3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE OF HUMAN LYMPHOCYTES: KINETIC PROPERTIES

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1. Introduction

The conversion of 3-hydroxy-3-methylglutaryl CoA to mevalonate, a reaction catalyzed by the 3-hydroxy-3-methylglutaryl CoA reductase (EC 1.1.1.34), is known to be the main rate-limiting step of cholesterol biosynthesis. This has been principally studied in the liver [1], but also in human cultured fibroblasts [2] and human leukocytes [3].

In order to study the mechanisms of regulation of the enzyme in human lymphocytes, it was necessary to know its kinetic properties. In the rat liver, discrepant K_m values for this enzyme have been reported from 1.1–27 μM for the D-isomer [4–8] but no data concerning the K_m of the reductase of human lymphocytes have been published.

Our first experiments have shown that the basal rate of 3-hydroxy-3-methylglutaryl CoA reductase activity in freshly isolated human lymphocytes was very low, for just before their isolation, these cells are in contact with the plasma low density lipoproteins which supply most of their cholesterol. It has been demonstrated with human leukocytes [3], that when lymphocytes are maintained for several hours in a cholesterol-depleted medium, the cholesterol synthesis and, concomitantly, the 3-hydroxy-3-methylglutaryl CoA reductase activity, are increased.

This increase could result from 'de novo' enzyme synthesis, enzyme activation, or both. The first mechanism is more likely to be involved if the kinetic properties of the enzyme remain unchanged after the rise of activity. K_m and V_{max} of 3-hydroxy-3-methylglutaryl CoA reductase were determined after preincubation of human lymphocytes in several media stimulating cholesterogenesis. According to the incubation medium used (RPMI-1640 alone or supplemented with lipid-free serum or with bovine serum albumin and lecithin dispersion), the K_m values ranged from 3.5–5.3 μM for the D-isomer, whereas important differences in V_{max} values were observed.

2. Materials and methods

2.1. *Materials*

Origin of reagents: DL-3-hydroxy-3-methyl- 3-[^{14}C glutaryl CoA (28 mCi/mmol), New England Nuclear; DL-mevalonic acid-2-[^{3}H]lactone (382 mCi/mmol), Amersham; 3-hydroxy-3-methylglutaryl CoA, sodium salt, PL Biochemicals; DL-mevalonic acid lactone, dithioerythritol, bovine serum albumin (fraction V), egg yolk lecithin (type VE) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (Hepes), Sigma; NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, Boehringer, Mannheim; gentamicine base, Unilabo (Caen); RPMI-1640 powdered medium, Flobio; Naphthalene, 2(4'-*t*-butylphenyl)-5-(4''-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) and phenylethylamine, Koch-Light Laboratories; organic solvents, Prolabo (RP grade) or Merck; detergent Kyro EOB, gift, Procter and Gamble; Kieselgel GF_{254} plates for thin-layer chromatography, Merck; radioactivity counting, Packard Tri-Carb liquid scintillation spectrometer Model 3320; human AB serums, gift, voluntary donors, Centre de Transfusion Sanguine, Montpellier.

2.2. *Preparation of lipid-free serum*

AB-serum was first centrifuged at 10 000 × *g* for

20 min in order to remove red cells and chylomicrons. We used the method in [9] to remove most of the lipids with freshly bidistilled diethyl ether. The remaining ether dissolved in the serum was evaporated under a nitrogen stream. The final concentrations of cholesterol and triglycerides were 0.20 g/l and 0.25 g/l, respectively. The serum was decomplemented by heating at 56°C for 30 min and sterilized by filtration through a 0.22 μm Millipore filter.

2.3. *Preparation of lecithin dispersions*

Lecithin dispersions were prepared as described [10]. Sonication duration was 50 min using 1 min pulses and 1 min coolings alternatively. Liposomes were centrifuged at 50 000 × *g* for 1 h and sterilized by filtration through a 0.22 μm Millipore filter. The final concentration of lipid phosphorus was assayed according to the method in [11].

