3-Hydroxy-3-methylglutaryl Coenzyme A Reductase from Spruce (*Picea abies*). Properties and Regulation

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Z. Naturforsch. **45c,** 973–979 (1990); received March 20/June 27, 1990

Picea abies, Cell Culture, HMGCoA Reductase, Enzyme Induction

HMGCoA reductase was identified in seedlings, callus cultures, cell suspension cultures and in needles of spruce (*Picea abies*) (L.) (Karst). Activity was found in both the 18 K pellet and in the 105 K pellet with different ratios between the two fractions from the various sources. The enzyme has a pH-optimum of 7.9 and an absolute requirement for NADPH. The presence of a thiol reagent such as dithiothreitol is required for activity. K_m for HMGCoA is 20–25 μM. Detergents have differential effects on the activity.

In seedlings, enzyme activity was considerably higher in the hypocotyls than in the cotyledons. Enzyme activity was high in dark-grown and low in light-grown seedlings. When the light conditions were reversed, levels of activity adapted to the respective new conditions (increase or decline of specific activity).

Aerobic incubations of seedlings, callus cultures or needles in medium containing a carbon source, resulted in a large (up to 20-fold) transient increase of HMGCoA reductase activity. Transfer of stationary phase cell suspension cultures into new medium caused a similarly large increase of activity. A number of carbohydrates induced the enzyme, glucose, fructose and sucrose being most effective. The increase of activity was prevented by cycloheximide. All changes of activity were much more pronounced in the 18 K pellet HMGCoA reductase.

Introduction

3-Hydroxy-3-methylglutaryl Coenzyme A reductase (EC 1.1.1.34) catalyzes the conversion of HMGCoA to mevalonic acid. This irreversible reaction occurs early in the biosynthesis of the large group of isoprenoid compounds. The enzyme, in mammalian cells, is assumed to be the major rate-determining activity for the synthesis of cholesterol [1, 2]. In yeast, HMGCoA reductase is the rate-controlling enzyme for the formation of ergosterol, being under the control of a number of regulatory factors [3, 4]. The isoprenoid pathway in plants is the source of a wide variety of essential compounds including sterols, carotinoids, the partially isoprenoid ubiquinone, plastoquinone, tocopherols and plant growth regulators such as the gibberellins and abscisic acid. A number of phytoalexins, important in plant disease resistance, also are isoprenoid compounds. Thus, some metabolites of the pathway are essential components in cell structure and function, while others are important regulators of cell growth and development.

To date, HMGCoA reductase was investigated in only a limited number of plant systems [5] (for a review see [6]). While most previous work in plants was carried out with cells or tissues from angiosperms, the present report, to our knowledge, for the first time presents data on the enzyme from a gymnosperm. Parts of the results have already been published in preliminary form [7, 8].

Abbreviations: HMGCoA, 3-Hydroxy-3-methylglutaryl Coenzyme A; DTE, dithioerythritol; DTT, dithiothreitol.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0900–0973 $ 01.30/0

Experimental

Plant Material

Seedlings

Seeds of *Picea abies* (L.) (Karst) were germinated and seedlings grown on humid Agriperl (plant perlite) under a normal daylight cycle at 20–22 °C and watered with tap water. For the experiments with light- and dark-grown seedlings (see Table III), the seedlings were cultivated under continuous white light (fluorescent tubes, Silvania GROLux 40 W, 400 μW/cm²) or in complete darkness for 16 days. Then the light conditions were reversed for 1 day and subsequently again reversed for 1 day.

Callus cultures and cell suspension cultures

These were prepared and propagated as described in detail [9].

Needles

These were taken during April and May from the tips of branches of 35–45 year old trees. The branches had been exposed to full light.

Incubations

Seedlings, callus cultures or needles were suspended in medium (3 g fresh weight/150 ml) (Murashige and Skoog plant salt mixture) (Flow Laboratories, Irvine, Scotland) and incubated in 1 liter fluted conical flasks on a rotary shaker (60 rpm) at 27 °C in continuous white light (see above).

Enzyme isolation

Plant material was homogenized (1 g fresh weight/10 ml buffer) with a Potter Elvehjem homogenizer in 0.05 M Tris-HCl buffer pH 7.5, containing 0.4 M sucrose, 0.01 M $MgCl_2$, 0.02 M EDTA, 0.001 M β-mercaptoethanol and 10 percent (w/v) polyvinyl pyrrolidone 40 (soluble) at medium speed for 30 sec. The homogenate was then sonicated for 30 sec.

