Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 530-537

THE LOCALIZATION, PARTIAL PURIFICATION AND REGULATION OF PEA PLASTID HMG-CoA REDUCTASE

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Received March 4, 1992

Summary: HMG-CoA reductase was located to the envelope membranes of pea etioplasts, the first report of the suborganelle localization of this key enzyme in isoprenoid synthesis. The enzyme was purified 156 fold from isolated envelope membranes. Purification was achieved by detergent solubilization, hydroxylapatite chromatography and glycerol gradient centrifugation. Membrane-bound etioplast HMG-CoA reductase was activated 4 to 5 fold by high concentrations of inorganic phosphate, this activation was prevented by arsenate, a structural analog of phosphate.

HMG-CoA reductase (3-hydroxy-3-methylglutaryl CoA reductase, E.C.1.1.1.34) catalyses the synthesis of mevalonic acid, the first committed step in the production of isoprenoid compounds. Investigations into the regulation of HMG-CoA reductase in mammals have revealed a range of control mechanisms (1,2), indicating that both short-term and long-term modifications are involved in the regulation of activity. The isoprenoids are a group of compounds that have important and varied roles in the function and metabolism of the plant cell. They are involved as growth regulators (such as abscisic acid and gibberellins), photosynthetic pigments and in cell structure and function (sterols and dolichols).

HMG-CoA reductase has been detected and studied in a range of plant species, being membrane-bound in all instances (for review, see Sabine (3). There has been some controversy concerning the subcellular localization of the enzymes for the isoprenoid pathway. One hypothesis envisaged that the different compartments are separated by membranes impermeable to the intermediates of the pathway, originally proposed by Goodwin (4), which would require the same enzymatic reaction in each compartment. And a second (5) suggested that the formation of an intermediate, isopentenyl pyrophosphate (IPP), occurs solely in the cytosol and that the IPP then enters the different subcellular compartments. However, subcellular localization studies of HMG-CoA reductase from etiolated peas have revealed the presence of the enzyme in the endoplasmic reticulum, the mitochondrion, and the plastid (6). Both the microsomal and the plastid forms of the pea reductase have been well studied and distinct properties have been found (7,8).

The partial purification of plant HMG-CoA reductase has been reported from potato (9) and radish (10). The cellular location of these enzymes were the endoplasmic reticulum for potato and a 16,000g pellet for the radish which would contain plastids and mitochondria. No plastid specific HMG-CoA reductase has been purified.

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0006-291X/92 $1.50

Early studies into the regulation of plant reductases are beginning to reveal the complex nature of this enzyme. Seasonal variation (11), induction of activity by fungal infection (12,13), phytochrome (7,14), phosphorylation (14), and hormonal regulation (15) have been found to affect different species of plant HMG-CoA reductases. Such a variety of responses suggest the enzyme is finely tuned to respond to a variety of external stimuli.

MATERIALS AND METHODS

Plant Growth: Seeds of *Pisum sativum* cv. Greenfeast (Otago Farmers, Dunedin) were grown in complete darkness at 27°C and used at the third internode stage, and harvested under dim green light.

Extraction of plastid HMG-CoA reductase: Method 1. Purification of enzyme: The etiolated apical buds were harvested on ice and hand homogenized with 10% (w/w) washed polyclar-AT and 3 vol of homogenizing medium (10 mM MOPS, pH 7.9; 10 mM KCl; 0.1% (w/v) BSA; 10 mM mercaptoethanol; 20 mM EDTA) and 0.35 M sucrose. The slurry was squeezed through two layers of muslin and then one layer of miracloth. The filtrate was centrifuged at 500g for 2 min and the pellet of nuclei was discarded; the supernatant was layered onto a 0.5 M sucrose cushion in the same buffer as described above and centrifuged at 5000g for 10 min to sediment the plastids. After decanting the supernatant, the plastids were osmotically ruptured by resuspending in 5 to 10 volumes of rupture medium (10 mM MOPS; pH 7.9; 10 mM DTT; 10 mM KCl; and 50 mM EDTA). This was left on ice for 15 min; the prolamellar bodies were removed by centrifugation at 4500g for 8 min and the supernatant containing the envelopes was then centrifuged at 100,000g for 60 min to pellet them. The membrane pellet was resuspended in resuspension buffer (50 mM MOPS, pH 7.9; 10 mM DTT and 20% (v/v) glycerol) using 40 μl/g of original fresh weight tissue.

