Isolation and Purification of a Rat Liver 3-Hydroxy-3-methylglutaryl-Coenzyme Reductase Activating Protein (RAP)*

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Iris Dotan and Ishaiahu Shechter†
From the Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

A protein with an estimated subunit mass of 19 kDa was isolated and purified from perfused rat liver cytosol. This protein activates hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase (NADPH) (EC 1.1.1.34), the rate-limiting enzyme in the cholesterol biosynthetic pathway. The activation process by this HMG-CoA reductase activating protein (RAP) is time-dependent and requires NADPH. Maximal activity of HMG-CoA reductase induced by RAP is comparable to that obtained in the presence of thiols, such as GSH, and can exceed 100-fold the activity obtained when thiols are omitted. Purified RAP lacks ability to reduce 5,5′-dithiobis-(2-nitrobenzoic acid).

RAP was purified to homogeneity utilizing DEAE- and phenyl-Sepharose CL-4B column chromatography. The purified RAP migrates as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and shows multiple interconvertible aggregational forms on native polyacrylamide gel electrophoresis.

A monospecific antibody against RAP was prepared by immunization of hens and extracted from either their egg yolks or serum.

The catalytic activity of RAP might be responsible for the physiological activation of HMG-CoA reductase and regulation of its activity.

Hydroxymethylglutaryl-coenzyme A reductase (EC 1.1.1.34) is the rate-determining enzyme in the cholesterol biosynthetic pathway in most mammalian cells (Rodwell et al., 1976). It is now well agreed upon that variations in the rate of sterol synthesis are mainly as outcome of changes in the rate of transcription of the HMG-CoA reductase gene (Chin et al., 1982; Clarke et al., 1983, 1984; Liscum et al., 1983). Changes in the rate of both synthesis and degradation of the HMG-CoA reductase protein are also regarded as major mechanisms of regulation (Faust et al., 1982; Edwards et al., 1983a, 1983b; Sinensky and Logel, 1983; Tanaka et al., 1983). Previous results from this laboratory and others have shown that, in order to express HMG-CoA reductase activity, a thiol such as GSH or dithiothreitol must be included in the assay (Kawachi and Rudney, 1970; Tormann and Scallen, 1981; Gilbert and Stewart, 1981; Dotan and Shechter, 1982, 1983).

The presence of thiol facilitates binding of the substrate HMG-CoA to the enzyme, as studied by the aid of affinity resins (Dotan and Shechter, 1982).

Under cellular conditions, there still lies the question of how the activity of the nascent protein is expressed. We have formerly suggested that the cellular ratio of reduced to oxidized glutathione may play a role in the activation and deactivation process of hepatic HMG-CoA reductase (Dotan and Shechter, 1983). However, we have not ruled out the possibility that specific enzyme(s) might be involved in maintaining HMG-CoA reductase in its reduced state.

In this work, we present evidence indicating that the cytosol of rat liver contains an enzymatic activity which is capable of activating HMG-CoA reductase at least as efficiently as GSH. The protein associated with this activity was purified to homogeneity. The relationship between this protein, the thioredoxin system, thioltransferases, and carrier proteins is discussed.

EXPERIMENTAL PROCEDURES

Materials—The following items were purchased from Sigma: NADH, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, 4-chloro-1-naphthol, 5,5′-dithiobis-(2-nitrobenzoic acid), HMG-CoA, MVA, GSH, bovine serum albumin, SDS, polyethylene glycol (8000), Coomassie Brilliant Blue R and G, cholestyramine, Amido Blue R, and Ponceau S. Radiolabeled 2-[14C]HMG-CoA (RS) was from Amersham Corp. Phenyl-Sepharose CL-4B and Sephadex G-75 were from Pharmacia Biotechnology, Inc., and DE52 from Whatman. Mevinolin was a gift of Dr. Alberts from Merck Sharp and Dohme. Nitrocellulose paper was from Gelman Sciences, Inc., Anne Arbor, MI. Other chemicals and reagents were of analytical grade and purchased from local sources.

Protein—Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

PAGE—SDS-PAGE was performed according to Laemmli (1970) using 12.5% acrylamide in the separating gel. Thickness of gels was 0.75 mm and sample buffer contained 4% urea. Coomassie Brilliant Blue R was used for staining. Native PAGE was performed essentially as described by Davis (1964).

