Aspects Related to Mevalonate Biosynthesis in Plants

Thomas J. Bach1,*, Albert Bonora2, Carme Caelles2, Albert Ferrer2, Thomas Weber2 and Annette Wetstein2

1Botanisches Institut (Pflanzenphysiologie und Pflanzenbiochemie), Universität Karlsruhe, D-7500 Karlsruhe 1, Germany and
2Universitat de Barcelona, Facultat de Farmacia, Unitat de Bioquimica, E-08028 Barcelona, Spain

We purified and characterized a membrane-associated enzyme system from radish (Raphanus sativus L.) that is capable of converting acetyl-CoA into 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA). The enzyme system apparently comprises acetoacetyl-CoA thiolase (EC 2.3.1.9) and HMG-CoA synthase (EC 4.1.3.3). Its activity in vitro can be strongly stimulated by Fe2+. When ferrous ions are applied chelated with ethylenediaminetetraacetate, citrate or adenosine 5'-triphosphate (ATP), the stimulation is further increased. Stimulation is due to a higher catalytic efficiency as indicated by an increase in $V_{max}$, whereas the affinity of the enzyme towards acetyl-CoA remains constant ($K_{m} = 6 \mu M$). A considerable portion of HMG-CoA lyase activity is associated with the same membranes. HMG-CoA lyase (EC 4.1.3.4) is also solubilized and partially co-purified. Its activity requires comparatively high concentrations of Mg2+. The conversion of HMG-CoA to mevalonic acid is catalyzed by HMG-CoA reductase (EC 1.1.1.34) that is associated with the same membranes. By cDNA encoding the Arabidopsis HMG-CoA reductase, we isolated a corresponding gene from a cDNA library newly established from etiolated radish seedlings. This full-length cDNA, referred to as acRS3, encodes a polypeptide of 583 amino acids with a molecular mass of about 63 kDa. The hydropathy profile suggests the presence of two hydrophobic membrane-spanning domains within the N-terminal 165 amino acids. The carboxy-terminal part, where the catalytic site resides, is highly conserved in all eukaryotic HMG-CoA reductase genes sequenced so far. Lipids 26, 637–648 (1991).

Plants are capable of synthesizing a myriad of isoprenoid derivatives which are biochemically functional in cells. The basic building block of all these compounds, despite their variety in structure and intracellular location, is derived from mevalonic acid (MVA, see refs. 1–3 for literature). The synthesis of MVA, which is generally considered the first step in isoprenoid biosynthesis, is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMGR, (R)-mevalonate:NADP+ oxidoreductase (CoA-acylating), EC 1.1.1.34]. This particular enzyme has been the subject of a great number of studies, mainly because of its key role in the regulation of cholesterol biosynthesis (4,5). HMGR has recently become a target in the treatment of hypercholesterolemia, a prevalent risk factor of cardiovascular disease in Western societies (6–12).

In plants, HMGR is apparently regulated by various environmental factors and stimuli, including light (13–16), growth regulators and phytohormones (16–18), as well as herbicides (19). Regulation by feedback mechanisms (17), by wounding and infection with pathogens or by fungal elicitor treatment (20–25), and by Hg (26) have also been reported. The cell-free synthesis of MVA has been demonstrated in a variety of plant species and in tissue cultures (1,3). Generally, the reaction is catalyzed by a membrane-bound enzyme. Recently, the solubilization of HMGR from the membranes either by detergent treatment (3,27–29) or by partial trypsinization (30) and subsequent purification (32–30) was shown to be possible. The data available on plant HMGR suggest its occurrence in plastids and mitochondria, in addition to the cytoplasm. However, this model, originally based on incorporation experiments (31), is still a matter of debate (32). The model appears difficult to prove due to the low activity and the susceptibility of HMGR towards rapid inactivation in vitro. However, the results from inhibition studies (1,3,2,33–35) strongly support the existence of HMGR isozymes in plant cells. Further evidence in favor of this hypothesis can be gathered using the powerful techniques of molecular biology and immunology. Genes encoding HMGRs from several plants have been characterized (36–40); some of the latter findings will be summarized and discussed in this contribution.

