Mechanisms of Inhibition by Mevinolin (MK 803) of Microsome-Bound Radish and of Partially Purified Yeast HMG-CoA Reductase (EC.1.1.1.34)*

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1) In kinetic studies, mevinolin proved to be a highly specific inhibitor of partially purified yeast HMG-CoA reductase ($K_i = 3.5$ nM towards HMG-CoA) and of microsomal HMG-CoA reductase from etiolated radish seedlings ($K_i = 2.2$ nM). At low concentrations of NADPH, the inhibitor counteracts the sigmoidal response of plant HMG-CoA reductase activity towards the cosubstrate. At higher concentrations of NADPH, the inhibition pattern is of non-competitive type.

2) Our results are extensively compared with that obtained by the use of animal tissue and yeast as an enzyme source in order to discuss model systems probably valid to evaluate properties and regulation of plant as well as yeast HMG-CoA reductase.

Introduction

The fungal metabolite mevinolin, isolated from the ascomycete *Aspergillus terreus* [1] has proved to be a potent hypocholesterolemic agent in rats and dogs [1] and in rabbits [2] as well as in men [3, 4]. This effect, like that of structurally related entities (Fig. 1) isolated from different strains of ascomycetes such as compactin [5] or ML-236 B [6], ML-236 A and C [6], dihydromevinolin [7] and dihydrocompactin [8], is caused by a highly specific inhibition of mammalian 3-hydroxy-3-methylglutaryl coenzyme A reductase [1, 9–15]. Mevinolin proved to be the most effective inhibitor. Compactin has been extensively used as a powerful research tool in studies on the regulation of mammalian isoprenoid synthesis [13–26], as well as mevinolin (= monacolin K) [1, 27–29].

HMG-CoA reductase represents the first major, committed step in isoprenoid synthesis and consequently it has been the object of a great deal of

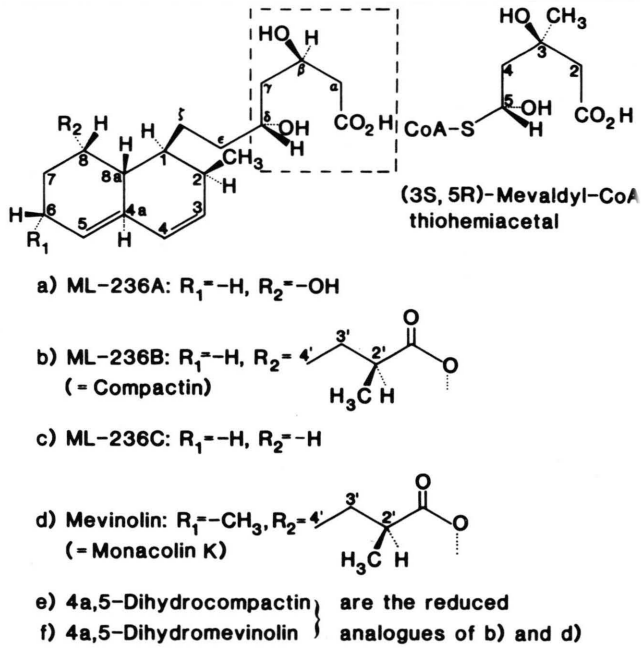

Fig. 1. Structures of the free-acid forms of mevinolin and of related entities showing the correct absolute configuration using the nomenclature originally given by Alberts *et al.* [1]. Note these compounds include a region (encircled) that resembles the mevaldyl moiety of (3S, 5R)-mevaldyl-CoA thiohemiacetal, the enzyme-bound intermediate in the two-step reduction of (S)-HMG-CoA to (R)-MVA as proposed by Rogers *et al.* [36].

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Abbreviations: DTE, dithioerythritol = Cleland's reagent; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; Mevinolin, 1,2,6,7,8,8a-hexahydro-β-δ-dihydroxy-2,6-dimethyl-8-(2-methyl-1-oxobutoxy)-1-naphthalene-heptanoic acid-δ-lactone; MVA, mevalonic acid; DTNB, 5,5′-dithiobis(2-nitro-benzoic acid) = Ellman's Reagent; IgG, immunoglobulin G; IPP, isopentenyl pyrophosphate.