Thiol Content—Thiol content was determined with 5,5′-dithiobis-(2-nitrobenzoic acid), essentially as described by Ellman and Lysco (1967) except trichloracetic acid treatment of the protein was omitted. Protein concentration for such determinations did not exceed 0.5 mg/ml in order to prevent turbidity due to protein aggregation and polymerization. Determination of acid-soluble thiols liberated after activation of HMG-CoA reductase by RAP was performed by reacting supernatants of HCl-acidified reaction mixtures with 5,5′-dithiobis-(2-nitrobenzoic acid).

Preparation of Microsomal and Solubilized Thiol-deficient HMG-CoA Reductase—Thiol-deficient microsomal HMG-CoA reductase was prepared by filtration of microsomes on Sepharose 4B as previously described (Roelman and Shechter, 1984). Thiol-deficient solubilized HMG-CoA reductase was prepared from rats fed for 1 week with 5% cholestyramine and then for an additional 3 days with 5% cholestyramine and 0.15% mevinolin. Microsomes prepared from these rats were subjected to three cycles of freezing and thawing, ammonium sulfate precipitation between 30–60%, heat treatment at

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† To whom correspondence should be addressed.

‡ The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; PBS, phosphate-buffered saline; RAP, HMG-CoA reductase activating protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ETTA, [ethylenedioxythieleninertil]tetrasceic acid.
65 °C for 15 min, and dialysis against PBS. Aliquots of both microsomal and solubilized HMG-CoA reductase were frozen and stored in liquid N2.

Isolation and Purification of RAP—Five to ten Charles River rats were usually taken for each preparation. Rats were anesthetized and their livers were perfused sequentially with 150 ml/liver of ice-cold PBS containing 2.5 mM EGTA. The freshly ligated liver was then homogenized (25 ml/liver) in PBS containing 2.5 mM EGTA, 2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin. Differential centrifugation was carried out to yield a 105,000 g supernatant (S90) which was then fractionated with ammonium sulfate between 50-80%. The ammonium sulfate pellet was suspended and then extensively dialyzed against 5 mM KH2PO4, 0.5 mM EGTA, pH 8 (hypotonic buffer). The dialyzed protein was loaded onto a DE52 column at a ratio of 8 (hypotonic buffer after which RAP was eluted as bulk with 0.3 M KCl. The ratio of loaded material was otherwise stated. The column was chromatographed with the hypotonic buffer after which RAP was eluted as bulk with 0.3 M KCl. The protein obtained by the 0.3 M KCl desorption was made 20% saturated with ammonium sulfate and loaded onto a phenyl-Sepharose CL-4B column preequilibrated with 20% saturation of ammonium sulfate containing 20 mM KH2PO4 at pH 8. The ratio of loaded material was 0.5 mg of protein/ml of packed resin. The nonadsorbed effluent of the phenyl-Sepharose column was collected and dialyzed once more against the hypotonic buffer. Finally, the dialyzed protein was loaded onto a small DE52 column (3-5 ml) preequilibrated with the hypotonic buffer. A KCl gradient up to 0.3 M KCl eluted RAP as the first emerging protein. Precise location of RAP was determined by running SDS-PAGE and collecting the gradient.

Assay of RAP—RAP assay was performed by incubating RAP, HMG-CoA reductase, and an NADPH regenerating system (3 mM NADPH, 15 mg glucose 6-phosphate, 0.5 IU glucose 6-phosphate dehydrogenase) in a total volume of 0.08 ml at 37 °C in 0.1 mM phosphate buffer, pH 8, containing 2.5 mM EGTA. After a preincubation period, 0.02 ml of [14C]HMG-CoA (5,000-10,000 dpm/nmol, 2-12 nmol) were added and incubation proceeded. At the end of incubation, 0.01 ml of concentrated HCl and 0.01 ml of 0.5 M non-labeled carrier MVA were added. Further isolation and quantitation of the product by thin layer chromatography was similar to the procedure described by Shapiro et al. (1979). Preincubation and incubation periods are indicated specifically in the legends.