Another problem to be addressed is how HMGR-CoA synthesis is regulated in plants. HMG-CoA plays a pivotal role not only as a precursor of the isoprenoid pathway, but it additionally serves as a putative intermediate in the degradation of branched-chain amino acids. Until recently, surprisingly little had been known about the properties as well as the intracellular localization of the enzymes involved (cf. 2,3). The synthesis of HMG-CoA from acetyl-CoA units comprises: i) a Claisen-type condensation that is catalyzed by acetoacetyl-coenzyme A thiolase (AACT, acetoacetyl-CoA acetyltransferase, EC 2.3.1.9); and ii) a subsequent aldol condensation catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A synthase [HMGS, (S)-3-hydroxy-3-methylglutaryl-Coenzyme A:acetoacetyl-coenzyme A lyase (CoA acylating), EC 4.1.3.5]. The presence of HMGS was largely inferred from the formation of an uncharacterized HMG-derivative from acetate in a cell-free extract of flax seedlings (41), the same plant tissue from which HMG-acid had first been isolated (42). Lynen (43) determined the activity of several enzymes of the rubber biosynthetic pathway in the latex of Hevea brasiliensis, among them AACT and HMGS; their properties were not investigated further. The synthesis of a HMG-derivative, most likely HMG-CoA, from acetyl-CoA

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*To whom correspondence should be addressed at: Universitat Karlsruhe, Botanik II, Kaiserstrasse 12, D-7500 Karlsruhe 1, Germany.

Abbreviations: AACT, acetoacetyl-CoA thiolase; ATP, adenosine 5'-triphosphate; Brij W-1, polyoxyethylene ether Brij W-1; CoA, coenzyme A; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetate; FPLC, fast-performance liquid chromatography; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMG, HMG-CoA lyase; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; HPLC, high-performance liquid chromatography; MVA, mevalonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
in a cell-free system of sweet potato was reported (44). This biosynthetic capacity was found in cytosolic and microsomal preparations and did not require co-factors such as ATP, Mg$^{2+}$, HCO$_3^-$ or NADPH (44). An iodoacetamide-sensitive AACT activity has been identified in vesicles from mature orange fruits (45); attempts to assay HMGS and HMGR activities were not successful. Quite recently, AACT activity has been detected in plastid preparations from Parthenium argentatum and Phaseolus radiatus (46). The presence of HMGS activity in osmophores of the orchid Stanhopea anfracta was detected by a cytochemical approach (47). Our experience with cytosolic and membrane-associated AACT/HMGS activity (3,48) support the view of a variable intracellular location of these enzymes.

Once synthesized, HMG-CoA instead of entering the isopenid pathway can be cleaved by HMG-CoA lyase (HMGL, (S)-3-hydroxy-3-methylglutaryl coenzyme A lyase, EC 4.1.3.4) in a stereocchemically controlled Claisen-type retrocondensation to yield acetoacetate and acetyl-CoA (49). This important reaction contributes to the formation of ketone bodies in vertebrate cells (50,51). Indeed, Hepper and Audley (52) provided some evidence for the presence of this enzyme activity in the latex of the rubber tree. An enzyme activity that interferes with the HMGR assay in membrane preparations from Ipomoea batatas, most probably HMGL, was also reported (53). Skrukrud et al. (54) have assayed HMGL in the soluble fraction of latex collected from Euphorbia lathyris. The presence of HMGL in latex explains, in part, the failure to detect any significant incorporation of acetyl-CoA, HMG-acid or of HMG-CoA into triterpenoids (55). HMGL might also be involved in the MVA shunt pathway (56,57), a cyclic retroconversion of MVA to HMG-CoA and a mechanism of routing away isoprene-units from their inclusion into the isopenid pathway. Convincing evidence has been presented of the MVA shunt existing in plants (58,59). We have started to characterize from plant tissue the enzymes that are involved in the synthesis and metabolism of HMG-CoA (3,48). Some improvements in the purification protocols and further in vitro-properties of these enzyme systems are reported here.

MATERIALS AND METHODS

**Chemicals.** [1-$^{14}$C]Acetyl-CoA, (R,S)-[3-$^{14}$C]HMG-CoA, [2-$^{3}$H]mevalonic acid and [$^{35}$P]GTP were purchased from Amersham (Braunschweig, Germany); [$^{35}$S]ATP was from New England Nuclear (Bad Homburg, Germany). Brij W-1 (polyoxyethylene ether), HMG-CoA, acetyl-CoA and molecular weight standards were from Sigma (Deisenhofen, Germany); HS-CoA, NADPH, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and a DNA random priming kit were from Boehringer (Mannheim, Germany). Superose 6 and oligo(dT) cellulose were from Pharmacia (Freiburg, Germany); Cellufine GC 200 and GC 700 were from Amicon (Witten, Germany). Fractogel materials (anion exchange and affinity chromatography) were purchased from Merck (Darmstadt, Germany); sorbitol and DTE from Serva (Heidelberg, Germany); and scintillation vials plus cocktails from Zinsser (Frankfurt, Germany). The sources of materials used for experiments in the cloning and expression of plant HMGR have been described elsewhere (38,60). All other chemicals purchased from commercial sources were of the highest purity available.

