Evidence for phosphorylation/dephosphorylation of rat liver acyl-CoA:cholesterol acyltransferase

(cholesterol metabolism/enzyme regulation)

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ABSTRACT Acyl-coenzyme A:cholesterol O-acyltransferase (ACATase; EC 2.3.1.20) is a membrane-bound microsomal enzyme that catalyzes the formation of long-chain fatty-acid cholesterol esters in rat liver and other tissues. This enzyme is important in regulating the concentration of unesterified cholesterol in the cell. Having recently demonstrated that rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.34), the major regulatory enzyme in cholesterol biosynthesis, undergoes in vivo phosphorylation and inactivation after a single cholesterol meal, we decided to test the hypothesis that the enzyme ACATase, important in cholesterol utilization and storage, is also subject to regulation by phosphorylation/dephosphorylation. The results show that rat liver ACATase can be reversibly inactivated/activated, in vitro, by incubation conditions that favor dephosphorylation/phosphorylation. Activation was also achieved by using a partially purified protein kinase extracted from microsomes. It is significant that HMG-CoA reductase is inactivated by phosphorylation whereas ACATase is activated by phosphorylation. ACATase is, therefore, regulated by phosphorylation in a manner exactly opposite to that of HMG-CoA reductase. We propose that the coordinate regulation of ACATase and HMG-CoA reductase by phosphorylation/dephosphorylation provides a mechanism for short-term intracellular cholesterol homeostasis.

Acyl-coenzyme A:cholesterol O-acyltransferase (ACATase) is a membrane bound microsomal enzyme that catalyzes the formation of long-chain fatty-acid cholesterol esters in rat liver and in other tissues. It has been proposed that esterification is a mechanism for the removal of a potentially harmful excess of unesterified cholesterol by conversion to a form that can be stored intracellularly without deleterious effects to the cell (1, 2).

Alternatively, at least for liver, esterified cholesterol may be utilized in the formation of lipoproteins, which are secreted from the liver and pass into the circulation (3–5). The total amount of cholesterol in the hepatic cell changes in response to changes in dietary cholesterol content and to other manipulations that affect the rate of endogenous cholesterol biosynthesis. However, the concentration of unesterified cholesterol present in the liver remains within a relatively narrow range, whereas the amount of esterified cholesterol can be remarkably increased (6–8). This effect is also clearly seen in hepatocytes from rats that have been fed fat and cholesterol (5), as well as in hepatocytes exposed to high concentrations of the sterol precursor mevalonic acid (9, 10).

The activity of microsomal ACATase has been found to be greater when the amount of total cholesterol in the cell is increased (8, 11). Thus ACATase appears to be important in regulating the amount of unesterified cholesterol in the cell. Previously, we have demonstrated the in vivo relevance of the phosphorylation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) as a short-term regulatory event after a single meal containing 2% cholesterol (12). In the present investigation, studies were initiated to determine whether or not evidence could be obtained to support the hypothesis that ACATase could also be regulated by phosphorylation/dephosphorylation. Microsomal fractions from rat liver homogenates were exposed to conditions that favor either dephosphorylation or phosphorylation. Results described here suggest that dephosphorylation inactivates the enzyme, whereas enzyme activity can be restored by conditions that favor phosphorylation. Our findings support the proposal that the short-term regulation of this key enzyme in cholesterol utilization and storage may occur by phosphorylation/dephosphorylation (activation/inactivation). Regulatory events such as these could occur rapidly and therefore facilitate the maintenance of intracellular unesterified cholesterol concentration within a narrow range.

MATERIALS AND METHODS

Materials. [1-14C]Oleoyl-CoA was obtained from New England Nuclear; unlabeled oleoyl-CoA, ATP, and Whatman DEAE-cellulose (DE-52) were from Sigma; diphenyloxazole was from Packard, silica Woelm 63-100, from Universal Scientific; dye reagent for protein determinations, from Bio-Rad; ammonium sulfate (ultra-pure), from Schwarz/Mann. All other chemicals and solvents were of reagent grade quality and were obtained from commercial sources.

Preparation of Substrate. [1-14C]Oleoyl-CoA (1,000 cpm/nmol) was prepared and stored as described (13), except that sodium acetate buffer (0.01 M, pH 6.0) was used.

Preparation of Microsomes and High-Speed Supernatant. Microsomes and supernatant (S30) were prepared by centrifugation of rat liver homogenates at 303,000 × g as described (13). Rats used for these preparations were 250- to 350-g Sprague-Dawley males from Simonsen Laboratories (Gilroy, CA). Protein determinations were conducted by the method of Bradford (14).

