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PURIFICATION AND CHARACTERIZATION OF TWO MEMBRANE BOUND SERINE PROTEINASES FROM RAT LIVER MICROSOMES ACTIVE IN DEGRADATION OF CYTOCHROME P450

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Two serine proteinases capable of digesting cytochrome P4502E1 (CYP2E1) have been purified from sodium cholate solubilized rat liver microsomal membranes. After chromatography on hydroxyapatite, DEAE-Sepharose chromatography resolved the CYP2E1-degrading activity into two peaks, and the two proteinases were finally purified on benzamidine-Sepharose. Both have a Mr of 32,000 on SDS-PAGE, are optimally active at pH 8, and show a susceptibility to inhibitors typical of serine proteinases. CYP2E1 degradation patterns exhibited by the proteinases are identical to each other and similar to that observed during the proteolysis of endogenous CYP2E1 in the microsomal membranes, which indicates that the proteinases can degrade CYP2E1 in its native environment. We suggest a role of these proteinases in the rapid phase of cytochrome P450 degradation in the endoplasmic reticulum. © 1993 Academic Press, Inc.

Liver microsomal cytochromes P450 are membrane-bound enzymes catalyzing the oxygenation of a wide variety of foreign chemicals and physiologically important substances. The amounts of individual isozymes are subject to regulation at the transcriptional, translational, and posttranslational levels (1). As shown earlier in this laboratory, the ethanol-inducible form of cytochrome P450 (CYP2E1) is to a great extent regulated by its substrates at the posttranslational level. In the absence of substrate, the enzyme undergoes particularly fast degradation, which can be triggered by phosphorylation of the apoprotein on Ser 129. Substrate prevents this phosphorylation and the isozyme is instead degraded via the autophagosomal-lysosomal pathway (2-4).

Evidence exists that the rapid degradation of CYP2E1 takes place within the endoplasmic reticulum (4, 5), but the responsible proteinase has not been identified yet. Recently, the involvement of cytosolic ATP/ubiquitin-dependent proteolytic system has been reported (6, 7), and

Abbreviations: CBZ, carbobenzoxy; CHAPS, (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; MCA, 7-amino-4-methylcoumarin; PCMB, p-chloromercuribenzoate; PMSF, phenylmethylsulfonylfluoride; TLCK, tosyl lysine chloromethyl ketone; TPCK, tosyl phenylalanine chloromethylketone.

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a ubiquitin-conjugating enzyme has been found in the endoplasmic reticulum of yeast as an integral membrane protein (8). On the other hand, data obtained in our laboratory suggest the involvement of another, ubiquitin-independent, membrane proteinase (4).

Several serine, cysteine, and metalloproteinases have been isolated from microsomes thus far. Some of them are believed responsible for preprotein processing (9-11) or cleavage of signal peptides (12, 13), while the function of others is unknown (14, 15). To purify the CYP2E1-degrading proteinase, an assay system based on the *in vitro* fragmentation of the enzyme in the presence of small amounts of detergents was developed. We now report on the purification of two similar serine proteinases with possible roles in the *in vivo* degradation of cytochrome P450 in the microsomal membrane.

MATERIALS AND METHODS

Materials - p-Aminobenzamidine Sepharose 6B, PMSF, PCMB, TLCK, TPCK, leupeptin, antipain, diamide, chymostatin, E-64, octyl glucoside and CHAPS were from Sigma. Lubrol 17A17 was from Serva. DEAE-Sepharose CL6B was from Pharmacia. Hydroxyapatite Bio-Gel HT was from Bio-Rad. CBZ-Ala-Arg-Arg-MCA was from Bachem. [1,3-^{3}H]DFP was from NEN. CYP2E1 was isolated from the liver of starved/acetone-treated rats as described earlier (16).

Microsomal membranes - Microsomes were isolated from livers of male Sprague-Dawley rats as described previously (16), including a wash with 0.1 M potassium pyrophosphate, pH 7.5. Untreated rats were used for the purification of the proteinase and rats treated with acetone and starvation (16) in studies on the degradation of endogenous CYP2E1 in the membrane. Microsomal membranes free of the lumenal content (ghosts) were prepared by treatment of the microsomes with 0.05% sodium deoxycholate followed by centrifugation and wash (17).