**Plant cultivation and membrane isolation.** Radish (Raphanus sativus, var. Saxa Knacker) seedlings were grown in tap water in complete darkness for four days. Membrane fractions (P 16,000 and P 140,000) and cytosol were isolated as described (16). Membranes were resuspended in small volumes of isolation buffer “A” (28). DTE was added to the suspended membranes to a final concentration of at least 20 mM. The suspensions were kept on ice until use or immediately deep-frozen and stored at $-20^\circ$C. The enzyme activities determined remained stable under these conditions.

**Enzyme assays.** The assay of HMGR activity has been described in detail (28).

Assay of HMG-CoA synthesis from acetyl-CoA (coupled assay of AACT and HMGS) using a crude enzyme preparation. Fifteen µL Tris/HCl [pH 7.5, 0.2 M, containing 10 mM ethylenediaminetetracetate (EDTA)] was mixed with 25 µL of enzyme solution (in 0.2 M K$_2$PO$_4$, 10 mM EDTA, 5 mM MgCl$_2$, 20 mM DTE, supplemented with 1% w/v Brij W-1) and kept on ice. After preincubation at 30°C for 5 min the reaction was started by addition of a 10 µL mixture containing 1.25 µL [1-$^{14}$C]acetyl-CoA (10 µCi/µL, specific activity 52 mCi/mmol), 1.2 µL acetyl-CoA (2 mM), and 7.75 µL 0.2 M Tris/HCl, pH 7.5, containing 10 mM Na$_2$ EDTA. The incubation at 30°C was continued for 10 min and the reaction was terminated by addition of 150 µL of 6N HCl. After incubation at 30°C for 30 min the reaction mixture was transferred into plastic scintillation vials (5-mL) that were kept at 105°C for more than 5 hr. Under such conditions, thioesters are hydrolyzed, acetate evaporates, and HMG acid remains in the vial (51,61). In order to avoid any contamination, the samples were evaporated in a closed metal box inside a drying oven. Vapors from the box were continuously removed by pumping and the radioactive material was trapped by passage through three wash bottles containing water, 1N NaOH and paraffin. Water (250 µL) was then added to the vials, and the samples were vigorously vortexed. Quickzint 2000 (4 mL) was added, and after vortexing the samples were assayed for radioactivity by liquid scintillation counting (Packard 2000 CA, Frankfurt, Germany). For time course and kinetic studies the enzyme assay volume was increased to 150 µL. At various time points, 50 µL aliquots of the reaction mixture were withdrawn, transferred to 150 µL 6N HCl and treated further as described above. The data were computed using spline functions to calculate best fit curves (62), which allowed calculation of approximate $V_\text{m}$ values. In kinetic studies, substrate concentrations were varied as appropriate (substrate dilution, cf. 16,28) and data were plotted as described (16,28). When the separation of AACT and HMGS activities was attempted, assays were run in duplicate, either in the presence or in the absence of 50 µM acetoacetyl-CoA. HMGS activity in fractions not containing AACT should be measurable only in the presence of acetoacetyl-CoA.
[1-14C]acetyl-CoA (10 µCi/mL, 52 mCi/mmol), 0.6 µL acetyl-CoA (2 mM), and 8.2 µL 0.2 M Tris/HCl pH 7.5. Further processing of the assay mixtures was as described above. When the effects of various cations on the activity of AACT/HMGS were studied, the cation tested (if required, complexed in the presence of a double concentration of EDTA) was first added to the Tris buffer, immediately followed by addition of the enzyme solution.

**Assay of HMGL activity.** The standard assay system in a final volume of 50 µL contained 20 µL of enzyme solution, and 20 µL of 0.2 M Tris/HCl, buffer pH 7.5-8.0, supplied with 40 mM MgCl₂. After preincubation for 2 min at 30°C the reaction was started by addition of 10 µL of starting mixture: 1.25 µL (R,S)-[3-14C]HMG-CoA (ca. 30,000 dpm, specific activity 52 mCi/mmol), 0.6 µL unlabeled (R,S)-HMG-CoA (4 mM stock solution in 50 mM KH₂PO₄), and 8.15 µL of 200 mM Tris/HCl pH 7.5. The final concentration of (R,S)-HMG-CoA thus obtained was ca. 50 µM. After incubation at 30°C the reaction was stopped by addition of 150 µL 6N HCl. The mixture was transferred to minivials and kept at 115°C for a minimum of 3-4 hr. Labeled acetoacetate evaporated and non-volatile HMG-CoA remained in the vial (51, 61). Further processing of samples was as described for the AACT/HMGS assay. Because HMGL cleaves only the (S)-isomer of synthetic HMG-CoA (49), a maximum of 50% of the initial radiolabeled material was used up by the enzyme.

