INACTIVATION OF SOLUBLE 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE BY ATP

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Received 2 August 1977

1. Introduction

Endoplasmic reticulum-bound HMG CoA reductase catalyzes the rate-limiting reaction of cholesterol synthesis in hepatocytes and other cells [1]. The activity of the enzyme in isolated rat liver microsomes is profoundly reduced by incubation with ATP–Mg prior to assay [2]. Inactivation is prevented or blocked by addition of EDTA [3]. Washing of the microsomes by repeated centrifugation prevents [2], and a protein fraction separated from the 100 000 × *g* supernatant of rat liver [2] or human fibroblasts [4] homogenates restores, the ATP–Mg-dependent inactivation. Beg et al. [2] indicated the possibility that HMG CoA reductase activity is subject to modulation through enzyme phosphorylation. However, Chow et al. [5] could not find phosphorylation or adenylation of the enzyme protein under conditions leading to inactivation. According to Brown et al. [4] the inactivation factor in the 100 000 × *g* supernatant would promote the conversion of the microsomal enzyme from an active to an inactive form. Both ATP and ADP are equally effective. Preparations of HMG CoA reductase solubilized by freezing and glycerol extraction are not susceptible to inactivation.

The present paper reports evidence that inactivation by ATP is a characteristic property of HMG CoA reductase protein both in the membrane-bound and in the soluble form. The presence of the inactivation factor in the 100 000 × *g* supernatant and of Mg^{2+} during the preincubation step is required to bring about inactivation by ATP of the membrane-bound enzyme, but is not necessary for the phenomenon to take place with the soluble preparation used. With such a preparation, ADP is ineffective.

Abbreviations: HMG CoA, 3-hydroxy-3-methylglutaryl CoA; HMG CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase (mevalonate: $NADP^+$ oxidoreductase (CoA acylating), EC 1.1.1.34); DTT, dithiothreitol

2. Materials and methods

Triton WR 1339 was purchased from Serva; DL-HMG CoA from P-L Biochemicals, Inc.; DL[3-^{14}C]-HMG CoA from New England Nuclear Corp.; ATP, ADP, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and hexokinase from Boehringer Mannheim.

Female rats (200–250 g) of the Wistar strain were used. The animals, fed ad libitum on an ordinary laboratory chow, were killed at 12.00 p.m., at the zenith of the diurnal rhythm of HMG CoA reductase of liver. Alternatively, the rats were injected intraperitoneally with 250 mg/kg of Triton WR 1339 (100 mg/ml in 0.9% NaCl) at 9.00 a.m. and sacrified 24 h later, when the activity of the Triton-stimulated HMG CoA reductase activity was at its peak [6,7]. Results obtained with preparations from either type of animal as source of enzyme were analogous.

2.1. *Preparation of soluble inactivation factor (S_{100}) and microsomes*

All operations were performed at 0–4°C. Livers were homogenized in a Potter homogenizer with a Teflon pestle with 3 vol. solution composed of 0.05 M potassium phosphate, pH 7.0, 0.3 M sucrose,

0.075 M nicotinamide, 2.5 mM EDTA and 5 mM mercaptoethanol. The homogenate was centrifuged at 5000 × *g* for 10 min; the supernatant at 8500 × *g* for 10 min; the second supernatant at 12 000 × *g*; the third supernatant at 100 000 × *g* for 60 min. The microsomal pellets from one liver were resuspended with 10 ml 0.05 M potassium phosphate, pH 7.0, and 2 mM DTT, and homogenized in a 20 ml tight-fitting all-glass homogenizer with 30 strokes. The suspension was centrifuged at 100 000 × *g* for 60 min. The supernatant (which will be called S_{100} hereafter) contained 3–6 mg protein/ml and was used as the source of the soluble inactivation factor. The microsomal pellet was resuspended in 0.05 M potassium phosphate, pH 7.0, and 2 mM DTT, to a protein concentration of about 5 mg/ml, and was used as the source of the membrane-bound HMG CoA reductase.

2.2. *Preparation of soluble HMG CoA reductase*

Preparation of soluble HMG CoA reductase was performed by subjecting the microsomal pellet from the second 100 000 × *g* sedimentation described above to the slow-freezing and thawing procedure described by Heller and Gould [8]. The enzyme was extracted and partially purified through ammonium sulphate fractionation, as described [8]. For the present work, the 35–50% ammonium sulphate fraction was used, following desalting through a column of Sephadex G-25, equilibrated with 10 mM potassium phosphate, pH 7.0, and 10 mM DTT. The ammonium sulphate fractionation and desalting steps were performed at room temperature (20°C). The enzyme preparation was diluted to a protein concentration of 4–6 mg/ml.

