Some Properties and a Suggested Reclassification of Mevaldate Reductase

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Mevaldate reductase was purified 70-fold from rat liver. The partly purified enzyme had a molecular weight of 27000–30000, reduced certain aromatic aldehydes and was inhibited by barbiturates. These properties are similar to those of other animal tissue aldehyde reductases (EC 1.1.1.2) and it is suggested that mevaldate reductase be reclassified as one of this group.

The pathway of mevalonate biosynthesis in rat liver has now been fairly well established. The existence and subcellular localization of the enzymes between acetyl-CoA and mevalonate have been determined (White & Rudney, 1970) and the minimal physiological significance of the 'malonyl-CoA' pathway (Brodie *et al.*, 1962, 1963) in this tissue has been demonstrated (Fimognari & Rodwell, 1970; Higgins & Kekwick, 1973).

One problem still outstanding is the role of the enzyme mevaldate reductase in this pathway. This enzyme, present in relatively high amounts in the cytosol of rat liver, catalyses the reduction of mevaldic acid (I) to mevalonic acid (II) by using NADH or, preferably for the rat liver enzyme, NADPH. Both 3*R*- and 3*S*-mevaldate are reduced, although the 3*R*-form is the preferred substrate, giving rise to the naturally active 3*R*-mevalonate. (Knauss *et al.*, 1962)). The high activity of this enzyme caused early workers in the field to obtain good

incorporation of labelled mevaldic acid into non-saponifiable lipids in rat liver homogenates, which lead to the view that free mevaldate was an intermediate in sterol biosynthesis (Folkers *et al.*, 1959). However, it was later shown that mevalonate is formed by the direct reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (III) to mevalonic acid by the microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase and that free mevaldate is not an intermediate (Ferguson *et al.*, 1959; Brodie & Porter, 1960; Linn, 1967). However, mevaldate, as its hemithioacetal with coenzyme A (IV), occurs as an intermediate in the 3-hydroxy-3-methylglutaryl-coenzyme A reductase reaction, with the enzyme from both yeast and rat liver (Rétey *et al.*, 1970; Beedle *et al.*, 1972a).

The reaction catalysed by mevaldate reductase is formally similar to the reduction of compound (IV) by 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and it was considered possible that the former enzyme could be related to 3-hydroxy-3-methylglutaryl-coenzyme A reductase. However, the stereospecificity of the reaction is different; although both enzymes utilize the 4-*pro-R* H atom of NADPH (Donninger & Popják, 1966; Beedle *et al.*, 1972b) this H atom becomes the 5-*pro-R* atom of 3*R*-mevalonate produced by mevaldate reductase (Donninger & Popják, 1966) but becomes the 5-*pro-S* H atom of the 3*R*-mevalonate resulting from the reduction of mevaldate-coenzyme A hemithioacetal by rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Beedle *et al.*, 1972a).

It seemed appropriate therefore to look for another role for mevaldate reductase. A number of reports have appeared (Bosron & Prairie, 1973; Turner & Tipton, 1972a,b) characterizing aldehyde reductases present in animal tissues. These enzymes have been implicated in the degradation of biogenetic amines, and share the following characteristics: (i) ability to reduce aromatic aldehydes; (ii) low (approx. 30000) molecular weight; (iii) inhibition by barbiturates

(Bosron & Prairie, 1973). The purpose of the present work was to characterize 'mevaldate reductase' as an enzyme of this type, and to show that reduction of mevaldate is purely incidental.

Experimental

Materials

Mevaldic acid dimethyl acetal, *NN'*-dibenzyl-ethylenediammonium salt, was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. and the free acid was prepared for use by the method of Knauss *et al.* (1962). Pyridine-3-carboxaldehyde and 4-cyanobenzaldehyde were obtained from R. N. Emanuel Ltd., Wembley, Middx., U.K. The following were obtained from the Sigma (London) Chemical Co., London S.W.6, U.K.: NADPH, NADH, Nitro Blue Tetrazolium, phenazine methosulphate, bovine heart cytochrome *c*, pepsin (2×crystallized), yeast glucose 6-phosphate dehydrogenase, yeast alcohol dehydrogenase, Coomassie Brilliant Blue. Bovine serum albumin was from Pentex Inc., Kankakee, Ill., U.S.A. and yeast hexokinase from Boehringer Corp. (London) Ltd., London W.5., U.K. Sephadex G-200 and Blue Dextran were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. Rats were of the CFBH strain from Carworth (Europe) Ltd., Alconbury, Hunts., U.K.

