Activation of Rat Liver Microsomal 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase by NADPH

EFFECTS OF DIETARY TREATMENTS

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The sigmoidal curves observed for rat liver microsomal 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase with NADPH as the varied substrate were markedly affected by feeding the animals diets containing colestipol, mevinolin and colestipol or cholesterol. Feeding of mevinolin and colestipol decreased the S0.5 for NADPH from 270 to 40 μM, while cholesterol feeding increased the value to 1.3 mM. Immuno blotting analysis revealed that the M, 100,000 form of HMG-CoA reductase predominated in cases where the S0.5 value was lowest, and the M, 200,000 species was the major form where the S0.5 values were highest. Activation of HMG-CoA reductase by NADPH was not due to conversion of the M, 200,000 form to the 100,000 form.

It has been demonstrated that rat liver microsomal HMG-CoA reductase displays sigmoidal kinetics when NADPH is the varied substrate (1). At low glutathione concentrations, less than 4 mM, a Hill coefficient of 2 was observed. This value approached 1 as the glutathione concentration was increased to 25 mM. It appears that NADPH exerted this effect by allosteric interactions rather than by donating electrons, as various structural analogues of NADPH could substitute. The minimal structure required was that of 5'-AMP (2). Using radiation inactivation and immunoblotting techniques it was recently demonstrated that microsomal reductase exists in media containing 10 mM dithiothreitol.

The objectives of this study were to determine whether the observed NADPH-dependent allosteric kinetics of HMG-CoA reductase are affected by various dietary treatments which are known to markedly affect reductase activity, and whether an interconversion of the M, 100,000 and 200,000 forms might contribute to the allosteric kinetics.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats weighing 125-150 g were purchased from Harlan Industries of Madison, WI. The rats were housed under a 14-h light, 10-h dark cycle and were fed ad libitum Purina Rodent Laboratory Chow 5001, ground lab chow with 0.04% mevinolin and 2% colestipol, ground lab chow with colestipol only, or ground lab chow with 2% cholesterol, for 2 days. Colestipol is a bile sequestrant which acts to decrease the cholesterol feedback regulation of reductase by increasing conversion to bile acids, leading to a 3-5 fold increase in activity. Mevinolin is a potent inhibitor of HMG-CoA reductase which acts to decrease effectively all metabolites derived from mevalonate, leading to a large compensatory increase in reductase protein. Given together, these drugs act synergistically to decrease reductase activity 30-50-fold. All rats except the diurnal low group were killed at the 4th h of the dark period (diurnal high). The diurnal low group was killed at the 4th h of the light period. Livers were quickly removed, and lysosome-free microsomes were prepared in 0.25 M sucrose as previously described (3).

HMG-CoA Reductase Assay—Activity was determined in microsomes containing intact reductase as a function of NADPH concentration using an NADPH generating system. Reaction mixtures contained potassium phosphate, pH 7.1, 100 mM; potassium chloride, 200 mM; glucose 6-phosphate, 30 mM; glucose-6-phosphate dehydrogenase, 1 unit; 60 μM R,S-[14C]HMG-CoA (specific activity 4400 cpm-nmol-1); and varying concentrations of NADPH ranging from 10 μM to 20 mM. The mixtures were preincubated at 37 °C for 10 min in order to generate the NADPH. The reactions were started by the addition of microsomes. The final volume of the reaction mixture was 300 μl. In some experiments (Table II), the microsomes were preincubated for 10 min, and the reactions were started by the addition of [14C]HMG-CoA. Incubation times ranged from 5 to 30 min. Reactions were terminated by the addition of 30 μl of 2.4 N HCl. Incubation was continued for another 20 min to ensure lactonization of the [14C] mevalonate. The reactions were centrifuged to remove denatured protein. Then 100-μl aliquots were applied to 2.5-cm wide lanes on 0.75-mm thick Silica Gel G plates, and the mevalonolactone was separated using a solvent system of acetonitrile-water-glacial acetic acid (1:1:1). The area containing the mevalonolactone (RF = 0.7) was scraped from the plate into a glass mini-counting vial. Four ml of ACS scintillation counting fluid was added. The amount of radioactivity present was determined by liquid scintillation counting. The nanomole of mevalonate formed was determined from the specific activity of the substrate determined under identical counting conditions and by correction for recovery using parallel reactions containing [3H]mevalonate. Protein concentrations of the microsomal suspensions were determined by a biuret method (4).

Data were analyzed by V versus S and Hill plots (log V/Vmax - V versus log S). Maximum velocities were obtained from V versus S plots employing sufficiently high concentrations of NADPH. Linear regression analysis was used to construct the best fit line to the data and thus determine the Hill coefficient (nH) and the S0.5 for NADPH.

Immunoblotting—The physical size of reductase present in microsomes was determined by immunoblotting analysis in the absence of β-mercaptoethanol as previously described (5).

