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Characteristics of rat liver microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase

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1. A procedure for the preparation of rat liver microsomal fractions essentially devoid of contaminating lysosomes is described. 2. When this preparation was examined by immunoblotting with a rabbit antiserum to rat 3-hydroxy-3-methylglutaryl-CoA reductase, a single band corresponding to an M_r of 100000 was observed. 3. No evidence was found for glycosylation of rat liver-3-hydroxy-3-methylglutaryl-CoA reductase. 4. Native rat liver microsomal 3-hydroxy-3-methylglutaryl-CoA reductase differs from the purified proteolytically modified species in that it displays allosteric kinetics towards NADPH.

INTRODUCTION

Microsomal HMG-CoA reductase is now known to be quite sensitive to proteinases (Ness *et al.*, 1981; Phillips & Ness, 1984). This was first realized by Ness *et al.* (1981), who showed that the solubilization of hepatic microsomal HMG-CoA reductase by the usual freeze–thaw procedure was due to the action of contaminating lysosomal proteinases. Addition of leupeptin, an inhibitor of cysteine proteinases, prevented this solubilization. In an attempt to determine the native subunit M_r of the reductase, Chin *et al.* (1982*a*) solubilized reductase from a line of CHO (Chinese-hamster ovary) cells designated UT-1 by the use of Zwittergent 3–14 in the presence of leupeptin and examined such preparations by immunoblotting. A value of 62000 was observed instead of the usual 50000–55000 (Rogers *et al.*, 1983). Subsequently, Chin *et al.* (1982*b*) found that, when EGTA was included together with leupeptin in the solubilization buffer, a single band at an M_r of about 90000 was observed. This is in reasonable agreement with the value of 97092 obtained from the amino acid sequence (Chin *et al.*, 1984), and indicates that the combination of EGTA and leupeptin effectively prevents proteolysis of reductase in these cells.

Unfortunately, addition of EGTA and leupeption does not completely prevent proteolysis of rat liver microsomal HMG-CoA reductase. As shown by Liscum *et al.* (1983), substantial amounts of reductase with an M_r of 52000–58000 in addition to material with an M_r of 90000 is present in rat liver microsomal fractions (referred to below simply as 'microsomes') isolated under these conditions. This indicated that considerable proteolysis had occurred. In order to define the properties and regulation of native rat liver HMG-CoA reductase, it is necessary to prepare microsomes with intact enzyme. In the present paper we describe and document such preparations and examine various characteristics of the intact enzyme from rat liver.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats weighing 125–150 g were purchased from Harlan Industries (Madison, WI, U.S.A.). Colestid (Colestipol hydrochloride) was furnished by Upjohn Co. (Kalamazoo, MI, U.S.A.). Mevinolin was kindly provided by A. W. Alberts of Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). Horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibodies and peroxidase colour-development reagent were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). M_r standards (30000–200000), β-glycerophosphate, leupeptin and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Prestained high-M_r standards were purchased from Bethesda Research Laboratories (Bethesda, MD, U.S.A.). Endoglycosidase H was purchased from New England Nuclear (Boston, MA, U.S.A.).

Isolation of microsomal and lysosomal fractions

The rats were housed in a reverse-cycle light-controlled room with the lights on for 14 h and off for 10 h. The animals were fed on ground Purina rodent laboratory chow 5001 containing 2% Colestid either with or without 0.04% Mevinolin. Animals were given Colestid for 5 days and Mevinolin for 2 days. Colestid sequesters bile acids and thereby diminishes the feedback regulation of HMG-CoA reductase by cholesterol. Mevinolin is a potent inhibitor of HMG-CoA reductase and thus results in decreases of all metabolites produced from mevalonate. In combination these drugs result in a marked 50-fold increase in HMG-CoA reductase. Rats were killed by decapitation 5 h after the dark period commenced. For the preparation of lysosome-free microsomes, livers were quickly removed, placed in cold 0.25 M-sucrose, minced finely with scissors, weighed and then homogenized in 10 vol. of 0.25 M-sucrose with the use of the loose-fitting pestle in a Dounce homogenizer. The homogenate was

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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then centrifuged at 16000 $g_{max.}$ for 15 min in an SS-34 rotor at 4 °C. The supernatant was removed and centrifuged again at 16000 $g_{max.}$ for 15 min. The upper two-thirds of this supernatant was carefully removed by using a syringe fitted with a length of Tygon tubing. This step was found to be most critical. This supernatant was centrifuged at 100000 $g_{max.}$ for 1 h to pellet the microsomes. The pellet was resuspended in one-half the original volume of 0.25 M-sucrose and centrifuged again to yield a washed microsomal pellet.

