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TOPOGRAPHY OF RAT LIVER MICROSOMAL 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE

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The orientation of 3-hydroxy-3-methylglutaryl coenzyme A reductase within the endoplasmic reticulum was investigated. Microsomal reductase activity was not latent, as addition of various detergents failed to activate the enzyme. Reductase activity was readily inhibited in intact microsomes by impermeable inhibitors such as trypsin, mercury dextran and anti-reductase IgG. Under the conditions used, these agents did not affect the intactness of microsomes as determined by latency of mannose-6-phosphate phosphohydrolase activity. The sensitivity to these inhibitors was not increased in disrupted microsomes. It is concluded that the domain containing the active site of the reductase is situated on the cytosolic surface of the endoplasmic reticulum.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is tightly bound to the hepatic endoplasmic reticulum. The orientation of the active site of the reductase within the membrane has not been established. Several lines of reasoning suggest a cytosolic orientation (1). One is that the enzymes responsible for the synthesis of HMG-CoA and for the phosphorylation of mevalonate are located in the cytosol. Thus, if the active site is situated on the cytosolic surface, a transport system would not be required. A second line of reasoning is based on the finding that anti-reductase IgG readily inhibits reductase activity in microsomes (2-8). However, the intactness of the microsome preparations used was not demonstrated and in some studies the microsomes were disrupted with detergents. Finally, it was generally held that the reductase is a loosely associated peripheral protein. This was based on its solubilization by mild

The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IgG, γ-immunoglobulins.

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freeze-thaw procedures (9,10). However, it is now known that this solubilization was due to contaminating lysosomal proteinases (11-13). The intact, native reductase can only be solubilized with non-ionic detergents (14-16). Thus, the existing evidence does not provide compelling support for the view that the active site of the reductase faces the cytosol.

MATERIALS AND METHODS

Materials - Mercury-dextran (average Mr 10,500) was a generous gift from Dr. W.L. Adair of this Department (17). Rabbit anti-reductase IgG used in this study was kindly provided by Dr. J.W. Porter (4). This antibody was raised against affinity purified reductase (50,000 Mr form). The IgG was purified from the sera by ammonium sulfate fractionation and DEAE cellulose chromatography (4). Colestid (colestipol hydrochloride) was furnished by Upjohn. Trypsin inhibitor and mannose 6-phosphate were purchased from Sigma Chemical Company.

Methods - Male Sprague-Dawley rats weighing 125 to 150g were purchased from Harlan Industries of Madison, Wisconsin. The rats were fed ground Wayne Lab Blox containing 2% Colestid for 4 to 10 days and were killed by decapitation at the diurnal high point of reductase activity (4 hours after the lights are turned off). Livers were rapidly removed, minced and homogenized in 10 volumes of cold 0.25M sucrose using ten stokes of the loose-fitting pestle in a Dounce Homogenizer. The homogenate was centrifuged at 16,000 g for 15 min. The supernatant solution was removed and centrifuged again at 16,000 g for 15 min. The upper three-quarters of this supernatant was carefully removed and centrifuged at 99,000 g for 60 min. The resulting pellets were resuspended in one-half the original volume of sucrose and centrifuged again at 99,000 g for 60 min. Microsomes prepared in this manner are essentially free of contaminating lysosomes based on immunoblotting studies which show a single band of Mr 105,000, lack of solubilization by the freeze-thaw procedure and the low constant level of acid phosphatase activity. Intact microsomes were resuspended in 1 ml of 0.25 M sucrose per g of liver. Disrupted microsomes were prepared by resuspending washed microsomal pellets in 0.25 M sucrose containing 0.5% Triton X-100. These preparations were used fresh. The degree of intactness of microsomes was ascertained by measuring the latency of mannose 6-phosphate phosphohydrolase activity of glucose 6-phosphatase (18). Phosphate release was determined by a modification (19) of the method of Fiske and Subbarow (20). Using this assay, we found that undisrupted microsomes had very low activity which was increased 10 to 15-fold by the addition of deoxycholate. With Triton X-100 disrupted microsomes, the addition of deoxycholate did not further increase the activity. Thus it was concluded that the intact microsomes were 90% or more intact and that the disrupted microsomes were essentially completely disrupted. The effect of trypsin, mercury dextran and anti-reductase IgG on the intactness of microsomes was also determined by this method. It was found that even at the highest concentrations used, these agents did not alter the intactness of the microsomes.