Method 2. Suborganelle localization: To obtain envelopes of greater purity an alteration of the previous procedure was used. The ruptured etioplasts were layered onto a stepwise gradient formed in an SW 28 tube (Sorvall), consisting of 7 ml of a 1.2 M sucrose solution, 7 ml of 0.9 M and 7 ml of 0.6 M solution. The solutions all contained 100 mM K-phosphate buffer pH 7.9, 10 mM DTT, 10 mM EDTA, and 2 mM $MgCl_2$. This was centrifuged for 90 min at 50,000g at 4°C. The bands were removed and centrifuged at 100,000g for 60 min. The pellets were resuspended as above.

Solubilization: HMG-CoA reductase was solubilized from isolated envelope membranes (method 1) by a three step procedure. Membranes (in resuspension buffer) were incubated with 0.1 M KCl, 0.1% (w/v) digitonin for 30 min at room temperature followed by centrifugation at 100,000g for 60 min. Supernatant was discarded and the pellet resuspended (as above) and incubated in 0.1 M KCl and 0.05% (w/v) taurodeoxycholate for 30 min at room temperature; centrifuged again at 100,000g for 60 min. The supernatant was discarded and the pellet resuspended. The third solubilization involved incubating the resuspended pellet with 0.3 M KCl and 1.0% (w/v) taurodeoxycholate, for a final 30 min at room temperature. After centrifugation at 100,000g for 60 min, the supernatant containing the solubilized HMG-CoA reductase is collected and routinely stored at -80°C.

Hydroxylapatite chromatography: The final supernatant from the triple solubilization was loaded batchwise onto hydroxylapatite resin (Sigma) equilibrated in 5 mM K-phosphate pH 7.9, 0.4% (w/v) TDOC and 10 mM DTT, 10% (v/v) glycerol and 2 mM EDTA. The resin was mixed for 20 min at 4°C, centrifuged and resuspended in 200 mM K-phosphate, 0.2% (v/v) Triton X-100, 10 mM DTT, 10% (v/v) glycerol, 2 mM EDTA, pH 7.9. The resin was mixed again, centrifuged briefly and the supernatant containing the HMG-CoA reductase was concentrated using Centricon 30's (Amicon).

Glycerol gradient: The sample from Hydroxylapatite elution was loaded onto a 15-55% (v/v) glycerol gradient set up in 5% increments in a SW40 tube (Sorvall) in buffer containing 1% (v/v) Triton X-100, 50 mM K-phosphate pH 7.9, 10 mM DTT, 10 mM EDTA, and 5 mM EGTA. The sample was then layered on the top and centrifuged at 110,000g for 60 hours at 4°C. After the run the gradient was fractionated by piercing a hole in the bottom of the tube and collecting fractions dropwise; usually 25 x 0.45 ml fractions were collected.

Enzyme assays: HMG-CoA reductase was assayed as in the microassay reported by Russell *et al* (16). Silica gel plates separated the product MVA (R_f 0.8) from the substrate [methyl^{3}H]-HMG-CoA (R_f 0.1) which were scraped and counted. One unit is defined as 1 nmol of MVA produced per hour. Galactosyl transferase was assayed as reported (17). Acyl-CoA thioesterase was assayed by the procedure reported by Andrews and Keegstra (18).

Protein determination: Protein was assayed by the Lowry procedure as modified by Peterson (19). The protein standard used was bovine serum albumin.