2.3. *Inactivation of HMG CoA reductase: Assay of activity*

The inactivation of HMG CoA reductase was tested by preincubation of a standard mixture, containing the following components in final vol. 120 μl: potassium phosphate, pH 7.2, 10 μmol; DTT, 2 μmol; EDTA, 0.75 μmol; ATP or ADP, 0.8 μmol; $MgCl_2$, 1.6 μmol; 30–60 μg soluble enzyme preparation, or 50–100 μg microsomal protein; 100–200 μg S_{100}. EDTA is required in the preincubation mixture to stabilize HMG CoA reductase [3], but its concentration at this step is low enough to allow sufficient Mg^{2+} to interact with adenine nucleotides. Samples were preincubated at 37°C for 60 min. The inactivation process was blocked by adding an excess amount of EDTA (2.5 μmol). When the effect of EDTA on ATP inactivation will be discussed in Section 3, this excess amount of EDTA will always be referred to.

The presence of excess EDTA in the subsequent assay of the reductase activity also prevents phosphorylation of mevalonic acid by possible contaminating enzymes. The determination of HMG CoA activity was started by adding $NADP^+$, 0.6 μmol; glucose-6-phosphate, 4 μmol; glucose-6-phosphate dehydrogenase, 0.9 unit; DL[3-^{14}C]HMG CoA, 0.02 μmol (6.5×10^6 cpm/μmol). Final volume was 200 μl. After incubation at 37°C for 15–30 min, [^{14}C]mevalonic acid formed was converted into lactone, isolated and counted according to Shapiro et al. [3]. In control reactions the amount of EDTA used to block the inactivation (2.5 μmol) was added at the start of the preincubation period. Activity was expressed as nmol mevalonic acid formed/min/mg HMG CoA reductase preparation protein. S_{100} and microsome protein was determined by the biuret method [9], following precipitation with 10% trichloroacetic acid and resuspension in 0.5 M NaOH [10]. Bovine serum albumin was used as standard. The protein concentration of the soluble preparation of HMG CoA reductase was estimated from the 280/260 nm absorbancy ratio [11].

3. Results and discussion

Preliminary experiments performed in this laboratory showed that HMG CoA reductase solubilized by the slow-freezing and thawing procedure of Heller and Gould [8] exhibits an excellent response to the inactivating effect of ATP. This indicated that the resistance to ATP inactivation is not a characteristic property of the soluble enzyme, as suggested by Brown et al [4], but a particular feature of the glycerol-extracted enzyme preparation employed in [4]. The experiments reported in table 1 were designed to examine whether the soluble HMG CoA reductase is inactivated under conditions comparable to those which are effective on the microsomal enzyme. The results show that the enzyme, preincubated in the presence of ATP, Mg^{2+} and S_{100} (expt c), is inactivated at an extent similar to that reported [2–4] for the membrane-bound enzyme (88%). ADP can replace ATP (expt d). The inactivating effect of ATP

Table 1
Inactivation of soluble HMG CoA reductase by ATP

Expt	Additions	Activity	
		EDTA added at start of preincubation	EDTA added after preincubation
a	Soluble enzyme	3.22	3.31
b	Soluble enzyme + S_{100}	3.71	3.70
c	Soluble enzyme + S_{100} + ATP	0.35	0.38
d	Soluble enzyme + S_{100} + ADP	3.31	0.40
e	Soluble enzyme + ATP	0.99	1.15
f	Soluble enzyme + ADP	2.95	2.07
g	Soluble enzyme + ADP + glucose + hexokinase	3.05	3.25

The experiments were performed as described in Section 2.3, with the following exceptions: S_{100}, ATP and ADP were present only where indicated; when present, hexokinase was 0.8 unit and glucose 2 μmol.

is evident on the soluble enzyme also when the preincubation is carried out in the presence of EDTA (expt c) and when S_{100} is deleted from the preincubation mixture (expt e). Being ATP effective in the presence of excess EDTA, it is compelling to conclude that also ATP, and not necessarily the complex ATP–Mg, can be an active species participating in the process. This should positively exclude phosphorylation or adenylation as the molecular basis of the inactivation. In the absence of S_{100}, ADP was only partially effective. When EDTA was present during preincubation, ADP was ineffective (expt f). Addition of the ATP-dephosphorylating system hexokinase plus glucose during the preincubation renders ADP ineffective as well (expt g).

The experiment described in table 2 was carried

Table 2
Changes of adenine nucleotide concentrations during preincubation of soluble HMG CoA reductase and S_{100}

Time (min)	Additions	Adenine nucleotide found (mM)		
		ATP	ADP	AMP
0	Soluble enzyme + ATP	3.25	0.36	0.23
	Soluble enzyme + ADP	0.09	3.35	0.66
	Soluble enzyme + S_{100} + ATP	3.22	0.39	0.20
	Soluble enzyme + S_{100} + ADP	0.10	3.40	0.65
60	Soluble enzyme + ATP	3.19	0.36	0.29
	Soluble enzyme + ADP	0.43	2.50	1.29
	Soluble enzyme + S_{100} + ATP	2.69	1.00	0.56
	Soluble enzyme + S_{100} + ADP	0.95	1.34	1.89

