Partial Purification and Characterization of Membrane-Associated 3-Hydroxy-3-methylglutaryl-Coenzyme A Lyase from Radish Seedlings*

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We solubilized from radish membranes and purified by 154-fold 3-hydroxy-3-methylglutaryl-CoA lyase (HMGL, EC 4.1.3.4) catalyzing the conversion of 3-hydroxy-3-methylglutaryl-(HMG-CoA) into acetyl-CoA and acetoacetate. The apparent molecular mass under non-denaturing conditions is 70 kDa. The enzyme has a broad pH optimum around 8.0 and its activation energy as determined from the linear part of an Arrhenius plot is 137.1 kJ/mol. The $K_m$ with respect to (S)-HMG-CoA is 40 $\mu$M. The enzyme is extremely unstable and rapidly loses activity even when kept on ice, but retains some activity over several weeks when stored at $-80^\circ$C.

Introduction

HMG-CoA serves two functions: as the substrate for mevalonate biosynthesis, catalyzed by HMG-CoA reductase (HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, mevalonate: NADP$^+$ oxidoreductase, CoA acylating, EC 1.1.1.34) and as a putative intermediate in the degradation of branched-chain amino acids via HMG-CoA lyase (HMGL, (S)-3-hydroxy-3-methylglutaryl coenzyme A lyase, EC 4.1.3.4). While the synthesis and degradation of HMG-CoA in mammals and yeast has been well examined and documented, only little was known until recently as to the situation in plants ([1 – 3] and literature cited therein). Hepper and Audley [4] provided some evidence for the existence of HMGL in the latex of *Euphorbia lathyris* [6]. At least in part, the presence of HMGL in latex would explain the failure of detecting any significant incorporation of acetyl-CoA, HMG-acid or of HMG-CoA into tri-terpenoids [7]. Nevertheless, the exact physiological role HMGL plays in the plant cell remains obscure at present. As yet there is no clear data available that would indicate the presence of a so-called HMG-CoA cycle and ketone body formation as described for vertebrate systems [8 – 10]. However, besides being involved in leucine catabolism, the enzyme might also participate in the process of shunting carbon units away from their inclusion into the isoprenoid pathway via the trans-methylglutaryl-CoA or mevalonate shunt mechanism as originally envisaged by Popjäk [11]. The existence of this shunt, which combines the synthetic pathways leading to fatty acids and to isoprenoids with the catabolism of leucine, has been demonstrated in vertebrates (see ref. [12] for review of literature), in insects [13] and in wheat seedlings, as demonstrated by incorporation of $[2-^3]H$mevalonate, but not of $[5-^3]H$mevalonate into long-chain fatty alcohols [14]. Tritium from $[2-^3]H$mevalonate, if routed through the mevalonate shunt, must appear in the acetoacetate fragment of the *Hevea brasiliensis*, Yu-Ito et al. [5] described an enzyme activity, most likely HMGL, that interfered with the HMGR assay in crude fractions evidently enriched in mitochondria. HMGL activity has also been found in the soluble fraction of latex collected from *Euphorbia lathyris* [6]. At least in part, the presence of HMGL in latex would explain the failure of detecting any significant incorporation of acetyl-CoA, HMG-acid or of HMG-CoA into tri-terpenoids [7]. Nevertheless, the exact physiological role HMGL plays in the plant cell remains obscure at present. As yet there is no clear data available that would indicate the presence of a so-called HMG-CoA cycle and ketone body formation as described for vertebrate systems [8 – 10]. However, besides being involved in leucine catabolism, the enzyme might also participate in the process of shunting carbon units away from their inclusion into the isoprenoid pathway via the trans-methylglutaryl-CoA or mevalonate shunt mechanism as originally envisaged by Popjäk [11]. The existence of this shunt, which combines the synthetic pathways leading to fatty acids and to isoprenoids with the catabolism of leucine, has been demonstrated in vertebrates (see ref. [12] for review of literature), in insects [13] and in wheat seedlings, as demonstrated by incorporation of $[2-^3]H$mevalonate, but not of $[5-^3]H$mevalonate into long-chain fatty alcohols [14]. Tritium from $[2-^3]H$mevalonate, if routed through the mevalonate shunt, must appear in the acetoacetate fragment of the