HMG-CoA reductase activity was measured as previously described using thin layer chromatography for the isolation of mevalonolactone (21).

Protein was assayed by a Biuret method (22) using bovine serum albumin as the standard.

RESULTS

Impermeable inhibitors used in conjunction with an assessment of microsomal integrity have become valuable tools for the investigation of the topography of proteins located in the endoplasmic reticulum (23). In this study, trypsin, mercury-dextran and anti-HMG-CoA reductase IgG were utilized as impermeable inhibitors of microsomal reductase. In this approach, it is critically important to verify the intactness of the isolated microsomes. This was done by measuring mannose 6-phosphate phosphohydrolase activity ± deoxycholate as described under "Methods."

The effects of trypsin on reductase activity in intact and disrupted microsomes is illustrated in Fig. 1. It is readily apparent that reductase activity is quite sensitive to trypsin and this sensitivity is not enhanced by disruption of the microsomes. Actually, reductase

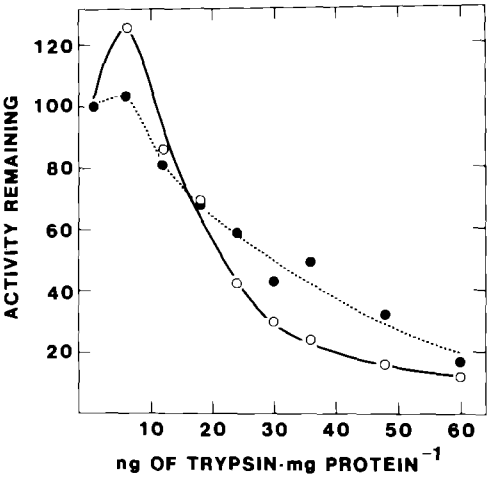

Fig. 1. Effect of trypsin on HMG-CoA Reductase Activity of Intact (o —— o) and Disrupted (●-----●) Microsomes. Microsomes, 0.4mg, were incubated in 150µl of 50mM Tris · HCl, pH 8.0 with varying amounts of trypsin at 37° for 20 min. The action of trypsin was terminated by adding 5µg of soybean trypsin inhibitor. Residual reductase activity was then determined. A solution, 100µl, containing 30µmol potassium phosphate, pH 7.1; 3µmol DTT; 60µmol potassium chloride; 1.2µmol glucose 6-phosphate, 0.3µmol NADP$^+$ and 0.5 units glucose 6-phosphate dehydrogenase was added. After 3 min, reactions were started by adding [^{14}C] HMG-CoA (specific activity 3500 dpm · nmol^{-1}) to a final concentration of 70µM. Reactions were terminated by adding 30µl of 2.4N HCl and the [^{14}C] mevalonolactone was isolated by thin layer chromatography. Reductase activity in untreated intact microsomes was 1.03 nmol·min^{-1}·mg^{-1}·mg^{-1}· and 0.78 nmol·mg^{-1} in disrupted microsomes incubated without trypsin.

activity in disrupted microsomes is less sensitive to trypsin. This may relate to the increased amount of protein available to trypsin in disrupted microsomes.

As shown in Fig. 1, a small increase in reductase activity was observed with low levels of trypsin. To determine whether this increase was real, we examined several trypsin concentrations in this range. It was found that concentrations ranging from 1 to 8ng per mg of protein all resulted in increased reductase activity, by as much as 50%. This increase in activity may be an inherent property of proteolytically modified reductase. Reductase solubilized by a procedure (24) now known to be dependent on proteinases (11) has previously been shown to have higher activity than the microsomal enzyme (24).

Since HMG-CoA reductase activity is readily inhibited by sulfhydryl agents (25), mercury-dextran was used as an impermeable inhibitor. As shown in Fig. 2, reductase was found to be extremely sensitive to mercury-dextran in both intact and disrupted microsomes. The incubations were carried out in the absence of DTT as addition of DTT after incubation with mercury-dextran was found to fully restore reductase activity.