The homogenate was centrifuged for 10 min at 2000 × *g* and the resulting supernatant was further centrifuged for 40 min at 18,000 × *g*. The pellet of this centrifugation was washed in the above buffer and recentrifuged. The supernatant of the first 18,000 × *g* centrifugation was further centrifuged for 60 min at 105,000 × *g*. The pellet as well as the 18,000 × *g* pellet were each suspended in 0.4–0.8 ml of KH_2PO_4, containing 0.01 M EDTA, 0.005 M $MgCl_2$, adjusted to pH 7.5, and 0.05 M DTE. The suspensions were sonicated for 30 sec. The complete procedure was carried out at 4 °C.

Assay for HMGCoA reductase

The radioisotopic assay was performed as described [10]. Analyses were carried out in triplicate, variations being less than 10 percent. The activity is expressed as nmol of mevalonate formed per hour per mg protein.

Protein determination

Protein was determined by the method of [11], using bovine serun albumin as standard.

Results and Comments

Properties of HMGCoA reductase

HMGCoA reductase in plants is a membrane bound activity (for its localization see [6]). To identify the HMGCoA reductase in spruce, subcellular fractions of the plant material (seedlings, callus cultures, cell suspension cultures and needles) were prepared. It was found that there always was an 18 K as well as a 105 K pellet activity present, although the ratio of these activities in the two fractions varied among the examined plant material. The specific activities are summarized in Table I (see also section on Regulation).

Of the commonly used buffers, Tris-HCl pH 7.5 was most suited for the extraction of the enzyme. Bovine serum albumin, which can stabilize or activate plant HMGCoA reductase [12, 13], had no effect on the activity of the enzyme from spruce. The pH-optimum of the reaction was at 7.9, with a sharp decline above and a gradual decrease below this value. Thus, the spruce enzyme has a somewhat higher pH-optimum than that reported for the enzyme from other plant sources being between 6.8 and 7.5 [5, 14]. HMGCoA reductase from the alga

Table I. Specific activity of HMGCoA reductase in spruce.

	105 K pellet activity	18 K pellet activity
Seedlings		
A light grown	0.10 ± 0.011	0.17 ± 0.013
B dark grown	0.28 ± 0.020	0.46 ± 0.031
C cotyledons (dark grown)	0.15 ± 0.020	0.28 ± 0.030
D hypocotyls (dark grown)	0.39 ± 0.031	0.63 ± 0.048
Callus culture	2.6 ± 0.29	1.3 ± 0.11
Cell suspension culture	0.65 ± 0.045	0.41 ± 0.05
Needles		
A 0.2–0.3 cm	1.8 ± 0.2	0.8 ± 0.09
B 0.6–0.9 cm	0.9 ± 0.11	0.35 ± 0.025

Values are means ± SEM of 8–10 experiments.

Ochromonas malhamensis also has a pH-optimum of about 8.0 [12]. As in all other eucaryotic systems, the spruce HMGCoA reductase has an absolute requirement for NADPH. Only two bacterial strains are known, which exhibit a specific requirement for NADH (see [4]). The K_m for HMGCoA was 25 μM for the 18 K pellet activity and 20 μM for the 105 K pellet activity. Incubations of the extracts prior to assay did not stimulate the activity. There is thus no evidence for a reversible cold inactivation as has been reported for the mammalian enzyme [15].

HMGCoA reductase required reduced thiol groups for maximal activity [16–18]. Activation of the enzyme from spruce by different thiol compounds is shown in Fig. 1. DTT and DTE were most effective, both stimulating the activity 4–5-fold. Glutathione and β-mercaptoethanol were much less effective, while cysteine had almost no effect. Preincubation with the thiol compounds was not necessary for the activation. A partially inactivated enzyme preparation (freezing and thawing of enzyme fractions, prepared without a sulphhydryl-protecting agent) could be reactivated by excess amounts of DTT or DTE. A similar reactivation by DTT was reported for the HMGCoA reductase from radish seedlings [18]. Spruce HMGCoA reductase was inhibited by SH-inhibitors: In the presence of 10^{-7} M *p*-chloromercuribenzoate or 10^{-6} M iodoacetamide, the activity was completely inhibited.

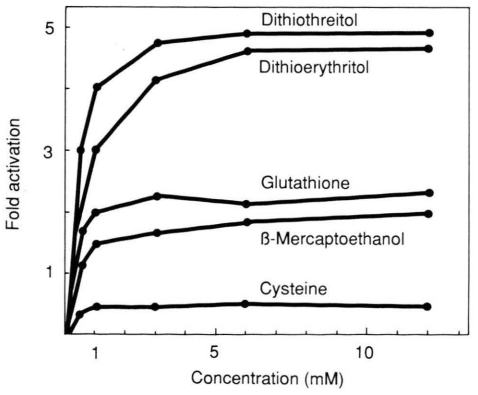

Fig. 1. Effect of thiol-protecting agents on the activity of HMGCoA reductase. Enzyme extract (callus culture) was prepared in the absence of a thiol compound. Activity was determined immediately after the preparation with the different SH-reagents present.