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research. There have been a number of recent reviews on the role of HMG-CoA reductase in the biosynthesis of MVA and its products in animal tissue [30–36] which should be consulted to orient the reader to the large amount of work done on regulation and characterization of this enzyme. HMG-CoA reductase is also considered to be a key-regulating enzyme of isoprenoid biosynthesis in plants [31, 37–46] as well as in fungi [70]. Therefore, it became interesting to establish the inhibitory potency of mevinolin on yeast [47, 48] and plant HMG-CoA reductase preparations [47, 49]. Besides such *in vitro* properties, mevinolin was proved to be a specific *in vivo* inhibitor of plant root elongation growth of seedlings [47–50] and might be regarded as a new type of herbicide.

In this publication, we present more detailed data about the *in vitro* inhibition kinetics of mevinolin on partially purified yeast and microsomal radish HMG-CoA reductase.

Methods

1. Conversion of mevinolin in its Na-salt

Mevinolin was converted to the water-soluble Na-salt [1, 13, 14] as described by Kita *et al.* [27], and stored in aliquots of 4 mg per ml at – 20 °C. For the *in vitro* inhibition studies, aliquots of mevinolin solution were thawed to 0 °C and subsequently diluted with ice-cold water immediately before use to yield the appropriate concentrations.

2. Isolation of membrane pellets from etiolated radish seedlings

For the isolation of microsomal membranes, 4 day old seedlings including roots were harvested in dim green light and homogenized by the aid of a Waring Blender (3 strokes à 5 s) in an ice-cold buffer system (2 ml/g fresh weight) containing 0.2 M K-phosphate pH 7.5, 0.35 M sorbitol, 0.01 M Na-EDTA, 0.005 M $MgCl_2$, 0.02 M 2-mercaptoethanol (added freshly), and insoluble polyvinylpyrrolidone (PVP, 5 g/100 ml, added to the buffer 30 min before use). The homogenate was filtered through four layers of cheese cloth. Cell debris, nuclei, and PVP-particles were revomed by a short centrifugation step at $1200 \times g$, for 4 to 5 min. The supernatant was centrifuged at $16\,000 \times g$ (40 min) to yield the $16\,000 \times g$ pellet, followed by a further centrifugation at $105\,000 \times g$ (60 min). Membrane pellets were resuspended in the buffer mentioned above and an excess amount of DTE was added (final concentration $\geqq$ 50 mM) to stabilize the HMG-CoA reductase activity [31, 45]. All these steps were performed at 0–4 °C under dim green safety light. Membrane suspensions were kept in ice for use the same day or frozen and stored at – 20 °C with a loss of activity of about 20–40% per month.

3. Radioactive assay and estimation of HMG-CoA reductase activity

The system for the assay of microsomal HMG-CoA reductase activity was according to that described elsewhere [31, 45] with little modifications, and consisted of 0.2 ml 0.3 M K-phosphate pH 7.5, 0.025 ml 0.1 M Na-EDTA, 0.025 ml 0.2 M DTE, 1 mg BSA, 0.05 ml NADPH (stock solution: 16 mg NADPH-Na_2, 40 mg glucose-6-phosphate and 14 U glucose-6-phosphate dehydrogenase, buffered with sodium bicarbonate in a total volume of 2.5 ml; this solution was diluted with water to yield the final standard concentration of 300 µM in the assay system), 0.05 to 0.1 µCi (R,S)-[3-^{14}C]-HMG-CoA (56.6 mCi/mmol) in 25 to 50 µl 0.01 M KH_2PO_4, and facultatively various amounts of unlabeled (R,S)-HMG-CoA to yield a maximal concentration in the assay of about 64 µM (isotope dilution). The inhibitor was added in 50 µl solution to yield the final concentrations of 4, 8, 16 nM. Including water or inhibitor and 100 µl membrane suspension (up to 0.6 mg protein), the final volume in the reagent vials was 0.605 ml. Because of the complicated experimental design required for the estimation of inhibition patterns by mevinolin, it was necesssary to modify the assay technique. The enzyme was pipetted into reagent vials stored on ice containing the complete cofactor-substrate system. The reaction was then started by incubating the samples at 37 °C in a temperature-regulated water bath (20 to 30 min). The reaction was terminated by the addition of 100 µl 25% HCl and 250,000 dpm [5–^{3}H] mevalonic acid (0.8 mg in 200 µl methanol) was added as an internal standard. The samples were allowed to lactonize for an additional 30 min period, then, without centrifugation of the denaturated protein, supplied with anhydrous sodium sulphate (4 g each) as suggested by Huber *et al.* [51] and stored for at least 24 h. The radiolabeled meva-