Microsomes were also prepared in accordance with the method of Heller & Gould (1973) with PESK buffer (40 mM-potassium phosphate, 30 mM-EDTA, 100 mM-sucrose, 50 mM-KCl and 1 mM-dithiothreitol at pH 7.2). Microsomes prepared by this method are contaminated with lysosomes, which leads to solubilization of the reductase. Where indicated, 50 μM-leupeptin and/or 5 mM-EGTA, pH 7.2, were added to the microsomal-isolation media.

Lysosomes were isolated by the procedure described by Fleischer & Kervina (1974). Rats were injected with 75 mg of Triton WR-1339/100 g body wt. 3 days before being used. The lysosomal fraction was separated from contaminating mitochondria and peroxisomes on a linear sucrose gradient (density 1.15–1.27 g/ml).

Antisera

Proteolytically modified (subunit M_r 50000) HMG-CoA reductase was purified to homogeneity as previously described (Ness *et al.*, 1979). Approx. 500 μg of this enzyme was concentrated to a volume of 400 μl in 50 mM-potassium phosphate buffer, pH 7.1, emulsified with 1 ml of Freund's complete adjuvant and injected into the toe pads of a 3-month-old male New Zealand White rabbit. Then 2 weeks later, 100 μg of purified enzyme in a volume of 400 μl was mixed with 1 ml of Freund's incomplete adjuvant and injected subcutaneously in several sites along the back of the rabbit. At 2 weeks later and at weekly intervals thereafter blood was collected from an ear vein, and IgG was prepared (Palacios *et al.*, 1972). The IgG was stored at −20 °C. Non-immune IgG was prepared in an identical manner. Ouchterlony double-diffusion analysis of this serum revealed a single preciptin line when diffused against reductase obtained from all stages in the purification procedure, indicating that the antiserum specifically recognizes HMG-CoA reductase.

Electrophoresis and immunoblotting

Electrophoresis in 7.5% (w/v) polyacrylamide slab gels containing 0.1% sodium dodecyl sulphate was conducted at 5 °C with a constant current of 30 mA/gel (Laemmli, 1970). Portions of microsomal suspensions in 100 μl of sample buffer (30 mM-Tris/HCl, pH 6.8, containing 1% sodium dodecyl sulphate, 5% 2-mercaptoethanol, 0.1 M-sucrose and 0.0005% Bromophenol Blue) with 8 M-urea were heated in a boiling-water bath for 3 min before application to the slab gel. A mixture of M_r standards was also routinely applied to the gel. These included: myosin, 205000; β-galactosidase, 116000; phosphorylase *b*, 97400; bovine albumin, 66000; ovalbumin, 45000; carbonic anhydrase, 29000. In some cases prestained M_r standards were used instead. These included: myosin, 205000; phosphorylase *b*, 97400; bovine albumin, 66000; ovalbumin, 45000; chymotrypsinogen, 26000; β-lactoglobulin, 18000; cytochrome *c*, 12000.

Proteins were electrophoretically transferred from the slab gels to nitrocellulose paper in 25 mM-Tris/192 mM-glycine/20% (v/v) methanol, pH 8.3. The paper was then incubated at room temperature for 30 min in 3% (w/v) gelatin containing 0.5 M-NaCl and 29 mM-Tris/HCl buffer, pH 7.5, with gentle agitation on a rocker platform. This was followed by a 2 h incubation with anti-(HMG-CoA reductase) IgG (30 μg/ml) and three washes as described in the Bio-Rad Immun-Blot (GAR-HRP) assay kit instructions. The paper was then incubated with horseradish-peroxidase-conjugated goat anti-(rabbit IgG antibodies), followed by washing and a 20 min incubation with peroxidase colour-development reagent (4-chloro-1-naphthol).

Digestion with endoglycosidase H

A 50 μg portion of microsomal protein was incubated with 0.135 μg of endoglycosidase H in 50 μl of 100 mM-sodium citrate buffer, pH 5.5, containing 1 mM-L-7-amino-1-chloro-3-tosylamidoheptan-2-one ('TLCK'), 5 mM-EGTA and 50 μM-leupeptin for 1.5 h at 37 °C. Under these conditions, digestion of the protein standards led to the expected decrease in the apparent M_r of ovalbumin. Where indicated, reaction mixtures also contained 0.1% sodium dodecyl sulphate.