Measurement of NADPH-dependent 5,5'-Dithiobis-(2-nitrobenzoic acid)-reducing Activity—Reaction mixtures (1 ml) contained phosphate buffer, pH 8, 120 μM NADPH, 0.5 IU glucose 6-phosphate dehydrogenase, 1 mM glucose 6-phosphate, 0.25 mM 5,5'-dithiobis-(2-nitrobenzoic acid), and 50 μg of RAP preparation. Reducing activity was calculated from the increase in absorbance at 412 nm. Proper corrections were made for NADPH-independent reduction of 5,5'-dithiobis-(2-nitrobenzoic acid).

Preparation of Chick Anti-RAP Antibody—At weekly intervals, 30-70 cm of the eggs, RAP prepared in Freund's incomplete adjuvant were injected to the breast muscle of egg laying hens (Rhode Island Red × White Leghorn). Purification of antibody from yolks was carried out by fractionation with polyethylene glycol (Polson et al., 1980a, 1980b) except ammonium sulfate fractionation was omitted. Serum antibodies were prepared by bleeding the hens through the wing vein, clotting the blood, and separating the serum. Repeated freezing and thawing of either the yolk or serum did not affect the efficiency of its binding to the antigen.

Immunoblotting—A partially purified preparation of RAP obtained by dialysis of a 60-90% ammonium sulfate precipitate of S90 was subjected to SDS-PAGE. Transfer and immunoblotting were performed essentially as described by Towbin et al. (1979) with a few minor modifications. After SDS-PAGE, the gel was equilibrated with the transfer buffer (192 mM glycine, 25 mM Tris, pH 8.3) for 15 min. Transfer to nitrocellulose paper was performed at 90 V (constant voltage) for 60 min in a Bio-Rad Trans-blot cell. The nitrocellulose paper was then fixed in 3% trichloroacetic acid for 10 min. Occasionally, the dye Ponceau S was included in the trichloroacetic acid solution in order to visualize transferred proteins. For permanent staining of the transferred proteins, Amido Blue-Black was used, as described by Schaffner and Weissmann (1973). After transfer, processing of the nitrocellulose was carried out at 37 °C. Bleeding of nonspecific binding sites was performed for 20 min, using a PBS solution containing 2.5% (w/v) of bovine serum albumin and 2.5% (w/v) low fat dry milk. First antibody at an appropriate dilution from either egg yolks or hen sera was prepared. The nitrocellulose paper was overlayed on the nitrocellulose paper, and agitation gently for 60 min. Next, 5 x 3 min washes were performed with PBS. A second antibody composed of horseradish peroxidase-linked rabbit anti-chick (affinity purified) at a dilution of 1:1000 in the blocking solution was agitated with the nitrocellulose paper for another 60 min. At the end, 5 x 3 min washes were performed and color was then developed using the substrate 4-chloro-1-naphthol according to recommendations given by Bio-Rad. For immunoblotting of native gels, gels were first stained with Coomassie Brilliant Blue R prepared in 50% methanol, 7% acetic acid and then destained totally in 50% methanol. For an unknown reason, preliminary staining with Coomassie was a prerequisite for successful processing of the native gel with the antibodies. A solution of 50% methanol, 7% acetic acid devoid of Coomassie dye was unsatisfactory for this purpose. Following destaining, proteins were transferred to nitrocellulose paper at 60 V (constant voltage) for 60 min. Further processing of the nitrocellulose was performed as described above.

RESULTS

Activation of Microsomal and Solubilized HMG-CoA Reductase by RAP—When thiol-deficient rat liver microsomes are assayed for HMG-CoA reductase activity, either a low molecular weight thiol or a rat hepatic cytosol preparation containing RAP activity must be included in the reaction mixture for detection of enzyme activity. Omission of these components results in a very low basal rate of mevalonate production. As shown in Fig. 1, a concentration-dependent rise in activity of microsomal HMG-CoA reductase occurs in the presence of RAP. Maximal activity of the microsomal HMG-CoA reductase with RAP reaches approximately 70% of the maximal activity obtained by GSH. The enhancement of microsomal HMG-CoA reductase activity in the presence of RAP can exceed 100-fold. When solubilized HMG-CoA reductase is assayed, RAP exhibits an even steeper activation curve than GSH, reaching approximately the same maximal plateau of activation (see Fig. 2).