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Abbreviations: AACT, acetoacetyl-coenzyme A thiolase; BrIJ, polyoxyethylene ether W-1; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMGL, HMG-CoA lyase; HMGR, HMG-CoA reductase; HMGs, HMG-CoA synthase; PQQ, pyrroloquinoline quinone; PVP, polyvinylpolypyrrolidone.

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HMGL-catalyzed cleavage of HMG-CoA. Because the label appeared in the long-chain fatty alcohols, the acetoacetate had to be used as a precursor of fatty acid biosynthesis, whether through conversion to acetate or not remains uncertain.

Over the past several years we have been interested in purifying and characterizing the enzymes involved in the conversion of acetyl-CoA to HMG-CoA [2, 3, 15, 16], the NADPH-dependent two-step reduction of HM G-CoA to mevalonate as catalyzed by HMGR [17], and HMG-CoA cleavage to acetoacetate and acetyl-CoA catalyzed by HMGL [2, 3]. This communication reports on the partial purification and characterization of a membrane-associated enzyme from 4-day-old etiolated radish seedlings (Raphanus sativus L.), capable of converting HMG-CoA into acetyl-CoA and acetoacetate.

**Experimental Procedures**

**Materials**

The sources of chemicals and of radiochemicals have been described [3, 17].

**Isolation of membranes**

Radish seedlings were grown in the dark and membranes (P 16,000 x g) were isolated as described [17]. In brief, 300 g of intact seedlings were homogenized portionwise by the aid of a Waring blender in ice-cold buffer A (200 mM K₃PO₄ pH 7.5, 350 mM sorbitol, 10 mM Na₂EDTA, 5 mM MgCl₂; before use this buffer system was supplied with 3 mM dithioerythritol and 5 g/100 ml insoluble polyvinylpyrrolidone) by three strokes of 5 s. The homogenate was squeezed through 15 layers of cheese cloth. The resulting homogenate was then centrifuged for 10 min at 2000 x g to remove cell debris and PVP particles (Hermle centrifuge type ZK 400, rotor A 6.14, 4 °C). The supernatant was immediately decanted and the pellet was washed three times with buffer B additionally containing 0.3% Brij W-1 (buffer C). The washed sediment was homogenized and redissolved in 10 to 15 ml buffer D (like C, but containing only 3 mM dithioerythritol (cf. [3]). Insoluble proteins were removed by centrifugation (5 min, 5000 x g, 2 - 4 °C). Preparations thus obtained remained unstable and had to be immediately further purified by column chromatography.

**Column chromatography**

All steps were performed with a HPLC/FPLC system (Pharmacia-LKB).

a) *Gel filtration:* A column type 16/70 (Pharmacia, volume 110 ml) was used, filled with Superose 6 prep. grade (max. loading volume 10 ml). The column was washed with a double void volume of buffer D. After stabilization of the base line at 280 nm, resuspended acetone precipitate (usually 5.5 ml when working on a preparative scale, 2 ml on a analytical scale) were loaded at a flow rate of 0.75 ml/min. The column was eluted under the same conditions. The molecular mass was determined by comparison to appropriate molecular weight standards.

b) *Anion exchange chromatography:* The column (type 150/10, Merck, Darmstadt, volume 10 ml, filled with Fractogel EMD TMAE 650, Merck) was firstly cleaned up by a passage of 10 ml 1.5 M KCl, followed by equilibration with >60 ml of buffer D. After stabilization of the base-line of absorption (280 nm), up to 12 ml of the pooled active...
fractions from the gel filtration were loaded at a flow rate of 0.5 ml/min. After 24 min (end of loading phase) the flow rate was increased to 1.5 ml/min and the column was washed for an additional 20 min with buffer system D. Proteins were eluted by a gradient of 0 to 1 M KCl in buffer D over 30 min. HMGL activity started to elute at >600 mM KCl.

