Allosteric Activation of Rat Liver Microsomal 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase by Nicotinamide Adenine Dinucleotides*

Joseph Roitelman and Ishaiahu Shechter
From the Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

NADH and NAD⁺ are neither substrates nor inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in concentrations up to 1 mM. Addition of either NADH or NAD⁺ enhanced the activity of rat liver microsomal reductase, yet NADH failed to affect the activity of the freeze-thaw solubilized enzyme. The degree of enhancement of enzyme activity by NADH decreased as GSH concentration in the assay increased. Addition of 500 μM NADH to the assay converted the sigmoidal (Hill coefficient = 2.0) NADPH-dependent kinetic curve of the microsomal reductase into Michaelis-Menten kinetics (Hill coefficient = 1.1). Furthermore, the kinetic curves were shifted to the right, resulting in an up to 35% decrease in the concentration of NADH required to obtain half-maximal velocity (S₀.₅) in the presence of 500 μM NADH. Again, this effect of NADH was diminished as GSH concentrations increased. These results demonstrate that NAD(H) is an allosteric activator of HMG-CoA reductase. These results also indicate that HMG-CoA reductase has NAD(H) binding site(s) distinct from the catalytic NADPH site(s).

The activity of hydroxymethylglutaryl-CoA reductase (NADPH) (EC 1.1.1.34) determines the rate of synthesis of cholesterol and other isoprenoids in cells (1–3). The long-term regulation of HMG-CoA reductase activity is achieved by alterations of the cellular content of its mRNA and by changes in the rates of enzyme synthesis and degradation (4–8). The short-term regulation of HMG-CoA reductase activity in vivo is proposed to involve a cascade of phosphorylation/dephosphorylation processes (reviewed in Ref. 9).

There is a growing number of reports concerning the role that thiols and disulfides play in determining the activity of HMG-CoA reductase in vitro (10–14). Taken together with earlier reports from this laboratory (15, 16), it was proposed that the cellular thiol status of the cell (17) may serve as a predominant factor in the short-term regulation of HMG-CoA reductase activity and, hence, in cholesterol synthesis (15, 16).

We have recently shown that at low GSH concentrations (2–4 mM) rat liver microsomal HMG-CoA reductase displays sigmoidal NADPH-dependent kinetics with a Hill coefficient of 2. Upon increasing the concentration of the activating thiol, the NADPH-dependent kinetic curves gradually acquired the characteristics of normal Michaelis-Menten-type hyperbola (18). Thus, at 25 mM GSH we calculated a Hill coefficient of 1. These phenomena were not observed with the freeze-thaw solubilized reductase which is an active proteolytic fragment (Mᵣ = 50,000–55,000) of the intact, membrane-bound reductase (Mᵣ = 97,000) (19). This fragmented form of the enzyme displayed normal Michaelis-Menten-type NADPH kinetics, regardless of the GSH concentrations used for its activation. These data indicated that, depending on the thiol concentration, the activity of HMG-CoA reductase may be allosterically modulated. The purpose of the present work was to search for a low molecular weight substance that can specifically affect the allosteric site(s) of this enzyme and thus serve as a modulator of microsomal reductase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—GSH, NADPH, NAD(H), Glc-6-P, HMG-CoA, yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (200 units/mg of protein), leupeptin and MVA were obtained from Sigma. Radiolabeled pro-[3-¹⁴C]HMG-CoA (55.1 Ci/mol) was from New England Nuclear. All other chemicals were of reagent grade or better.

**Enzyme Preparations**—Microsomal fraction was obtained from livers of 200–250-g female rats (Wistar, Tel-Aviv) that were fed ad libitum with normal diet containing 10% (w/w) ground Amberlite XAD-2 resin for 2 weeks (20). Animals were killed 4 h after onset of the dark phase of a 12-h light cycle. Microsomes were prepared in 0.3 m sucrose, 30 μM leupeptin, 10 mM K⁺-Hepes, pH 7.5, solution and filtered through a Sepharose 4B column (3 × 48 cm) as described (18). Solubilized HMG-CoA reductase was prepared by the freeze-thawing procedure and was partially purified as previously described (18).