Assay of Enzyme Activity. Mixtures for assays of ACATase activity contained microsomal protein (0.5 mg/ml), potassium/phosphate buffer (20 mM, pH 7.2) or imidazole-HCl buffer (20 mM, pH 7.2), and [1-14C]oleoyl-CoA (10 μM) in a total incubation volume of 1.0 or 2.0 ml. Other components were added as described for the various experiments. Assays were conducted in glass scintillation counting vials (20 ml) in a Dubnoff shaker (1.5 reciprocations per sec) at 37°C. The reaction was...
initiated by the addition of \([1,14]C\)oleoyl-CoA, after either warming (37°C, 3 min) or preincubation as indicated at 37°C. ACATase assays, after the addition of substrate, were conducted for 3 min at 37°C. The reaction was linear during this time. The reaction was stopped by the addition of 2:1 (vol/vol) methanol/chloroform. Extraction, silicic acid column chromatography, and determination of the amount of radioactive cholesterol ester formed were performed as described (13), except that silica Woelm 63-100 was used for the chromatographic separation.

Partial Purification of a Microsomal Protein Kinase. In a typical preparation, eight male Sprague-Dawley rats (350–400 g) were killed at the midpoint of the dark cycle, and their livers (96 g) were chilled and homogenized in sucrose (0.3 M) containing 2-mercaptoethanol (10 mM). Homogenization was in 2 ml of sucrose/2-mercaptoethanol per g of liver. Microsomes were isolated as described (13) and washed once in the homogenizing medium. After this, a procedure that has been described for the partial purification of a microsomal protein kinase (15) was used. The microsomes were extracted four times with a phosphate/sodium chloride buffer (50 mM potassium phosphate, pH 7.4/250 mM NaCl/1 mM EDTA/5 mM dithiothreitol). Protein from the washes was precipitated with ammonium sulfate (25–45% saturated fraction), and the precipitate was dissolved and dialyzed in a glycerol/Tris buffer [10% (vol/vol) glycerol/5 mM Tris-HCl, pH 7.4/0.1 mM EDTA], and passed through two DEAE-cellulose columns in this buffer. Fractions from the first column with conductivities between 1.3 and 3.5 mS were pooled, dialyzed in the above buffer, and applied to the second column. Protein was eluted from the second column (1.5 × 12 cm, bed volume 20 ml) by a gradient of glycerol/Tris buffer and sodium chloride (0.27 M) in the same buffer (100 ml each). Fractions eluted from the second column between 2.0 and 3.0 mS were capable of reactivating ACATase in the presence of ATP. The reactivation factor (putative protein kinase) did not survive freezing.

RESULTS

Effect of Magnesium Ion. Cholesterol ester formation by rat liver microsomes was substantially reduced by incubation of the microsomes with Mg\(^{2+}\) (Fig. 1). Incubation without Mg\(^{2+}\) caused only a small decrease in activity (12% in 30 min). In contrast, much greater inactivation (69% in 30 min) was seen when Mg\(^{2+}\) (5 mM) was present during preincubation.

Potassium fluoride, a known protein phosphatase inhibitor, partially blocked the inhibitory effect of Mg\(^{2+}\) on ACATase activity. Addition of both potassium fluoride and EDTA to incubation mixtures containing Mg\(^{2+}\) completely prevented the inactivation of ACATase activity by the metal ion (Fig. 1).

Inactivation of ACATase by Mg\(^{2+}\) was dependent on time (Fig. 1) and concentration (Fig. 2).

Cyclic Activation/Inactivation of ACATase. As shown in Figs. 1 and 2, the magnesium-ion-caused inactivation of ACATase is blocked, but not reversed, by EDTA or potassium fluoride. Furthermore, this inactivation is not reversed by dilution of the magnesium ion. The ACATase inactivation can, however, be reversed by subsequent incubation of the microsomes with ATP and S\(_{303}\) in the presence of KF. Such an experiment is shown in Fig. 3. Microsomal ACATase (point A) was inactivated by preincubation with Mg\(^{2+}\) (point B), followed by preincubation with ATP and rat liver S\(_{303}\). After two washes by ultracentrifugation to remove soluble protein and cofactors, the microsomes were assayed for ACATase activity. Inactivated ACATase formed 0.11 nmol of cholesterol oleate per mg of protein per 3 min (point B), and incubation of this preparation with ATP and
ductivity.