**Enzyme assays**

a) **HMG-CoA lyase**: The enzyme assay was based on a method developed by Clinkenbeard et al. [10] and was modified as described [2, 3]. In brief: 25 μl enzyme solution were added to 15 μl of a mix containing 14 μl 200 mM Tris-HCl pH 8 and 1 μl 1 M MgCl₂ in H₂O. The reaction was started by addition of 10 μl of a solution consisting of 2 μl (R,S)-[3-¹⁴C]HMG-CoA in 50 mM K₃PO₄, pH 4.5 (ca. 40,000 dpm, 8 μM final concentration in the assay (only 50% of this synthetic substrate can be used up by HMGL, due to its exclusive stereospecificity towards (S)-HMG-CoA), and of 8 μl 200 mM Tris-HCl pH 8. After up to 20 min of incubation the reaction was stopped by addition of 125 μl 6 N HCl. In kinetic experiments the incubation was shortened to 2 min, and substrate concentrations were varied as appropriate (isotope dilution). After transfer to scintillation minivials, the samples were heated to 110 °C for 3 h. Under these conditions thioesters are hydrolyzed, enzymatically formed [¹⁴C]acetoacetate is volatile, and only unreacted HMG-acid remains in the vials (see ref. [3] for further details). To redissolve the HMG-acid 0.25 ml of water was added followed by incubation at RT for 10 min. After addition of 4 ml scintillation cocktail (Aquasafe, Zinsser, Frankfurt) the samples were vigorously vortexed. After 10 min radioactivity was determined by the aid of a liquid scintillation counter type 2000 CA (Packard) with automatic quench correction by the external standard method. Appropriate blanks were run in the absence of enzyme protein. Kinetic data were calculated and plotted as described [17, 18].

b) **AACT/HMG-S**: Enzyme activity was assayed radiochemically as described in detail [3] but additionally in the presence of 10 μM PQQ [16].

c) **HMG-R**: Activity was radiochemically determined as described in detail [17].

**Protein determination**: Protein was determined by a modified Lowry method [19] with some modifications [17].

**Results**

**Enzyme purification**

We developed a purification protocol for membrane-associated HMGL. The purification factor reached is 154, with a specific activity of the protein of 9.8 nmol mg⁻¹ min⁻¹ (standard assay conditions) (Table I).

The main steps of this purification protocol consist of a) acetone precipitation and b) anion exchange chromatography. The enzyme was revealed to be very sensitive and rapidly lost activity even when kept on ice. Among a number of methods tested [2, 3, 15], the introduction of a precipitation step with cold acetone was a good possibility when the enzyme without a complete or nearly complete loss in activity. Anion exchange chromatography on Fractogel EMD TMAE 650 (Fig. 1) reliably removed an enzyme system termed AACT/HMG-S [2, 3, 16], which catalyzes the conversion of acetyl-CoA to HMG-CoA and which might interfere with HMGL activity. The preceding gel filtration step where HMGL eluted at an apparent molecular mass of 70 kDa and by which the separation from AACT/HMG-S (having a molecular mass of about 56 kDa) is incomplete (data not shown), led to some activation of the protein, possibly due to the removal of traces of acetone remaining from the precipitation step.