**Enzyme solubilization and purification.** AACT/HMGS activity was solubilized from a heavy-membrane pellet (P 16,000) by thawing of frozen samples followed by incubation at 30°C for 30 min in the presence of 2% (w/v) Brij W-1. The turbid yellow suspension was homogenized by aid of a teflon homogenizer (15 to 20 strokes) and centrifuged at 100,000 × g for 45 min at 15°C. In the supernatant 80-90% of the initial enzyme activity was found. The clear solution was brought to a final concentration of higher than 10 mM with DTE. A 1.5-fold volume of 3-4 hr. cold acetone was added. After incubation on ice for 10 min, the turbid solution was centrifuged for 10 min at 10,000 × g (2°C). The resulting pellet was washed three times with cold acetone and resuspended in a buffer system consisting of 50 mM K₂PO₄ pH 7.5, 0.3 M sorbitol, 5 mM EDTA, 5 mM MgCl₂ and 0.3% Brij W-1, 3 mM DTE (buffer system "D") (28). The volume of resuspension buffer was 50% of the enzyme solution used for acetone precipitation. Material that could not be redissolved by 15 strokes with a teflon homogenizer was removed by centrifugation for 5 min at 5,000 × g. After this step about 80% of the initial enzyme activity was found in the supernatant.

**Gel filtration.** Superose 6 prep grade (90 cm²) in an HR 16/50 column (Pharmacia-LKB, Freiburg, Germany) was equilibrated overnight with buffer system "D" with a biocompatible high-performance liquid chromatography (HPLC) system (Pharmacia-LKB) at a flow rate of 0.1 mL/min. Partially purified enzyme (3.3 mL, resuspended in buffer D) was loaded on the column and elution was continued with buffer D (0.75 mL/min). The fractions collected (2.25 mL) were tested for AACT/HMGS activity as described above. The column was calibrated with standard proteins with molecular masses from 29 to 150 kDa.

**Anion exchange chromatography.** Fractogel EMD TMAE 650 S (11 cm²) in a Superformance glass column (Merck, Darmstadt, Germany) was equilibrated with 50 mL of buffer D at a flow rate of 1.5 mL/min by using the same HPLC system. The three peak fractions from the gel filtration with highest apparent AACT/HMGS activity were pooled, and 6.6 mL were loaded on the column. The column was washed with the same buffer system. AACT/HMGS activity eluted with the breakthrough fraction.

**Determination of molecular weight by classical gel filtration.** Cellufine 200 (or 700, 130 cm³) in a C 18/70 column (Pharmacia-LKB) was equilibrated with 200 mL of modified isolation buffer A (0.05 M K₂PO₄, 0.35 M sorbitol, 10 mM Na₂EDTA, 5 mM MgCl₂, 3 mM DTE), containing 0.2% Brij W-1. Solubilized enzyme (7.5 mL; see above) was loaded and the column was eluted with the same buffer system. The fractions collected (2.25 mL) were tested for AACT/HMGS activity as described above. The column was calibrated with standard proteins with a molecular mass ranging from 29 to 150 kDa.

**Protein assay.** Proteins were assayed by a modified Lowry assay (63) with some additional modifications, as described elsewhere (28).

**Molecular cloning of radish HMGR.** Total RNA from either etiolated or green (light regime: 16 hr white light, ca. 4500 lux, 8 hr darkness, 22-24°C) four-day-old radish seedlings was isolated as described (64), with minor modifications (28). Poly(A)⁺ RNA was purified by oligo(dT) cellulose chromatography according to Aviv and Leder (65). RNA samples were fractionated on 1% agarose/formaldehyde gels, transferred and UV-cross-linked to Zeta-Probe membranes and hybridized for Northern blot analysis as described (28). A cDNA library from mRNA isolated from etiolated radish seedlings was constructed by the aid of a cDNA synthesis kit (Bethesda Research Laboratories, Eggenstein, Germany) according to the manufacturer's manual and cloned into the expression vector pgt11 (EcoRI restriction site) following established protocols (66, 67). The radish cDNA library was screened by hybridization with a 32P-labeled cDNA probe encoding HMGR from Arabidopsis thaliana (38). Repeated screening yielded seven positive clones referred to as λcRS1-7. λcRS3 comprised the entire protein-coding transcription unit of a HMGR gene of radish.

**DNA sequencing and analysis.** For DNA sequencing, the DNA fragments generated by digestion of the EcoRI insert with various restriction enzymes were subcloned into appropriate Bluescript plasmids (Stratagene, San Diego, CA). The DNA was sequenced by the dideoxy chain termination method (68) as modified by Biggen et al. (69) by the aid of the T7-Sequencing kit (Pharmacia-LKB). Microgen software from Beckman (München, Germany) was used to analyze DNA sequences. Hydrophathy profiles were calculated according to the algorithm of Kyte and Doolittle (70).

