PHOSPHORYLATION–DEPHOSPHORYLATION OF RAT LIVER 3-HYDROXY 3-METHYLGLUTARYL COENZYME A REDUCTASE ASSOCIATED WITH CHANGES IN ACTIVITY

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Received 14 December 1979

1. Introduction

3-Hydroxy 3-methylglutaryl coenzyme A reductase (mevalonate:$NADP^+$ oxidoreductase (CoA acylating) EC 1.1.1.34) the major regulatory enzyme of cholesterol biosynthesis, appears to be regulated by reversible modulation [1–6]. We reported the incorporation of ^{32}P into microsomal protein from [γ-^{32}P]ATP accompanied by inactivation of HMG-CoA reductase [7]. Similar studies have shown the incorporation of ^{32}P into solubilized rat liver HMG-CoA reductase precipitated by specific antibodies [8,9].

Here we demonstrate covalent binding of ^{32}P into the HMG-CoA reductase molecule upon incubation of microsomes with [γ-^{32}P]ATP and concomitant inactivation of the enzymatic activity. Furthermore, we have partially purified a phosphatase (HMG-CoA reductase phosphatase) which leads to the release of ^{32}P from ^{32}P-labelled HMG-CoA reductase accompanied by restoration of reductase activity. Thus, it appears now definitely established that HMG-CoA reductase can undergo a phosphorylation–dephosphorylation cycle, in vitro, as a possible mechanism of modulation of its activity, in vivo.

2. Material and methods

2.1. *Reagents*

DL-Hydroxy methyl [3-^{14}C]glutaryl CoA (spec. act. 3000 cpm/nmol) was prepared from hydroxymethyl [3-^{14}C]glutaric acid (New England Nuclear, Dreieichenhain), from the same unlabelled acid, and CoA (Sigma Chem., St Louis, MO) by the method in [10]. The product was determined as in [11]. [γ-^{32}P]-ATP (spec. act. 25 cpm/pmol) was prepared from unlabelled ATP (Sigma) and ^{32}P (Amersham, Buckinghamshire) as in [12]. Blue dextran Sepharose 4B was prepared from Sepharose 4B and blue dextran (Sigma) as in [13]. Chromatography sheet (ITLC type SG) was the product of Gelman Instr. Co., Ann Arbor, MI. All other chemicals were obtained from the standard sources listed in [7].

Abbreviations: HMG-CoA, 3-hydroxy 3-methylglutaryl coenzyme A; DTT, dithiothreitol; PMSF, phenyl methyl sulphonyl fluoride; ITLC, instant thin-layer chromatography

2.2. *Buffered solutions*

Buffer A contained 100 mM sucrose, 30 mM EDTA, 50 mM KCl, 5 mM DTT and 40 mM K_xPO_4 (pH 7.2). In addition, buffer A-F_{50} contained 50 mM KF and buffer A-F_{100} 100 mM KF. Buffer B contained 300 mM sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 0.5 mM PMSF and 40 mM Tris–HCl (pH 7.2). In addition, buffer B-M contained 5 mM $MnCl_2$.

2.3. *Purification of HMG-CoA reductase*

Sprague Dawley rats (12 at 200–250 g) fed ad libitum with laboratory chow and maintained in a room darkened 04:00–16:00 h and illuminated 16:00–04:00 h for $\geqslant$15 days prior to sacrifice were decapitated at 10 h (mid-dark phase). Microsomes were obtained as in [7]. Inactivation of microsomal fractions by endogenous protein kinase was carried out in the presence of 6 mM [γ-^{32}P]ATP and 20 mM $MgCl_2$ as in [7]. The microsomal concentration (35 mg/ml) and the time of incubation (10 min) were adjusted to ensure measurable residual HMG-CoA

reductase activity (~50–75% of the initial activity). Immediately after incubation, the microsomal suspension was made 100 mM in KF. The inactivated preparation was filtered through Sephadex G-25 equilibrated with buffer A-F_{100}, to separate microsomes from [γ-^{32}P]ATP. The filtrates were placed in a dry ice–alcohol bath and temperature decreased to −50°C at 6°C/min as in [13]. Then, they were thawed and centrifuged at 105 000 × *g* for 90 min. The clear supernatant solution (65 ml) containing 35% of the HMG-CoA reductase activity present in ATP-treated microsomes, was supplemented with 90 mg NADPH, heated at 37°C for 20 min, followed by heating at 60°C for 10 min. The heated extract was centrifuged at 30 000 × *g* for 15 min at 25°C and the supernatant was purified on a blue dextran Sepharose 4B column as in [13] except that all the buffers contained 100 mM KF.

