Degradation of Stearoyl-Coenzyme A Desaturase: Endoproteolytic Cleavage by an Integral Membrane Protease

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Stearoyl-coenzyme A desaturase (SCD) is a key regulator of membrane fluidity, turns over rapidly, and represents a prototype for selective degradation of resident proteins of the endoplasmic reticulum. Using detergent-solubilized, desaturase-induced rat liver microsomes we have characterized a protease that degrades SCD. Degradation of SCD in vitro is highly selective, has a half-life of 3–4 h, and generates a 20-kDa C-terminal fragment of SCD. The N terminus of the 20-kDa fragment was identified as Phe177. The cleavage site occurs in a conserved 12-residue hydrophobic segment of SCD flanked by clusters of basic residues. The SCD protease remains associated with microsomal membranes after peripheral and lumenal proteins have been selectively removed. SCD protease is present in normal rat liver microsomes and cleaves purified SCD. We conclude that rapid turnover of SCD involves a constitutive microsomal protease with properties of an integral membrane protein.

INTRODUCTION

The lipid composition of cellular membranes is regulated to maintain membrane fluidity. A key enzyme in this process is stearoyl coenzyme A (CoA) desaturase (SCD), a Δ⁹ desaturase and the rate-limiting enzyme in the synthesis of monounsaturated fatty acids. Poikilothermal animals maintain membrane fluidity in response to cold by up-regulating SCD activity and increasing the unsaturation of membrane phospholipids (Tiku et al., 1996). SCD activity is induced in mammals when dietary fat is restricted (Oshino and Sato, 1972) and also by insulin, carbohydrates, and peroxisome proliferators (reviewed in Ntambi, 1995). Animals preferentially use exogenous unsaturated fatty acids and express low levels of SCD when sufficient unsaturated fat is available from dietary sources. The mechanism of SCD induction has been studied extensively and involves transcriptional as well as posttranscriptional controls (reviewed in Ntambi, 1995). Recently, a family of membrane-bound, proteolytically activated transcription factors was identified that regulates multiple genes involved in cholesterol and fatty acid metabolism, including SCD (Brown and Goldstein, 1997). By contrast, little is known about SCD degradation. It was shown some 25 years ago that SCD is degraded rapidly in vivo (Oshino and Sato, 1972). Although most resident proteins of the endoplasmic reticulum have half-lives of 2–6 d (Oura et al., 1967; Arias et al., 1969), the half-life of SCD is 3–4 h (Oshino and Sato, 1972). SCD is selectively degraded in vitro when microsomes from rats induced for SCD are incubated at 37°C (Ozols, 1997). Degradation of SCD in vitro is not inhibited by a wide variety of protease inhibitors (Ozols, 1997), suggesting that SCD may be degraded by a unique protease.

We have extended our previous observations by developing procedures to solubilize SCD and the microsomal components that degrade it. Using purified SCD and detergent-solubilized microsomes we have reconstituted the microsomal system that degrades SCD in vitro. We show that the microsomal proteases that degrade SCD are highly selective and resist solubilization by procedures that solubilize peripheral and
lumenal endoplasmic reticulum (ER) proteins. During degradation, a 20-kDa C-terminal fragment of SCD is generated. The cleavage site was identified as the Phe–Phe bond at residues 176–177.

MATERIALS AND METHODS

Preparation of Microsomes

Desaturase-induced microsomes (DSIMs) were prepared as described (Strittmatter and Enoch, 1978) with modifications. Ten male Sprague Dawley rats (Charles River, Wilmington, MA) were starved for 48 h, fed normal laboratory chow for 48 h, starved for a second 48-h period, and refed with Nutritional Biochemical (Cleveland, OH) Fat Free test diet for 20 h on a schedule that permits the animals to be killed at the beginning of a day. All procedures were carried out at 0–5°C. Livers were quickly excised and placed in 800 ml 0.25 M sucrose, 10 mM Tris-acetate (pH 8.1), 1 mM EDTA, and 1 mM dithiothreitol (DTT). The livers were blotted, weighed, minced, and homogenized with 5 ml of sucrose buffer per gram of liver in a Potter-Elvehjem (Kontes, NJ) tissue grinder. The homogenate was centrifuged at 960 × g for 10 min. The first supernatant was centrifuged at 18,000 × g for 15 min. The second supernatant was carefully separated from the mitochondrial pellet and centrifuged at 92,000 × g in a Beckman type 45 rotor for 90 min. The microsomal pellets were suspended in 20% glycerol, 10 mM Tris-acetate (pH 8.1), 1 mM EDTA, and 1 mM DTT using a buffer equal to the original weight of liver. “Control” microsomes were prepared in the same manner from rats that were refed normal laboratory chow for 20 h after a 48-h period of starvation. Microsomes were used immediately or after brief periods of storage at −80°C.