**Assay of HMG-CoA Reductase**—Reductase activity was assayed in Hepes buffer (160 mM K⁺-Hepes, 200 mM KCl, pH 7.9) in a total volume of 100 μl by a modification (18) of the procedure described by Shapiro et al. (21). [¹⁴C]MVA was measured as described (18).

Other Assays—Protein was measured according to Lowry et al. (22) on acid-precipitated protein. Concentrations of pyridine nucleotides were measured spectrophotometrically at 259 and 339 nm, using molar extinction coefficient (εᵥ) of 16,900 and 6,200, respectively, for NADPH and 16,900 and 6,220, respectively, for NADH. Concentrations of NAD⁺ were determined at 259 nm using εᵥ of 17,800 (23). GSH was determined by the Ellman procedure (24).

**Data Analysis**—Vmax was determined from double reciprocal plots of rate versus the first and second powers of NADPH concentrations. Hill coefficients and S₀.₅ values were calculated from Hill plots (log V/Vmax – o) versus log [NADPH]. Significance of the differences between constants was calculated by Student's t-test procedure.

**RESULTS**

The effect of pyridine nucleotides which are not substrates for the reaction was investigated. Fig. 1 shows the effect of increasing concentrations of NADH on the activity of both microsomal and freeze-thaw solubilized HMG-CoA reductase.
Modulation of HMG-CoA Reductase Activity by NAD(H)

FIG. 1. The effect of NADH on the activity of HMG-CoA reductase. Microsomal (circles) or solubilized (squares) HMG-CoA reductase was preincubated in Hepes buffer with NADPH-generating system (7.5 mM Glc-6-P, 0.25 units of Glc-6-P dehydrogenase), 4 mM GSH, and either NAD' or NADH to the indicated concentrations with (open symbols) or without (closed symbols) NADPH. Preincubation was about its concentration equal to its NADPH-generating system (7.5 mM Glc-6-P, 0.25 units of Glc-6-P dehydrogenase), 4.5 mM GSH, and either NAD' (open symbols) or NADH (closed symbols) to the specified concentrations, in a volume of 90 µl. Reactions were initiated by adding 10 µl of [14C]HMG-CoA and were terminated after 30 min.

FIG. 2. The effect of NAD' and NADH on the activity of microsomal HMG-CoA reductase. Column-filtered microsomes, suspended in Hepes buffer, were preincubated with 60 µM NADPH, NADPH-generating system (7.5 mM Glc-6-P, 0.25 units of Glc-6-P dehydrogenase), 4.5 mM GSH, and either NAD' (open symbols) or NADH (closed symbols) to the specified concentrations, in a volume of 90 µl. After 35 min at 37 °C, reactions were initiated by adding 10 µl of [14C]HMG-CoA and ran for 35 min at 37 °C.

These experiments were performed under assay conditions (Hepes buffer and low GSH) in which the microsomal reductase displays sigmoidal NADPH-dependent kinetics (18). Microsomal HMG-CoA reductase was activated with 4 mM GSH and the NADPH concentration that was used for assaying its activity was about its S0.5 value (60 µM) (18). In the assay of the solubilized enzyme we used 35 µM NADPH which is the concentration equal to its Km at 4 mM GSH (18). It is clearly seen that upon increasing the NADH concentration up to 250 µM, the activity of the microsomal reductase is drastically enhanced whereas that of the solubilized enzyme remains unaffected. Furthermore, for both enzyme species, NADH is neither a substrate nor does it inhibit the enzymatic activity at concentrations up to 1 mM.

In the experiments depicted in Fig. 2 we compared the effects of reduced and oxidized forms of nicotinamide adenine dinucleotide on the activity of microsomal HMG-CoA reductase. Clearly, both NADH and NAD' enhance the activity of the enzyme to a similar extent, within the same range of concentrations.

The relative degree of enhancement of microsomal reductase activity by NADH is dependent on the GSH concentration, as demonstrated in Fig. 3. The -fold increase in enzyme activity by saturating concentrations of NADH and the NADH concentrations necessary to produce 50% of maximal enhancement (NADH0.5) are summarized in Table I. As shown, both the -fold increase in enzyme activity and the NADH0.5 values decrease with the increase of GSH concentration, whereas the maximal activity increases (Fig. 3).