lonolactone was extracted by anhydrous diethylether (1 × 5 ml, 2 × 2.5 ml), each step after a short centrifugation. The combined extracts were evaporated to dryness and resuspended in 0.5 ml water. Aliquots of 0.4 ml were loaded on pasteur pipettes filled with anion exchange resin (Dowex AG 1 × 8, Cl^-, 100–200 mesh, 1.4 meq/ml resin bed), bedvolume 2.5 ml, and eluted with water to give a volume of 5 ml [51] collected in scintillation vials. Ten ml of Unisolve 100 as a scintillation cocktail was added. After vigorous shaking and a dark period of at least 60 min, the samples were assayed for radioactivity (Packard 2425, separate channels for ^3H- and ^{14}C, with no spillover of ^3H into the ^{14}C channel). Recoveries were estimated by use of quench curve coefficients (AES-ratio method) and calculated by the aid of computer programs as described in detail elsewhere [31]. When the radioisotope dilution method mentioned above was employed, kinetic parameters were estimated by modified Lineweaver-Burk plots as suggested by Lee and Wilson [52] and Glick *et al.* [53] with refinements of Bach [31] who used the following equation:

$$1/\bar{v} = (1/V) + (K_m/V) * (1/S_0) * [(-\ln(S/S_0)/(1 - S/S_0)]$$

The term in brackets yields a correction factor for the transition of $1/S_0$ into $1/\bar{S}$. $\bar{S}$ is the average substrate concentration used by the enzyme during the incubation period, where S_0 is continuously diminished (for details see ref. [31]).

4. Isolation and optical assay of yeast HMG-CoA reductase

Yeast HMG-CoA reductase was purified through the Zn-acetate precipitation step according to Kirtley and Rudney [54] with some modifications as described elsewhere [31]. The optical assay system for yeast HMG-CoA reductase was based on that described by Rétey *et al.* [55] and consisted of 0.25 ml K-phosphate (0.3 M, pH 7.0), 50 µl 0.2 M DTE, 1 mg BSA, 50 µl 3 mM NADPH, 50 µl 0.1 M Na-EDTA, 0.1 ml enzyme solution (in 0.3 M K-phosphate pH 7.6 + 0.05 M DTE + 0.025 M NaEDTA), and water or water supplied with Na-mevinolin. The higher pH-value of the assay system as compared to that of Rétey *et al.* [55] was taken in order to avoid any lactonization of mevinolin. The reaction was started by addition of 100 µl HMG-CoA solution of the appropriate concentration (final volume: 1.2 ml) and the disappearance of NADPH was monitored at 340 nm and 25 °C (Shimadzu UV-200). There was no other NADPH-utilizing activity detected. It was therefore assumed that upon addition of HMG-CoA, only HMG-CoA reductase was measured.

5. Protein determination

Protein content was determined by means of a modified Lowry procedure [56] after sodium deoxycholate solubilization and precipitation with trichloracetic acid to avoid any interference in the assay with compounds such as DTE, sorbitol or pigments, with bovine serum albumine as a standard (for details, see ref. [31]).

Results and Discussion

The primary double reciprocal plot (Fig. 2a) $1/\bar{v}$ *versus* $1/\bar{S}$ clearly demonstrates the competitive type of inhibition by mevinolin of plant HMG-CoA reductase. The secondary plot (Fig. 2b) of slopes versus inhibitor concentration yields a value for K_i of 2.2 nM. This highly specific inhibition is comparable to that obtained with mammalian tissue or yeast cells as an enzyme source (Table I).