RESULTS

Localization. The exact location of the HMG-CoA reductase protein in the plastid was of interest to gain an understanding into the role of the isoprenoid pathway in the plastid. The envelope membranes were separated from the prolamellar bodies by density gradient centrifugation and the location of the reductase was determined. By monitoring the activities of galactosyl transferase and acyl-CoA thioesterase, previously located to the envelope (18,20), the location of HMG-CoA reductase was ascertained (Table 1). The specific activity of HMG-CoA reductase was 7.4 fold greater in the envelopes than in the prolamellar bodies. There was only 57% of the total activity in the envelope indicating that the prolamellar bodies were probably heavily contaminated with envelope membranes.

Purification. HMG-CoA reductase proved to be a firmly membrane-bound protein as high concentrations of salts, EDTA, and a range of proteases and low detergent concentrations would not remove the reductase from the membrane (data not shown). The finding that low concentrations of detergents would not remove the enzyme was exploited to develop a technique in which the membranes were stripped of extrinsic proteins by a low concentration of digitonin followed by taurodeoxycholate. To render the reductase soluble the concentration of taurodeoxycholate was increased to 1.0% (w/v). This resulted in an 8.1 fold purification with 97% recovery of activity.

A variety of resins were tested for use in further purification of HMG-CoA reductase. Ion-exchange, dye-ligand, affinity and hydrophobic resins were all unsuccessful; the principal problem being the inability to elute the

Table 1. Separation of envelope from prolamellar membranes by a three step sucrose density gradient centrifugation

	Enzyme units nmol/hr	Specific activity nmol/hr/mg protein	% of total activity
HMG-CoA reductase			
0.9/1.2 M interface (prolamellar)	10.3	1.9	43
0.6/0.9 M interface (envelope)	13.9	14.0	57
Galactosyl transferase			
0.9/1.2 M interface (prolamellar)	9.6	3.2	40
0.6/0.9 M interface (envelope)	14.2	19.6	60
Acyl-CoA thioesterase			
0.9/1.2 M interface (prolamellar)	8.3	2.0	41
0.6/0.9 M interface (envelope)	12.0	15.0	59

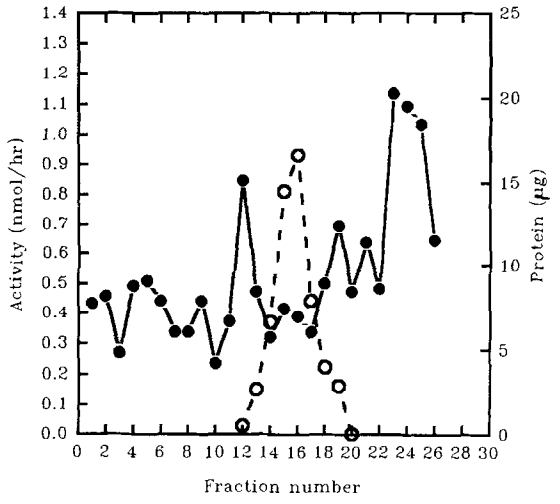


Figure 1. Profile of glycerol gradient purification; (○-○) protein, (● ●) HMG-CoA reductase activity. Fraction 1 is at 55% v/v glycerol and fraction 26 is at 15% v/v glycerol.

reductase once bound. Hydroxylapatite was able to achieve separation where other chromatography techniques failed. It was found through repeated trials that the reductase would bind in low concentrations of phosphate, and elute in higher Pi concentrations only if a non-ionic detergent such as Triton X-100 replaced the taurodeoxycholate detergent in the binding buffer. It was also found that a batchwise procedure gave better recovery than a column approach. The other technique that proved to be successful was glycerol gradient density centrifugation. By careful selection of the detergent and the concentration range of glycerol the conditions were optimized to 1% Triton X-100 and 15-55% glycerol (Figure 1). The reductase peaked at Fraction 16 and the SDS gel profile of the final purification step revealed 6 bands after silver staining (data not shown) yielding a final purification of 156 fold with 2% recovery (Table 2).

