The Functional Size of Acyl-coenzyme A (CoA):Cholesterol Acyltransferase and Acyl-CoA Hydrolase as Determined by Radiation Inactivation*

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Frozen rat liver microsomes and rough endoplasmic reticulum were irradiated with high energy electrons. The surviving enzymatic activity of acyl-CoA:cholesterol acyltransferase and activity for esterification of 25-hydroxycholesterol decreased as a simple exponential function of radiation exposure, leading to a target size of 170–180 kDa. The loss of acyl-CoA hydrolase activity with a radiation dose was complex and resolved as a 45-kDa enzyme associated with a large inhibitor. It is interpreted that acyl-CoA hydrolase is the acyl-CoA-binding component and the inhibitor is the cholesterol-binding component of acyl-CoA:cholesterol acyltransferase.

Cholesterol is required by all mammalian cells for the proper functioning of cellular membranes. Similarly, almost all eucaryotic organisms require cholesterol or related sterol for cell viability. In addition to the free sterol, cholesterol is found esterified to long chain fatty acids. Cholesteryl ester is thought to be a storage form of cholesterol. In general, the concentration of cholesteryl ester is greatest in those tissues (liver, intestine, ovary) which play a major role in the synthesis and metabolism of cholesterol. Large amounts of cholesteryl esters are known to accumulate in certain tissues as a result of diseases in lipid metabolism such as atherosclerosis.

The intracellular esterification of cholesterol is catalyzed by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), which is a membrane-bound enzyme localized in the endoplasmic reticulum of the cell (1–3). Because of its central role in cellular cholesterol metabolism, there has been great interest in the properties and regulation of ACAT. Although ACAT has been solubilized from microsomes (4, 5), the purification of ACAT has been unsuccessful. Therefore, little is known about the physical properties of ACAT.

Radiation inactivation by high energy electrons is a unique method for determination of the molecular size of a membrane-bound protein without prior purification. It requires only that the activities of interest survive freezing and thawing. We have utilized this method on rat liver microsomes and report here the functional molecular mass of ACAT and of the enzyme acyl-CoA hydrolase (ACH). A possible interrelationship between ACAT and ACH is discussed.

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The abbreviations used are: ACAT, acyl-CoA: cholesterol acyltransferase; ACH, acyl-CoA hydrolase; RER, rough endoplasmic reticulum.

MATERIALS AND METHODS

Bovine serum albumin, oleoyl-CoA, glutathione, leupeptin, cholesteryl oleate, porcine pancreatic cholesterol esterase, and glucose-6-phosphate dehydrogenase were purchased from Sigma. Cholesterol was obtained from Eastman, 25-hydroxycholesterol from Steroloids, and Triton WR-1339 from River Chemical Co. [14C]Oleoyl-CoA was purchased from Amersham Corp; [3H]cholesteryl oleate and 25-[3H]hydroxycholesterol were from Du Pont-New England Nuclear. 25-Hydroxycholesterol 3-monooctadecene was prepared enzymatically using pancreatic cholesterol esterase as described previously (6).

Male Sprague-Dawley rats (200–250 g) fed commercial chow ad libitum were fasted 18 h prior to being killed by decapitation. Livers were perfused with 50 ml of ice-cold 0.25 M sucrose and homogenized in 0.05 M phosphate buffer (pH 7.4) containing 0.25 M sucrose, 1 M glutathione, and 20 μM leupeptin. Microsomes were prepared by differential centrifugation and washed once with homogenization buffer, and aliquots (with a final protein concentration of ~20 mg/ml) were taken for irradiation experiments. Rough endoplasmic reticulum (RER) was prepared using the technique of Kreibich et al. (7).

Samples (200 μl) of freshly prepared microsomes and RER were aliquoted into long necked vials and frozen on dry ice, and the vials were sealed under a flame. Frozen samples were held at ~80 °C, except during irradiation at ~135 °C as described by Harmon et al. (8). In some experiments, glucose-6-phosphate dehydrogenase (12 units) was added as an internal standard, and its activity was determined according to Olive and Levy (9).

ACAT activity was determined as described by Billheimer et al. (10). The standard assay in a final volume of 200 μl contained 100 μg of protein, 1 mg of fatty acid-free bovine serum albumin in 0.1 M potassium phosphate buffer (pH 7.4), 1 μM glutathione, and 100 μM [1-14C]Oleoyl-CoA (10,000 dpm/nmol). Where designated, exogenous cholesterol (20 μg) was added as an aqueous dispersion in Triton WR-1339 (600 μg). All components except oleoyl-CoA were preincubated for 15 min; the reaction was initiated by the addition of 4 ml of chloroform:methanol (2:1, v/v); 10 μg of cholesteryl oleate and oleic acid were added as carriers and [3H]Cholesteryl oleate (30,000 dpm) was added as an internal standard. After separation into two layers by the addition of 0.8 ml of water, the chloroform layer was removed, and the lipids were separated by thin-layer chromatography on Gelman ITLC-SA polysilicic acid-impregnated sheets using hexane:ethyl acetate (80:20, v/v). The spots corresponding to cholesteryl ester and oleate were cut out and placed directly into scintillation vials for counting.