There is some evidence that microsomal HMG-CoA reductase of radish seedlings exhibits a type of sigmoidal kinetics versus NADPH cosubstrate concentration at nonsaturating HMG-CoA concentrations [31, 45]. This type of kinetics can also be observed at higher initial concentrations of HMG-CoA (unpublished results). Therefore, it is a surprising result (Fig. 3) that this type of sigmoidity (in the double-reciprocal plot demonstrated as an upward shape of the control curve) disappears in the presence of 8 nM mevinolin (linear shape) and even leads to a positive sigmoidal response at the higher inhibitor concentration of 16 nM. At higher amounts of NADPH in the assay mixture, however, mevinolin acts as a clear non-competitive inhibitor of HMG-CoA reductase towards NADPH.

A comparison of K_i values obtained with different mevinolin-type entities and enzyme sources (Table I) reveals that enzyme from animal tissue or from human fibroblasts – mostly in solubilized or partially purified preparations – is to some extent

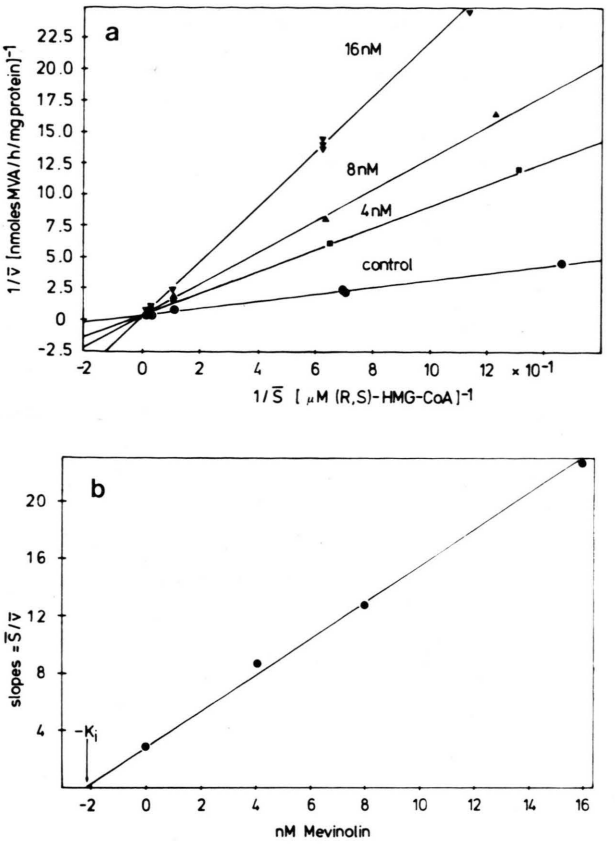

Fig. 2a, b. Competitive inhibition of microsomal HMG-CoA reductase from 4 day-old etiolated radish seedlings *in vitro* estimated by the use of a modified Lineweaver-Burke plot. The K_i against HMG-CoA as evaluated from the secondary plot of slopes versus inhibitor concentration was 2.2×10^{-9} M.

more susceptible to those inhibitors. This might be explained by, a) the fact that only microsomal preparations from radish plants were used in our assay, and b) because of the slightly higher K_m value towards S-HMG-CoA of about 2.5 μM for the plant enzyme [31, 57]. But even in partially purified preparations of HMG-CoA reductase of yeast, we obtain a K_i of about 3.5 nM (Fig. 4). Therefore, it might be interesting to compare the inhibition of yeast or animal HMG-CoA reductase by mevinolin with that caused by citrinin, a fungal metabolite that has been shown to be a potent inhibitor of sterol biosynthesis in both rat liver and yeast enzyme systems [58], also with the liver enzyme preparation being more sensitive to the inhibitor as compared to the yeast enzyme system ($I_{50} = 0.5$ mM and 2.4 mM, respectively). Of the enzymes involved in cholesterol biosynthesis from acetyl-CoA, acetoacetyl-CoA thiolase (EC 2.3.1.9) and HMG-CoA reductase were inhibited [58]. The inhibition pattern by citrinin of solubilized rat liver HMG-CA reductase that was inhibited to the same degree as the membrane-bound enzyme, was of a mixed type with respect to HMG-CoA, and non-competitive towards NADPH [59]. Dixon-plots, however, curved upwards, suggesting that the inhibition by citrinin might be either cooperative or time-dependent [59]. These authors concluded from their results that the time-dependent and irreversible inhibition of rat liver HMG-CoA reductase upon preincubation with citrinin is due to the binding of the inhibitor to a site distinct from the active center of the enzyme