Purification of proteinase - Microsomal CYP2E1 proteinase was purified in two different manners. The initial approach employed DEAE-Sepharose chromatography of CHAPS-solubilized microsomal membranes followed by chromatography on heparin-agarose and benzamidine-Sepharose. The second method was largely based on the protocol described in (15) with certain modifications. Pelleted microsomal membranes corresponding to 1400 mg total protein were resuspended in 10 mM K-phosphate buffer, pH 7.5, containing 0.15 M NaCl and 1% Na-cholate (buffer A) to a concentration of 3 mg/ml. The mixture was stirred for 1 h and centrifuged at 105,000 g for 70 min. One third of the supernatant (cholate extract) was applied on a 17 x 4.8 cm hydroxyapatite column equilibrated with buffer A. The column was washed with 0.5 l of buffer A and eluted with a linear 0.01-0.3 M K-phosphate gradient in 1 l of buffer A at a flow rate of 47 ml/h. This step was repeated three times. The active fractions were pooled, dialyzed twice against 10 mM Tris-HCl buffer, pH 8.2, containing 0.06% Lubrol (buffer B), and applied on a 22 x 4.8 cm DEAE-Sepharose column equilibrated with buffer B. The column was washed with 0.3 l of buffer B and eluted with a linear 0-0.3 M NaCl gradient in 2 l of buffer B at a flow rate of 105 ml/h. Fractions corresponding to the two peaks of CYP2E1-degrading activity were pooled separately and applied on two 8 x 1.4 cm benzamidine-Sepharose columns equilibrated with buffer B containing 0.1 M NaCl. The columns were washed with 10 column volumes of 10 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl and 10 mM CHAPS and eluted with the same buffer supplied with 0.1 M benzamidine. Fractions of 3 ml were collected, the active fractions pooled and dialyzed three times against 10 mM Tris-HCl, pH 7.5.

Assay of proteolytic activity - 4 µl of fractions from different chromatographic steps or 2 µl of purified proteinases was incubated at 37 °C with specified amounts of CYP2E1 in 10 mM Tris-HCl buffer, pH 7.5, containing 0.005% SDS for 19 h. The total volume of the incubation mixture was 20 µl. The samples were then subjected to SDS-PAGE followed by immunoblotting

which employed a polyclonal CYP2E1 antiserum (16) and alkaline phosphatase conjugate assay. Proteolytic activity was detected by the formation of immunoreactive CYP2E1 fragments and expressed as percent degraded CYP2E1 after densitometry on a Personal Densitometer (Molecular Dynamics). Activity towards CBZ-Ala-Arg-Arg-MCA (0.1 mM) was determined fluorometrically at 37 °C in10 mM Tris-HCl buffer, pH 7.5 using excitation and emission wavelengths of 380 and 460 nm, respectively. One unit of activity was defined as the amount of the enzyme releasing 1 pmol of MCA per minute.

[^{3}H]DFP labeling - 10 μl of sample was incubated with 10 μCi of [^{3}H]DFP (specific activity, 111 GBq/mmol) for 2 h at 37 °C followed by SDS-PAGE. The gel was soaked in Amplify (Amersham), dried and subjected to autoradiography.

RESULTS

Purification of microsomal P450 proteinase. Our initial attempts to purify the CYP2E1-degrading proteinase employed the DEAE-Sepharose chromatography of CHAPS-solubilized microsomal membranes. The fractions were incubated with purified CYP2E1 in the presence of 0.005% sodium dodecyl sulfate to render the enzyme more susceptible to proteolysis. The proteolytic activity was eluted as a single peak at about 0.09 M sodium chloride (Fig. 1) indi-