Preincubation mixtures were prepared as described in Section 2.3, with the exception that all quantities were multiplied by five. No EDTA was added. Water was added to final vol. 1 ml. At zero time and after incubation for 60 min at 37°C, convenient aliquots were withdrawn and diluted with cold perchloric acid to stop the reaction and to precipitate protein. Denaturated protein was sedimented by centrifugation. The supernatants were neutralized with KOH in the cold and centrifuged. On aliquots of the neutralized supernatants the adenine nucleotides were determined by routine enzymatic analysis [13,14]. S_{100}, ATP and ADP were present only where indicated

out to ascertain how the adenine nucleotides are altered during preincubation. The concentrations of the nucleotides present at the end of the preincubation period were determined and compared with those present at zero time. The determinations were carried out on mixtures in which soluble enzyme alone or soluble enzyme plus S_{100} were present. It appears that, when ADP is the starting nucleotide, the soluble enzyme preparation can catalyze a modest, but significant formation of ATP, while S_{100} promotes the formation of a substantial amount of ATP. These effects are caused probably by adenylate kinase. So the apparent effectiveness of ADP, when present, can be attributed to ATP formed during the preincubation.

To ascertain whether the observed characteristics are compatible with those known for the phenomenon as it occurs with the microsomal HMG CoA reductase, the experiments of table 3 were performed. Microsomes were preincubated in the presence of ATP, Mg^{2+} and S_{100} (expt c) and optimal inactivation was observed (76%). ADP could replace ATP (expt d). Neither of the nucleotides was effective when S_{100} was omitted (expt e, f) or if EDTA was present during the preincubation period. However, inactivation by ATP (not ADP) was obtained also when the nucleotide was added to the reaction mixture after excess EDTA, provided the microsomes had been preincubated in the presence of S_{100} and free Mg^{2+} (expt g, h). The results suggest that during the preincubation period S_{100} and free Mg^{2+} promote some alteration in the microsomal structure, which renders the reductase susceptible to inactivation by ATP. In this respect the role of S_{100} might be essentially that of a mixture of lipolytic and proteolytic enzymes, acting on the microsomal lipoprotein.

Table 4 shows that ATP inactivation cannot be obtained omitting preincubation (expt c). Once the inactivation has taken place, it cannot be reversed by dephosphorylating ATP with the use of hexokinase plus glucose (expt e). This seems to exclude that ATP acts as an inhibitor of the competitive type or as a negative allosteric effector.

In conclusion, inactivation by ATP appears to be a characteristic property of HMG CoA reductase, which is masked in the membrane-bound enzyme and becomes apparent following preincubation with S_{100} and Mg^{2+}. Possibly a previously inaccessible ATP-binding site of the enzyme is exposed during preincubation. Thus the transition of the protein to an inactive conformation is made possible. In the enzyme solubilized by slow-freezing and thawing, ATP binding takes place more directly. Conformational changes promoted by ATP could be the bases for a slow irreversible degradation, spontaneous or catalyzed, of the enzyme. This is supposedly the explanation of the fact that ATP can inactive the soluble HMG CoA reductase also independently of S_{100}.

Recently, Tormanen et al. [12] observed a profound

Table 3
Inactivation of membrane-bound HMG CoA reductase by ATP

Expt	Additions	Activity	
		EDTA added at start of preincubation	EDTA added after preincubation
a	Microsomes	0.63	0.65
b	Microsomes + S_{100}	0.66	0.69
c	Microsomes + S_{100} + ATP	0.62	0.15
d	Microsomes + S_{100} + ADP	0.64	0.12
e	Microsomes + ATP	0.67	0.64
f	Microsomes + ADP	0.65	0.65
g	Microsomes + S_{100} + ATP added after preincubation	–	0.18
h	Microsomes + S_{100} + ADP added after preincubation	–	0.69

The experiments were performed as indicated in Section 2.3, with the following exceptions: S_{100}, ATP and ADP were present only where indicated; in expts g, h, S_{100} was present from the start of the preincubation, while ATP and ADP were added at the end of the preincubation period, after excess EDTA

Table 4
Necessity of preincubation for ATP inactivation of HMG CoA reductase irreversibility of the reaction

Expt	Additions	Activity	
		EDTA added at start of preincubation	EDTA added after preincubation
a	Soluble enzyme	2.57	2.55
b	Soluble enzyme + ATP	0.55	0.50
c	Soluble enzyme added to the complete mixture (with ATP) after excess EDTA	–	2.60
d	Soluble enzyme + ATP + hexokinase + + glucose	2.55	2.51
e	Soluble enzyme + ATP; hexokinase and glucose added 5 min before excess EDTA	–	0.75

The experiments were performed as described in Section 2.3, with the following exceptions: S_{100} was omitted; ATP was present where indicated; when present hexokinase was 1.4 unit and glucose 2 μmol

inhibition of the rat liver HMG CoA reductase solubilized by the slow-freezing and thawing method [8]. Inhibition was attributed the 105 000 × *g* supernatant fraction (S_{105}) + ATP + Mg^{2+}. However, the reported inhibition was due for the greatest part (more than 75%) to S_{105} alone.

Acknowledgements

Thanks are due to Professor N. Siliprandi for encouragement and helpful suggestions during this work. The secretarial aid of Mrs Maurizia Cuccia is gratefully acknowledged.

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