. for three (A), six (B) or five (C) different cellular preparations. *Significantly different ($P \leq 0.05$) from the value at 0 min. Significantly different ($P \leq 0.05$) from the respective control value.

### 3.3. *Separation of HMGCoA reductase phosphatase from GAPP by chromatography on DEAE-Sephadex-A50*

No effect of glutamine on HMGCoA reductase activity was detected in hepatocytes. This suggests that glutamate does not stimulate HMGCoA reductase activation or, alternatively, that HMGCoA reductase phosphatase differs from GAPP. Indeed, several trimeric forms of PP-2A have been identified. They have a common core structure consisting of a 36–38 kDa catalytic subunit (PP-$2A_C$) and a 61–65 kDa regulatory subunit (subunit A). This dimeric species (PP-$2A_D$) can also associate with a third (B) subunit. The trimeric forms of PP-2A are named according to the size of their B subunit [29], e.g. PP-$2A_{T54}$, PP-$2A_{T55}$, PP-$2A_{T72}$, PP-$2A_{T74}$ in cardiac or skeletal muscle. This B subunit is thought to regulate the activity and substrate specificity of type-2A protein phosphatases [30]. In liver, two species of PP-2A were first described and were named PP-$2A_1$ and PP-$2A_2$ [31]. PP-$2A_2$ was identified as PP-$2A_D$ [32], whereas PP-$2A_1$ has a trimeric structure and was tentatively identified as the hepatic homolog of PP-$2A_{T55}$ [33]. GAPP is a newly discovered hepatic type-2A phosphatase with a relative mass of 285 000 which suggests a trimeric structure [8].

If the HMGCoA reductase phosphatase and GAPP are indeed different type-2A protein phosphatases, they might be

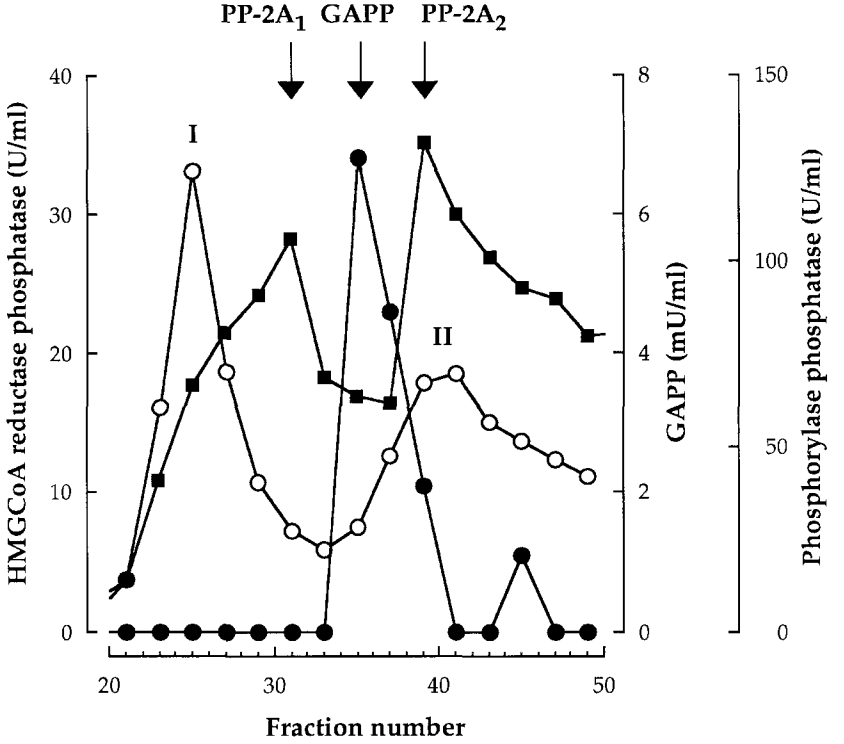


Fig. 3. Elution profile of HMGCoA reductase phosphatase, GAPP and phosphorylase phosphatase activities from DEAE-Sephadex-A50. A liver cytosolic fraction was applied on a DEAE-Sephadex-A50 column, as detailed in [9]. The fractions were assayed for HMGCoA reductase phosphatase (○), GAPP (●), or protamine-stimulated phosphorylase phosphatase (■) activity. I and II refer to the two peaks of HMGCoA reductase phosphatase.

separated by chromatography. A cytosol from rat liver was therefore fractionated by chromatography on DEAE-Sephadex-A50. Two peaks of HMGCoA reductase phosphatase (peaks I and II in Fig. 3), as measured by the dephosphorylation of the catalytic domain of HMGCoA reductase, were detected. This is in agreement with previous observations [34,35]. The fractions were also tested for their ability to dephosphorylate phosphorylase *a* in the presence of protamine, a well known stimulator of the phosphorylase phosphatase activity of PP-2A [36]. Two peaks of activity corresponding to PP-$2A_1$ and PP-$2A_2$ were detected (Fig. 3). As observed by others [34], the peak of PP-$2A_1$ was eluted immediately after the peak I of HMGCoA reductase phosphatase although the elution profile of these two protein phosphatases were overlapping (Fig. 3). PP-$2A_2$ co-eluted with the peak II of HMGCoA reductase phosphatase. GAPP was eluted as a single peak between PP-$2A_1$ and PP-$2A_2$ as already observed [8] and between the two peaks of HMGCoA reductase phosphatase (Fig. 3).

## 4. Discussion

In isolated hepatocytes incubated in the presence of glutamine to stimulate GAPP, no change in HMGCoA reductase activity was detected, whereas a 3-fold activation of ACC was observed within the same experiments. Reoxygenation of hepatocytes following a period of anoxia elicited an activation of HMGCoA reductase that was not influenced by glutamine addition. This suggests that hepatocytes contain an active HMGCoA reductase phosphatase that differs from GAPP. These two protein phosphatases were indeed found to be separated by chromatography on DEAE-Sephadex-A50 (Fig. 3).

By comparison with the work of Ingebritsen et al. [34] we propose that peak I of HMGCoA reductase phosphatase is largely accounted for by PP-2C, with perhaps a small contribution from PP-$2A_1$. However, in hepatocytes, HMGCoA reductase is markedly inactivated by okadaic acid treatment [12], ruling out a major role for PP-2C in the regulation of HMGCoA reductase in intact cells. Peak II of HMGCoA reductase phosphatase co-eluted with PP-$2A_2$. Therefore, the HMGCoA reductase phosphatase activity in hepatocytes could result from both PP-$2A_1$ in peak I and PP-$2A_2$ in peak II. The HMGCoA reductase phosphatase expresses its activity in hepatocytes when AMPK is inactive. This is not the case for GAPP which requires glutamine.

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