Enzyme assays

Acid phosphatase activity was determined by measuring the release of inorganic phosphate from β-glycerophosphate (Trouet, 1974). In order to assay this activity in microsomes isolated in PESK buffer, extensive dialysis against 0.25 M-sucrose was carried out and a zero-time blank containing the same amount of this dialysed microsomal preparation was included. A similar blank for microsomes isolated in sucrose was also used. HMG-CoA reductase activity and protein were determined as previously described (Ness *et al.*, 1979).

RESULTS AND DISCUSSION

Preparation of 'lysosome-free' microsomes

Perhaps the major obstacle to the characterization of native rat liver HMG-CoA reductase is the presence of small quantities of lysosome-derived proteinases in microsomes prepared by the methods currently used. This is perhaps most clearly illustrated by the experiment depicted in Fig. 1. Addition of small amounts of lysosomes to microsomal suspensions (1–6 μg/mg) followed by freezing and thawing resulted in solubilization. Further increases in the ratio of lysosomal to microsomal protein resulted in inactivation. Thus differing degrees of lysosomal contamination could account for the reported differences in the percentage solubilization and yield of reductase obtained with the standard freeze–thaw extraction procedure (Ness & Heller, 1983).

We found that breakage of liver cells in 0.25 M-sucrose in a Dounce homogenizer followed by repeated centrifugation at 16000 $g_{max.}$ before the microsomes were pelleted could yield preparations essentially devoid of lysosomes. To confirm that such preparations are substantially free of contaminating lysosomes, three criteria were employed. First, the activity of acid phosphatase, a standard marker enzyme for lysosomes,

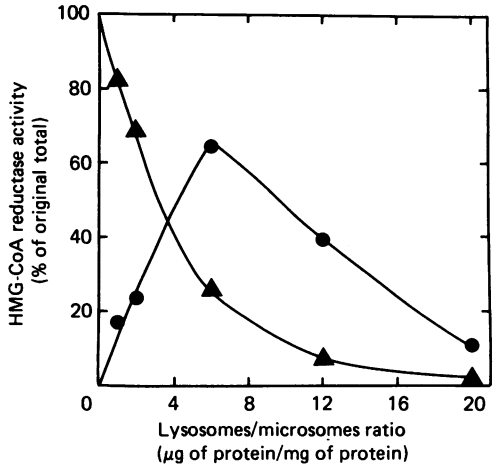

Fig. 1. Solubilization of microsomal HMG-CoA reductase by lysosomes

The indicated amounts of lysosomal protein were added to microsomal suspensions, frozen overnight, thawed, homogenized and centrifuged at 100000 $g_{max.}$ for 60 min. HMG-CoA reductase activity was then determined in the microsomal pellet (▲) and the extract (●).

was determined in various fractions. Secondly, since it is known that the freeze–thaw-mediated solubilization of microsomal HMG-CoA reductase is dependent on lysosomal proteinases (Ness *et al.*, 1981); the percentage of reductase solubilized by a single freeze–thaw buffer-extraction cycle was determined. Thirdly, microsomes were examined by immunoblotting for proteolysis products. As shown in Table 1, substantial differences between microsomes isolated in 0.25 M-sucrose and those isolated in PESK buffer were noted. After two centrifugations at 16000 $g_{max.}$, the degree of solubilization of HMG-CoA reductase from microsomes isolated in sucrose was decreased to undetectable levels and the specific activity of acid phosphatase reached a constant value. In contrast, microsomes isolated in PESK buffer by the method of Heller & Gould (1973) showed a high degree of solubilization and had somewhat higher acid phosphatase activity. The remaining acid phosphatase activity found in microsomes isolated in sucrose is probably due to microsomal phosphatases, as significant amounts have been reported previously (Trouet, 1974). Thus measurement of acid phosphatase activity is not a particularly sensitive method for detecting lysosomal contamination. When examined by immunoblotting, HMG-CoA reductase present in microsomes isolated in sucrose migrated as a single sharp band, with no evidence of proteolysis (Fig. 2, lane 4). Microsomes isolated in PESK buffer (Fig. 2, lane 2) did show some proteolysis products, i.e. faint bands at M_r about 70000 and less. These faint bands were markedly increased when the microsomes were frozen and thawed before analysis (Fig. 3). Inclusion of leupeptin and EGTA in the PESK buffer prevented proteolysis (Fig. 2, lane 1). When 'lysosome-free' microsomes were prepared, addition of these proteinase inhibitors was not necessary.