Fractions showing HMG-CoA reductase activity were pooled, precipitated with solid ammonium sulphate (to 60% saturation) and centrifuged at 7500 × *g* for 10 min in a refrigerated centrifuge, the pellet being dissolved in buffer A-F_{100}.

2.4. *Gel electrophoresis*

Samples containing 40 μg purified enzyme were applied on 5% polyacrylamide gels at pH 8.9 and gel electrophoresis performed at 4 mA/tube for 2 h as in [14] (gel system no. 1), except that polyacrylamide was 5% and bis-acrylamide 0.13%. Gels after electrophoresis were measured for both HMG-CoA reductase activity and ^{32}P bound to protein. Gels were sliced vertically into 3 mm segments and stored overnight at 4°C in small test tubes covered with 0.2 ml buffer A for extraction. HMG-CoA reductase activity of the extracts was assayed as in [7]. Other gels were also sliced into 3 mm segments and counted for ^{32}P using a scintillation mixture of toluene/triton/PPO (2:1:0.5%). Protein on the remaining gels was stained with Coomasie blue as in [15].

2.5. *Purification and assay of HMG-CoA reductase phosphatase*

Two rat livers were homogenized in buffer B and processed as in [7] all steps being carried out at 4°C. The 105 000 × *g* supernatant was loaded onto a DEAE-cellulose column (4.5 × 7 cm) equilibrated with buffer B. The column was washed with buffer B until the A_{280} of the fractions returned to the base line (~120 ml), and then with 120 ml buffer B containing

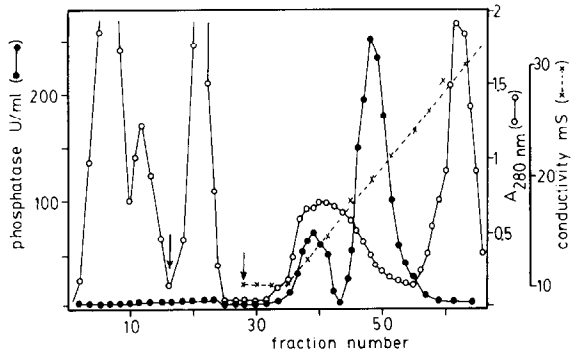

Fig.1. Chromatography of rat liver HMG-CoA reductase phosphatase on DEAE-cellulose. Conditions are described in the text. The points at which 100 mM KCl and linear gradient 100–500 mM KCl in buffer B are applied are indicated by the arrows. Optical density (○–○) and enzymatic activity (●–●) were represented, respectively, in right and left ordinate.

100 mM KCl. None of the fractions collected showed HMG-CoA reductase phosphatase activity. The column was then eluted with 400 ml of a linear gradient of 100–500 mM KCl in buffer B. Fractions (10 ml) were collected at 100 ml/h. Two different protein phosphatases which activated inactive microsomal HMG-CoA reductase were obtained (fig.1). Fractions corresponding to the second peak (≃200 mM KCl), were pooled, brought to 60% saturation with solid ammonium sulphate, and the precipitate collected by centrifugation (30 000 × *g*, 15 min). The pellet was dissolved in a minimum volume of buffer B-M and dialyzed overnight with 2 changes of the same buffer. The dialyzed solution was poured into 5 vol. 95% ethanol at room temperature and the mixture was immediately centrifuged (5000 × *g*, 5 min, 4°C). The supernatants were dialyzed overnight at 4°C against buffer B-M, then concentrated by ultrafiltration using a millipore cell Pellicon filter PTGC and stored at −40°C until used. By this procedure the enzyme was purified 590-fold with respect to the 105 000 × *g* supernatant.