The effect of NADH on the NADPH-dependent kinetics of microsomal HMG-CoA reductase is shown in Fig. 4. Addition of NADH, to the reaction mixture containing 4.5 mM GSH, gradually converted the sigmoidal NADPH-dependent kinetics towards normal hyperbolic curves (Panel A). This is clearly demonstrated by the linearization of the double reciprocal plots (Panel B) and is further substantiated by the decrease in the Hill coefficient for NADPH from 2.0 in the absence of NADH to 1.1 in the presence of 500 µM NADH (Table II).

Furthermore, the kinetic curves are shifted to the left, resulting in the decreased NADH0.5 values, when the GSH concentration is raised from 3.6 to 8.3-fold PM.

**TABLE I**
The effect of NADH on the activity of microsomal HMG-CoA reductase at various concentrations of GSH

<table>
<thead>
<tr>
<th>GSH (mM)</th>
<th>Increase*</th>
<th>NADH0.5 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>8.3</td>
<td>65</td>
</tr>
<tr>
<td>9.0</td>
<td>3.6</td>
<td>65</td>
</tr>
<tr>
<td>27.0</td>
<td>1.6</td>
<td>50</td>
</tr>
</tbody>
</table>

* The maximal enhancement of enzyme activity by NADH at each concentration of GSH.

**NADH0.5** is the concentration of NADH which promotes 50% of maximal enhancement of enzyme activity.
ing in an up to 35% decrease in the \( S_{0.5} \) value for NADPH in the presence of 500 \( \mu M \) NADH. Again, at high GSH concentrations the drastic effect of NADH on HMG-CoA reductase kinetic pattern is diminished. Thus, at 27 mM GSH, the enzyme kinetics observed are of Michaelis-Menten type (Fig. 4) and the double reciprocal plots are almost completely linear. Under these conditions the Hill coefficient observed is near unity, regardless of the amount of the NADH present. It should also be noted that while variations in the GSH concentrations resulted in a drastic change of the \( V_{\text{max}} \) of the reaction, NADH has no effect on it (Table II).

DISCUSSION

In mammalian cells, the conversion of HMG-CoA to mevalonate is catalyzed by the microsomal enzyme HMG-CoA reductase. This reaction utilizes NADPH exclusively as the reductant (1, 25) and requires thiols as essential cofactors (15, 16, 18). The data presented in this communication demonstrate that both NADH and NAD\(^+\), which are not substrates for the reaction, similarly enhance the activity of microsomal HMG-CoA reductase. This enhancement of activity cannot be attributed to a direct effect on the enzyme catalytic site(s), since such an effect of NADH would have been equally expressed in the solubilized reductase. Any possible NADPH contamination in the NADH preparation is also ruled out, since in the absence of added NADPH to the reaction, no enzyme activity is observed (Figs. 1 and 3). Thus, it appears that NAD(H) affects microsomal HMG-CoA reductase activity by interacting with the enzyme at a distinct, noncatalytic, regulatory site(s).

Our recent evidence for sigmoidal kinetics of HMG-CoA reductase with respect to NADPH (18) suggested that the enzyme binds the substrate NADPH to its catalytic sites in a cooperative fashion. Alternatively, NADPH, although being a substrate, may also interact with noncatalytic, regulatory site(s) on the enzyme molecule. During the studies described here, NADPH concentrations were kept relatively constant by the Glc-6-P/Glc-6-P dehydrogenase NADPH-regenerating system (18). This cannot be stated about NADH since NADH-regenerating system was not employed in our experiments. Therefore, it is possible that during the experiments a modification, most likely oxidation, of NADH may have occurred. However, even if such oxidation of NADH to NAD\(^+\) has occurred, it would not have significant effect on the observed results since the enzyme is activated by both NADH and NAD\(^+\) to a similar extent (Fig. 2).

Based on these observations, we propose that HMG-CoA reductase possesses regulatory binding site(s) for pyridine nucleotide(s). Whether these nucleotides bind to common regulatory site(s) still remains to be established.