Solubilization of SCD and SCD Protease (SCDP)

10% (wt/vol) stock solutions of 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS; Pierce Chemical, Rockford, IL), Triton X-100 (TX100; Bio-Rad, Hercules, CA), digitonin (Wako, Richmond, VA), and deoxycholate (DOC; Sigma, St. Louis, MO) were prepared in 10 mM Tris-acetate (pH 8.1). A mixed detergent stock solution (10% DOC/TX100) was prepared by combining equal volumes of the DOC and TX100 stock solutions. One milliliter of microsomes was diluted with 0.25 ml of 10% detergent. After 30 min at 4°C, the insoluble material was pelleted by centrifugation at 105,000 × g for 60 min in a Beckman (Palo Alto, CA) TLA 100.2 rotor.

Time Course of SCD Degradation

Ten milliliters of DSIMs were diluted with 2.5 ml of 10% DOC/TX100. After 30 min at 4°C, insoluble proteins were pelleted by centrifugation at 105,000 × g in a Beckman type 60 rotor for 60 min. One hundred-microliter aliquots of the supernatant were incubated at 37°C.

Purification of SCD

SCD was purified from DSIMs by differential detergent extraction and ion exchange chromatography as described (Strittmatter et al., 1974; Strittmatter and Enoch, 1978).

Preparation of Salt-washed DSIMs

Twenty-five milliliters of DSIMs were diluted with 25 ml of 1 M NaCl, 10 mM Tris-acetate (pH 8.1) 30 mM EDTA, and 1 mM DTT. After 10 min at 4°C, the membranes were pelleted by centrifugation in a Beckman type 45 rotor at 125,000 × g for 65 min. The supernatant was adjusted to 2% DOC/TX100. The sample was incubated on ice for 15 min and centrifuged at 114,000 × g for 30 min. Fifty milliliters of TX100 extract were incubated with 6–8°C overnight. The extract was passed through a DEAE-cellulose column (volume, 75 ml) equilibrated in 10 mM Tris-acetate (pH 8.1), 20% glycerol, 2% TX100, 1 mM EDTA, and 0.1 mM DTT. The column was washed with 100 ml equilibration buffer, and 2-ml fractions were collected. The 20-kDa fragment in effluent fractions was identified by immunoblotting with polyclonal antibody against the C-terminus of SCD.

Micro sequence analysis of the 20-kDa band, eluted from a preparative electrophoresis gel with 40 mM ammonium bicarbonate and 0.02% SDS buffer using the Elutrap chamber (Schleicher & Schuell, Keene, NH), according to their instructions, was carried out on an Applied Biosystems (Foster City, CA) 470A gas phase sequenator equipped with a 120A PTH analyzer, according to the manufacturer’s instructions.

Antibodies and Immunoblots

Monoclonal antibody to Golgi 58 kDa was purchased from Sigma (G-2404). Polyclonal antiserum against SCD was prepared as described (Ozols, 1997). Polyclonal antiserum against the C-terminus of SCD was prepared using a 26-residue synthetic peptide corresponding to residues 338–358 of SCD as antigen. The sequence of the synthetic peptide was based on the cDNA sequence of rat SCD (Thiede et al., 1986). Protein samples were resolved by SDS-PAGE and electrophoretically transferred to sheets of nitrocellulose. The
blots were blocked with 3% albumin. The blots were incubated with antibody overnight at 4°C. Bound antibody was detected with an anti-IgG-alkaline phosphatase conjugate (Sigma A-3687) and a phosphatase detection kit (Kirkegaard and Perry Laboratory, Gaithersburg, MD).

RESULTS

Solubilization and Reconstitution of SCD Degradation

The current studies were designed to establish the solubility characteristics of the microsomal proteolytic system that degrades SCD, to reconstitute SCD degradation using purified SCD and solubilized microsomal protease, and to identify proteolytic fragments of SCD that result from degradation. Initially we investigated the effect of various detergents and salts on SCD degradation. None of the detergents investigated inhibited SCD degradation; however, 500 mM NaCl appeared to inhibit degradation. We therefore performed our solubilization studies in low-ionic-strength buffer. Figure 1 shows an experiment in which DSIMs were solubilized with various detergents. The insoluble fraction was removed by ultracentrifugation, and the detergent supernatants were incubated at 37°C. The SDS-PAGE protein profiles of the detergent extracts before and after incubation are compared in adjacent lanes. Only the detergent combination of DOC/TX100 effectively solubilized SCD (Figure 1, lane 8). After incubation, SCD was depleted from the DOC/TX100 detergent extract indicating that the SCDP was also solubilized (Figure 1, compare lanes 8 and 9). Unexpectedly, a 96-kDa protein was also degraded upon incubation at 37°C (Figure 1). This protein is not degraded when DSIMs are incubated in the absence of detergent (Ozols, 1997).