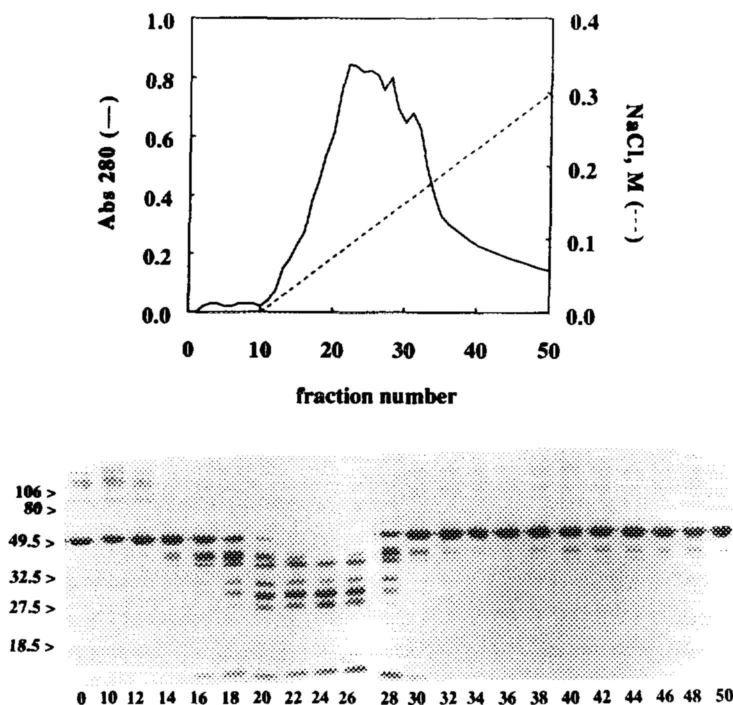

Fig. 1. DEAE-Sepharose chromatography of CHAPS-solubilized microsomal membranes. The membranes were extracted with 10 mM CHAPS in 10 mM Tris-HCl buffer containing 20% glycerol, 1 mM EDTA and 0.1 mM dithioerythritol. The extract was applied on a 17x4.8 cm DEAE-Sepharose column and eluted with a 0-0.3 M NaCl gradient in the above buffer (upper panel). Lower panel - fractions were assayed for CYP2E1-degrading activity (5 pmol CYP2E1 per 20 μl incubation mixture) followed by SDS-PAGE/Western blot; given below are fraction numbers; 0 - enzyme blank.

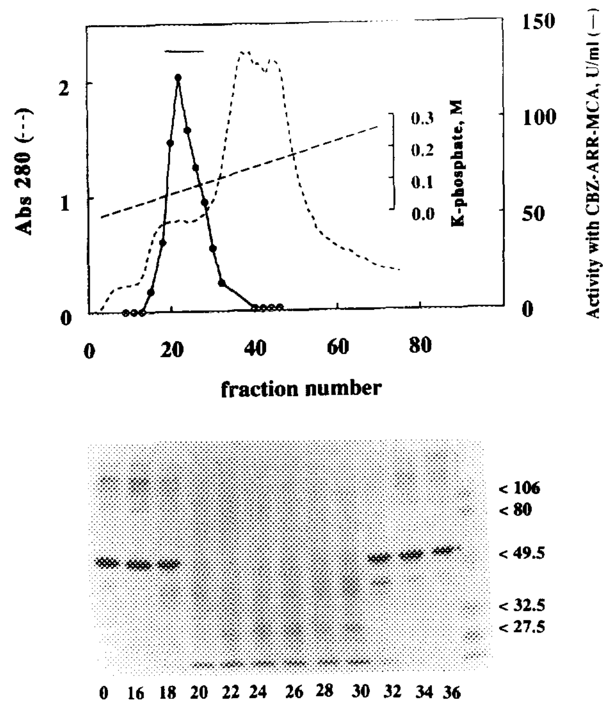

Fig. 2. Chromatography of cholate-solubilized microsomal membranes on hydroxyapatite. Upper panel - Elution profile and activity towards CBZ-Ala-Ala-Arg-MCA. Lower panel - Activity towards CYP2E1 (5 pmol per 20 μl of incubation mixture) as determined by Western blot (given below are fraction numbers; 0 - enzyme blank). Fractions under the bar were pooled. No activity was detected in fractions to the left and right of the presented region (not shown).

cating the presence of only one type of microsomal proteinase active on CYP2E1 under the conditions used. The activity was further purified on heparin-agarose and, owing to its inhibition by benzamidine, benzamidine-Sepharose. The labelling of the resulting partially purified preparation with [^{3}H]DFP showed the protease to be a 32 kDa protein (data not shown). A serine protease of the same molecular weight has been recently purified from liver microsomes by Tamanoue et al. (15). To check the possible identity of the two proteinases and to purify the CYP2E1-degrading proteinase completely we employed a protocol similar to that of Tamanoue et al. (15).

Cholate solubilized microsomes were subjected to hydroxylapatite chromatography and CYP2E1 proteinase eluted as a single peak at 0.08 M potassium phosphate (Fig.2). The activity cleaving on the carboxy side of the Arg-Arg doublet, which has been described by Tamanoue et al. (15), was eluted at the same salt concentration. However, subsequent DEAE-Sepharose chromatography resolved the two activities from each other and further resolved the CYP2E1-degrading activity into two peaks (Fig. 3). After benzamidine-Sepharose chromatography as the last step, essentially pure preparations of the two forms of a CYP2E1-active microsomal membrane-bound serine proteinase were obtained, both having a M_r of about 32,000 on SDS-PAGE under reducing conditions (Fig. 4A). The preparations still contain a few minor bands of lower M_r. At present it is unclear whether these are contaminating proteins or autodegradation products of the proteinases.