Subunit M_r and glycosylation

From Fig. 2 it appears that the subunit M_r of intact rat liver microsomal HMG-CoA is somewhat greater

Table 1. Effects of procedure used for isolation of microsomes on lysosomal contamination

For experimental details see the text. Heavy particles refer to the pellet obtained after the first 16000 $g_{max.}$ centrifugation step. A 10 min spin at 800 $g_{max.}$ preceded this. 'Sucrose' microsomes 1, 2 and 3 refer to the number of 16000 $g_{max.}$-centrifugation steps used before the microsomes were pelleted. All values are presented as means ± S.D. for data from four rats.

Fraction	Acid phosphatase activity (nmol/min per mg)	HMG-CoA reductase solubilized (% of total)
Homogenate	8.9±0.7	—
Heavy particles	16.9±0.8	—
'Sucrose' microsomes 1	4.5±0.2	2.3±0.4
'Sucrose' microsomes 2	4.8±0.7	< 0.5
'Sucrose' microsomes 3	4.6±0.9	< 0.5
'PESK buffer' microsomes	6.2±0.5	30.4±5.2

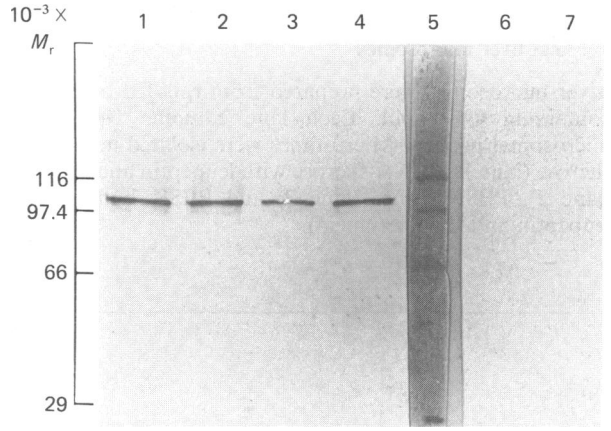

Fig. 2. Effect of isolation procedure on immunoblotting of rat liver microsomal HMG-CoA reductase

Liver microsomes were prepared from rats fed on diet containing Colestipol and Mevinolin. Microsomal protein was solubilized in sample buffer containing 8 M-urea and separated by electrophoresis on a sodium dodecyl sulphate/7.5%-polyacrylamide slab gel. Microsomes were isolated in PESK buffer with (lane 1) and without leupeptin and EGTA (lane 2) or in 0.25 M-sucrose with (lane 3) and without leupeptin and EGTA (lane 4). M_r standards are shown in lane 5. Blotting of 'PESK buffer' microsomes and 'sucrose' microsomes with non-immune IgG is shown in lanes 6 and 7. Samples were diluted so that 50 μg of microsomal protein was applied to each lane.

than that of phosphorylase *b*, which, on the basis of its amino acid sequence, has an M_r of 97400 (Koide *et al.*, 1978). To assess the subunit M_r more accurately, immunoblots of fixed-percentage sodium dodecyl sulphate/polyacrylamide gels, with the use of prestained M_r standards, were performed (Fig. 4). When the relative mobility of the reductase subunit in such gels was plotted against the logarithm of the M_r, a value of 100000±3000 was obtained. This value is slightly larger than the 90000

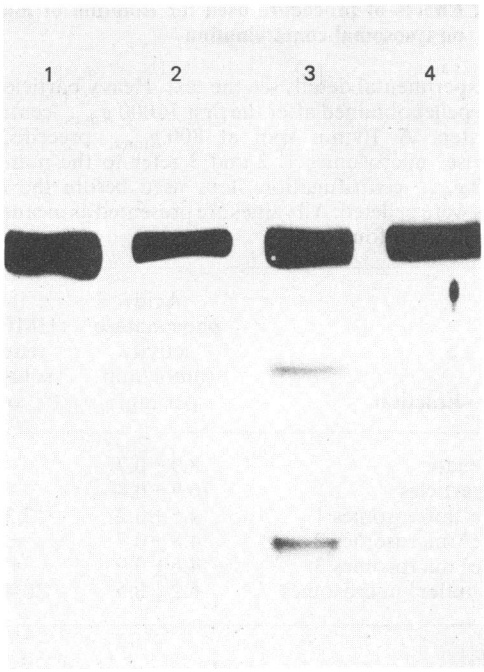

Fig. 3. Immunoblotting of HMG-CoA reductase in frozen–thawed rat liver microsomes

Liver microsomes were prepared from rats fed on a diet containing Colestipol. Each lane contains 100 μg of microsomal protein. Microsomes were isolated in 0.25 M-sucrose (lane 1), 0.25 M-sucrose with leupeptin and EGTA (lane 2), PESK buffer (lane 3) and PESK buffer with leupeptin and EGTA (lane 4).