HMG-CoA reductase phosphatase activity was assayed using inactive microsomal HMG-CoA reductase as substrate. As the phosphatase promotes the activation of the HMG-CoA reductase activity, it was of interest to obtain microsomes which contained as low HMG-CoA reductase activity as possible (i.e., highly inactivated). In vivo microsomal HMG-CoA reductase has ~15% of the maximum activity

which can be obtained by incubation with supernatant [5]. Therefore, microsomes were isolated at 0–4°C as in [7] in the presence of 50 mM KF to avoid activation of HMG-CoA reductase during homogenization and centrifugation by phosphatases present in cytosol. These highly inactivated microsomes were resuspended in buffer B, KF in the mixture being diminished to 12.5 mM. Aliquots of this suspension (20 μl, ~300 μg protein) were incubated with 30 μl of HMG-CoA reductase phosphatase at 37°C for 10 min; thereafter the reductase assay was started by adding 170 μl of substrate and cofactor mixture and further incubated for 30 min. The labelled mevalonolactone was isolated and quantified as in [7]. The residual 5 mM KF did not produce significant inactivation of the protein phosphatase (data not shown).

In the experiments with ^{32}P-labelled HMG-CoA reductase as substrate, incubation with partially purified HMG-CoA reductase phosphatase was performed in buffer B (pH 8.5) for the indicated times. The [^{32}P]protein was separated from the released ^{32}P by ITLC chromatography as in [16]. The reaction mixture (30 μl) was spotted on the chromatographic strips (1.5 X 9.8 cm) 1.5 cm above the bottom edge at a site which had received 10 μl of 20% trichloroacetic acid. Chromatography was performed using 5% trichloroacetic acid in 0.2 M KCl. The papers were dried with warm air, a 2.5 cm segment of each strip, from 1.5 cm above to 1.0 cm below the starting line was cut out, inserted into a scintillation vial, and counted for [^{32}P]protein in toluene PPO. Analogous determinations of ^{32}P bound to protein were done with fractions eluting from the blue dextran Sepharose 4B column (see section 2.3). Protein was measured as in [17].

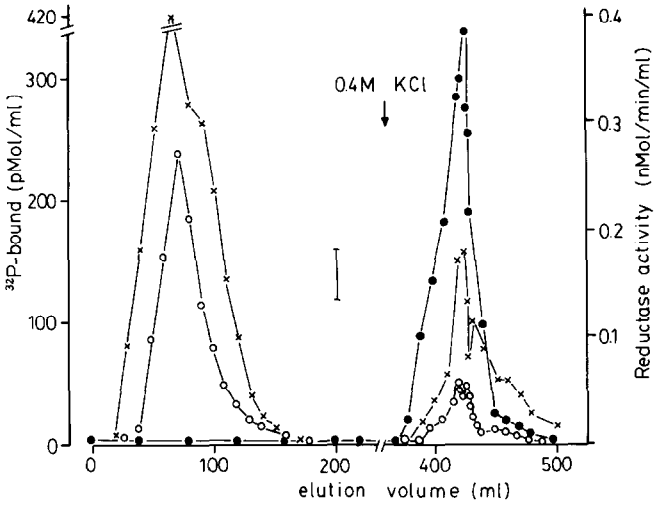

Fig.2. Resolution of HMG-CoA reductase from other ^{32}P-labelled microsomal proteins by chromatography on blue dextran Sepharose 4B. The crude extract from solubilized microsomes partially inactivated by [γ-^{32}P]ATP (60 ml, 1.4 mg/ml) was applied to a 4.2 X 7.0 cm column of blue dextran Sepharose 4B equilibrated with buffer A-F_{100}. Elution was first with buffer A-F_{100} (300 ml), then with buffer A-F_{100} containing 400 mM KCl (150 ml). (○) Protein; (×) ^{32}P (left ordinate); (●) HMG-CoA reductase activity (right ordinate). The bar in the center represents 0.2 mg protein/ml.

3. Results

3.1. *Phosphorylation of HMG-CoA reductase*

When rat liver microsomes, prepared in the absence of KF, were preincubated with 6 mM [γ-^{32}P]ATP, 20 mM $MgCl_2$ as described, the HMG-CoA reductase activity was reduced to 50–75% of the initial activity in 10 min. After filtration through Sephadex G-25, 99.9% of the radioactivity eluted with the protein fractions was precipitable by trichloroacetic acid.

