(3*R,S*)-3-HYDROXY-3-METHYL-4-CARBOXYBUTYL-CoA, A SPECIFIC INHIBITOR OF 3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE

Tran-Giac NGUYEN, Horst AIGNER and Hermann EGGERER
Institut für Physiologische Chemie der Technischen Universität München, Biedersteiner Straße 29, 8000 München 40, FRG

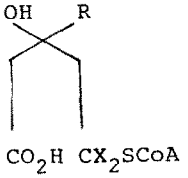
Received 1 April 1981

1. Introduction

The regulatory role of HMG-CoA reductase (EC 1.1.1.88) was first demonstrated for hepatic cholesterol biosynthesis from acetate in fasted [1] and in cholesterol-fed rats [2]. Much progress has since been made towards an understanding of the control of this enzyme by a cholesterol-dependent feedback mechanism, mediated through low density lipoprotein. The use of a specific inhibitor, compactin [3], has furthermore led to the discovery of multivalent feedback inhibition of HMG–CoA reductase and to insights into the coordination of isoprenoid biosynthesis and cell growth (reviews [4,5]).

During studies on the mechanism of action of citrate synthase (EC 4.1.3.7) we synthesized (3*R,S*)-3,4-dicarboxy-3-hydroxybutyl-CoA (I), an inhibitory intermediate analogue of the synthase reaction [6,7]. To establish the specificity of (I) we have also prepared the title compound (II), a substrate analogue for HMG–CoA reductase.

If (I) represents a specific inhibitor of citrate synthase, (II) should not be inhibitory; the reverse should be found with HMG-CoA reductase. This mode of inhibition provided, (II) should further not affect the rest of the enzymes involved in cholesterol biosynthesis. The results presented here demonstrate this specificity.



HMG-CoA : R = CH_3; X_2 = O

I : R = CO_2H; X = H

II : R = CH_3; X = H

2. Material and methods

(3*R,S*)-3-Hydroxy-3-methyl[3-^{14}C]glutaryl-CoA (54 mCi/mmol), (3*R,S*)-[5-^{3}H]mevalolactone 5.7 mCi/mmol), (3*R,S*)-[2-^{14}C]mevalolactone (47 mCi/mmol), [1-^{14}C]acetate (44 mCi/mmol) and [1-^{14}C]-acetyl-CoA (57 mCi/mmol) were purchased from New England Nuclear. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49; 6 μkat/mg protein), citrate synthase and other biochemicals were from Boehringer Mannheim GmbH. Cholestyramine was from Serva, Heidelberg; Biorad (1 X 8; Cl^-; 100–200 mesh) was from Biorad Laboratories and Unisolve 100 from Zinsser, Frankfurt. The inhibitor (I) was prepared as in [6]; (II) was similarly obtained in the sequence methyl (*R,S*)-mevalonate → methyl (*R,S*)-3-hydroxy-3-hydroxy-3-methyl-4-carboxybutylbromide → II (in preparation).

2.1. *Animals*

Male Sprague-Dawley rats (~180 g body wt) were housed in a light-controlled room on an alternate 12 h light–dark schedule (dark phase 4 a.m.–4 p.m.). The animals were fed ad libitum for 5 days a 2% cholestyramine-containing diet (Rattendiät-Zuchtfutter; J. Stetter, Munich). The rats were sacrificed at 10 a.m.

2.2. *Purification of HMG-CoA reductase*

Purification of microsomes from excised livers (~100 g) and solubilisation of enzyme were performed as in [8]. The reductase was purified through the heat step and ammonium sulfate precipitation (0–50% saturation) as in [8] to yield 6 mg (45 nkat) protein dissolved in 1.0 ml buffer A [8].

2.3. *Assays*

Activity of HMG-CoA reductase was determined

as in [9]. The reaction mixture which was kept at 37°C in a total volume of 0.2 ml contained 0.1 mM (*R,S*)-[3-^{14}C]HMG-CoA (1.32 × 10^5 cpm), 0.23–0.45 nkat reductase (which was used to start the reaction) and (assay 1; in work with crude extracts) 0.1 M Tris buffer (pH 7.2); 20 mM EDTA; 0.1 M neutralized cysteine hydrochloride; 10 mM glucose-6-phosphate; 1 mM NADP and 12 nkat glucose-6-phosphate dehydrogenase or (assay 2; in work with purified enzyme samples) 25 mM phosphate buffer (K^+) (pH 7.0); 1 mM dithiothreitol; 10 mM EDTA and 0.5 mM NADPH. With purified enzyme samples both assays yielded identical results.

The samples after start of the reaction were incubated for 10 min; (*R,S*)-[5-^{3}H]mevalonate (5 μmol; 8 × 10^4 cpm) was then added and the reaction was terminated by adding 0.10 ml 2 N H_2SO_4. Mevalonic acid lactone was isolated as in [9]; samples (5 ml each) were mixed with 15 ml Unisolve 100 and the radioactivity was determined in a Berthold-Betascint 5000/300 scintillation counter.

2.4. *Protein determination*

This was done by the biuret method; enzyme units are expressed in kat.