Table 2. Purification of HMG-CoA reductase from pea etioplasts

	Total units nmol/hr	Specific activity nmol/hr/ mg protein	Protein mg	Fold purification	Yield %
Etioplast membranes	78.3	0.78	480	1	100
Envelope membranes	48.1	4.03	120	5.2	61
Solubilized sample	46.4	32.5	22	41.6	59
Hydroxylapatite chromatography	5.3	61.1	0.25	78.1	7
Glycerol gradient centrifugation	1.5	122.0	0.008	156	2

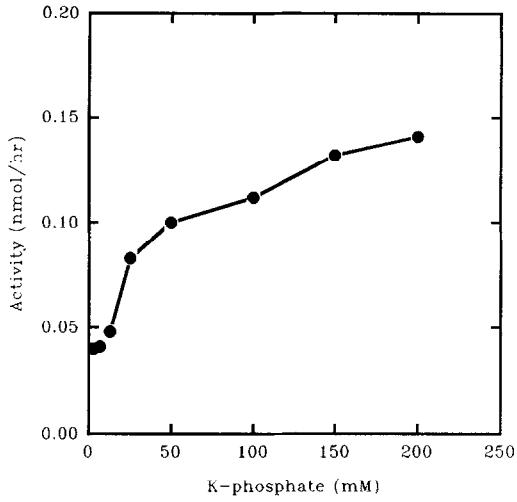


Figure 2. Membrane-bound HMG-CoA reductase (10 µg) was incubated with increasing concentrations of potassium phosphate.

REGULATION. Membrane-bound HMG-CoA reductase was activated up to four fold by the addition of potassium phosphate buffer; the curve was sigmoidal suggesting a form of positive cooperativity (Figure 2). A variety of other ions were also tested for their ability to activate membrane-bound reductase activity (Table 3); all gave linear increases in activity except for sodium phosphate, indicating that the inorganic phosphate ion was giving the

Table 3. Effect of ions on HMG-CoA reductase activity

Ion tested	Concentration range (mM)	Highest fold activation	Type curve
Potassium sulphate	0-200	3	linear
Potassium nitrate	0-200	2	linear
Sodium nitrate	0-200	2	linear
Sodium phosphate	0-200	4	Sigmoidal
Potassium chloride	0-200	4	linear
Sodium chloride	0-200	1.6	linear
Sodium pyrophosphate	0-50	2	linear
ATP	0-20	1	linear
Fructose-6-phosphate	0-50	1	linear
Phosphoric acid	0-20	1.3	linear
cyclic AMP	0-2	1	linear
cyclic GMP	0-2	1	linear
Dihydroxyacetone phosphate	0-5	1	linear
Sodium azide	0-20	4	linear

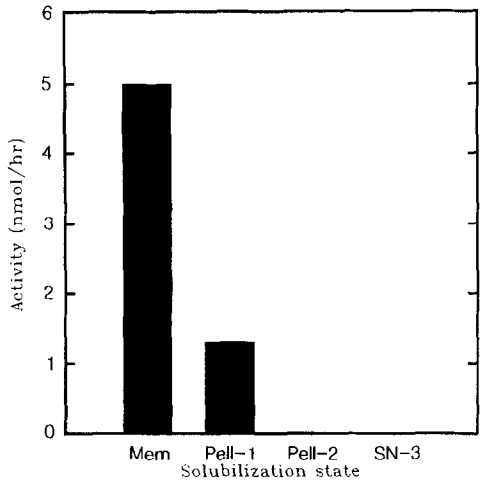


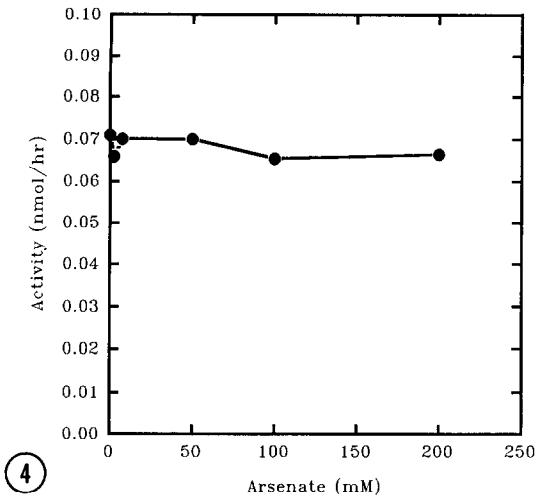
Figure 3. Loss of activation by phosphate at different stages of the solubilization of HMG-CoA reductase from the etioplast envelopes. Mem, envelope membranes; Pell 1, pellet from the 0.1% (w/v) digitonin solubilization; Pell 2, pellet from the 0.05% (w/v) taurodeoxycholate solubilization; SN 3, supernatant from the final 1.0% (w/v) taurodeoxycholate solubilization.