Figure 2 shows a time course of SCD degradation in the DOC/TX100-soluble fraction of DSIMs. SCD is noticeably depleted after 4 h and nearly gone after 12 h. The 96-kDa protein appears completely degraded in 4 h. Quantitation of the data in Figure 2 by image analysis indicated that the half-life of SCD is 3–4 h and the half-life of the 96-kDa protein is 1–2 h. The selectivity of SCD degradation after solubilization with DOC/TX100 suggests that this in vitro system represents a physiologically relevant process. Furthermore, the half-life of SCD in vitro is identical to the half-life reported for in vivo degradation (Oshino and Sato, 1972).

The experiments of Figure 3 were conducted to determine whether proteolytic intermediates of SCD could be detected during degradation and whether degradation could be reconstituted from solubilized components. DSIMs and control rat liver microsomes (CRLMs) were solubilized with DOC/TX100, and the insoluble fraction was removed by ultracentrifugation. Where indicated, the soluble fraction of CRLM was supplemented with purified SCD. After incubation the preparations were subjected to electrophoresis and blotted with antisera to SCD. This antisera detected three protein bands in solubilized DSIMs before incubation (Figure 3, lane 1). The largest and most abundant of these represents intact SCD; two minor
bands with apparent molecular masses of 35 and 32 kDa result from loss of material at the N terminus of SCD. Several residues from the N terminus of the SCD molecule can be deleted without affecting the catalytic activity of the enzyme (Ozols, 1997). After incubation, three new bands were detected with apparent molecular masses of 24, 23, and 20 kDa (Figure 3, lane 2). CRLMs contained low levels of SCD (Figure 3, lane 3). No proteolytic intermediates were detected in solubilized CRLMs before or after incubation (Figure 3, lane 4). However, when the CRLM preparation was supplemented with purified SCD, the 20-kDa proteolytic fragment was detected after incubation (Figure 3, lane 6). To further characterize the proteolytic fragments of SCD, antisera were prepared against a C-terminal peptide of SCD. The C-peptide antisera detected the 20-kDa fragment of SCD generated during degradation (Figure 3B, lane 8). These results suggest that a constitutive microsomal protease cleaves SCD and generates a 20-kDa C-terminal fragment. To detect the 20-kDa intermediate by immunoblot, we loaded a relatively large amount of sample on the gel. Under such conditions, there is no visible difference in the amount of 37-kDa band detected by immunoblot after incubation, even though there is a marked decrease in the amount of 37-kDa band detected by Coomassie blue staining. If a smaller amount of sample is loaded, a decrease in the 37-kDa band can be demonstrated by immunoblot, but then the 20-kDa fragment is not detected. The ranges in which the two methods are quantitative do not even overlap. In fact, the maximum immunoblot signal is produced by a quantity of SCD that is below the limit of detection by Coomassie blue staining.

SCDP Is an Integral Membrane Protein

Microsomal proteins can be separated into three groups based on their solubility characteristics. Peripheral proteins bound to the cytosolic membrane surface by ionic interactions are selectively solubilized by aqueous buffers that contain sufficient salts. For example, the signal recognition particle is efficiently extracted from rough microsomes with solutions of high ionic strength (Warren and Dobberstein, 1978). Lumenal proteins are selectively solubilized by freezing and thawing microsomes or by detergents used below their critical micelle concentration (Kreibich and Sabatini, 1974). Integral membrane proteins typically require detergent at concentrations above the critical micelle concentration for solubilization (Hjelmeland, 1990). The experiment of Figure 4 was conducted to determine whether SCDP is a peripheral protein. DSIMs were washed with 500 mM NaCl/15 mM EDTA to strip off peripheral proteins. The salt-washed microsomes were then washed with buffer to reduce the NaCl concentration and incubated overnight at 37°C. Incubation of salt-washed DSIMs led to the complete disappearance of the 37-kDa band from the SDS-PAGE gel profile (Figure 4A). Immunoblot analysis of salt-washed DSIMs with SCD antibody

### Figure 2

Time course of SCD degradation. DSIMs were solubilized with DOC/TX100. Insoluble proteins were removed by ultracentrifugation. The soluble protein fraction was incubated at 37°C for the indicated times, subjected to SDS-PAGE, and stained with Coomassie blue. Time points analyzed were 0 h (lane 1), 0.5 h (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), and 12 h (lane 6). The SCD protein band is marked with an asterisk to the left of lane 1.