RESULTS AND DISCUSSION

The effects of varying NADPH concentration on hepatic microsomal HMG-CoA reductase activity is shown in Fig. 1. Marked differences in the degree of sigmoidicity as a function of dietary treatment were noted. Microsomes with higher reductase activity required lower concentrations of NADPH to achieve maximal activity and exhibited less sigmoidicity. To define more precisely the changes in the sigmoidal curves caused by the various dietary factors, data were analyzed by Hill plots (Fig. 2). Table I presents a summary of these data. The S0.5 for NADPH progressively increases as the specific activity decreases. These differences in S0.5 values

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1 The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; S0.5, substrate concentration required to achieve 50% of maximal velocity.

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in the assay. Thus assays with microsomes from colestipol-treated 5 mM glutathione and were started by the addition of microsomes from colestipol-fed activity.

It is of interest that the reported concentration of NADPH was not reflective of the observed Hill coefficient. Although the concentration of NADPH does not appear to change in response to fasting or feeding (5), NADPH could still affect the rate of the reductase reaction in vivo owing to large changes in the S_{0.5} values (Table I).

In contrast with the effects on S_{0.5}, the Hill coefficient, n_H, was not affected as much by dietary manipulations. In most cases the n_H was somewhat greater than 1 but always less than 2, indicating a degree of positive cooperativity. An exception was seen in microsomes from cholesterol-fed animals, where n_H was consistently less than 1, suggesting negative cooperativity. Inclusion of glutathione in the assay always resulted in an increase in the Hill coefficient (Tables I and II).

When microsomes were preincubated with NADPH substantial differences in the sigmoidal curves were noted (Table II). A 4–6-fold increase in the S_{0.5} values was seen. Inclusion of glutathione up to 25 mM reduced these values to near those seen without preincubation. This also resulted in Hill coefficients of 3 and greater with microsomes containing lower levels of reductase, i.e. diurnal high, diurnal low, and cholesterol-fed. Since, intracellularly, the reductase is exposed to both HMG-CoA and NADPH, it is felt that these values are not reflective of the in vivo situation. This experiment may,
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FIG. 3. Immunoblotting of hepatic microsomal HMG-CoA reductase. Each lane contained 100 μg of microsomal protein from 48-h-fasted (F48); 24-h-fasted (F24); diurnal high (DH); colestipol-fed (C); and mevinolin and colestipol-fed (MC) rats. The positions of prestained protein standards are indicated. Electrophoresis was conducted in the absence of β-mercaptoethanol.

FIG. 4. Effect of NADPH concentration on immunoblots of HMG-CoA reductase from diurnal high rats. Reactions with the indicated millimolar concentrations of NADPH, 5 mM glutathione, and 60 μM R,S-HMG-CoA were initiated by the addition of microsomes. Five min after starting the reactions, 100-μg aliquots were removed and mixed with 2 × Laemmli sample buffer. Electrophoresis was conducted in the absence of β-mercaptoethanol.

However, suggest a possible role for HMG-CoA in protecting the reductase from NADPH-mediated inactivation.

Comparing the results of Table II with those in Table I may provide some insight into certain reported discrepancies between changes in reductase activity and those in cholesterologenesis (6, 7). Often the observed change in reductase activity exceeded that of acetate or water incorporation into cholesterol. This could be due to NADPH-mediated inhibition in microsomes with lower levels of reductase. Preincubation of reductase with NADPH has become standard practice largely as a result of the observation that the proteolytically modified form of reductase is cold-labile and that NADPH could protect the enzyme (8). Intact native microsomal reductase is not cold-labile (9).

To determine whether the observed differences in allosteric properties of microsomal reductase might relate to the portion of enzyme present in the M, 100,000 and 200,000 forms, immunoblots in the absence of β-mercaptoethanol were performed (Fig. 3). The portion of enzyme present in the M, 100,000 form increases as the S_{0.5} for NADPH decreases. In Fig. 3 equal amounts of microsomal protein were applied. Thus the lane containing protein from rats fed mevinolin and colestipol is overloaded with respect to reductase immunoreactive enzyme. When less protein was applied, it was apparent that more than 80% of the reductase was present in the M, 100,000 species. It is striking that immunoreactive protein is not decreased even after 48 h of fasting when reductase activity was undetectable.

The possibility that the allosteric activation of reductase by NADPH might result from conversion of the M, 200,000 form to the M, 100,000 form was examined by immunoblotting in the absence of β-mercaptoethanol (Fig. 4). Samples were taken from reaction mixtures containing varying concentrations of NADPH. There was no evidence for formation of the M, 100,000 form even at NADPH concentrations where maximal activity was obtained. These data suggest that both forms might be capable of enzyme activity but have different requirements for NADPH. This finding is also consistent with NADPH acting at an allosteric site rather than providing electrons to effect the reduction of disulfide linkages in the M, 200,000 species.

The present data suggest that dietary treatments profoundly affect the allosteric kinetics of HMG-CoA reductase, and that this appears to be due to changes in the ratio of the M, 200,000 species to the M, 100,000 species. How the interconversion of these two forms is accomplished in vivo in response to needs for mevalonate-derived products remains to be investigated.

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