Enzyme purification

All steps were performed at 0–2°C. The initial steps of the purification were modified from that of Knauss *et al.* (1962) as described by Popják (1969). Protein from the second $(\text{NH}_4)_2\text{SO}_4$ precipitation step was dissolved in 50mM-potassium phosphate buffer, pH6.5, containing 0.5mM-EDTA, and dialysed for 3h against 100vol. of the same buffer. The enzyme solution (131mg of protein) was concentrated to 5.6ml by vacuum dialysis and applied to a column (78.5cm×2.5cm) of Sephadex G-200. Elution in an upward-flow mode was at the rate of 8ml/h with 50mM-potassium phosphate buffer, pH6.5, containing 0.5mM-EDTA, and 5ml fractions were collected. Protein concentration (E_{280}) and the ability to reduce mevaldate and 4-cyanobenzaldehyde were measured for each fraction.

A symmetrical peak of activity was obtained between 280 and 320ml. The fractions showing high activity were pooled and concentrated by vacuum dialysis.

Enzyme assay

The standard assay contained, in a 1cm path-length cell: NADPH, 0.2μmol; substrate, 1.0μmol;

enzyme; 50mM-potassium phosphate buffer, pH6.5, containing 0.5mM-EDTA, to 1.0ml. The reaction was initiated by the addition of substrate and was followed by the decrease in E_{340} as measured in a Unicam SP1800 recording spectrophotometer. Cells and contents were maintained at 25°C. 4-Cyanobenzaldehyde was added in 10μl of ethanol, which was shown to have no effect on the reaction rate. Inhibitors were added in a volume of 0.1ml. Rates were linear for at least 4–5min.

Determination of protein

Protein was measured by the biuret method (Gornall *et al.*, 1949) with dry bovine serum albumin as standard or by absorption at 280nm in the later stages of purification (Warburg & Christian, 1941). Enzyme activities were measured as μmol of nicotinamide nucleotide oxidized/min. Specific activities are μmol/min per mg of protein.

Gel filtration

The column (78.5cm×2.5cm) of Sephadex G-200 was calibrated with the following proteins whose molecular weights were taken from the literature: bovine heart cytochrome *c* (13370); pepsin (36000); bovine serum albumin (67000); yeast hexokinase (99000, but dissociates at pH6.5 to units of 49500); yeast glucose 6-phosphate dehydrogenase (128000); yeast alcohol dehydrogenase (148000). Blue Dextran was used to determine the column void volume of 117ml. In all cases the column was eluted with the buffer previously described. Proteins were detected by absorption at 280nm and by enzyme activity where appropriate. A plot of the ratio of elution volume to void volume versus the logarithm of the molecular weight of the protein was used to determine the molecular weight of mevaldate reductase.

Polyacrylamide-disc-gel electrophoresis

Gels were run at 0–2°C, and contained 7.5% (w/v) acrylamide and 0.18% (w/v) methylenebisacrylamide. The gel buffer was 0.37M-Tris-chloride, pH9.6, at 0°C, and the electrode buffer was 0.005M-Tris with 0.038M-glycine (pH9.6 at 0°C). Chemical polymerization was used and the gels were then pre-electrophoresed for 2h at 8mA/gel to remove excess of ammonium persulphate. Approx. 50μg of protein in 10% glycerol was layered on top of each gel, then the sample was run toward the anode at 5mA/gel until the Bromophenol Blue tracking dye approached the bottom of the tube. Gels were stained for protein with 0.1% Coomassie Brilliant Blue in methanol-acetic acid-water (3:1:6, by vol.) for 6–12h, and then destained in 7.5% (v/v) acetic acid

for 24h. For detection of enzyme activity, each gel was incubated in 12ml of a solution containing 0.5mM-NADPH and 2mM-substrate (mevaldate or 4-cyanobenzaldehyde) in 50mM-potassium phosphate buffer (pH 6.5) with 0.5mM-EDTA. Incubations were for 60min at 20°C. Gels were then removed from this solution, rinsed with 50mM-Tris-chloride buffer (pH 8.5) and incubated for 60min at 38°C with 12ml of 50mM-Tris-chloride buffer (pH 8.5) containing 0.5mg of Nitro Blue Tetrazolium and 0.05mg of phenazine methosulphate/ml. Enzyme activity was revealed as a colourless band where the NADPH had been oxidized, the background of the gel being purple.