2.4. *Collection and separation of lymphocytes*

Collection and separation of lymphocytes were achieved by Dr Serrou and his colleagues in the Département d'Immunologie Clinique et Expérimentale, Centre Paul Lamarque, Montpellier. The cells were isolated by a Haemonetics-Model 30 blood cell separator from voluntary normal donors, and further purified on a Ficoll-Hypaque gradient [12,13]. An additional centrifugation (1200 rev/min for 10 min) removed most of the remaining platelets. The lymphocytes were counted and their viability checked by the blue trypan exclusion test. They were then transferred to culture flasks, adjusted to conc. 4×10^6 cells/ml medium and incubated at 37°C for 16 h. The culture medium was sterile RPMI-1640 containing 40 mM Hepes (pH 7.2) and gentamicine (8 mg/100 ml). In some cases, this medium was supplemented with 20% lipid-free serum or 1.5% bovine serum albumin and lecithin dispersion (final concentration: 60–100 μg lecithin/ml medium).

2.5. *Assay of 3-hydroxy-3-methylglutaryl CoA rèductase*

3-Hydroxy-3-methylglutaryl CoA reductase activity was determined by the techniques described in [14,15] and modified as follows: The cells ($5–7 \times 10^7$ cells for one assay in duplicate) were washed twice in phosphate buffered saline (pH 7.2) and disrupted in 150 μl 50 mM potassium phosphate buffer containing 5 mM EDTA, 5 mM dithioerythritol, 0.2 M KCl and 0.25% Kyro-EOB (W/V). The suspension was incubated at 37°C for 15 min, centrifuged at 12 000 × *g* for 12 min and the protein content of supernatant determined [16]. The reaction mixtures contained 100–300 μg protein, 5 mM dithioerythritol, 10 mM glucose-6-phosphate, 2.5 mM NADP and 0.14 IU of glucose-6-phosphate dehydrogenase in final vol. 0.2 ml. After preincubation at 37°C for 15 min, the reaction was started by the addition of 1.67–80 μM DL-3-hydroxy-3-methyl- 3-[^{14}C]glutaryl CoA (unless otherwise stated, spec. act. was 28 mCi/mmol). After various periods of incubation, the reaction was stopped by 20 μl 6 N HCl. 100 000 dpm and 0.2 mg [^{3}H]mevalonic acid lactone were added and lactonization was performed at 37°C for 30 min. In blank assays, proteins were heated at 100°C for 5 min and 20 μl 6 N HCl were added before the incubation started. The incubation mixture was extracted 3 times with 2 ml diethyl ether in presence of saturating Na_2SO_3. The extracts were collected, dried, evaporated and chromatographied on silica gel plates in benzene–acetone 1/1. The mevalonic acid lactone zone was scraped off and the gel burnt in an oxydizer (Oxymat-Intertechnique). ^3H_2O and $^{14}CO_2$ were counted with the following scintillation fluids: [^{3}H], 7 $^0/_{00}$ butyl-PBD and 20$^0/_{00}$ naphthalene in dioxan/toluene, 70/30; [^{14}C], 7$^0/_{00}$ butyl-PBD in bidistilled water/phenylethylamine/methanol/toluene, 5/33/22/40. The lactone recovery averaged 65%.

Each assay was made in duplicate and blank values were subtracted from the results.

3. Results and discussion

First it was checked that, for substrate concentrations from 2.5–15 μM (D-isomer) there was a linear relationship between the rate of mevalonate synthesis and the incubation duration for at least 40 min. Such was the case when the preincubation in culture medium resulted in either a low or a high enzyme activity.

A similar linear relationship was observed between the rate of mevalonate synthesis and the protein concentration in the sample from 0–300 μg.

Thus, K_m were determined using 200 μg protein in each assay during incubations of 40 min.

It has been shown that preincubation of leukocytes

Table 1
Influence of preincubation medium on K_m and V_{max} of 3-hydroxy-3-methylglutaryl CoA reductase of human lymphocytes

Preincubation medium	K_m (μM)	V_{max} (pmol/min/mg protein)
None	–	1.2[a]
RPMI-1640	5.3	5.3
RPMI-1640 + lipid-free serum	3.5	25
RPMI-1640 + bovine serum albumin and lecithin dispersion	3.5	34

[a] K_m was not determined but the activity was measured using a saturating concentration of substrate

in a buffer supplemented with lipid-free serum of with bovine serum albumin and lecithin dispersion involved a sterol loss from the cell, resulting in an induction of 3-hydroxy-3-methylglutaryl CoA reductase [17]. To see if this kind of induction modified the K_m of 3-hydroxy-3-methylglutaryl CoA reductase in human lymphocytes, we determined the K_m of the reductase in lymphocytes activated by preincubation in three different mediums. One medium, RPMI-1640, was a poor inductor of 3-hydroxy-3-methylglutaryl CoA reductase and the other ones, RPMI-1640 supplemented with 20% lipid-free serum or with 1.5% bovine serum albumin and lecithin dispersion, were efficient inductors. The results are shown in table 1 and figures 1a,b. Slight differences are observed between the K_m values (3.5 μM and 5.3 μM for the D-isomer).