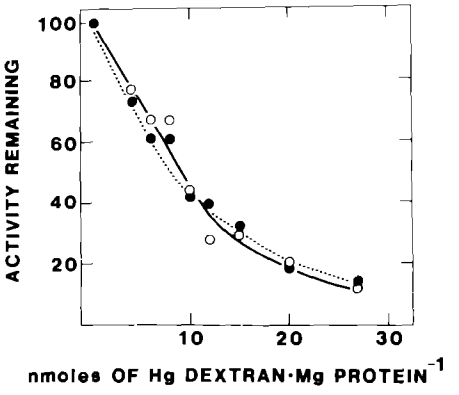

Fig. 2. Effect of Mercury-Dextran on HMG-CoA Reductase Activity of Intact (o —— o) and Disrupted (● -----●) Microsomes. Microsomes, 0.3mg, were incubated in 150µl of potassium phosphate buffer pH 7.1 with varying amounts of mercury-dextran at 37° for 20 min. Residual reductase activity was determined as described in Fig. 1 except that DTT was excluded. Reductase activity of untreated intact and disrupted microsomes was 0.29 and 0.28 nmol·min^{-1}·mg^{-1} respectively.

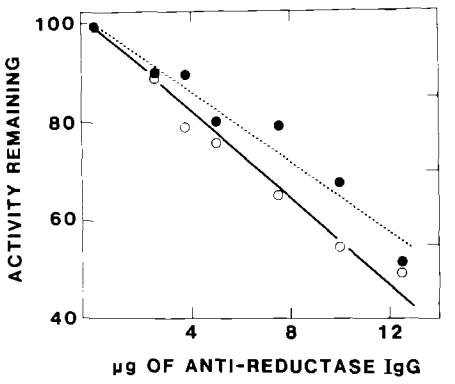

Fig. 3. Effect of Anti-Reductase IgG on HMG-CoA Reductase Activity of Intact (o —— o) and Disrupted (●-----●) Microsomes. Microsomes, 0.2mg, were incubated in 90µl of 0.15M sodium chloride with varying amount of antibody on ice for 5 min. Residual reductase activity was then determined as described in Fig. 1. The incubation was carried out at 37° for 10 min. Reductase activity in intact and disrupted microsomes incubated without antibody was 5.7 and 3.6 nmol·min^{-1}·mg^{-1} respectively.

The most specific impermeable inhibitor of HMG-CoA reductase is anti-reductase IgG. In Fig. 3, it can be seen that reductase activity is inhibited by the antibody in intact microsomes. The sensitivity to anti-reductase IgG was not increased in detergent disrupted microsomes.

DISCUSSION

These studies with impermeable inhibitors demonstrate that the catalytic site of HMG-CoA reductase or a domain essential for activity is located on the cytoplasmic surface of the endoplasmic reticulum. Additional support for this view is provided by the fact that microsomal reductase activity does not exhibit latency. Rather the activity was inhibited to varying extents by all detergents tested. These included: Triton X-100, Nonidet P-40, octyl glucoside, CHAPS (3-[(3-cholamidopropyl) dimethylammoniol-1-propane-sulfonate) and deoxycholate. Inhibition was observed at all levels of detergents used; thus ruling out the possibility of a biphasic response. When taken together, these observations provide a strong argument for a cytoplasmic surface location for the catalytic site.

Location of the active site of the reductase on the cytosolic surface of the endoplasmic reticulum fits well with the cytosolic location of the enzymes which synthesize HMG-CoA and those which further metabolize mevalonate. However, this location does not provide an explanation for the export of mevalonate from the hepatocyte. Since plasma mevalonate levels undergo a diurnal variation just as does hepatic reductase activity (12), it seems reasonable that mevalonate may be exported from liver and then taken up by various tissues including liver for synthesis of cholesterol, dolichol, ubiquinone, isopentenyladenine and heme A. Perhaps a separate protein or protein domain may be responsible.

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