Time Kinetics of Activation by RAP—Fig. 3 shows that the time kinetics of activation of microsomal HMG-CoA reductase by RAP resembles that obtained by 5 mM GSH. A linear increase in activity occurs during the first 90 min of preincubation. When GSH is used as an activator, an increase in activation is also observed for the first 90 min. However, beyond that, an inactivation process of HMG-CoA reductase commences, resulting in lower rates of mevalonate production. The decline in activity of HMG-CoA reductase may be explained by spontaneous oxidation of GSH to GSGG, a potent inhibitor of the enzyme. This decline was never observed with RAP, even when preincubations were extended to 150 min. Both RAP and HMG-CoA reductase activities were stable after prolonged incubations at 37 °C.

![Fig. 1. Activation of microsomal HMG-CoA reductase by RAP.](image-url)
continued for dialysis, liberation of protein-bound thiols could in turn result in a nonenzymic activation of HMG-CoA reductase. Although the preparations were subjected to extensive reduction by RAP.

CoA reductase by RAP raised the possibility that the preparations used contained high concentrations of free thiols. The purified RAP from a dialyzed 60-90% ammonium sulfate fraction (0) nmol was added at the specified time intervals and the reaction was incubated with increasing concentration of either 100 pg NADH and NADPH for the HMG-CoA reduction of HMG-CoA reductase, resulting in an almost 3-fold rise in activity of the microsomal enzyme (compare assays 3 and 4). Such an increase in activity can not be demonstrated when NADPH is replaced by NADH during the preincubation (see assay 10). The apparent ineffectiveness of NADH to support the activation by RAP does not result from an inhibitory effect of NADH on HMG-CoA reductase. This is demonstrated by the simultaneous incubation of GSH and NADH which does not lead to a significant decrease in the reductase activity as compared to GSH alone (compare assays 6 and 9).

Unlike the requirement of NADPH for HMG-CoA reductase activation by RAP, the activation of HMG-CoA reductase by GSH is not affected by the presence of NADPH during the preincubation (see assays 7, 8, and 10). Therefore, the activation of HMG-CoA reductase by RAP requires NADPH exclusively.

**Relationship between HMG-CoA Reductase Activation by RAP and Protein Dephosphorylation.**—Since reversible inactivation of HMG-CoA reductase by the protein phosphorylation-dephosphorylation process was reported (Ingebritsen et al., 1981; Beg et al., 1984), we decided to study any possible connection between the newly discovered HMG-CoA reductase activating protein and the latter. Any relationship between the protein phosphorylation-dephosphorylation system of HMG-CoA reductase and activation by RAP was eliminated by the use of NaF as general phosphatase inhibitor. Fig. 4 shows that when microsomal HMG-CoA reductase is activated with RAP, the presence or absence of 50 mM NaF does not alter the extent of activation. Thus, even the active, dephosphorylated form of microsomal HMG-CoA reductase, in the absence of thiols, is dependent upon RAP for expression of its activity.

**Purification of RAP.**—Attempts to purify RAP by gel filtration chromatography were unsuccessful. Invariably, the active material was spread over many column fractions, regardless of the resin or eluting buffer used. This may be explained by the appearance of multiggregational forms of RAP on native polyacrylamide gels (see below Fig. 8). The sulfonated cation exchanger SP-Sephadex did not bind RAP at pH 5. However, the anion exchanger DEAE-cellulose adsorbed RAP only at very low salt buffers. Exhibiting such features, RAP was assumed to be a poorly surface-charged protein. Following this assumption, the hydrophobic resin phenyl-Sepharose was introduced. Again, RAP proved to be unique in that it did not adsorb to the resin. Whatever the reasons for this anomaly, these properties of RAP enabled us to devise a purification procedure leading to a final homogeneous preparation, as described under "Experimental Procedures."

Fig. 5 shows chromatography of RAP on a DEAE-cellulose (DE52) column. A gradient of up to 0.2 M KCl eluted RAP from the column at approximately 40 mm KCl. Chromatography of RAP on phenyl-Sepharose CL-4B is shown in Fig. 6. The protein loaded was eluted with 20% saturation of ammonium sulfate. Under these conditions over 90% of the proteins were adsorbed to the column. Two major proteins appeared in the nonadsorbed effluent. These were RAP and an additional protein with a subunit mass of approximately 24 kDa. These two proteins were later separated on a DEAE-cellulose column by use of an eluting salt gradient.