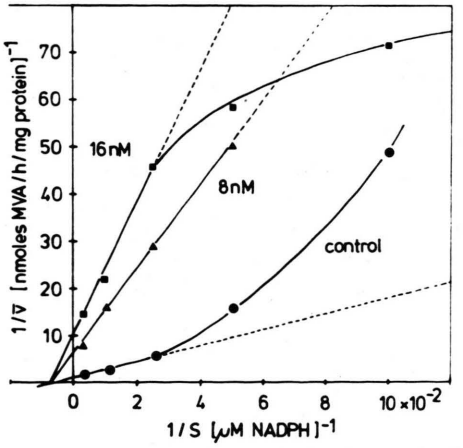

Fig. 3. Non-competitive inhibition by mevinolin of microsomal radish HMG-CoA reductase against the cosubstrate NADPH. (R, S)-HMG-CoA concentration (1.45 μM).

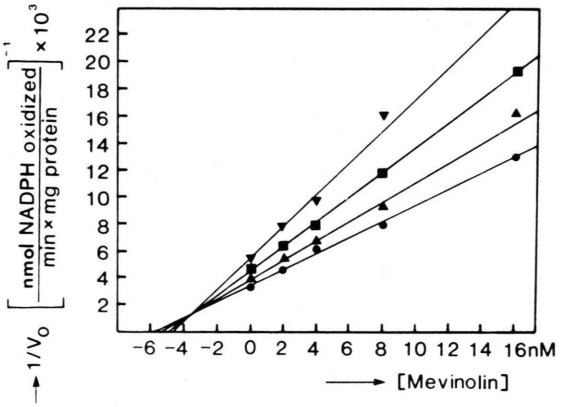

Fig. 4. Dixon-plot of the inhibition by mevinolin of yeast HMG-CoA reductase. The concentrations of (S)-HMG-CoA used in the assay were a) 19.47, b) 9.74, c) 4.87 and d) 2.92×10^{-6} M, respectively.

Table I. Comparison of inhibition constants (K_i values) of compactin/mevinolin-type metabolites against HMG-CoA reductase preparations from different enzyme sources.

Compound	Enzyme source	K_i [nM]	Reference
ML-236 A	rat liver microsomes, partially purified	220	Endo *et al.* 1976 [9]
ML-236 B (= Compactin)	rat liver microsomes, partially purified	10	Endo *et al.* 1976 [9]
ML-236 B	rat liver microsomes, partially purified	2.66	Tanzawa and Endo, 1979 [10]
ML-236 B	rat liver microsomes, partially purified	1.4	Alberts *et al.* 1980 [1]
ML-236 B	human fibroblasts, detergent solubilized	1.1	Brown *et al.* 1978 [15]
ML-236 B	homogenates of the corpora allata of the tobacco hornworm (Manduca sexta)	0.9	Monger *et al.*, 1982 [69]
Mevinolin	rat liver microsomes, partially purified	0.64	Alberts *et al.*, 1980 [1]
Mevinolin (= Monacolin K)	rat liver microsomes, partially purified	0.50	Endo, 1980 [12]
Mevinolin	yeast cells, partially purified	3.5	Bach and Lichtenthaler, 1982 [48]
Mevinolin	radish seedlings, microsomal-bound	2.2	Bach and Lichtenthaler, this paper
4a, 5-Dihydro-compactin	rat liver microsomes, partially purified	3.7	Tony Lam *et al.*, 1981 [8]
4a, 5-Dihydro-mevinolin	rat liver microsomes, partially purified	? (50% inhibition at 2.7 nM)	Albers-Schönberg, *et al.*, 1981 [7]