**Table I. Purification of HMGL from a heavy-membrane pellet isolated from 4-day-old etiolated radish seedlings.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein [mg]</th>
<th>Specific activity [nmol mg⁻¹ min⁻¹]</th>
<th>Purification factor</th>
<th>Yield [%]</th>
<th>Total activity [nmol min⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 16,000 x g</td>
<td>552</td>
<td>0.064</td>
<td>1</td>
<td>100</td>
<td>32</td>
</tr>
<tr>
<td>P 16,000 x g + Brij</td>
<td>303</td>
<td>0.17</td>
<td>2.7</td>
<td>164</td>
<td>52</td>
</tr>
<tr>
<td>Acetone precipitate</td>
<td>136</td>
<td>0.24</td>
<td>3.8</td>
<td>103</td>
<td>33</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>3.6</td>
<td>3.6</td>
<td>59.7</td>
<td>45</td>
<td>14</td>
</tr>
<tr>
<td>Anion exchange</td>
<td>0.4</td>
<td>9.8</td>
<td>154</td>
<td>13</td>
<td>4.1</td>
</tr>
</tbody>
</table>
Validity of the assay method

We have shown earlier, even with rather crude HMGL preparations, that the $[^{14}C]$-labeled product of incubations with $(R,S)$-$[^{3,14}C]$HMG-CoA, was volatile in the presence of HCl, while remaining radioactivity indicated the presence of unreacted substrate. Maximum 50% of the initial radioactivity could be used up since the enzyme reacts exclusively with its natural enantiomer $(S)$-HMG-CoA [19a, 20]. Utilization of $(S)$-HMG-CoA was also demonstrated by the concomitant loss in the NADPH-dependent conversion of $(R,S)$-HMG-CoA to mevalonate if aliquots of the reaction mixture were further incubated in the presence of HMGR either purified from yeast or from *E. coli* overexpressing radish HMGR1 [21] (data not shown).

Cofactor requirements

Studies on the requirement of cofactors, e.g. to overcome disactivation effects during the development of purification protocols (cf. [2]), revealed that for maximum in vitro activity the enzyme needs bivalent cations (Bach *et al.*, 1990). The assay conditions had been modified accordingly. This partial dependence on bivalent cations is one of the reasons why interference of HMGL with the assay of HMGR can be precluded by addition of EDTA.

Temperature dependence

The enzyme has a temperature optimum of 30 °C and is rapidly inactivated above 30 °C (Fig. 2a). The apparent activation energy as determined from the linearly ascending part of an Arrhenius plot is 137.1 kJ/mol (Fig. 2b). This temperature-sensitivity rendered the detergent-solu-
bibilization method at 37 °C unacceptable that was developed for radish HMGR occurring in the same membrane fraction [17]. In contrast to HMGL, HMGR is rather heat-stable [2, 17]. This holds also true for other plants such as maize [17] or tobacco [22], where a preincubation in the presence of detergent (Brij W-1) at 37 °C leads to a nearly complete solubilization of HMGR activity, this under considerable activation, while HMGL rapidly loses activity.

Kinetic properties

For the determination of the $K_m$ with respect to HMG-CoA substrate concentrations were varied as was appropriate (isotope dilution). Incubation time was 2 to 10 min (see Fig. 3). Especially under condition of longer incubation times, a considerable proportion of substrate will be used up by the enzyme, which requires the application of correction factors derived from the integrated Michaelis-Menten equation [17, 18], based on a approximation procedure as originally proposed by Lee and Wilson [23] and further refined by Glick et al. [24]. By this correction method inflation of $K_m$ values can be avoided since average substrate concentrations during the time-stop assay are calculated. The enzyme assay conditions were designed in such a way that a concentration of product that should have led to a significant inhibition was most likely never achieved. Thus, such an effect was neglected when the value of 40 μM with respect to (S)-HMG-CoA was calculated. The pH optimum we determined was around 8.0 (Fig. 4), which is slightly lower than found earlier with a crude preparation from radish seedlings [2].