Figure 2 shows the results of the purification of HMG-CoA reductase conducted as in [13]. Most of the protein applied to the column was eluted in the first peak that contained ^{32}P bound to protein but no HMG-CoA reductase activity. The second minor peak contained all the HMG-CoA reductase activity and also ^{32}P bound into protein. The purification of the HMG-CoA reductase with respect to the inactivated microsomes was 750-fold with a recovery of 9%.

Gel electrophoresis of the purified enzyme is shown in fig.3. A major band (mobility 0.59–0.67) coincides with both the HMG-CoA reductase activity peak (mobility 0.57–0.66) and with the ^{32}P radioactivity peak (mobility 0.58–0.67). A minor peak (mobility 0.42) showed neither HMG-CoA reductase activity nor ^{32}P radioactivity.

3.2. *Properties of HMG-CoA reductase phosphatase*

Partially purified HMG-CoA reductase phosphatase was found to be mol. wt 32 000. Determination was done by gel filtration on a column of Sephadex G-100 (1.6 X 80 cm) using as marker proteins, seroalbumin (mol. wt 68 000), ovoalbumin (mol. wt 43 000) and

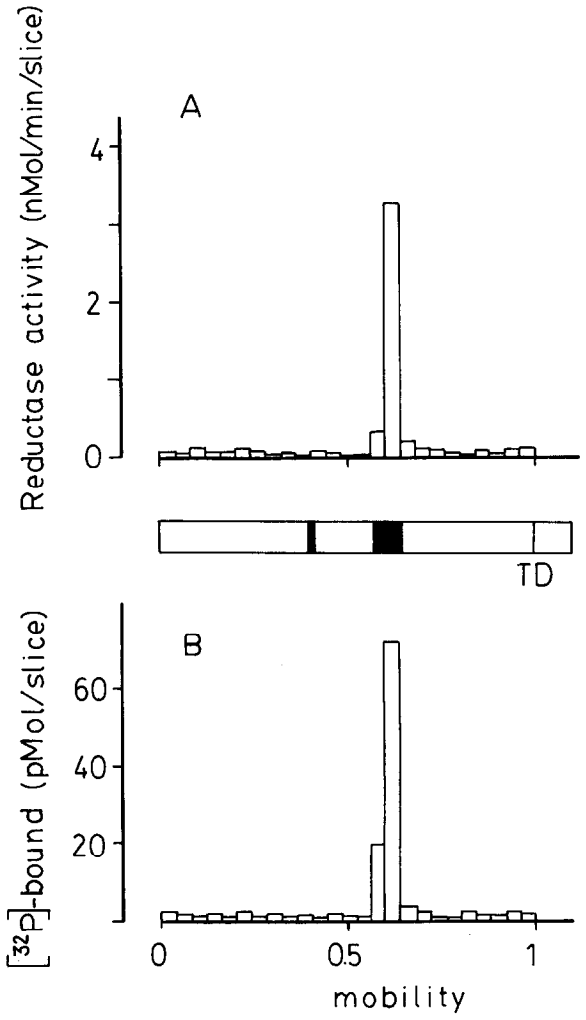

Fig.3. Polyacrylamide gel electrophoresis of purified rat liver HMG-CoA reductase. The experiments were performed in triplicate. One of the gels was stained with Coomasie blue, while the second and third were sliced in 3 mm segments. Each segment of the second gel was eluted for HMG-CoA reductase as in section 2, and its activity represented in (A). Each segment of the third was measured for ^{32}P radioactivity and the radioactive profile represented in (B). The location of bands in the gel stained with Coomasie blue is shown in the center. TD, tracking dye.

myoglobin (mol. wt 17 500). The pH optimum when purified HMG-CoA reductase was used as substrate, was found to be pH 8.5 (fig.4). The activity of the phosphatase was completely blocked by 100 mM KF.