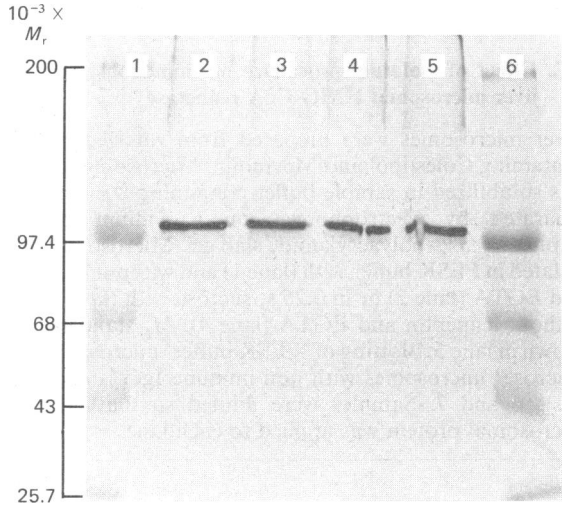

Fig. 4. Endoglycosidase H digestion of HMG-CoA reductase in the presence of proteinase inhibitors

Lanes 1 and 6 contain prestained protein standards. Samples containing 50 μg of microsomal protein with 50 μM-leupeptin, 5 mM-EGTA and 1 mM-L-amino-1-chloro-3-tosylamidoheptan-2-one incubated without (lanes 3 and 5) or with (lane 4) endoglycosidase H were applied to the gel. Lane 2 contains samples of digested and undigested microsomes. M_r values are indicated.

reported by others (Chin *et al.*, 1982*b*; Hardeman *et al.*, 1983; Edwards *et al.*, 1983; Clarke *et al.*, 1983; Liscum *et al.*, 1983) for the rat liver enzyme. It also exceeds the 97092 based on the amino acid sequence for HMG-CoA reductase from UT-1 cells (Chin *et al.*, 1984).

It is well established that HMG-CoA reductase from UT-1 cells is glycosylated (Liscum *et al.*, 1983). With rat liver it has only been reported that the enzyme binds to concanavalin A–Sepharose and can be released by α-methyl D-mannoside. This approach depends on the native reductase actually being solubilized from the microsomes by Zwittergent 3-14. We elected to investigate the question whether rat liver HMG-CoA reductase is glycosylated by using the endoglycosidase H digestion approach. Endoglycosidase H was chosen because it effectively releases high-mannose oligosaccaride chains, the type present on HMG-CoA reductase from UT-1 cells, from various glycoproteins. As shown in Fig. 4, endoglycosidase H digestion had no effect on the M_r of the reductase. The possibility that the microsomal reductase might not be accessible to endoglycosidase H was addressed by carrying out the digestion in the presence of sodium dodecyl sulphate. Again no change in M_r was observed. From these experiments it does not appear that rat liver HMG-CoA reductase contains high-mannose oligosaccharide chains.

The fact that rat liver HMG-CoA reductase differs somewhat from UT-1-cell HMG-CoA reductase should not be viewed as surprising. UT-1 cells are a line of tumour cells selected for their resistance to compactin, a potent inhibitor of HMG-CoA reductase. These cells have undergone gene amplification (Luskey *et al.*, 1983). Typically, tumour cells express different isoenzymes from those of differentiated tissues. Interestingly, the transcription initiation sites of the HMG-CoA reductase gene used by UT-1 cells differ from those used in hamster liver (Reynolds *et al.*, 1984). The use of different initiation sites would give rise to different primary RNA transcripts, which may well be processed differently, giving rise to distinct mature mRNAs and hence generate different proteins. The different fast-myosin light chains are produced by such a mechanism (Periasamy *et al.*, 1984).