The characteristics of the NADPH-dependent kinetics change from sigmoidal curves to normal Michaelis-Menten-type hyperbolas as GSH concentrations increase (see Fig. 4 and Ref. 18). Furthermore, the relative enhancement of HMG-CoA reductase activity by NADH and its concentrations required to achieve this enhancement both diminish as the GSH concentrations increase (see Table I). These data indicate that the interactions, either between the catalytic sites and/or between the catalytic and the regulatory site(s), may require the existence of disulfide bridge(s). These protein disulfides are gradually reduced by thiols, resulting in an enzyme which no longer binds NADPH cooperatively and thus loses its characteristic sigmoidal kinetics. This state of the enzyme is also characterized by higher catalytic rates (18).

---

**Table II**

The effect of NADH on the NADPH-dependent kinetics of microsomal HMG-CoA reductase

<table>
<thead>
<tr>
<th>GSH (mM)</th>
<th>NADH (( \mu M ))</th>
<th>( V_{\text{max}} ) (pmol/mg/min)</th>
<th>( S_{0.5} ) (( \mu M ))</th>
<th>Hill coefficient ( n_H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>0</td>
<td>4.5 ± 0.2*</td>
<td>92</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>100</td>
<td>100</td>
<td>2.0</td>
</tr>
<tr>
<td>27.0</td>
<td>75</td>
<td>8.4 ± 0.3*</td>
<td>78</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>65</td>
<td>65</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*Numbers represent standard deviation of the mean, \( n = 6 \), \( p < 0.001 \) was calculated for the significance in the difference in \( V_{\text{max}} \).

---

**Fig. 4.** The effect of NADH on the NADPH-dependent kinetics of microsomal HMG-CoA reductase. Column-filtered microsomes, suspended in Hapes buffer, were preincubated with NADPH generating system (7.5 mM Glc-6-P, 0.25 units of Glc-6-P dehydrogenase), 4.5 mM (closed symbols) or 27.0 mM (open symbols) of GSH, with 75.0 \( \mu M \) (triangles), 500 \( \mu M \) (squares) or without (circles) NADH. Preincubation was in a total volume of 80 \( \mu l \) for 40 min at 37 °C. Reactions were initiated by the simultaneous addition of both substrates in 20 \( \mu l \). Reactions ran for 40 min at 37 °C and then terminated. For clarity, the NADPH kinetic curves in the presence of 75 \( \mu M \) NADH are omitted for panel A.
Modulation of HMG-CoA Reductase Activity by NAD(H)

Since it has been established that the freeze-thaw solubilization procedure results in a lower molecular weight enzyme due to proteolytic processes (4–7, 19, 26, 27), it is possible that the active proteolytic fragment or corelated the freeze-thaw solubilized HMG-CoA reductase is devoid of the regulatory pyridine-nucleotide(s) binding site(s). Alternatively, the freeze-thaw solubilization may have destroyed the region(s) in the enzyme through which the allosteric transitions are mediated. In either situation this would result in the abolishment of the NADH-enhancing effect (Fig. 1) and, as was previously demonstrated, in normal Michaelis-Menten-type NADPH-dependent kinetics (18).

At saturating concentrations, NADH caused a decrease in the $S_{25}$ value for NADPH and changed the S-shaped NADPH-dependent kinetic curve to classical Michaelian hyperbola (Fig. 4 and Table II). These variations of the kinetics in the presence of a modulator are typical to a “K-enzyme” (28). Thus, HMG-CoA reductase should be classified as such.

The results presented here demonstrate that NADH and NAD$^+$ are allosteric effectors of HMG-CoA reductase. Whether other structurally related physiological compounds may serve the same function is presently under investigation. The present report does not attempt to point out a possible physiological role of either NADH or NAD$^+$ in the regulation of reductase activity. Furthermore, the finding that both NADH and NAD$^+$ exert similar effects (Fig. 2) suggests that the ratio NADH/(NADH + NAD$^+$) may not necessarily be a predominant factor in the regulation of HMG-CoA reductase. Rather, this study merely indicates the existence of one or more regulatory site(s) on the enzyme. Nevertheless, we do not overlook the possibility that the putative allosteric interactions may play a significant role in the short-term regulation of the activity of HMG-CoA reductase.

REFERENCES