molecule [59]. It is also known from the early studies of Kirtley and Rudney [54] that yeast HMG-CoA reductase is progressively inactivated by solutions of CoASH and even HMG-CoA, but CoASH acts also as an activator for the reduction of mevaldate to mevalonate [55, 60]. This inhibition by free CoASH of the overall reaction was confirmed to be time-dependent and to be not reversed by the addition of an excess amount of substrate [61]. The enzyme's response was rather specific to CoASH, because some derivatives of it were much less effective [61]. More recently it was demonstrated by Gilbert and Stewart [62] that the time-dependent inactivation of HMG-CoA reductase from yeast by CoASH or HMG-CoA is due to the rapid reaction of the enzyme with CoA disulfide as the oxidation product of CoASH present at trace levels in solutions of both the compounds. A thiol-disulfide exchange reaction with a sulfhydryl group of the enzyme leading to a mixed disulfide was regarded to account for the enzyme inhibition [62]. In contrast to this, an artificial analogue of HMG-CoA (3R,S)-3-hydroxy-3-methyl-4-carboxybutyl-CoA was proved to be a specific inhibitor of partially purified rat liver HMG-CoA reductase with a clear competitive inhibition pattern towards HMG-CoA and a non-competitive pattern towards NADPH [63]. Interestingly, the thioether analogue of acetoacetyl-CoA, S-(3-oxobutyl)-CoA, that interacts with acetoacetyl CoA utilizing enzymes [64] was used as a substrate by avian liver HMG-CoA synthetase to synthesize S-(4-carboxy-3-hydroxy-isoamyl)-CoA, identical with one enantiomer of the compound mentioned above.

Microsomal HMG-CoA reductase from etiolated radish seedlings is also inhibited by CoASH [31, 57, 65] with a mixed-type inhibition pattern [31]. A high concentration of DTE is needed for maximal enzyme activity in the organelle fraction (P 16 000×g) as well as in the microsomal fraction (P 105 000×g).

The addition of a thiol-group specific reagent such as DTNB resulted in a total loss of enzyme activity [31, 57]. Furthermore, in the absence of DTE as a thiol-group protecting compound, the enzyme activity decreased as a function of time and of temperature [45]. It was possible to reactivate the HMG-CoA reductase activity, especially in the organelle fraction (P 16 000×*g*), upon addition of excess amounts of DTE in a time and temperature dependent process [45]. This led us to conclude that activation/deactivation of plant HMG-CoA reductase might be regulated by direct reduction/oxidation of the enzyme molecules as a covalent modification process supposed for a series of plant enzymes such as glucose-6-phosphate dehydrogenase [66]. The mixed-type inhibiton pattern by CoASH of microsomal HMG-CoA reductase from radish seedlings [31] can perhaps be explained by assuming that the CoASH (or the CoA disulfide present in the solution) might not singularly bind to a single site of the enzyme molecule.

A comparison of the structures of citrinin and of mevinolin-type entities leads to the assumption that a bicyclic molecule moiety present in both compounds might primarily interfere with the binding site(s) of HMG-CoA reductase against CoASH (or CoA disulfide). For an exact stereo drawing of a mevinolin-type compound, dihydromevinolin, see ref. [7]. Whereas the inhibition of HMG-CoA reductase by citrinin requires concentrations in the 10^{-4} M range [59], that for the inhibition by mevinolin (identical with monacolin K) is several orders of magnitude lower [1, 12–14, 48, 49]. This can easily be explained because mevinolin-type metabolites bind at least at two distinct, probably neighboring sites of the HMG-CoA reductase molecules, one binding the HMG-like moiety of the inhibitor molecule, and the other binding the more unpolar bicyclic moiety resulting in a multiplication of the inhibition constants for HMG and CoASH.

