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PROTEINASE INVOLVEMENT IN THE SOLUBILIZATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE

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SUMMARY

This paper demonstrates that a heavy particle fraction, which contains lysosomes, is required for the solubilization of HMG-CoA reductase from rat liver microsomes by the widely-used slow freeze-thaw procedure. This solubilization is effectively inhibited by the proteinase inhibitors, leupeptin and antipain, but not by phenylmethylsulfonyl fluoride, pepstatin A or N-α-p-tosyl-L-lysine methyl ester. These results suggest that a thiol proteinase, possibly derived from lysosomes, is responsible for solubilizing the reductase.

The slow freeze-thaw procedure (1) for solubilizing HMG-CoA reductase from liver microsomes has been used by several investigators (2-5) and has made it possible to purify the enzyme to homogeneity. The basis for this solubilization is not understood. The possibility that proteinases might be responsible was considered previously (6); but was dismissed when it was shown that inclusion of phenylmethylsulfonyl fluoride in the homogenizing and extracting buffers did not alter the degree of solubilization or the properties of the enzyme (6). However, not all proteinases are inhibited by PMSF.

We noted that the degree of solubilization of reductase by the freeze-thaw procedure varied and appeared to depend upon the extent of heavy particle contamination. Thus, we have investigated the possibility that proteinases present in this fraction might be involved in solubilizing the reductase by this procedure.

MATERIALS AND METHODS

Materials - phenylmethylsulfonyl fluoride, N-α-p-tosyl-L-lysine methyl ester, pepstatin A, leupeptin, and antipain were purchased from Sigma Chemical Co.

The abbreviations used are HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLME, N-α-p-tosyl-L-lysine methyl ester; PMSF, phenylmethylsulfonyl fluoride.

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The PESK buffer used consists of 40 mM potassium phosphate, 30 mM EDTA, 100 mM sucrose, 50 mM KCl and 1 mM dithiothreitol at pH 7.2.

Methods - Male Sprague-Dawley rats weighing 125 to 150 g, purchased from Harlan Industries, Madison, WI, were housed in a light-controlled room with alternating periods of 10 hours of darkness and 14 hours of light. The rats received Wayne Lab Blox with or without 2% cholestyramine ad libitum. Rats were killed by guillotining at 4 hours into the dark period (diurnal high for reductase activity). The livers were rapidly removed and minced with scissors in cold PESK buffer. The mince was then homogenized with 4 volumes of fresh cold PESK buffer using three passes of a motor-driven Potter-Elvehjem Teflon glass homogenizer.

Reductase activity and protein concentrations were measured as previously described (5).

RESULTS

The degree of solubilization of reductase by the slow freeze-thaw procedure (1) is quite variable and in order to obtain 80 to 90% solubilization the procedure must be repeated 4 to 5 times (5). In this procedure, liver homogenates are centrifuged at 10,000 x g for 15 min prior to sedimenting the microsomes. Under such conditions, one would expect some contamination of the microsomes with lysosomes. To determine whether heavy particle (lysosomal) contamination affected reductase solubilization, microsomes with minimal contamination were prepared. As shown in Table I, less than 10% of the reductase present could be solubilized from these microsomes. However, when the heavy particle fraction was added to these microsomes, complete solubilization was obtained (Table 1).

To determine whether proteinases might be responsible for this enhanced solubilization, the solubilization of HMG-CoA reductase from microsomes containing the heavy particle fraction was carried out in the presence of various proteinase inhibitors. As shown in Table II, antipain and leupeptin, inhibitors of sulfhydryl proteinases, were quite effective in preventing the heavy particle-mediated solubilization of reductase. This effect was not due to inhibition of reductase activity as the reductase not solubilized was still present in the extracted microsomes. Furthermore, direct addition of these proteinase inhibitors to reaction mixtures did not inhibit the activity of solubilized reductase. In contrast, the serine proteinase inhibitors, phenylmethylsulfonyl

Table I

INVOLVEMENT OF THE HEAVY PARTICLE FRACTION IN THE SOLUBILIZATION OF HMG-CoA REDUCTASE

Treatment	Reductase Activity	
	Extract	Extracted Microsomes
	(nmol · min^{-1} · g^{-1})	
- heavy particles	0.89 ± 0.12	7.66 ± 0.06
+ heavy particles	9.98 ± 0.11	0.13 ± 0.02