Fig. 3. Cyclic activation/inactivation of ACATase by preincubation conditions favoring phosphorylation/dephosphorylation of the enzyme. The four sets of experiments were conducted as follows: Point A, control: Microsomes were thawed and aliquots were set aside (on ice) for assay. Point B, conditions favoring dephosphorylation of the enzyme: Microsomes (10 mg/ml, 6.0 ml) were preincubated (30 min) with Mg\(^{2+}\) (5 mM). Point C, conditions favoring phosphorylation of inactivated enzyme: Inactivated microsomal ACATase (12 mg of microsomal protein) from B above was preincubated (30 min) at 37°C with rat liver S\(_{50}\) (90 mg of protein), ATP (5 mM), Mg\(^{2+}\) (5 mM), and KF (50 mM) in Erlenmeyer flasks (50 ml) in a total preincubation volume of 12 ml. Controls done at the same time omitted either Mg\(^{2+}\) ATP or both S\(_{50}\) and Mg\(^{2+}\) ATP. At the end of the preincubation period, the flasks were chilled in ice water and EDTA (to 40 mM) was added to all. The microsomes were washed twice with phosphate buffer and resuspended by centrifugation at 303,000 \(\times\) g. Recovery of microsomal protein was 63–72%. Point D, conditions favoring phosphorylation of activated enzyme: Reactivated microsomal ACATase from C was preincubated (30 min) at 37°C with Mg\(^{2+}\) (5 mM) and then assayed for ACATase activity (3 min at 37°C). All assay mixtures for ACATase activity contained microsomes (1 mg of protein) in phosphate buffer (20 mM, pH 7.2) containing KF (50 mM) and EDTA (0.1 mM).

oleate from [1\(^{14}\)C]oleoyl-CoA into cholesterol olate by microsomal ACATase is reduced in the presence of S\(_{50}\) (1), preincubation of inactive microsomal ACATase (Fig. 3, point B).

![Consistent graph](Consistent graph)

Fig. 4. Elution profile of putative microsomal protein kinase from DEAE-cellulose. A soluble extract from rat liver microsomes was partially purified as described in Materials and Methods. The DEAE-cellulose column was 1.5 \(\times\) 12 cm; protein was eluted with a sodium chloride gradient (0–0.27 M). The fraction volume was 9.8 ml and the flow rate was 32 ml/hr. Microsomal ACATase was inactivated by preincubation with Mg\(^{2+}\) (5 mM) for 30 min at 37°C. ACATase activity at this point was 0.1 nmol of cholesterol olate formed per mg of microsomal protein per 3 min. Subsequent incubation mixtures contained inactivated microsomal ACATase (1 mg of microsomal protein), ATP (5 mM), Mg\(^{2+}\) (5 mM), KF (50 mM), and aliquot (0.5 ml) of the column fractions; preincubation was conducted for 30 min at 37°C. ACATase activity was then assayed. c, Protein; e, activation of ACATase; x, conductivity.

![Consistent graph](Consistent graph)

Fig. 5. Effect of concentration of partially purified microsomal protein kinase on the activation of ACATase. Rat liver microsomes were inactivated by preincubation with Mg\(^{2+}\) (5 mM) for 30 min at 37°C. ACATase activity at this point was 0.05 nmol/mg of protein per 3 min. Subsequent preincubation for activation of the enzyme (conducted for 30 min) was in the presence of microsomes (0.5 mg of protein), partially purified protein kinase in the amounts indicated, ATP (5 mM), Mg\(^{2+}\) (5 mM), and KF (50 mM) in a total volume of 1 ml. [1\(^{14}\)C]oleoyl-CoA (10 \(\mu\)M) was added after the second preincubation, and ACATase activity was assayed with ATP and S\(_{50}\), followed by washing, in a 4-fold activation (point C). Microsomes that were preincubated with only S\(_{50}\) were also slightly activated (2-fold). This amount of activation may be due to endogenous ATP present in S\(_{50}\). Those preincubated without either S\(_{50}\) or ATP were unchanged (0.12 nmol of cholesterol olate formed per mg per 3 min).

Reactivated microsomal ACATase (Fig. 3, point C) was inactivated a second time by subsequent preincubation with Mg\(^{2+}\) (Fig. 3, point D).

Effect of ATP and a Putative Microsomal Protein Kinase on ACATase Activity. Microsomal ACATase, which was inactivated by preincubation with Mg\(^{2+}\), showed a low-level variable response to the addition of ATP in the absence of S\(_{50}\). Therefore, protein was extracted from microsomes with salt washes, concentrated by precipitation with ammonium sulfate, and partially purified by passage through two DEAE-cellulose columns. This method, described by Beg et al. (15), partially purifies a microsomal protein kinase. Fractions eluted from the second DEAE-cellulose column between 2.0 and 3.0 mM conductivity (0.035–0.06 M NaCl) were found to activate ACATase when ATP was added and S\(_{50}\) was present.