specific sigmoidal response. A range of other chemicals containing bound phosphate were also tested for their affect on reductase activity (Table 3). Phosphoglyceric acid gave the highest activation of all the chemicals tested but none exhibited the same sigmoidal response as inorganic phosphate. This linear versus sigmoidal increase in activity suggests that their may be two different means of activation; one specific for phosphate and one general for almost any ion. These two effects may in fact coexist.

Alteration of properties. If phosphate was removed from the activated reductase by centrifugation of the membranes and resuspended in phosphate-free medium the activation was lost (data not shown). This suggested that the phosphate ion does not covalently bind to the enzyme. The values of Km and Vmax for both the substrates with membrane-bound reductase were altered in the presence of phosphate (Table 4). The Vmax being increased and the Km decreased for HMG-CoA and both being increased for NADPH. Arsenate, a structural analog of phosphate, did not alter the reductase activity over the concentration range tested (Figure 4) but increasing concentrations of arsenate did inhibit the activation by phosphate (Figure

Table 4. Alteration of Michaelis-Menten kinetics of HMG-CoA reductase by 200 mM K-phosphate

	Km (μM)		Vmax (nmol/hr)	
	Control	Phosphate	Control	Phosphate
Substrate				
HMG-CoA	29	14	0.033	0.071
NADPH	26	36	0.067	0.135



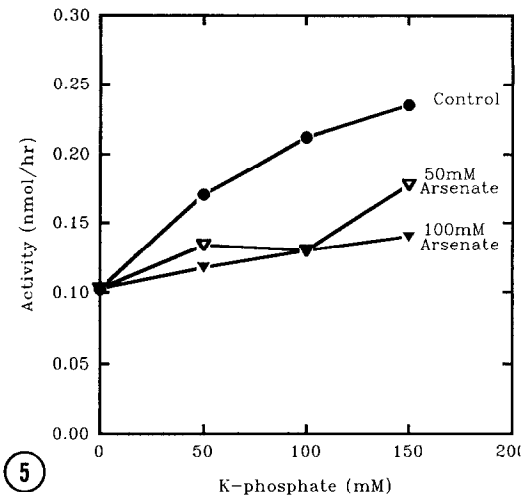


Figure 4. Effect of competition with arsenate. HMG-CoA reductase activity assayed with increasing concentrations of sodium arsenate.

Figure 5. HMG-CoA reductase activity with increasing K-phosphate concentrations plus arsenate; (●-●) no additions, (▽-▽) 50 mM sodium arsenate, (▼-▼) 100 mM sodium arsenate.

5). Hence the HMG-CoA reductase enzyme was able to differentiate between phosphate and arsenate.

The reductase was rendered soluble by a three step procedure which involved stripping the plastid envelope membranes of extrinsic proteins by two separate detergent extractions followed by the solubilization of the intrinsic proteins (including HMG-CoA reductase) in the final step. The ability to be activated by phosphate was lost as the solubilization went through the three steps (Figure 3). This lack of activation may be due to either the nature of the detergent (taurodeoxycholate forms a small charged micelle) or reflect the instability of the enzyme in the detergent micelle.

DISCUSSION

The localization of HMG-CoA reductase to the envelope of pea plastids supports the proposal that separate pathways for isoprenoid biosynthesis exist in pea. Early studies isolating the cDNA clone encoding the plant HMG-CoA reductase in Arabidopsis (21) and Tomato (22) have only revealed one form of the enzyme. But they used either a yeast probe (Arabidopsis) or an oligonucleotide sequence derived from sequence similarities from previously cloned genes (tomato) which would isolate only the gene encoding the endoplasmic reticulum enzyme and miss the plastid encoding gene.