3.3. *Dephosphorylation of HMG-CoA reductase*

When purified ^{32}P-labelled HMG-CoA reductase

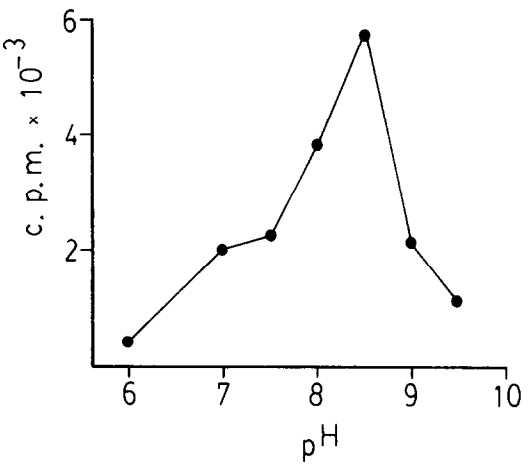

Fig.4. The pH optimum of HMG-CoA reductase phosphatase. Activity towards purified rat liver HMG-CoA reductase as substrate was determined in the assay system in section 2. The cpm of mevalonolactone from HMG-CoA reductase assay are represented in ordinate. Bis-Tris buffer (40 mM) was used at pH <7.2 and 40 mM TAPs buffer at pH >7.2.

was incubated with partially purified HMG-CoA reductase phosphatase, a time-dependent release of ^{32}P from ^{32}P-labelled reductase concomitant with an increase of HMG-CoA reductase activity was observed (fig.5). Under the conditions described, the reaction was not linear with respect to time of incubation in

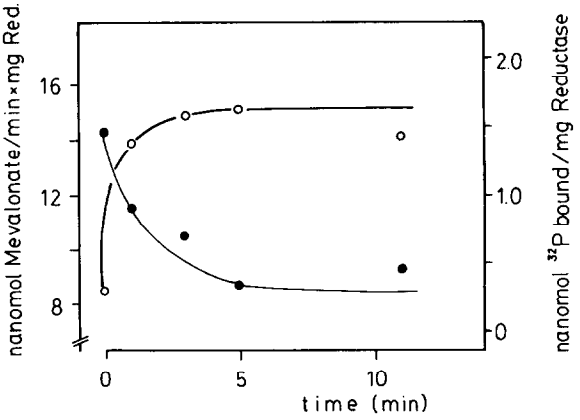

Fig.5. Time course of dephosphorylation of purified HMG-CoA reductase by HMG-CoA reductase phosphatase. Samples (5.7 μg) of purified ^{32}P-labelled HMG-CoA reductase were incubated with 6 μg HMG-CoA reductase phosphatase as in section 2. The HMG-CoA reductase activity was measured and the specific activity represented in left ordinate (open symbols). The ^{32}P bound to HMG-CoA reductase is expressed as nmol ^{32}P/mg HMG-CoA reductase and represented on the right ordinate (closed symbols).

the range of 5 min. However, the release of ^{32}P correlates perfectly with the reactivation of the enzyme. The release of ^{32}P as well as the increase of HMG-CoA reductase activity were halted when 100 mM KF was added to the incubation mixture (data not shown).

4. Discussion

The present study clearly shows that ^{32}P is incorporated in the HMG-CoA reductase molecule and that the enzyme is inactivated if microsomes are incubated in the presence of [γ-^{32}P]ATP. These observations are in full agreement with [9] which appeared when this work was completed. While no information is available in [9] concerning the reversibility of the inactivation reaction, our experiments provide conclusive proof that the phosphorylated and inactivated species of HMG-CoA reductase is dephosphorylated and reactivated by a phosphatase which we have purified some 600-fold from the cytosolic supernatant. Thus, from these in vitro studies it appears now clearly established that HMG-CoA reductase is an interconvertible enzyme whose activity is regulated by covalent phosphorylation and dephosphorylation. The physiological significance of this kind of regulation in vivo remains to be established.

Acknowledgements

The authors sincerely thank Drs J. J. Guinovart and E. Salsas for helpful discussions and Professor O. Wieland for his cooperation and criticism in the redaction of the manuscript. This work was supported by the grant 2597 from Comisión Asesora de Investigación Científica y Técnica, Spain and by US grant NSF-44115 (second part).

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