Results

Purification of mevaldate reductase

Mevaldate reductase was partially purified from rat liver essentially by the method of Knauss *et al.* (1962). The enzyme at this stage was able to reduce mevaldate and two of the aromatic aldehydes known to be substrates for aldehyde reductases, that is, 4-cyanobenzaldehyde and pyridine-3-carboxaldehyde. NADPH was the preferred cofactor, NADH giving only one-fifth the rate obtained with NADPH. However, the activities could be attributed to different proteins, and so the enzyme was further purified by gel filtration. The enzyme solution was placed on a column of Sephadex G-200 and eluted with 50mM-potassium phosphate buffer (pH 6.5) containing 0.5mM-EDTA. Mevaldate reductase activity was eluted in a symmetrical peak

Fig. 1. *Sephadex G-200 gel filtration of mevaldate reductase*

Enzyme (17.4 units; 131mg of protein) was applied to a column (78.5cm×2.5cm) of Sephadex G-200 and eluted with 50mM-potassium phosphate buffer, pH 6.5, containing 0.5mM-EDTA. Fractions (5ml) were collected and E_{280} (●—●) and activity with mevaldate (●---●) and with 4-cyanobenzaldehyde (■····■) measured for each fraction.

between 280 and 320ml. Reduction of 4-cyanobenzaldehyde also followed this peak (Fig. 1). Fractions showing high activity were pooled, concentrated by vacuum dialysis, and the protein content analysed by polyacrylamide-disc-gel electrophoresis. One major and several minor protein bands were observed (Fig. 2a). A summary of the purification is shown in Table 1. The enzyme was stored frozen in small portions at -20°C and was stable under these conditions for at least 3 months.

Molecular-weight determination

A portion of the enzyme was placed on the Sephadex G-200 column and its elution volume determined

Fig. 2. *Polyacrylamide-disc-gel electrophoresis of mevaldate reductase*

Protein (about 50µg) was placed on the gel and electrophoresed towards the anode. Conditions of electrophoresis and staining methods are described in the Experimental section. (a) Gel stained for protein with Coomassie Brilliant Blue; (b) gel stained for reductase activity by using mevaldate as substrate; (c) gel stained for reductase activity by using 4-cyanobenzaldehyde as substrate. The dotted line shows the position of the tracking dye. □, Clear; ■, blue (Coomassie Brilliant Blue); ■, purple (Nitro Blue Tetrazolium).

Table 1. *Purification of mevaldate reductase from rat liver*

Purification was achieved as described in the Experimental section. Enzyme activity was measured in the standard assay containing 1mM-3RS-mevaldate, 0.2mM-NADPH and 50mM-potassium phosphate buffer (pH6.5) containing 0.5mM-EDTA. Protein was measured by the biuret assay or by absorption at 280nm.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
105000g supernatant	190	4693	38.4	0.008	100
30–60% (NH ₄) ₂ SO ₄ fraction	55	1472	27.9	0.019	73
After calcium phosphate gel	68	371	23.0	0.062	60
(NH ₄) ₂ SO ₄ ppt.	11	131	17.4	0.132	45
After G-200 (peak fractions)	9	19	10.9	0.573	28

with 50mM-potassium phosphate buffer (pH6.5) containing 0.5mM-EDTA as eluent. Again, mevaldate reductase was eluted between 280 and 320ml. Elution volumes of calibration proteins were determined and Blue Dextran was used to determine the column void volume. The ratio of elution volume to void volume was calculated for each protein and plotted against the logarithm of the molecular weight. The molecular weight of mevaldate reductase was determined to be 27000–30000 from two experiments.

Polyacrylamide-disc-gel electrophoresis

In a further attempt to demonstrate that the reduction of both mevaldate and the aromatic aldehydes was due to the same protein, enzyme activity was measured after polyacrylamide-disc-gel electrophoresis. A two-stage detection procedure was used, based on that of Tarmy & Kaplan (1968). This consists essentially of incubation of the gel with substrate and NADPH, followed by incubation with phenazine methosulphate and Nitro Blue Tetrazolium. Enzyme activity is seen as a clear band on the purple background of the gel.

Separate gels were incubated with both mevaldate and 4-cyanobenzaldehyde as substrate, and another gel was stained for protein. The results are depicted in Fig. 2. It was found that the activity for reduction of mevaldate and 4-cyanobenzaldehyde, and the major protein band all coincided. The minor protein bands showed no activity; control gels incubated without substrate but with NADPH also showed no clear bands. Hence it would seem that the same protein is responsible for the reduction of both mevaldate and aromatic aldehydes.