These K_m values are in the range of the low value (1.12 μM) reported [4] but they contrast with the values usually reported (> 10 μM) [5–8]. Our assay conditions agree with [4] where deviations from linearity when substrate conversions exceeded 20% were reported. In fact, we observed linear relationships of mevalonate synthetized versus incubation

Fig.1a

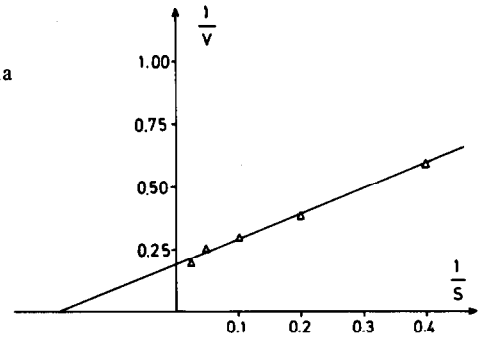

Fig.1b

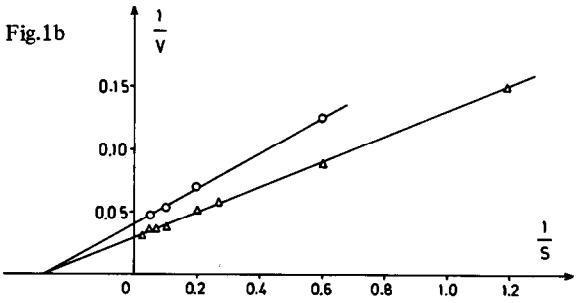

Fig.1. Lineweaver-Burk plot to determine K_m of the 3-hydroxy-3-methylglutaryl CoA reductase of human lymphocytes. V, pmol/min/mg protein. S, [D-3-hydroxy-3-methylglutaryl CoA] (μM). (1a) Cells were preincubated in RPMI-1640 for 16 h. (1b) Cells were preincubated for 16 h in RPMI-1640 supplemented with: (o——o) 20% lipid-free serum; (Δ——Δ) 1.5% bovine serum albumin and lecithin dispersion (95 μg lecithin/ml medium). DL-substrate spec. act. 7 mCi/mmol.

duration, and the rate of conversion was lower than 20% for substrate concentrations beyond 5 μM. For the two concentrations below 5 μM (fig.1b), the rate of conversion exceeded 20%, however the points fit perfectly on a linear Lineweaver-Burk plot. The slight difference between the K_m we obtained and those in [4] could be explained by a lower affinity of the lymphocyte enzyme compared with the hepatic enzyme.

In contrast with K_m, there was a drastic variation of V_{max} of 3-hydroxy-3-methylglutaryl CoA reductase when the lymphocytes were preincubated in the different culture mediums (table 1) which, according to their ability to pick up cholesterol, induced cholesterogenesis in the cells. Indeed, cholesterol assays made in the lymphocytes after incubation showed that the values were related to sterol loss: the more important the sterol loss, the higher V_{max} (table 2).

In these conditions, the stability of K_m seems to indicate that the active site working is not substantially modified and that changes in the membrane neighbourhood, allosteric activation or synthesis of a different form of enzyme are unlikely. If such is the case, the increase of 3-hydroxy-3-methylglutaryl CoA reductase could be related to enzyme 'de novo' synthesis.

The optimal conditions were so determined for 3-hydroxy-3-methylglutaryl CoA reductase assay in human lymphocytes: 40 μM DL-substrate; 200 μg protein; 40 min incubation. It will now be possible to study the variation of activity of this enzyme after induction or inhibition of the sterol synthesis in these cells.

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Table 2
Relationship between cellular cholesterol content and V_{max} of 3-hydroxy-3-methylglutaryl CoA reductase

Preincubation medium	Cholesterol (μg/3.10^7 cell)	V_{max} (pmol/min/mg protein)
RPMI-1640	1	28
+ lipid-free serum	4	21
	2.7	22
RPMI-1640	14	16
+ bovine serum albumin and lecithin dispersion	30	16
	38	4
RPMI-1640	42	1.5
	47	1.0

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