Table 1. Effect of ATP concentration on the reactivation of microsomal ACATase by a partially purified microsomal protein kinase

<table>
<thead>
<tr>
<th>ATP added, mM</th>
<th>Cholesterol olate formed, nmol/mg of protein per 3 min</th>
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<tbody>
<tr>
<td>None</td>
<td>0.05</td>
</tr>
<tr>
<td>0.2</td>
<td>0.11</td>
</tr>
<tr>
<td>2.0</td>
<td>0.42</td>
</tr>
<tr>
<td>5.0</td>
<td>0.45</td>
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Microsomal ACATase was inactivated by preincubation with Mg\(^{2+}\) (5 mM) for 30 min at 37°C. Subsequent preincubations (conducted for 30 min) were in the presence of inactivated microsomal ACATase (0.5 mg of microsomal protein), partially purified microsomal protein kinase (33 \(\mu\)g of protein), KF (50 mM), Mg\(^{2+}\) (5 mM), and ATP in the concentrations indicated, in phosphate buffer (20 mM, pH 7.2). After the second preincubation, [1\(^{14}\)C]oleoyl-CoA (10 \(\mu\)M) was added and ACATase activity was assayed.
was present (Fig. 4). The activation was time dependent (data not shown). It was also dependent on the amount of protein present (Fig. 5) and on ATP concentration (Table 1).

**DISCUSSION**

The results obtained in the present investigation support the conclusion that rat liver microsomal ACATase activity can be modulated by phosphorylation/depolymeration (Fig. 6). Preincubations conducted in the presence of Mg\(^{2+}\) produced enzyme inactivation. However, this inactivation was blocked, but not reversed, by the addition of EDTA and potassium fluoride, a known protein phosphatase inhibitor (Figs. 1 and 2). Microsomes that were washed after preincubation with Mg\(^{2+}\) did not regain activity. These results are consistent with the conclusion that a membrane-bound protein phosphatase, Mg\(^{2+}\)-activated, is responsible for the inactivation of the enzyme.

ACATase activity in microsomes that had been inactivated could be restored with preincubation conditions that favor phosphorylation of the enzyme (from point B to point C, Fig. 3). Reactivation occurred in the presence of S\(_{300}\), ATP, and KF. This result is consistent with the presence of a protein kinase present in S\(_{300}\). The experiment described in Fig. 3 also demonstrates the cyclic, reversible nature of the inactivation/activation of ACATase.

A putative microsomal protein kinase was freed from rat liver microsomes by repeated salt washes and was partially purified (15). This protein kinase preparation activated ACATase when preincubated with microsomes (Figs. 4 and 5). Activation was dependent on the amount of protein kinase added (Fig. 5) and ATP concentration (Table 1). These results and those presented above (Fig. 3) support the conclusion that both microsomes and S\(_{300}\) contain a protein kinase. Because the microsomal activator was extracted from microsomes with relative ease, it is possible that it is loosely bound and that the same protein is present in the (soluble) supernatant. Partition between the soluble and microsomal phases could occur during the homogenization procedure.

In a related study, we showed that the partially purified microsomal protein kinase not only was capable of activating ACATase (as in Figs. 4 and 5) but also was capable of inhibiting HMG-CoA reductase (data not shown). In addition, the ACAT-activating activity had exactly the same elution profile from a DEAE-cellulose column, developed as in Fig. 4, as the HMG-CoA reductase-inactivating activity. Both activities required ATP and Mg\(^{2+}\). These results support the hypothesis that a single microsomal protein kinase may be involved in the phosphorylation of both HMG-CoA reductase and ACATase.

Previously, we demonstrated the in vitro relevance of the phosphorylation of HMG-CoA reductase as a short-term regulatory event after a single meal of rat chow containing 2% cholesterol (12). It is of distinct importance that HMG-CoA reductase, the major regulatory enzyme in cholesterol biosynthesis, is inactivated by phosphorylation, whereas ACATase is activated by phosphorylation (Fig. 6). ACATase is therefore regulated by phosphorylation in a manner exactly opposite to that of HMG-CoA reductase. Therefore, we propose that the coordinate regulation of ACATase and HMG-CoA reductase by phosphorylation/depolymerization provides a mechanism for short-term intracellular cholesterol homeostasis. Further discussion of this proposal, as well as evidence concerning the phosphorylation/depolymerization of cholesterol 7a-hydroxylase (16, 17), will appear later (18).

Note Added in Proof. Results similar to those described in this paper have recently been reported by Basheeruddin et al. (19).

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