2.5. *Incorporation experiments*

Rat liver microsomes and cytosolic enzyme fraction were isolated according to [10], modified as in [11]. The reaction mixture which was kept at 37°C in a total volume of 0.20 ml contained 0.1 M Tris buffer (pH 7.2); 1 mM ATP; 10 mM glucose-6-phosphate; 6 mM glutathione; 6 mM $MgCl_2$; 40 μM CoASH; 0.25 mM NAD; 0.25 mM NADP; 0.05 mg microsome protein; 0.5 mg cytosolic fraction protein and 22.8 μM [1-^{14}C]acetate (44 mCi/mmol). In some experiments [1-^{14}C]acetate was replaced by 17.3 μM [1-^{14}C]acetyl-CoA (57 mCi/mmol), 18.5 μM (*R,S*)-[3-^{14}C]HMG-CoA or 32.4 μM (*R,S*)-[2-^{14}C]mevalonate (47 mCi/mmol). The reaction was stopped after 10 min by adding 1 ml 15% ethanolic KOH containing 1 μmol unlabelled carrier cholesterol and the products were hydrolyzed for 1 h at 75°C. Extraction was performed with petroleum ether (1 × 5, 2 × 2 ml) and the combined extracts were washed with 1 ml water; 10 ml Unisolve 100 was added and the radioactivity was determined as in section 2.3. The incorporations of the substrates were proportional to time for at least 30 min.

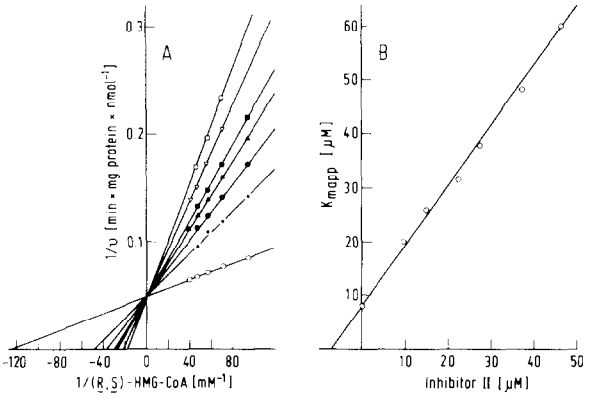


Fig.1. (A) Inhibition of HMG-CoA reductase by (II) *vs* HMG-CoA. Assays were performed with purified enzyme as in section 2 but with HMG-CoA varied as indicated. The concentration (μM) of (II) was: zero (○); 9.25 (*); 14.9 (●); 22.4 (▲); 27.8 (■); 37.3 (▽); and 46.3 (□). (B) Replot of app K_m *vs* [inhibitor].

3. Results

3.1. *Inhibition of HMG-CoA reductase*

The kinetics of the reductase reaction (HMG-CoA varied) without and in the presence of increasing concentrations of (II) are presented as double reciprocal plots in fig.1A and indicate competitive inhibition. A replot of app K_m taken from fig.1A *vs* inhibitor concentration is shown in fig.1B, from which K_i = 7 μM (*R,S*) was determined; K_m for HMG-CoA was 8 μM (*R,S*). The inhibition of the reductase by (II) on variation of NADPH was non-competitive (fig.2A)

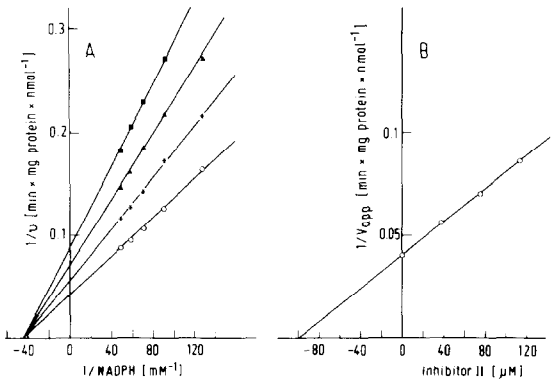


Fig.2. (A) Inhibition of HMG-CoA reductase by (II) *vs* NADPH. Assays were performed with purified enzyme as in section 2 but with NADPH varied as indicated. The concentration (μM) of II was: zero (○), 38 (*), 76 (▲) and 114 (■). (B) Replot of $1/V_{app}$ *vs* [inhibitor].

Table 1
Inhibitory effects of I, II and CoASH on HMG-CoA reductase

Inhibitor (μM)			Mevalonate (nmol)[a]	% of control
I	II	CoASH		
None	None	None	1.68	100
9.1	–	–	1.54	91
–	9.3	–	1.02	61
–	–	9.3	1.55	92
27.4	–	–	1.44	86
–	27.8	–	0.70	42
–	–	27.8	1.50	89

[a] nmol mevalonate × (10 min × mg protein)$^{-1}$ formed under assay conditions in section 2; 35 μg protein (0.26 nkat) was applied

and yielded K_i (II) = 100 μM (fig.2B) and K_m (NADPH) = 23 μM.