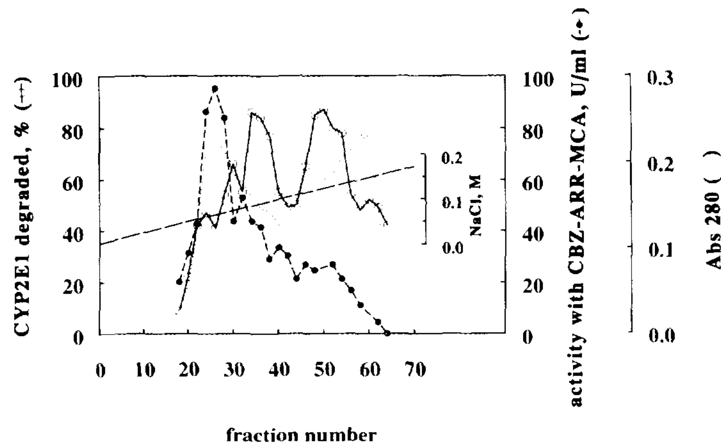

Fig. 3. ***Chromatography of fractions from hydroxyapatite column on DEAE-Sepharose.*** Activity was determined with 0.125 μM CYP2E1. Fractions under the bars were pooled separately. See Materials and Methods for details.

Characteristics of the microsomal P450 proteinases. Incubation of the purified proteinases with [^{3}H]DFP revealed the incorporation of radioactivity into the 32-kDa band, indicating the enzyme to be a serine proteinase (Fig.4A). The two forms of the proteinase were designated

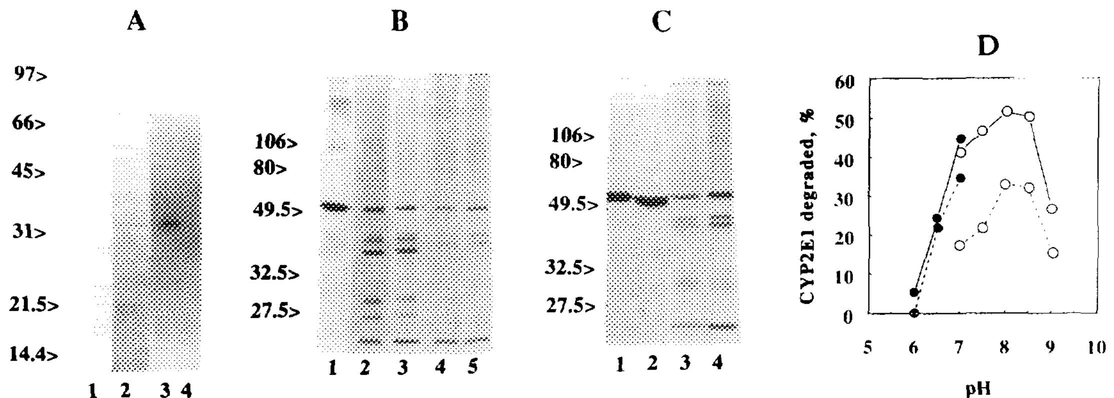

Fig. 4. Properties of purified MCPP1 and MCPP2. A. SDS-PAGE of purified MCPP1 (1, 3) and MCPP2 (2, 4) as detected by silver staining (1, 2) and autoradiography after labeling with [^{3}H]DFP (3, 4). **B.** Degradation pattern of CYP2E1 (2.5 pmol per 20 μl of incubation mixture) with MCPP1 (2, 4) and MCPP2 (3, 5) in the presence of 0.005% SDS (2, 3) and 0.3% octyl glucoside; 1 - enzyme blank. **C.** Comparison of degradation patterns of purified CYP2E1 by MCPP1 (4) and of endogenous CYP2E1 in microsomal membranes (3). 5 pmol of purified CYP2E1 was incubated with 4 μl of purified MCPP1 in 20 μl of 10 mM Tris-HCl, pH 7.5, in the presence of 0.35% octyl glucoside at 37 °C for 20 h. Microsomal membranes (1.7 mg/ml protein) were incubated in 0.1 M K-phosphate buffer, pH 7.5, containing 0.7% octyl glucoside at 37 °C for 20 h. The samples were then subjected to SDS-PAGE/Western blot. Lanes 1 and 2 show purified and microsomal CYP2E1 before incubation. **D.** Effect of pH on the activities of the proteinases. The activities of MCPP1 (solid line) and MCPP2 (dashed line) were assayed with 2.5 pmol CYP2E1 in a total of 20 μl of 0.1 M bis-Tris (closed circles) or 0.1 M Tris-HCl (open circles).