Therefore, it is of considerable interest to discuss the results of Rogers and Rudney [67], who used immunological techniques to evaluate the interaction of various compounds such as HMG-acid, CoASH, HMG-CoA, NADP, NADPH, compactin or mevinolin with rat liver HMG-CoA reductase molecules. By the aid of clean preparations of rabbit anti-HMG-CoA reductase IgG, these authors could demonstrate that upon preincubation of the HMG-CoA reductase, these compounds interfere strongly with the immunotitration curves of the free and membrane-bound enzyme. Preincubation of the enzyme with free CoASH together with HMG acid, being comparably effective as HMG-CoA itself, results in conformational changes in the enzyme, which might be expected to affect the ability of inhibitory antibodies to alter catalytic activity of HMG-CoA reductase [67]. Rogers and Rudney [67] also observed an apparent irreversibility of the effect of mevinolin or compactin on the immunoinhibition of enzyme activity which indicates that these compounds may affect anti-reductase IgG in a different manner than the substrates mentioned above, perhaps through a tight binding also to other regions of the HMG-CoA reductase molecule, inducing a change in enzyme conformation and causing a blockade of antigenic sites. Alberts *et al.* [1] already described that the inhibition type by mevinolin of rat liver HMG-CoA reductase slightly depends on the order of mixing enzyme, substrates and inhibitor in the test system. We made comparable observations when we used partially purified HMG-CoA reductase preparations from yeast. Especially at low HMG-CoA concentrations, we could observe a tendency for an upward shape of the curves in the Dixon-plot (Fig. 4), possibly indicating some non-linear inactivation of the enzyme by mevinolin which requires further investigations.

The product inhibition studies using microsomal-bound HMG-CoA reductase from radish seedlings with NADPH as an inhibitor yielded up to a concentration of 2×10^{-4} M, an uncompetitive pattern towards HMG-CoA; at higher concentrations, however, one of noncompetitive type [31]. Other products, substrates or analogues such as mevalonic acid, 3,3-dimethylglutaric acid or HMG-acid exhibited no clearly inhibitory effect even at unphysiologically high concentrations. It was therefore concluded that besides the redox state of the enzyme [45], the NADP/NADPH ratio within the cell might also be responsible for the regulation state of the plant enzyme activity [31]. This could probably also explain the slight sigmoidity of plant HMG-CoA reductase activity as a function of NADPH concentration. We cannot rule out that microsomal preparations from etiolated plant tissue might also contain further enzyme(s) such as ubiquitous thioredoxin(s) (*cf.* ref. [68] and literature cited herein) which use NADPH as a hydride donator for the reduction of disulfide bonds – possibly an inactive form of

HMG-CoA reductase molecules – and consequently, convert other enzymes into an active, reduced state. These aspects have to be further investigated by the use of solubilized and purified HMG-CoA reductase from plant tissue.

Conclusion

There is good reason to believe that there are common properties of HMG-CoA reductase molecules from widely different sources (on an evolutionary scale) which may reflect the existence of regulatory systems for isoprenoid biosynthesis already present in early eukaryotic cells (*cf.* ref. [30, 36]). Therefore, we have extensively considered related experiments with animal tissue or yeast as an enzyme source.

That the enzyme HMG-CoA reductase plays an important role in determining the flux of mevalonic acid throughout the branched isoprenoid pathway, is furthermore emphasized through the existence of highly specific and naturally produced antibiotics such as mevinolin (Fig. 1) which are directed exclusively against this enzyme.

In addition, our data demonstrating the inhibitory potency of mevinolin on plant growth and on isoprenoid biosynthesis [48] is a piece of evidence for the importance of a functioning biosynthetic pathway that directs the HMG-CoA to MVA to IPP and to all various isoprenoid compounds depending upon the needs of the plant cells for these materials. The regulation at this particular step might be thought of as a coarse-control, not ruling out that other steps in different branches of the pathway might be tuned for finer control depending on developmental stages and cell differentiation. Mevinolin promises to serve as a powerful tool to elucidate the regulation of this multibranched pathway in plants that is considered to be extraordinarily complex.

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