Catalytic properties

The apparent K_m values of native microsomal HMG-CoA reductase were determined. The apparent K_m for HMG-CoA was found to be 5 μM. This is significantly higher than the value previously determined for the purified proteolytically modified enzyme, which ranged from 0.5 to 2.6 μM (Rogers *et al.*, 1983) and is only slightly lower than the reported concentration of HMG-CoA in rat liver (Knappe, 1974). Although the relative concentrations of HMG-CoA in the cytosol and mitochondria are not known, it is conceivable that changes in the cytosolic concentration could exert a regulatory effect on flux through the HMG-CoA reductase reaction. Changes in cytosolic HMG-CoA concentration might occur when the activities of the enzymes preceding HMG-CoA reductase in the cholesterol-biosynthetic pathway are increased. Indeed, it has been demonstrated that these enzymes are co-ordinately induced with HMG-CoA reductase (Chang & Limanek, 1980). The possibility that changes in cytosolic HMG-CoA concentration may contribute to regulation of hepatic HMG-CoA reductase has also been suggested by the studies performed by Gibbons *et al.* (1984). Determination of cytosolic

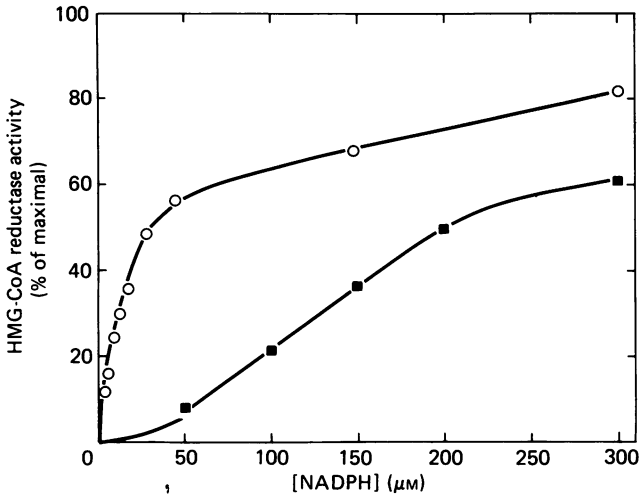

Fig. 5. Effect of NADPH concentration on the activity of the purified proteolytically modified form of HMG-CoA reductase and the native microsomal enzyme

Proteolytically modified HMG-CoA reductase was purified to homogeneity as described by Ness *et al.* (1979) (○). Microsomes containing intact HMG-CoA reductase were isolated, in 0.25 M-sucrose, from rats fed on normal chow (■). HMG-CoA reductase activity was assayed in 100 mM-potassium phosphate buffer, pH 7.1, containing 5 mM-glutathione. The NADPH concentrations were maintained by using a regenerating system of 4 mM-glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase and the indicated concentrations of NADPH.

HMG-CoA concentrations should be helpful in assessing the relative importance of this potential mode of regulating HMG-CoA reductase activity.

Perhaps the most striking difference between proteolytically modified and native HMG-CoA reductase is that seen when NADPH was the substrate whose concentration was varied (Fig. 5). The proteolytically modified enzyme, whether purified to homogeneity or in the crude extract, exhibited hyperbolic kinetics with an apparent K_m of 38 ± 11 μM. In contrast, the native intact microsomal enzyme displayed marked sigmoidal kinetics (Fig. 5) with an $[S]_{0.5}$ value (substrate concentration required to achieve 50% of apparent maximal velocity) for NADPH of 234 ± 74 μM. Hill plots of the data in Fig. 5 yield Hill coefficients of 1 for the proteolytically modified enzyme and about 2 for the native microsomal enzyme. Sigmoidal kinetics for microsomal HMG-CoA reductase with respect to NADPH concentration have also been reported by Roitelman & Schechter (1984). These investigators found that addition of glutathione to assay mixtures resulted in normal hyperbolic kinetics. We have found that addition of glutathione increases the Hill coefficient to 2, whereas in the absence of glutathione the Hill coefficient was typically 1.3. Preincubation of microsomes with NADPH in the absence of HMG-CoA and the use of a buffer other than phosphate, the natural intracellular buffer, seem to us to be most probably responsible for this discrepancy.

It is of interest to consider the implications of these sigmoidal kinetics in the light of the intracellular concentration of NADPH in rat liver, which is about 500 μM (Passonneau & Lowry, 1974). Since the $[S]_{0.5}$ for microsomal HMG-CoA reductase from rats fed on normal chow is about 230 μM, changes in the NADPH concentration could affect the flux through the reductase reaction. Similarly, if the $[S]_{0.5}$ value should change in response to dietary or hormonal manipulations and the NADPH concentration remain unchanged, this would also provide a means of regulation. Our recent studies suggest that this later possibility seems to be operative (Ness *et al.*, 1985).

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