Table 1. Effect of protease inhibitors and metal ions on the CYP2E1 degrading activity of MCPP1

Inhibitor	Relative activity, %	Inhibitor	Relative activity, %
none	100	0.1 mM antipain	0
2 mM DTT	0	0.1 mM TPCK	120
1 mM PCMB	0	0.1 mM TLCK	42
0.1 mM E-64	61	0.1 mM chymostatin	92
1 mM diamide	66	10 mM EDTA	45
1 mM benzamidine	42	1 mM $CaCl_2$	14
1 mM PMSF	64	1 mM $MgCl_2$	72
0.1 mM leupeptin	15	1 mM $ZnSO_4$	0

CYP2E1 (5 pmol per 20 µl incubation mixture) was incubated with MMSP1 in the presence of the inhibitors and activity was determined as described under Materials and Methods.

MCPP1 and MCPP2 (**M**icrosomal **C**ytochrome **P**450 **P**roteinase).They showed identical fragmentation patterns of CYP2E1 in the presence of SDS (Fig. 4B). CYP2E1 could also be degraded in the presence of octyl glucoside and the resulting pattern was the same as that of endogenous CYP2E1 in intact microsomal membranes (Fig. 4C). Both forms are neutral proteinases showing maximum activity at pH 8 (Fig. 4D).

MCPP1 was inhibited by benzamidine, leupeptin, antipain, as well as by thiol group directed agents (Table 1), whereas PMSF, E-64 and diamide were only weak to moderate inhibitors. Neither calcium nor magnesium activated the enzyme under the conditions used, whereas zinc strongly inhibited the MCPP-dependent CYP2E1 proteolysis. Inhibitors which blocked MCPP1 activity were active against MCPP2 as well (data not shown).

DISCUSSION

The data obtained demonstrate the existence of two similar enzymes that are likely to be isoforms of a microsomal membrane-bound neutral serine proteinase with cytochrome P450 as one of the substrates. Additional proof is however needed to conclude that the enzymes participate in cytochrome P450 turnover under physiological conditions. Although not conclusive, the available data are in line with this view. Thus, in addition to SDS used in the assay system in the course of purification CYP2E1 is degraded by the proteinase in the presence of octyl glucoside (Fig. 4). The latter, in contrast to SDS, does not disturb the catalytically active conformation of cytochrome P450 (18), which indicates that some forms of folded CYP2E1 in intact microsomes can be substrates for the enzyme. Furthermore, the degradation pattern of endogenous CYP2E1 in the microsomal membranes of starved/acetone-treated rats is similar to that of purified hemoprotein in the presence of purified proteinase (Fig. 4) demonstrating that the same proteinase can attack cytochrome P450 in its native environment. Data from our laboratory indicate that phosphorylation of CYP2E1 on Ser 129 causes heme loss (3) and makes the enzyme more susceptible for degradation (4). It might be suggested that MCPPs act as an intracellular protein quality control system and that only improperly folded variants of the enzyme can be its

substrates. In this respect phosphorylation is proposed to be the endogenous trigger reaction for modification of enzyme folding, thereby increasing the susceptibility to proteolytic attack. This process also appears to involve aggregation of the enzyme[1].

Some recent reports point to the involvement of the cytosolic ATP/ubiquitin-dependent system in the *in vivo* cytochrome P450 degradation (6, 7), and the proteinase under investigation can prove to be one of several enzymes catalyzing cytochrome P450 destruction in a concerted or sequential manner. If so, it is of importance to identify the proteinase which is the first to recognize hemoprotein molecules tagged for degradation and effect the primary cleavage. The localization of the MCPPs in the microsomal membrane makes them likely candidates for such 'proximal' enzymes.

MCPPs are different from the proteinase described by Tamanoue et al. (15), which cleaves short peptides on the carboxy side of the Arg-Arg doublet. The enzymes have the same source, molecular mass, pH optimum, and sensitivity to inhibitors, but differ in their affinity to DEAE-Sepharose. One might suggest that they are all members of the same family of microsomal membrane-bound serine proteinases. MCPPs are also similar to a 31-kDa serine proteinase partially purified by Shen and coworkers from bovine adrenal chromaffin granule membranes (19). Unlike the proteinase of Tamanoue et al. (15), this enzyme cleaves on the carboxy side of the Lys-Lys doublet. The cleavage site and substrate specificities of MCPP1 and MCPP2 as well as the distinct roles played by the multiple forms of the proteinase in the endoplasmic reticulum of various tissues remain to be established. Proteins other than cytochromes P450 can also prove to be substrates for the proteinases, and the purified enzymes might thus be part of a versatile intracellular protein quality control system.

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