**RESULTS**

**Solubilization and partial purification of plant AACT/HMGS.** A considerable portion of the total activity capable of converting acetyl-CoA to HMG-CoA appears to be associated with a heavy-membrane pellet (P 16,000) isolated from four-day-old etiolated radish (48) and maize seedlings (3). Since the activity found in the cytosolic fraction was shown to be salt-sensitive, concentration and...
fractionation by ammonium sulfate precipitation was not possible (48). However, in radish, the same membrane fraction (P 16,000) is the major source of HMGR activity (28) and was therefore used as a source of enzymes to further study the properties of AACT/HMGS. Previously, we successfully developed a protocol for solubilizing HMGR from membranes using the detergent Brij W-1 (27,28). For the enzyme system containing AACT and HMGS we used a similar, but simplified protocol. Prolonged incubation at 37°C was detrimental to the enzyme activity, whereas HMGR appeared to be quite heat-stable (3,28). Therefore, the temperature during the incubation in the presence of 2% Brij W-1 was lowered to 30°C. By application of the protocol, as outlined in Materials and Methods, the recovery of total AACT/HMGS activity was about 90% of the initial value (Table 1).

During the attempts to purify the enzyme(s) further, we always ran enzyme assays twice—either in the presence or absence of unlabeled acetoacetyl-CoA, in addition to [1-14C]acetyl-CoA. If a protein fraction exclusively would contain HMGS, but not AACT activity, only if both substrates were available should we have been able to observe the incorporation of radiolabel into HMG-CoA. We have not separated AACT from HMGS activity as yet, even though we have developed different protocols, including precipitation with polyethylenimine (3,48) and free solution IEF (3,71). As already mentioned above, the salt-sensitivity of this enzyme system, in contrast to the properties of HMGR (28), prevented the use of salt gradients higher than 200 mM for the elution of enzyme activity from various column materials. An important factor in maintaining enzyme activity during purification is to speed up the procedures, which is possible by using fast-performance liquid chromatography (FPLC). In this way we have already achieved a considerable purification factor by combination of mild detergent solubilization, gel filtration, acetone precipitation, and anion exchange chromatography (Table 1). If cation chromatography is included in the purification protocol (where AACT/HMGS is passing through the column, whereas many contaminating proteins bind) an additional purification by 1.5-2-fold is possible. It was possible to separate AACT/HMGS activity from HMGL (cf. Fig. 1). This is especially important when kinetic properties have to be determined, where any interference of HMGL with AACT/HMGS has to be excluded.

Some properties of AACT/HMGS. Recently it has been shown that both enzymes appear to cooperate closely in a way so that no intermediate acetoacetyl-CoA is released into the medium from AACT before it can bind to the active site of HMGS (3,48). During the course of these experiments we have also proved that our enzyme-assay system allows for the conversion of acetyl-CoA to HMG-CoA, which is quantitatively converted to MVA in the presence of NADPH and highly purified yeast HMGR (3,48). Recrystallization from diethyl ether of an enzymatically formed and acid-hydrolyzed product, supplied with additional unlabeled HMG-acid, resulted in greater than 90% radiochemical purity (data not shown). Complex formation between two enzymes could easily explain why any attempt to separate AACT from HMGS was unsuccessful. The apparent molecular mass of the coupled enzyme system AACT/HMGS as deduced from conventional gel filtration on Cellulose 200 and 700 was about 54 kDa (3). This value was confirmed by chromatography of a crude solubilisate from radish membranes (P 16,000) using a FPLC system with Superose 6 prep grade. Although the properties of this material do not permit a good separation of proteins with a difference in molecular mass of less than 20 kDa, some separation from HMGL activity (68-72 kDa, cf. 3) was achieved (Fig. 2). When the enzyme system was purified by anion exchange chromatography, especially after an additional passage through small PD-10 columns to remove low-molecular-weight molecules and ions, apparent activity was drastically decreased. Reconstitution of activity required the presence of bivalent cations in addition to Mg2+ (3). Fe2+ was the most active cofactor of various cations tested. Complex formation with EDTA led to an even higher efficacy of Fe2+ (Fig. 3) in stimulating AACT/HMGS activity in vitro. This stimulation of enzyme activity, e.g., a doubling of enzyme activity requiring less than 50 μM of Fe2+EDTA in the assay mixture, was apparently due to a Vmax effect (Fig. 4) without change in the affinity towards the substrate acetyl-CoA. The km value was calculated to be 6 μM. Since EDTA cannot shield all six coordination sites of Fe2+, additional Fe2+ complexes were tested for their ability to stimulate the in vitro conversion of acetyl-CoA to HMG-CoA. The data show that concomitant with a decrease in the dissociation constants of the complexes the stimulation disappeared (Fig. 3). Fe2+, either complexed with CN−,...
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where all six coordination sites are occupied, or sterically shielded, as in the heme moiety of reduced cytochrome c (not shown), was inactive. When EDTA was replaced by either ATP or citrate, which may act as natural chelators of Fe²⁺ (72), the activation of AACT/HMGS was relatively efficient (Fig. 3). When ferrous ions were prevented from becoming oxidized by the presence of excess ascorbic acid (a natural reductant), an even better stimulation of in vitro activity occurred (Fig. 5). Hydroxyurea that is known to scavenge radicals (73), inhibited the activation by ferrous ions of gel-filtrated AACT/HMGS, but it was slightly inhibitory to the enzyme(s), even in the absence of Fe²⁺ (Table 2).