A sample taken from the various purification steps was subjected to SDS-PAGE as shown in Fig. 7. RAP migrates as a single band with an estimated subunit size of 19 kDa.

**Loss of 5,5′-Dithiobis-(2-nitrobenzoic Acid)-Reducing Activity during Purification of RAP.**—Oxidoreductases such as the...
Three µg of thiol-deficient microsomes were preincubated for 125 min at 37°C and then incubated for 55 additional min at 37°C. In the preincubation period the activity of HMG-CoA reductase was tested, whereas in the incubation period the activity of the enzyme was assayed. Tested compounds that were added just prior to the preincubation and the incubation periods are marked (+). Reactions of HMG-CoA reductase in the incubation period were started by adding [14C]HMG-CoA. The concentrations of the tested compounds were: 200 µg of partially purified RAP (dialyzed ammonium sulfate precipitation step), 3 mM NADPH, 15 mM glucose 6-phosphate, 0.5 IU glucose-6-phosphate dehydrogenase, and 5 mM GSH. HMG-CoA added at the start of the 55-min incubation was 60,000 dpm/6 nmol. Termination of the reaction and isolation of the product was done as described under “Experimental Procedures.”

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**FIG. 4. Activation of HMG-CoA reductase by RAP in de-phosphorylated microsomes.** Aliquots of thiol-deficient microsomes (3.5 µg) were preincubated for 60 min with an NADPH regenerating system and the following additions: 100 µg of partially purified RAP (dialyzed ammonium sulfate precipitation step), 100 µg of partially purified RAP + 50 mM NaCl, 100 µg of partially purified RAP + 50 mM NaF and an incubation with none of the above. After the preincubation period, the substrate [14C]HMG-CoA (60,000 dpm/6 nmol) was added and the reaction was continued for 30 min. Termination of the reaction and isolation of the product were followed as described under “Experimental Procedures.”

**TABLE I**

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<th>Requirement of NADPH for the activation of HMG-CoA reductase by RAP</th>
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**FIG. 5. Purification of RAP on DEAE-cellulose column.** Partially purified RAP (12 mg of dialyzed 60-90% ammonium sulfate fraction) was loaded onto a DEAE-cellulose (DE52) column (1 x 8 cm) presoildified with 5 mM KH2PO4, 0.5 mM EGTA, pH 8. The column was eluted with 30 ml of the equilibration buffer after which a gradient of KCl was applied. Protein (•) was determined by the method of Bradford. Activity of RAP (○) was determined by incubating 2 µl of each fraction with 1.5 µg of solubilized HMG-CoA reductase and an NADPH regenerating system for 30 min. Radioactive HMG-CoA (60,000 dpm/6 nmol) was then added and incubation proceeded for an additional 30 min. The reaction was terminated by HCl, and radiolabeled MVA was determined as described under “Experimental Procedures.”

**Aggregational Forms of RAP—**When a homogeneous, by the SDS-PAGE criterion, sample of RAP is subjected to electrophoresis on a native gel, three evenly spaced bands are elicted. As shown in Fig. 8, the relative intensities of the 3 bands, after staining with Coomassie Brilliant Blue R, decreases when passing from the slow migrating form to the faster one. When the slow migrating band is excised from the gel and re-electrophoresed, a similar pattern of the three aggregational states is observed (data not shown). The redistribution of the excised band indicates that the observed triplet indeed represents different aggregational states of the same entities.

As noted before, attempts to purify RAP on gel filtration columns were unsuccessful due to the appearance of activity throughout the fractions collected. Considering the observed migration of RAP on native gel as three (or more) bands, this

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**Note:** The text appears to be cut off or incomplete at the end. Further information might be required for a complete understanding.
FIG. 6. Purification of RAP on phenyl-Sepharose column. Partially purified RAP (2.5 mg of dialyzed 60-90% ammonium sulfate fraction) was made 20% with ammonium sulfate and loaded onto a phenyl-Sepharose CL-4B column (1 x 5 cm) preequilibrated with 20% ammonium sulfate in phosphate buffer, pH 8. The column was then eluted with phosphate buffer devoid of ammonium sulfate and finally with 50% ethylene glycol. Protein profile (○) and RAP activity (△) were determined as described in the legend to Fig. 5.