Properties of the enzyme

The purified enzyme reduced mevaldate, pyridine-3-carboxyaldehyde and 4-cyanobenzaldehyde. No activity was seen with formaldehyde, acetaldehyde, or *n*-butyraldehyde, and neither was alcohol dehydrogenase activity present, since reduction of NAD⁺ in the presence of ethanol did not occur. The enzyme

Table 2. *Michaelis constants of mevaldate reductase*

K_m values for mevaldate and 4-cyanobenzaldehyde were determined in the presence of 0.2mM-NADPH, by using a range of substrate concentrations from 0.2 to 1mM. K_m values for NADPH were obtained by changing the NADPH concentration from 0.01 to 0.1mM in the presence of 5mM-mevaldate or 4-cyanobenzaldehyde.

Substrate	K_m value (μ M)
3RS-Mevaldate	260
4-Cyanobenzaldehyde	150
NADPH (with mevaldate)	9
NADPH (with 4-cyanobenzaldehyde)	10

was essentially irreversible because no NADPH production could be detected in the presence of high (10mM) concentrations of mevalonate and NADP⁺. With 1mM-substrate, the efficiency of substrate reduction was in the order 4-cyanobenzaldehyde > pyridine-3-carboxyaldehyde > mevaldate in the proportions 1.00: 0.43: 0.37. NADPH was the preferred cofactor, being oxidized five times faster than NADH when 1mM-3RS-mevaldate or 4-cyanobenzaldehyde were used as substrates. Michaelis constants were determined for 4-cyanobenzaldehyde, 3RS-mevaldate and NADPH (Table 2).

The similarity of the K_m value for NADPH when mevaldate or 4-cyanobenzaldehyde are reduced is further evidence that both reactions are due to a single protein.

Inhibitor studies

A characteristic feature of the aldehyde reductases mentioned above is inhibition by barbiturates. Studies with the enzyme described have also revealed this feature (Fig. 3). With mevaldate (1mM) as substrate, 1mM-sodium aminobarbitone gave 48% inhibition and 1mM-thiobarbituric acid gave 22% inhibition. The data of Fig. 3 suggest that the inhibition is non-competitive with respect to mevaldate.

Fig. 3. Inhibition of mevaldate reductase by sodium aminobarbitone

Rates of mevaldate reduction were determined in the presence of 0.2mM-NADPH and at substrate concentrations from 0.2 to 1mM. ●, 3RS-mevaldate; ■, 3RS-mevaldate in the presence of 1mM-sodium aminobarbitone.

Discussion

The results in the present paper demonstrate that the reduction of mevaldate is due to a protein that has many of the properties previously reported for aldehyde reductases. These properties include a molecular weight of around 30000, inhibition by barbiturates and the ability to reduce a number of aromatic aldehydes. Mevaldate reductase from rat liver has been studied by a number of workers, notably Nakamura & Greenberg (1960) and Knauss *et al.* (1962). Suggested functions have included its participation in an alternative pathway of terpenoid biosynthesis when the activity of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase pathway is decreased, and as a 'scavenging' enzyme to convert any mevaldate lost from the surface of 3-hydroxy-3-methylglutaryl-coenzyme A reductase into mevalonate. It is now suggested that reduction of mevaldate is probably not the biological function of this protein, but that it is an aldehyde reductase whose substrate specificity includes mevaldate. The enzyme reported here shares many of the characteristics of the low-molecular-weight (approx. 23000) aldehyde reductase reported in rat liver by Bosron & Prairie (1973) and, allowing for experimental variation in molecular-weight determination, may well be the same enzyme.

It can be argued that mevaldate reduction is the primary function of the protein and that the reduction of aromatic aldehydes is incidental. However, in view of the fact that significant production of free

mevaldate has never been reported in rat liver, that the direct reduction of 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate is well established and that the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase is generally rate-limiting for isoprenoid biosynthesis, this seems unlikely. The reported preference of 'mevaldate reductase' for the naturally occurring 3R-mevaldate must therefore be a consequence of arrangement of substrate-binding sites on the enzyme, and probably does not reflect the physiological function of the enzyme.

Reclassification of mevaldate reductase (EC 1.1.1.33) as aldehyde reductase (alcohol-NADP oxidoreductase, EC 1.1.1.2) is therefore suggested.

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