Molecular properties of radish HMGR. When HMGR

FIG. 1. Gel filtration on Superose 6 prep grade. Proteins from a heavy-membrane pellet isolated from four-day-old etiolated radish seedlings were solubilized in the presence of 2% (w/v) Brij W1. Solubilized proteins were concentrated by acetone precipitation. The precipitate was resuspended, and insoluble proteins were removed by centrifugation. The clear solution (3.3 mL) was loaded onto the column and eluted as described in Materials and Methods. Volume activity is expressed as percent of loaded fraction. Assay conditions: a) AACT/HMGS: 5 min at 30°C, 25 μM [1-14C]acetyl-CoA, 250 μM Fe and 500 μM EDTA. Assay volume was 55 μL; 20 μL fraction, 25 μL Tris/HCl (0.2 M, pH 7.5), immediately mixed with EDTA (stock solution: 40 mM, pH 7.0) and FeSO₄ (stock solution: 20 mM, pH 2). The Tris/HCl-Fe-EDTA system was immediately added to the enzyme solution and after a preincubation of 2 min, 10 μL of start mix containing [14C]acetyl-CoA were added. The reaction was stopped by addition of 6N HCl. b) HMGL: Similar to a), however, the Tris buffer was supplied with 30 mM Mg²⁺ instead of Fe-EDTA. Concentration of R,S-[3-14C]HMG-CoA, 25 μM.

FIG. 2. Anion exchange chromatography on Merck Fractogel EMD TMAE 650. The four peak fractions from gel filtration (Fig. 1) were loaded and the column was eluted as described in Materials and Methods. The assay conditions for AACT/HMGS and HMGL, respectively, were described in the legend to Figure 1 with the exception that 25 μL of fraction was mixed with 20 μL of Tris buffer.
was detergent-solubilized from radish membranes and purified further (28), a band of 45 kDa was detected in the peak fractions from the last purification step when analyzed by SDS-PAGE. Sucrose-density centrifugation suggested a molecular weight of 180 kDa for the active, non-denatured enzyme. Thus, a tetramer of 45 kDa subunits was suggested to represent the functional entity (28). However, in the first peak fraction we also observed an additional band of less than 66 kDa, which was first interpreted as a typical contaminant appearing in SDS gels loaded with mercaptoethanol-treated protein samples (cf. 74). We have characterized a full-length cDNA clone, referred to as \( \lambda cRS3 \) (39), from a newly established radish cDNA library. Northern blot analysis of poly(A)+RNA isolated from etiolated and light-grown radish seedlings revealed a total length of 2.3 kb for the primary transcript (39). The cDNA consists of a 5'-noncoding region of 45 nucleotides, an open reading frame of 1749 bases, and a 3'-untranslated region with two potential polyadenylation signals. \( \lambda cRS3 \) codes for a polypeptide of 583 amino acids with a molecular mass of 62,720 Da. The hydropathy profile of the amino-acid sequence (Fig. 6) suggests the presence of two membrane-spanning domains within the N-terminal 165 amino acids (residues 33 to 57, and 68 to 104) as was found for HMGR from \( \text{Arabidopsis} \) (38), pea (40) and tomato (J.O. Narita and W. Gruissem, personal communication). These domains and the spacer region in between are highly conserved between the \( \text{Arabidopsis} \) and radish HMGR (Fig. 7). The linker region (residues 105 to 165) that joins the N- and C-terminal parts of radish HMGR contains a proline-rich "PEST" sequence (cf. 75), which is slightly longer than in \( \text{Arabidopsis} \) and is less conserved. The C-terminal part of radish HMGR (residues 165 to 571), where the catalytic site resides, is highly conserved in all plant and other euakaryotic HMGR genes sequenced so far (36-40,76-82), indicating a stringent evolutionary pressure onto this region (Fig. 8). Since proline-rich sequences serve as recognition sites for certain proteases, a cleavage product presumably has given rise to the formation of the 45 kDa fragment seen on the SDS gel, although the solubilization efficiency was not affected in the presence of protease inhibitors {28,83). For the HMGR isolated from latex of \( \text{Hevea brasiliensis} \), by application of essentially the same protocol as was developed for radish, a subunit molecular mass of 44 kDa was determined by the aid of SDS-PAGE and, as deduced from native PAGE, a functional size of 176 kDa (29). The band of higher molecular mass as detected in the SDS gel loaded with purified HMGR from radish was most probably not caused by contamination, as was discussed earlier (28), but more likely represented undegraded, native HMGR protein.