FIG. 7. SDS-PAGE of various purification steps of RAP. SDS-PAGE in 12.5% acrylamide and 0.75-mm thick gels was performed. After the electrophoresis, the proteins were stained with Coomassie Brilliant Blue R. Molecular weight markers are indicated in kilodaltons. The following samples of the purification steps (see "Experimental Procedures") were loaded onto the gel: lane 1, perfused rat liver homogenate; lane 2, SIo; lane 3, SIo; lane 4, 60-90% ammonium sulfate fraction; lane 5, first DEAE column; lane 6, phenyl-Sepharose column, and lane 7, second DEAE column.

broadening might be due to the elution of different aggregational forms of the protein. If so, the data indicates that RAP is active at various aggregational states. We are currently investigating which of the visualized aggregational forms is indeed active and whether the pattern observed in native gels has a structural meaning.

Preparation of a Monospecific Antibody against RAP—Purified RAP injected to egg laying hens gave rise to a monospecific anti-RAP antibody both in egg yolks and in serum. Antibody was detected after 4 weeks from the first injection by the enzyme-linked immunosorbet assay method. Later, both serum and yolk antibody were used in the Western immunoblotting technique as described under "Experimental Procedures." Fig. 9 shows that the antibody reacts specifically with RAP. No cross-reaction with other proteins is observed. The same antibody also reacts with all three aggregational forms of RAP observed on native polyacrylamide gels (see Fig. 10).

FIG. 8. Aggregational forms of RAP. Electrophoresis of homogeneous RAP (approximately 5 µg) was performed using 12.5% acrylamide native gel at a thickness of 0.75 mm. Gels were run for 12 h at 200 V and then stained with Coomassie Brilliant Blue R.

FIG. 9. Monospecific antibody raised against RAP. An egg laying hen (Rhode Island Red × White Leghorn) was immunized with homogeneous RAP emulsified with incomplete Freund's adjuvant. Breast intramuscular injections containing approximately 30-70 µg of RAP were performed at weekly intervals for the first 4 weeks. Monospecific antibodies were detected in the serum by the Western immunoblotting technique after 6 weeks. For this procedure, a dialyzed 60-90% ammonium sulfate preparation was used for the electrophoresis. The Amido Blue-Black staining pattern of the transferred proteins to the nitrocellulose is shown on the right and the immunoblot using a 1:500 dilution of the hen serum is shown on the left.

DISCUSSION

The works of Brown, Goldstein, and others have delineated the process of induction and repression of the HMG-CoA reductase protein (Luskey et al., 1983; Clarke et al., 1983; Gil et al., 1985). Increase and decrease in the amount of HMG-CoA reductase mRNA is, undoubtedly, the main mechanism by which the rate of cholesterol synthesis can be controlled. Nevertheless, many investigators have raised the possibility
that the post-translationally regulated protein is enzymatically regulated (Ingebritsen et al., 1981; Beg et al., 1984) or that translational control of the mRNA exists (Kirsten and Watson, 1974; Krone et al., 1979; Koizumi et al., 1982; Peffley and Sinensky, 1985). A refined regulating mechanism at the level of the enzyme was needed in order to explain discrepancies between the rate of cholesterol production (as measured by incorporation of a radiolabeled precursor) and the activity of HMG-CoA reductase (as assayed in vitro under optimal conditions). In this work we evoke the basic question of how HMG-CoA reductase is activated under cellular conditions. We, as well as others, have already established the necessity for a thiol-mediated reducing environment for the binding of HMG-CoA to the enzyme and expression of enzyme activity under in vitro conditions (Kawachi and Rudney, 1970; Tormanen and Scallen, 1981; Gilbert and Stewart, 1981; Dotan and Shechter, 1982, 1983). The immediate implication of such an observation is that there must be a cellular system capable of maintaining HMG-CoA reductase constantly in its reduced form. A natural candidate for this reduction would be GSH, the most abundant free thiol in mammalian cells (Kosower and Kosower, 1978). However, in numerous experiments designed to change glutathione levels by exogenous means, we were unable to establish a correlation between its levels in cultured cells and the rate of cholesterol synthesis (Dotan and Shechter, 1985). In fact, other enzymatic systems which can be modulated in vitro by the ratio GSH/GSSG have not been proved to undergo a similar in vivo modulation as well (Ziegler, 1985). Therefore, it stands to reason that an additional HMG-CoA reductase activating mechanism may exist, independently of GSH. In this process, as well as in the one involving GSH, protein disulfides are reduced to the corresponding thiols. There are two possible rat liver cytosolic candidates for the reduction of protein disulfides, aside from GSH. These are the thioredoxin system and the thioredoxin reductases. Thioredoxin reductase is known to reduce low molecular weight disulfides, such as 5,5′-dithiobis-(2-nitrobenzoic acid), in the presence of NADPH (Holmgren, 1977; Latham and Holmgren, 1982a, 1982b). This 5,5′-dithiobis-(2-nitrobenzoic acid)-reducing property excludes RAP from being related to this system. As noted under “Results,” the majority of the 5,5′-dithiobis-(2-nitrobenzoic acid)-reducing activity present in the crude fraction is lost at the first DEAE-cellulose purification step. The second group, the thioredoxin reductases, are known to require a low molecular weight thiol/disulfide cofactor (usually glutathione) for the reversible oxidation/reduction of protein thiol/disulfides (Mannervik and Eriksson, 1974; Carmichael et al., 1977; Axelsson et al., 1978). Since there is no such requirement by RAP for the activation of HMG-CoA reductase, it is highly unlikely that RAP is such an oxidoreductase. Therefore, we conclude that RAP differs from these two reducing systems and exhibits unique properties.