Discussion

In our initial attempts to characterize HMGL we used a radioassay method adopted from Clinkenbeard et al. [10], which was then further optimized. Optical assay methods for HMGL exist (see e.g. [25]), but especially with crude preparations of plant proteins proved to be too unreliable. The establishment of purification protocols was hampered by the extreme instability of radish HMGL. For example, if only the ion exchange purification step preceded the gel filtration, practically all HMGL activity was lost. The apparent molecular mass of radish HMGL as determined by non-denaturing gel filtration on various materials [2] was approximately 70 kDa. For the purified avian enzyme a molecular mass of 48 kDa [26] and for the bovine liver HMGL of 49 kDa was determined, with the enzyme consisting of two identical subunits [27]. Meanwhile sequences encoding avian and human HMGLs have been characterized [28]. The bacterial enzyme, isolated from Pseudomonas mevalonii, an organism which uses mevalonic acid as the only carbon source and where HMGL, in conjunction with NAD-dependent HMGR, has a catabolic function [20, 29], has a mass of 31608 Da [30].
For the purification of plant HMGL we had also tried free-solution isoelectric focussing [2]. By this method the enzyme solubilized from membranes of radish and of maize was reasonably well separated from AACT/HMGS and HMGR, and was concentrated at a pH of 6.8 to 7, most likely indicating a similar value of its pI, a value not too far away from the pI of 6.1 found for avian HMGL [10].

The affinity of partially purified radish HMGL towards (S)-HMG-CoA is slightly lower than found for the corresponding vertebrate enzymes, but within the same order of magnitude. A pH optimum of 8 or more is also characteristic of all HMGLs studied so far (see ref. [31] for literature). The elegant studies of Hruz et al. [31] using bacterial HMGL and the affinity labeling agent 2-butynoyl-CoA, suggest that a deprotonated active-site cysteine serves as a base for catalysis, which might be formed under basic conditions. However, the stability of coenzyme A esters at pH values above 8.0 is drastically diminished. Therefore, during a stop-time assay as we use here, measurements could be prone to artefacts due to an elevated pH at which coenzyme A thioesters tend to be hydrolyzed.

Higgins et al. [32] compared the data obtained with enzyme from several mammalian tissues and assumed the enzyme to contain firmly bound Ca²⁺, which was not affected by chelators above pH 8. Our previous observations indicate a requirement of bivalent ions for plant HMGL that is not absolute [2]. Pseudomonas mevalonii HMGL, that had been expressed in Escherichia coli and purified to homogeneity, was stimulated by Mg²⁺, but much more specifically by Mn²⁺ [33]. Atomic absorption and EPR analyses indicate there the presence of a tightly bound type II copper, most likely with nitrogen ligands [33].

The membrane/organelle fraction used to isolate HMGL from radish is also a major source of AACT/HMGS, catalyzing the Fe(II)/quinone-stimulated double condensation of acetyl-CoA to HMG-CoA [16, 34]. The presence of HMGL, although less active than AACT/HMGS when measured in vitro (cf. Fig. 1), could indicate that in plants this enzyme plays a role in a ketogenic cycle, similar or identical to that one described for mammalian systems [8–10]. The intracellular compartmentation of such intervening and/or scavenging pathways in plants is not yet known in detail, nor is their regulation. The occurrence of a key enzyme of the mevalonate shunt or of the degradation pathway of leucine, biotin-containing methylcrotonyl-CoA carboxylase in mitochondria of green pea has been recently shown [35]. In contrast to AACT/HMGS, and especially to HMGR, repeated washing with buffers in the absence of detergent and centrifugation of membrane pellets resulted in a considerable enrichment of HMGL activity in the supernatant, which might indicate its being soluble but included in vesicles or organelles [2].

For the occurrence of a soluble HMGL in homogenates of radish seedlings represents an isozymic form or is simply the product of a rather harsh disruption of tissue and of organelles [15] remains to be investigated. So far the extreme instability of HMGL has prevented a further substantial purification of the enzyme found in the soluble protein fraction, but it is hoped that application of recent findings using bacterial HMGL [33] might help in increasing the stability of plant HMGL.

Our current attempts are thus directed towards further purifying radish (and maize) HMGL for a close molecular characterization and with the aim of cloning the corresponding gene(s).

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