The effect of detergents on the activity of HMGCoA reductase has been studied in a number of plant systems [12, 16, 19]. Enzyme activity was enhanced in the presence of Triton X-100 [12, 16] and Tween 40 [19], while deoxycholate strongly inactivated the enzyme [19]. HMGCoA reductase from radish seedlings was solubilized by Brij W-1 and could subsequently be purified 350-fold [19]. Solubilization of the enzyme from potato tubers was also achieved by digestion with trypsin [20]. The *in vitro* effects of a number of detergents on HMGCoA reductase from spruce are summarized in Table II. As can be seen, Triton X-100 and Brij W-1 were without effect, while deoxycholate completely inhibited the enzyme. Tween detergents also affected the enzyme, the 105 K pellet activity being much more sensitive than the 18 K pellet activity. There is an obvious relationship between the degree of inhibition of the 18 K pellet activity and the fatty acid moiety of the Tween detergents (see Table II). For example, with a detergent concentration of 0.45 percent, activity was inhibited 80 percent by Tween 40, which contains palmitic acid, while inhibition was only 30 percent with Tween 60 (stearic acid) and no inhibition was seen with

Table II. Effect of detergents on HMGCoA reductase.

	Percent in assay	Percent inhibition 105 K pellet activity	18 K pellet activity
Tween 40	0.075	80	20
	0.15	90	40
	0.45	>95	80
Tween 60	0.075	70	10
	0.15	85	18
	0.45	90	30
Tween 85	0.075	60	< 5
	0.15	75	< 5
	0.45	85	< 5
Brij W-1	0.2	15	< 5
	0.6	20	< 5
Triton X-100	0.6	< 5	< 5
	1.2	< 5	< 5
	2.4	< 5	< 5
Deoxycholate	0.025	80	80
	0.1	>95	>95
	0.5	>95	>95

Aliquots of enzyme preparations (callus cultures) were assayed in the presence of the different concentrations of the detergents.

Table III. HMGCoA reductase activity in light- and in dark-grown seedlings of spruce and changes upon reversing the light conditions.

	Cultivation A 16d light	B 16d light + 1d dark	C 16d light + 1d dark + 1d light	D 16d dark	E 16d dark + 1d light	F 16d dark + 1d light + 1d dark
105K pellet activity	0.10 ± 0.012	0.30 ± 0.02	0.15 ± 0.018	0.33 ± 0.02	0.17 ± 0.015	0.27 ± 0.03
18K pellet activity	0.15 ± 0.011	0.48 ± 0.05	0.20 ± 0.022	0.45 ± 0.04	0.20 ± 0.015	0.38 ± 0.033
Glucose-6-P dehydrogenase	180 ± 19	170 ± 24	175 ± 19	240 ± 21	220 ± 19	215 ± 20

For conditions of cultivation see Experimental section. Values are specific activities (means ± SEM) of 6–8 experiments (A, B, D, E) and 2–3 experiments (C and F).

Tween 85, which contains oleic acid. Thus, the fatty acid moiety probably is of importance for the effect of the detergent on enzyme activity, this being an interaction with microsomal and mitochondrial membranes which bind the enzyme. It might well be that a similar relationship will emerge for the 105K pellet activity, when lower Tween concentrations than those listed in Table II are used.

Regulation of HMGCoA reductase

Table I summarizes the specific activities of the 18K and 105K pellets in the investigated plant material:

In seedlings, specific activity of the 18K pellet enzyme was higher than that of the 105K pellet enzyme (A–D). Also the specific activity was higher in the hypocotyls than in the cotyledons (C and D). These differences between cotyledons and hypocotyls prevailed throughout a 7–9 week period of cultivation. HMGCoA reductase also was higher in dark- than in light-grown seedlings (A and B) (see also below).

In callus cultures, specific activities were much higher than in the seedlings and the ratio between the two activities was reversed, 18K pellet activity being lower here.

In cell suspension cultures, specific activities were considerably lower than in callus cultures, from which they were derived. The ratio was identical in both cultures.

In needles, which again had high HMGCoA reductase activities, the ratio was identical with that of callus and of cell suspension cultures. Activities declined with increasing length of growing needles.