Liver homogenate from rats fed a normal diet was centrifuged at 8,800 x g for 10 min. The supernatant was centrifuged twice at 16,000 x g for 15 min. The resulting pellets were used as the heavy particle fraction. The supernatant was then centrifuged at 144,000 x g for 45 min. to sediment the microsomes. These microsomes were then washed twice. The washed microsmal pellets and the heavy particle fraction were stored at -20° overnight. The microsomes were thawed and each pellet obtained from 1.5 g of liver was homogenized in 4 ml of cold PESK buffer with or without the heavy particle fractions derived from 1.5 g of liver. These homogenates were then centrifuged at 144,000 x g for 45 min. The resulting pellets were refrozen and reextracted. The combined extracts, the extracted microsomes and the original microsomes were assayed for reductase activity. Reductase activity is expressed in terms of nmol of mevalonate formed per min per g of liver. Means ± standard deviations are presented. Reductase activity in the original microsomes was 9.56 ± 0.44.

fluoride and N-α-p-tosyl-L-lysine methyl ester, and the carboxyl proteinase inhibitor, pepstatin A, did not significantly affect the degree of solubilization. Although the data presented in Table II was obtained with liver microsomes from cholestyramine fed rats similar results were obtained with microsomes from animals fed the normal diet.

DISCUSSION

From the data presented, the solubilization of rat liver microsomal HMG-CoA reductase by the slow freeze-thaw procedure (1) appears to be due to the action of a proteinase, possibly a lysosomal proteinase. This proteinase shares several properties with the recently described cathepsin T (7,8) which catalyzes the conversion of 52,000 M_r subunits of tyrosine aminotransferase to 48,000 M_r subunits. Cathepsin T is inhibited by leupeptin and antipain but

Table II

EFFECT OF PROTEINASE INHIBITORS ON THE SOLUBILIZATION OF HMG-CoA REDUCTASE

Additions to Extraction Buffer	Reductase Activity		
	Extract	Extracted Microsomes	Percent Extracted
	(nmol · min^{-1} · g^{-1})		
none	33.0 ± 0.8	9.4 ± 0.6	77.8
1% ethanol	35.1 ± 0.8	2.7 ± 0.1	92.9
100 μM antipain	2.3 ± 0.1	39.8 ± 0.2	5.5
10 μM antipain	5.0 ± 0.2	42.9 ± 1.9	10.4
100 μM leupeptin	1.9 ± 0.1	40.3 ± 2.5	4.5
10 μM leupeptin	3.4 ± 0.1	43.0 ± 0.1	7.5
10 μM pepstatin A	38.2 ± 1.3	2.7 ± 0.2	93.4
400 μM TLME	43.1 ± 1.8	4.2 ± 0.1	91.1
1 mM PMSF	24.0 ± 0.8	3.8 ± 0.2	86.3

Liver homogenate from cholestyramine-fed rats was centrifuged at 8,800 x g for 10 min. The supernatant was centrifuged at 144,000 x g for 45 min. The resulting microsomal pellets were then washed twice in PESK buffer. In the second wash, the PESK buffer was supplemented with the various proteinase inhibitors. Pepstatin A and PMSF were dissolved in ethanol and then added to PESK. The final ethanol concentration was 1%. The washed microsomal pellets were stored at -20° overnight. The pellets (each from 1.5 g of liver) were then thawed and homogenized with 4 ml of cold PESK extraction buffer with the indicated additions. The homogenate was centrifuged at 144,000 x g for 45 min. The resulting extracts and extracted microsomal pellets were assayed for reductase activity.

not by phenylmethylsulfonyl fluoride or pepstatin A (8); as is the solubilization of HMG-CoA reductase. Cathepsin T is activated by EDTA and by dithiothreitol (8) which are components of PESK buffer required for solubilization of the reductase. In contrast with many other lysosomal proteinases (9), cathepsin T has a neutral pH optimum (7) and thus would be expected to be active at the pH of PESK buffer which is 7.2. In addition cathepsin T is stabilized by glycerol (7), which has been found to enhance the solubilization of microsomal

reductase (10). Thus, the proteinase responsible for solubilizing HMG-CoA reductase appears to be similar to, or possibly identical with cathepsin T.

The present findings imply that HMG-CoA reductase solubilized by the freeze-thaw method may be a proteolytically modified form. Indeed, both we (5) and Edwards et al. (6) have noted two bands of reductase activity on polyacrylamide gels. Either one or both of these may be proteolytically modified forms. The question of whether the properties of the freeze-thaw solubilized reductase actually reflect those of the native enzyme naturally arises. In order to address this question, a proteinase independent method for solubilizing the reductase is needed.

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