### Figure 3

Immunoblot analysis of SCD degradation. Nitrocellulose blots were probed with rabbit antiserum to SCD (lanes 1–6) and rabbit antiserum to a C-terminal peptide of SCD (lanes 7–8). The samples analyzed were the DOC/TX100-soluble fraction of DSIMs before (lanes 1 and 7) and after (lanes 2 and 8) incubation at 37°C and the DOC/TX100-soluble fraction of CRLMs unsupplemented (lanes 3 and 4) and supplemented with 2 μg purified SCD (lanes 5 and 6) before (lanes 3 and 5) and after (lanes 4 and 6) incubation at 37°C.
detected three protein bands as expected (Figure 4B, lane 3). After incubation, the 20- and 24-kDa SCD fragments characteristic of SCDP cleavage were detected (Figure 4B, lane 5). A peripheral protein of the Golgi apparatus, Golgi 58 kDa (Bloom and Brashear, 1989), was present in DSIMs but not detected in salt-washed microsomes (Figure 4C), an indication that the 500 mM NaCl/15 mM EDTA washing procedure was effective. These results suggest that peripheral membrane proteins are not required for the proteolytic degradation of SCD described here.

We next asked whether SCD degradation requires lumenal proteins. Selective release of lumenal ER proteins by freeze–thawing or extraction with low concentrations of nonionic detergent did not affect SCD degradation. However, release of lumenal proteins by these methods is incomplete. We took advantage of our observations regarding the solubility of SCD to design a procedure that more completely solubilizes lumenal proteins without solubilizing SCD. In the experiment of Figure 5, DSIMs were incubated with [3H]DFP to label the microsomal esterases. The labeled DSIMs were solubilized with 2% CHAPS. The insoluble protein fraction was collected by ultracentrifugation, suspended in buffer containing 2% DOC/TX100, and incubated overnight at 37°C. The samples were analyzed by Coomassie blue-stained SDS-PAGE (A), autoradiography (B), and immunoblot (C) using rabbit antiserum to SCD. The samples analyzed were unfraccionated DSIMs (lanes 1 and 2) and the CHAPS-insoluble fraction of DSIMs (lanes 3 and 4) before (lanes 1 and 3) and after (lanes 2 and 4) incubation.

SCD was recovered with the CHAPS-insoluble material (Figure 5, lane 3). SCD was degraded, and the proteolytic fragments of SCD characteristic of SCDP cleavage were detected in the CHAPS-insoluble material after incubation (Figure 5, compare lanes 3 and 4). Autoradiography of the CHAPS-insoluble material showed only a trace of mi-
crosomal esterase (Figure 5B). These results suggest that lumenal proteins are not required for SCD degradation.

To confirm the results of the previous experiments, we examined preparations of peripheral proteins and lumenal proteins of DSIMs for proteolytic activity against SCD. Peripheral proteins were prepared by extracting DSIMs with 500 mM NaCl/15 mM EDTA. The peripheral protein preparation was then dialyzed to lower the NaCl concentration, and DOC/TX100 was added. Lumenal proteins were selectively extracted with 0.1% TX100. The lumenal protein preparation and the peripheral protein preparation contained only traces of SCD (Figure 6), an indication that the preparations were substantially free of integral membrane proteins. When supplemented with purified SCD and incubated overnight, neither preparation generated detectable quantities of the 20-kDa proteolytic fragment of SCD (Figure 6, lanes 3 and 6).

SCDP Cleaves SCD between Residues 176 and 177 in a Conserved Hydrophobic Segment

SCD was recovered in the TX100 extract of salt-washed DSIMs. SCD is one of a spectrum of microsomal proteins that are not solubilized by nonionic detergents from native microsomes at low ionic strength but are solubilized by nonionic detergents from salt-washed microsomes. The 20-kDa fragment was generated by incubating the extract overnight at 6–8°C. Thus, SCDP was also solubilized under these conditions. SCD and the 20-kDa fragment were partially purified by passing the extract through a DEAE column. SCD and the 20-kDa fragment were recovered in the DEAE flow-through fractions (Figure 7). Sequence analysis of the 20-kDa fragment purified by SDS-
Degradation of Stearoyl-CoA Desaturase

Table 1. N-terminal sequence analysis of the 20-kDa fragment

<table>
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<th>Position</th>
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<tr>
<td>1</td>
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<td>177</td>
</tr>
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* X, Not identified.

PAGE and isolated by electroelution revealed the sequence shown in Table 1.