To determine the esterification of 25-hydroxycholesterol, 25-[3H]hydroxycholesterol (20,000 dpm/nmol, 10 μg) was substituted for cholesterol, and unlabeled oleoyl-CoA was employed (11). 25-Hydroxycholesterol 3-monooctadecene was separated from 25-hydroxycholesterol by thin-layer chromatography on Gelman ITLC-ASA polysilicic acid-impregnated sheets using hexane:ethyl acetate (80:20, v/v). The activity of ACH was determined using the identical assay as for ACAT except that the radioactivity in oleic acid was quantitated (12).

Protein was determined by the Bradford method (13) using bovine serum albumin as a standard.

Irradiation Conditions—Frozen samples were irradiated at ~135 °C with 13 MeV electrons as described (8).

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RESULTS AND DISCUSSION

Initial experiments (data not shown) demonstrated that ACAT and ACH activities were not significantly affected by freezing (at -80 °C) and thawing the microsomal or RER preparations; similar stability has been reported previously (15). Glucose-6-phosphate dehydrogenase added to these preparations as an internal standard yielded a target size of 117 kDa (data not shown), in agreement with the value (104 ± 16 kDa) obtained by McIntyre and Churchill (16).

ACAT activity was measured by the incorporation of [3H]oleoyl-CoA into cholesteryl oleate. Radiation inactivation of these preparations was expressed as a fraction of that observed in the nonirradiated control. The logarithm of surviving fraction was plotted as a function of radiation dose (the inactivation curve). For simple exponential inactivation curves, a least-squares analysis constrained to 1.0 at zero dose was performed, and target size calculations were as previously described (8). Complex inactivation curves were analyzed as described elsewhere (14) and reported here as averages ± S.D.

Table I

<table>
<thead>
<tr>
<th>Assay Without Exogenous Cholesterol</th>
<th>Assay With Exogenous Cholesterol</th>
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<tbody>
<tr>
<td>kDa</td>
<td>kDa</td>
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<tr>
<td>RER</td>
<td>137 ± 14 (n = 5)</td>
</tr>
<tr>
<td>Microsomes</td>
<td>137 ± 17 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>173 ± 11 (n = 6)</td>
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<td>161 ± 13 (n = 7)</td>
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purification of ACAT from rat liver, ACH activity was always
error in calculating the size of the inhibitor protein is neces-
sarily large because it is based on a difference measurement.
The radiation data yields a target size of 180 kDa (Table II). The
known about its biological function. During attempts at the
mitochondrial, and cytosolic cell fractions (21, 22). Little is
obtained for ACH purified from bovine heart (M, 46,000) (19, 20).
The larger component in the
achieved by extrapolating the linear portion of the graph (Fig. 3) to zero radiation dose. The ratio of the theoretically maximal activity to ob-
erved activity is given by: (ACAT activity + ACH activity)/
ACH activity. The present data yield a value of 1.4 ± 0.2. In
the model, the same value should be obtained by extrapolating
the linear portion of the graph (Fig. 3) to zero radiation dose
the maximal ACH activity expressed in the absence of all
inhibitor molecules); this yields A/A0 = 1.5 ± 0.6. Although
the intercept error is large, the data are consistent with a two-
component model for ACAT/ACH.
Microsomes contain other acyltransferases which are in-
volved in triglyceride and phospholipid synthesis. These en-
zymes also may be hetero-oligomers in which a common acyl-
CoA-binding subunit (ACH) is associated with an additional
subunit which binds the second substrate (e.g. glycerophos-
phate). If substrates for these other acyltransferases were not
available, only the hydrolysis of acyl-CoA would occur. This
would explain the high ACH activity relative to ACAT activity
observed in the microsomes.
The proposed model predicts that maximum acyl-CoA hy-
drolase activity will be observed in the absence of cholesterol
substrate for esterification. Since there is always a small endogenous supply of cholesterol, the acyl-CoA hydrolase
activities in these membrane preparations will be somewhat
less. With the addition of endogenous cholesterol, there should
be a progressive decrease in acyl-CoA hydrolase activity and
a progressive increase in ACAT activity. Beyond a cholesterol
concentration which saturates ACAT, there will be no further
increase in transferase activity and no further decrease in
acyl-CoA hydrolase activity (the remaining hydrolase activity
is presumably due to other transferases and could be reduced
still further by the addition of the other transferase sub-
strates). Fig. 4 shows ACH and ACAT activities as a function
of increasing exogenous cholesterol supplied in the assay.
Over the testable range of cholesterol concentrations, both
activities behave as predicted, and the decrease in ACH activ-
ity is comparable to the increase in ACAT activity.
These results show that the size of the functional unit for
ACAT is much larger than that for ACH, whereas the ester-
fication of 25-hydroxycholesterol requires a structure whose
size is indistinguishable from that of ACAT. The complex
inactivation curve of ACH is consistent with a two-component
model consisting of a 45-kDa subunit which binds acyl-CoA
together with a larger structure which inhibits its activity. An
acyl-CoA-binding unit and a cholesterol-binding unit are both
required for ACAT activity, corresponding to the two struc-
tures revealed by radiation inactivation of ACH. This model
accurately predicts the behavior of both ACH and ACAT
activities when microsomes are assayed in the presence of
exogenous cholesterol.

REFERENCES
Functional Size of ACAT and ACH

The functional size of acyl-coenzyme A (CoA):cholesterol acyltransferase and acyl-CoA hydrolase as determined by radiation inactivation.
J T Billheimer, D A Cromley and E S Kempner


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