**DISCUSSION**

**Synthesis and metabolism of HMG-CoA.** In mammalian systems it is well documented that regulation of HMGR is mediated by rapid processes both at the translational and the post-translational level (cf. refs. 4–6). HMGS appears to be regulated in parallel (84–92). Similar processes can be expected to occur in plant cells. A thorough study using plant tissue requires a profound knowledge of the properties of the enzymes involved in the formation of HMG-CoA.
Our data (3,38) imply the formation of a tightly fitted complex between AACT and HMGS. The acetoacetyl-CoA arising from the AACT reaction was immediately transferred to the active site of the consecutive enzyme HMGS without any substantial diffusion into or exchange with the medium (3,38). We have some further evidence for this hypothesis since the antibiotic F-244 is completely inactive in our system (3). F-244 has been described as a highly specific inhibitor of HMGS both from yeast (93) and rat liver (94), with \( K_i \) values in the range of 100 nM. Through the formation of this enzyme complex, the active site of HMGS may somehow be shielded, preventing the binding of the inhibitor, but otherwise facilitating the direct transfer of acetoacetyl-CoA to enter the HMGS catalyzed reaction. In view of the numerous enzymes competing for the central intermediate acetyl-CoA (cf. 1), a directed flux of carbon units into the isoprenoid pathway makes sense. However, despite the differences in the reaction mechanisms in both condensations leading from acetyl-CoA to HMG-CoA, viz. Claisen vs. aldol condensation, it cannot be excluded that a single polypeptide catalyzes both partial reactions.

Separation of HMGL from AACT/HMGS has been achieved, e.g., by means of free-solution IEF, where the enzyme appears to band at high activity with an apparent \( p_I \) value of 7.0 (3,71), or by ion exchange chromatography. Considerable activity of HMGL is associated with the same heavy-membrane pellet (P 16,000) like AACT/HMGS (cf. 3,38). HMGL behaves rather unstably and loses ac-
FIG. 5. Effect of ascorbic acid on the efficiency of AACT/HMGS activation by Fe^{2+}-EDTA. AACT/HMGS was purified as indicated in previous legends. The assay volume was 70 μL (30 μL of enzyme solution, 30 μL of Tris buffer system). Cofactors were freshly added to this buffer system (sequence: EDTA, ascorbate, Fe^{2+}). After preincubation at 30°C for 2 min the reaction was started by addition of 10 μL start mix (final concentration of L-acetyl-CoA, 25 μM, Control 100%); without ascorbate (stock solution: 40 mM, pH 7.0) and Fe^{2+}-EDTA.

FIG. 6. Hydropathy index plot of radish HMGR. The algorithm of Kyte and Doolittle (70) was used with a window size of nine residues. Positive values indicate hydrophobic regions. Bars 1 and 2 indicate putative trans-membrane regions.

FIG. 7. Amino-acid sequence alignment of the putative trans-membrane regions of plant HMGRs. RS: Raphanus sativus, HMGI; AT1, AT2: Arabidopsis thaliana, corresponding to HMGI and HMG2 (data from ref. 37,38,40); PSa: Phaseolus vulgaris, HMGI. Dots indicate residues identical to radish enzyme.

FIG. 8. Amino-acid sequence alignment of the putative trans-membrane regions of plant HMGRs. RS: Raphanus sativus, HMGI; AT1, AT2: Arabidopsis thaliana, corresponding to HMGI and HMG2 (data from ref. 37,38,40); PSa: Phaseolus vulgaris, HMGI. Dots indicate residues identical to radish enzyme.

A series of cations have been tested for their ability to restore the initial activity. Mg^{2+}, Co^{2+}, Mn^{2+}, and Fe^{2+} were similarly effective in stimulating HMGL activity, whereas trivalent cations were inactive (3). However, it cannot be excluded that cations such as Ca^{2+} remain bound to the enzyme even after passage through a gel filtration column. Higgins et al. (97) compared the data obtained for several mammals and tissues as an enzyme source and assumed the enzyme to contain bound Ca^{2+}, which was not affected by chelators above pH 8.