The extent of activation of HMG-CoA reductase by RAP seems to be as efficient as the activation induced by low molecular weight thiols. In fact, solubilized HMG-CoA reductase is activated effectively at very low concentrations of RAP, as demonstrated in Fig. 2. Contrary to thiols, RAP requires NADPH for the activation of HMG-CoA reductase. This requirement might be significant for the possible regulatory role of RAP in cholesterogenesis. Changes in the cellular concentration of RAP at various cholesterogenic states may affect HMG-CoA reductase activity to different extents. Such changes may reflect the overall flux through the cholesterol biosynthetic pathway. Alternatively, it is possible that the cellular concentration of NADPH rather than the concentration of RAP determines the extent of activation of HMG-CoA reductase. In this model, variations in the cellular level of NADPH will modulate HMG-CoA reductase activity two ways: (a) by inducing different degrees of activation of the enzyme and (b) by varying the reaction rate of the active enzyme. This model does not necessarily call for cellular variations of the concentration of RAP. We also do not rule out the possibility that the appearance of multigregational forms of RAP may play a regulatory role in the process of activation of HMG-CoA reductase, similar to the process observed for phosphofructokinase (Hesterberg and Lee, 1980, 1981, 1982).

Early reports made by Gaylor and collaborators (Gaylor and Delwich, 1976; Spence and Gaylor, 1977) showed the existence of a rat liver cytosolic protein capable of activating HMG-CoA reductase. The capacity of this protein to enhance HMG-CoA reductase activity was examined in the presence of 1 mM dithiothreitol. Since dithiothreitol itself converts HMG-CoA reductase to an active form, the nature of the activation effect reported is unclear. These investigators have purified the activating protein to homogeneity, reporting a subunit size of approximately 10,300 kDa. Based on this reported subunit size we assume that the protein isolated by Gaylor and collaborators is a different protein than RAP. In fact, in a later report, the same investigators discuss the identity of their purified protein with the already known noncatalytic Z-protein (Billheimer and Gaylor, 1980).

There are various steps in the cholesterol biosynthetic pathway which are enhanced in the presence of cytosolic factors such as carrier proteins (Dempsey et al., 1981; Chin and Bloch, 1984; Scallen et al., 1985). There seems to be no relationship between RAP and these proteins. Unlike the enzymes which involve carrier proteins for their action, HMG-CoA reductase utilizes soluble substrates and does not require the participation of carrier proteins for translocation of substrates in the membrane. Furthermore, none of the carrier proteins involved in cholesterogenesis have been assigned with a catalytically reductive nature. We, therefore, propose that the newly isolated protein either belongs to a new class of proteins capable of activating enzymes which require reduced protein thiols or is a specific activator of HMG-CoA reductase. With the aid of the monoclonal antibody prepared, we hope to further understand the nature and function of this novel protein.

REFERENCES