The finding that phosphate ions activated membrane-bound HMG-CoA reductase 4-5 fold in a sigmoidal manner led to the investigation of other ions. It was concluded that the enzyme was more active in an environment of higher ionic strength but the phosphate ion was producing a more specific alteration in reductase activity. The half-maximal concentration required for activation was 20 mM. Estimates of the concentration of phosphate in the chloroplast stroma vary from 10-138 mM (23) and in the cytosol, values of 5-20 mM have

been reported (24). The activation by phosphate may be physiologically significant in vivo. The sigmoidal activation was specific to phosphate. Arsenate, a structural analog of phosphate did not activate the reductase itself but was able to inhibit the phosphate activation suggesting a specific site (or sites) on the reductase protein, the binding being non-covalent and reversible. The proposed model for the activation of membrane-bound HMG-CoA reductase involves the allosteric shift from a low activity to a high activity form. Such an allosteric shift could be mediated by non-covalent binding of inorganic phosphate ions resulting in a conformational shift in structure. Such a phosphate activation of HMG-CoA reductase has not been reported in other plant species. But regulation of plant enzyme activities by phosphate has been noted (25).

REFERENCES

(1) Beg ZH, Stonik J, Brewer Jr HB (1978) Proc. Natl. Acad. Sci. 75,3678-3682.
(2) Goldstein JL, Brown MS (1984) J. Lipid Res. 25,1450-1461.
(3) Sabine JR (1983) 3-Hydroxy-3-methylglutaryl coenzyme A reductase. Boco Raton, FL CRC Press.
(4) Goodwin TW (1965) In "Biosynthetic pathways in higher plants". Eds. J.B. Pridham and T. Swain Academic press, London. pp 57-71.
(5) Kreuz K, Kleinig H (1981) Planta 153,578-581
(6) Brooker JD, Russell DW (1975) Archives Biochem. Biophs. 167,730-737.
(7) Brooker JD, Russell DW (1979) Archives Biochem. Biophys. 198,323-334.
(8) Wong RJ, McCormack D, Russell DW (1982) Archives Biochem. Biophys. 216,631-638.
(9) Kondo K, Oba K (1986) J. Biochem. 100,967-974.
(10) Bach TJ, Rogers DH, Rudney H (1986) Eur. J. Biochem. 154,103-111.
(11) Arebalo RE, Mitchell ED (1984) Phytochemistry 23,13-18.
(12) Hepper CM, Audley BG (1969) Biochem. J. 114,379-386.
(13) Oba K, Kondo K, Doke N, Uritani I (1985) Plant Cell Physiol. 26,873-880.
(14) Russell DW, Knight JS, Wilson TM (1985) Current topics in Plant Biochemistry and Physiology 4,191-206.
(15) Russell DW, Davidson H (1982) Biochem. Biophys. Res. Commun. 104,1537-1543.
(16) Russell DW (1985) Methods Enzymol. 110,26-40.
(17) Lem NW, Williams JP (1987) Methods Enzymol. 148,346-350.
(18) Andrews J, Keegstra K (1983) Plant Physiol. 72,735-740.
(19) Peterson GL (1977) Anal. Biochem. 83,346-356.
(20) Cline C, Keegstra K (1983) Plant Physiol. 71,366-372.
(21) Learned RM, Fink GR (1989) Proc. Natl. Acad. Sci. 86,2779-2783.
(22) Narita JO, Gruissem W (1989) Plant Cell 1,181-190.
(23) Hall DO (1976) in: The Intact Chloroplast, Topics in Photosynthesis, volume 1, Editor J. Barber pp 135-170 Elsevier, Amersterdam.
(24) Bieleski RL (1973) Ann. Rev. Plant Physiol. 24,225-252.
(25) Preiss J (1982) Ann. Rev. Plant Physiol. 33,431-454.