DISCUSSION

Our data show that SCD is selectively degraded by a constitutive microsomal protease with properties of an integral membrane protein. Several observations support our view that the in vitro proteolytic system described here is responsible for the rapid turnover of desaturase observed in vivo. First, the degradation of SCD in isolated microsomes is highly selective. When DSIMs are incubated intact at 37°C, SCD is the only major microsomal protein significantly degraded after 12 h (Ozols, 1997). When DSIMs are incubated in the presence of detergent, one other protein, an unidentified 96-kDa protein, is also rapidly degraded. All other abundant microsomal proteins in DSIMs appear to be relatively resistant to proteolysis. These results correlate with seminal observations regarding the turnover of microsomal proteins in vivo. Microsomal proteins are degraded independently, and most microsomal proteins have half-lives of 2–6 d (Omura et al., 1967; Arias et al., 1969). Second, the rate of SCD degradation in vitro is identical to the rate observed in vivo. The half-life of SCD in vivo was determined by inhibiting protein synthesis during dietary induction of SCD and measuring desaturase activity in microsomes isolated from treated animals (Oshino and Sato, 1972). Under these conditions desaturase activity declines, with a half-life of 3–4 h. The half-life of SCD in detergent-solubilized DSIMs at 37°C is also 3–4 h. Third, our results suggest that the protease responsible for SCD degradation (SCDP) is a resident protein of the endoplasmic reticulum. Degradation proceeds in microsomal vesicles in the absence of ATP or cytosol, a condition that prevents membrane fusion events (Stafford and Bonifacino, 1991; Wikstrom and Lodish, 1992). If SCDP were confined to a non-ER component of microsomes, e.g., an endosomal or trans-Golgi compartment, it would probably not have access to SCD in vesicles derived from ER before solubilization. The fact that SCD is degraded in microsomal vesicles suggests that SCDP is in close proximity to SCD before vesiculation of the ER during homogenization. Several observations make unlikely the possibility that SCDP is a cytosolic protease or lysosomal protease artifically adsorbed on the surface of microsomes. Incubation of cytosol with microsomes does not accelerate degradation. Extensive washing procedures to remove trapped cytosolic proteins do not diminish SCD degradation. Procedures that selectively remove extrinsic proteins from the cytosolic ER surface and soluble proteins from the ER lumen fail to inhibit SCD degradation. These results provide strong evidence that SCDP is an integral membrane protein or protein complex of the ER.

We have shown that purified SCD is degraded by normal rat liver microsomes and that this results in the generation of proteolytic SCD fragments similar to those produced by DSIMs. Therefore, it appears that SCDP is constitutively expressed rather than coinduced with SCD. This is consistent with the observation that desaturase is degraded in vivo at the same rate 5 and 16 h after dietary induction (Oshino and Sato, 1972). If the protease were coinduced, we would expect SCD to be degraded more rapidly later in the induction. Thus, degradation of SCD does not appear to require a molecular trigger to activate proteolysis. This contrasts with another target of ER degradation, 3-hydroxy-3-methylglutamyl-CoA (HMG-CoA) reductase. HMG-CoA reductase is a relatively stable enzyme under conditions of sterol depletion but is rapidly degraded in the presence of mevalonate (Brown and Goldstein, 1980). The protease that degrades HMG-CoA reductase is also constitutively expressed but differs from SCDP in its sensitivity to protease inhibitors. Degradation of HMG-CoA reductase is inhibited by cysteine protease inhibitors (Inoue et al., 1991) and the proteosome inhibitor lactacystin (McGee et al., 1996). Neither of these types of protease inhibitor affect SCD degradation (Ozols, 1997).

During degradation in vitro, proteolytic fragments of SCD with apparent molecular masses of 20 and 24 kDa accumulate. The 20-kDa fragment is recognized by antisera against a C-terminal peptide of SCD. The 24-kDa fragment is not recognized by the C-peptide antisera. These results suggest that during in vitro degradation SCD is cleaved near the middle of the polypeptide chain, generating a 24-kDa N-terminal fragment and a 20-kDa C-terminal fragment. This was confirmed by isolating the 20-kDa fragment and identifying its N terminus as Phe177. The quantity of the 20-kDa fragment in the digests is never >1% of the original SCD starting material. Presumably this reflects the activity of the system for degrading the 20-kDa fragment. SCD is 358 amino acids in length and contains several hydrophobic domains (Thiede et
Hydropathy analysis of rat liver SCD. Hydropathy plot of the amino acid sequence (Thiede et al., 1986) was analyzed by the method of Kyte and Doolittle (1982). The position of the identified cleavage site is indicated by an arrow.

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