3.2. *Specificity of inhibitors (I) and (II)*

The activity of citrate synthase is strongly depressed in the presence of I [7] and so is that of ATP citrate lyase (EC 4.1.3.8) (U. Lill, unpublished). Substitution of (I) for (II) in the assays of the two enzymes yielded an inhibition as low as that effected by CoASH. Citrate synthase: K_i (CoASH) ~ K_i (II) ~ 250 μM; ATP citrate lyase: 25 μM CoASH or (II) caused 10% inhibition.

The reversed specificity of the inhibitors (I) and (II) for HMG-CoA reductase is shown in table 1. In the presence of 28 μM (II) the reductase was strongly inhibited, to ~60%, whereas 28 μM (I) or CoASH yielded only ~10% inhibition.

3.3. *Inhibition of non-saponifiable lipid biosynthesis*

The inhibitory effect of (II) on the incorporation of several different precursors of cholesterol biosynthesis into nonsaponifiable lipids is shown in table 2. Similar to the action of compactin [3], albeit at higher concentrations, (II) suppressed the incorporation of acetate, acetyl-CoA and HMG-CoA but was without effect towards the incorporation of mevalonate into non-saponifiable lipids.

4. Discussion

These results demonstrate the specificity of inhibitors (I) and (II) and therefore strengthen the conclusion that (I) represents an intermediate analogue of the citrate synthase reaction [7]. Replacement of the 3-carboxyl group in (I) by a methyl group yields (II) concomitant with the loss of inhibitory power *vs* this enzyme. Similarly, also (3*R*)-citryl-CoA is not inhibitory towards citrate synthase (in preparation).

Table 2
Effects of II on the incorporation of ^{14}C-labelled precursors of cholesterol biosynthesis into non-saponifiable lipids

Substrate	Inhibitor II (μM)	incorporation[a] (cpm)	% of control
[1-^{14}C]Acetate	None	5200	
	30	3660	70
	90	2710	52
[1-^{14}C]Acetyl-CoA	None	9440	
	30	7240	77
	90	4180	43
(*R,S*)-[3-^{14}C]HMG-CoA	None	13 060	
	30	8940	68
	90	5500	42
(*R,S*)-[2-^{14}C]Mevalonate	None	11 500	
	30	10 520	92
	90	10 600	92

[a] Experiments were performed as in section 2

The intermediate analogue (II) of the HMG-CoA reductase reaction specifically inhibited the reductase which was hardly influenced by (I). The results match those obtained with the corresponding acyl-CoA derivatives. The substrate of the reductase, (3*R,S*)-HMG-CoA, is not inhibitory towards citrate synthase and the substrate for the latter enzyme, (3*S*)-citryl-CoA, is not inhibitory towards the reductase. ATP citrate lyase is likewise neither inhibited by HMG-CoA nor by (II) but by (I), as expected if (3*S*)-citryl-CoA is formed as an intermediate on this enzyme [12]. The conclusion in [7] can therefore be extended in that not only the affinity but also the specificity appears to be unaffected in substrate analogues formed by replacement of oxygen next to sulfur of acyl-CoA derivatives by hydrogen.

As judged from incorporation studies of radioactively labelled precursors of cholesterol biosynthesis into non-saponifiable lipids, HMG-CoA reductase was the target enzyme for (II), which had no effect on other enzymes involved in this biosynthesis. The affinity of (II) towards the reductase is nearly identical to that of HMG-CoA but ~3 powers of 10 lower

than that of compactin [3]. Nevertheless the chemical and enzymological properties of (II) suggest that it may basically represent a useful component in future attempts to repress hypercholesterinaemia.

Acknowledgements

We thank Miss Gabriele Günther for handling the animals and Professor Melchior Reiter for providing the facilities. This work was supported by the Fonds der Chemischen Industrie.

References

[1] Bucher, N. L. R., Overath, P. and Lynen, F. (1960) Biochim. Biophys. Acta 40, 491–501.
[2] Siperstein, M. D. and Fagan, V. M. (1966) J. Biol. Chem. 241, 602–609.
[3] Endo, A., Kurada, M. and Tanzawa, K. (1976) FEBS Lett. 72, 323–326.
[4] Brown, M. S. and Goldstein, J. L. (1980) J. Lipid Res. 21, 505–516.
[5] Endo, A. (1981) Trends Biochem. Sci. 6, 10–13.
[6] Eggerer, H., Giesemann, W. and Aigner, H. (1980) Angew. Chem. 92, 133–134; I.E. 19, 136.
[7] Bayer, E., Bauer, B. and Eggerer, H. (1981) submitted.
[8] Beg, Z. H., Stonik, J. A. and Brewer, H. B. (1980) J. Biol. Chem. 255, 8541–8545.
[9] Huber, J., Latzin, S. and Hamprecht, B. (1973) Hoppe-Seyler's Z. physiol. Chem. 354, 1645–1647.
[10] Bucher, N. L. R. and McGarrahan, K. (1956) J. Biol. Chem. 222, 1–15.
[11] Kurada, M. and Endo, A. (1976) Biochim. Biophys. Acta 486, 70–81.
[12] Eggerer, H. and Remberger, U. (1963) Biochem. Z. 339, 62–76.