The effects of cations have been studied in great detail for AACT/HMGS. Although required for some basal activity, Mg^{2+} alone stimulated enzyme activity in vitro only slightly. This was demonstrated when the enzyme system that was purified about 240-fold was additionally...
filtered through PD-10 columns previously equilibrated with 100 mM phosphate buffer without any Mg\(^{2+}\). In previous experiments, it was shown that AACT/HMGS can be separated from HMGL by free-solution IEF, forming a peak at a rather acidic pH. The reason for this apparent acidic pI value may be due to complex formation of the enzyme system with phospholipids that are detergent-solubilized in parallel. Under such conditions AACT/HMGS lost most of its activity. A series of cations were tested for their ability to restore activity, with Fe\(^{2+}\) being the most effective (3). The even higher activity observed in the presence of a double concentration of EDTA (at pH 7.5 to 8.0) can be readily explained by stabilization of Fe\(^{2+}\) which, as a free cation, is rapidly oxidized to Fe\(^{3+}\) and does not stimulate enzyme activity. Which amino acids of the protein(s) are involved in the reaction of Fe\(^{2+}\), presumably histidine residues (cf. 98), is not yet known.

When Fe\(^{2+}\) was added as a complex where all six coordination sites are occupied (e.g., with CN\(^{-}\)) the enzyme
system was not activated. There remains the possibility that Fe²⁺ is involved in the formation of an organic free radical, as it is essential for ribonucleotide reductase activity (cf. 73, and literature cited therein). Addition of hydroxyurea, which is capable of scavenging such radicals, partially prevented activation of AACT/HMGS (Table 2).

Other bivalent cations such as Co²⁺, Mn²⁺, Sn²⁺ or Ni²⁺, which are more stable than Fe²⁺, were much less effective (3). When the ferrous ions were prevented from being oxidized because of the presence of ascorbic acid, activation of AACT/HMGS was increased further. Iodoacetamide capable of alkylating thiol groups did not drastically affect catalytic efficiency. A mechanism that only involves the shielding by Fe²⁺-EDTA of reactive thiol groups from becoming oxidized seems unlikely. It was reported that Fe²⁺-EDTA, but not unchelated ferrous and ferric ions, efficiently inhibited porcine brain lipoamidase, possibly due to interaction with the active center (99). Anaerobically induced NAD-linked glycerol dehydrogenase of Klebsiella pneumoniae was inactivated in the presence of O₂, Fe²⁺ and ascorbate (100). This was interpreted to mean that O₂ and ascorbate generated H₂O₂, and that H₂O₂ reacted with Fe²⁺ to produce an activated species of oxygen, which attacked the enzyme (100). In the cell Fe²⁺ possibly forms complexes with ATP or citrate (101). These complexes, capable of generating oxygen radicals, might lock plant AACT/HMGS into a conformation that results in a higher catalytic efficiency, viz. in an increased turnover rate, whereas the apparent affinity towards the substrate is not affected; Vₘₐₓ, but not kₘₐₓ, changes. A doubling of apparent activity can already be achieved at concentrations less than 50 μM Fe²⁺, which is well within physiological range.

**Cloning and characterization of radish HMGR.** Recently, by the aid of a cDNA encoding hamster HMGR (76), it has been possible to isolate a corresponding gene from Arabidopsis thaliana (38). The isolation of the corresponding gene from radish has been facilitated because of the close phylogenetic relationship between Raphanus sativus and A. thaliana, both belonging to the same family of Brassicaceae (40). The radish HMGR was the first enzyme comprising nearly E. coli, comprising nearly 1.6 kb in length, has a different restriction pattern than itcRS3, which has been completely sequenced. Thus, in radish seedlings at least two genes are actively expressed. In Arabidopsis two genes have been identified (38,40), which are referred to as HMG1 and HMG2. Since HMG2 is apparently expressed at a very low level and only in certain parts of seedlings, in contrast to HMG2, its complete sequence has been determined from genomic, but not from cDNA clones (Enjuto, M., and Boronat, A., unpublished observations). In tomato, only a single HMGR gene had been initially isolated (36); however, a second HMGR gene also has been identified (Narita, J.O., and Gruissem, W., personal communication). Based on the presumably variable localization of HMGR isozymes in the plant cell, the presence of three HMGR genes has been postulated (1). Recently it has been shown that in the pea three closely related genes appear to be active (40).

The expression system we have used to produce radish HMGR45 in E. coli (60) should facilitate isolation of HMGR protein from other cDNA clones for identification of kinetic and molecular properties. Since this enzyme can be considered a promising target for plant growth regulators (1,33,34,106), such as the antibiotic mevinolin (lovastatin) and its natural and synthetic analogues (cf. 7), structural modeling requiring the crystallization of plant HMGR could be envisaged. It is possible to obtain greater than 95% pure radish HMGR45 by just two purification steps (Bach, T.J., unpublished observations).

Clear information on the differences of HMGR isozymes may finally provide us with the clue as to the intracellular location, and of the expression of the corresponding genes in space and time. Antibodies that are raised against oligopeptides comprising non-homologous parts of the isozymes could be used for immuno-cytochemical studies. The ultimate aim must be to isolate the genes coding for AACT, HMGS, HMGL and for enzymes beyond the HMGR step, to get a better understanding of how MVA biosynthesis and utilization is regulated in plants.

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