In seedlings, HMGCoA reductase activity was considerably higher in the hypocotyls than in the cotyledons (see Table I). The reason for this difference is not clear. Although elevated enzyme levels in the hypocotyls might indicate an increased sterol production for cell membrane synthesis, this seems unlikely as the high enzyme levels still prevail when the period of elongation is terminated. In addition, other enzymes of spruce seedlings also show an irregular distribution: phenylalanine ammonia-lyase, the regulatory enzyme for the synthesis of phenolic compounds, like HMGCoA reductase, has a much higher activity in the hypocotyls than in the cotyledons [8]. The enzymes of glycolysis also are not uniformly distributed: In spruce seedlings, grown autotrophically with minerals, all enzymes of glycolysis except fructosebisphosphate aldolase, glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase had 50 to 200 percent higher activities in the hypocotyls, while the activity of the latter enzymes was considerably higher in the cotyledons [21]. This distribution did not change up to 4 months of cultivation of the seedlings. Stilbensynthase of *Pinus silvestris* had a much higher activity in isolated roots than in needles [22].

As mentioned above, 18K and 105K pellet HMGCoA reductase were higher in dark- than in light-grown seedlings (see Table I, A and B). When the light conditions were reversed (dark ↔ light), levels of enzyme activity readily adapted themselves to the respective new conditions (increase or decline of specific activity). This is shown in Table III. When growth for 16 days in the light was

followed by a 1 day growth period in the dark, specific activities increased (A and B). Likewise, activity declined after 1 day growth in the light when it followed cultivation in the dark (D and E). When the light conditions were once again reversed, enzyme levels again adapted to the new conditions (B and C, E and F). Glucose-6-phosphate dehydrogenase, as control, had slightly higher levels of activity in dark-grown seedlings (A and D), however, no changes were observed when the light conditions were changed (A–F).

In radish [18] and pea [23], HMGCoA reductase also was high in dark-grown and low in light-grown seedlings and light-mediated variations have been reported. Changes of activity occurring in response to reversing the light conditions were also found with phenylalanine ammonia-lyase of spruce seedlings [8]. In contrast to HMGCoA reductase, this activity was low in dark-grown and high in light-grown seedlings [8].

The observations made in spruce suggest a reversible activation/inactivation of enzyme activity mediated by light. HMGCoA reductase of pea seedlings is known to be post-translationally controlled by phytochrome [23–25] (see also [26]). Other post-translational control of activity of this enzyme is by abscisic acid (negative control) [27] and by gibberellic acid and zeatin (positive control) [28]. However, from the present results, a phytochrome-mediated control of the spruce enzyme cannot be deduced.

When seedlings, calli or needles were suspended in medium containing a carbon source and were aerated, the activity of HMGCoA reductase increased drastically. This is shown in Fig. 2 for a callus culture, in Fig. 3 for needles and in Fig. 4 for seedlings. A similarly large (up to 20-fold) increase of HMGCoA reductase occurred after transfer of a cell suspension culture from the late linear growth phase into new medium. It can be seen that both 18K pellet and 105K pellet activity rapidly increased. This increase, which is shown in Fig. 2 for the callus culture, in all cases was transient and the activities declined again soon after having reached a maximum. 18K pellet activity always increased considerably more than 105K pellet activity (see Fig. 2, 3 and 4). The precise position of the peak levels of activity could vary from 24 to 48 h after initiation of induction. Also, maximal inducible levels of the same enzyme fraction could differ between identically performed experiments.

Similar rapidly occurring changes have already been reported for the activities of the nitrate reductase [29, 30], the nitrate uptake system [31], for phenylalanine ammonia-lyase and *p*-coumarate: CoA ligase [32] and for other enzymes related to the phenylpropanoid metabolism [33].

The induction of spruce HMGCoA reductase activity required the presence of carbohydrate and

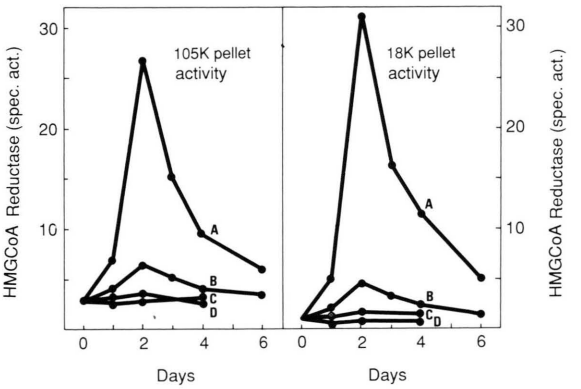

Fig. 2. Induction of HMGCoA reductase in callus cultures. Callus cells were suspended in medium (3 g fresh weight/150 ml) and incubated (see Experimental section). Sucrose, when present, was 1% (w/v).
A Oxygen, sucrose;
B oxygen, no sucrose;
C nitrogen, sucrose;
D oxygen, sucrose, 15 µg/ml cycloheximide.

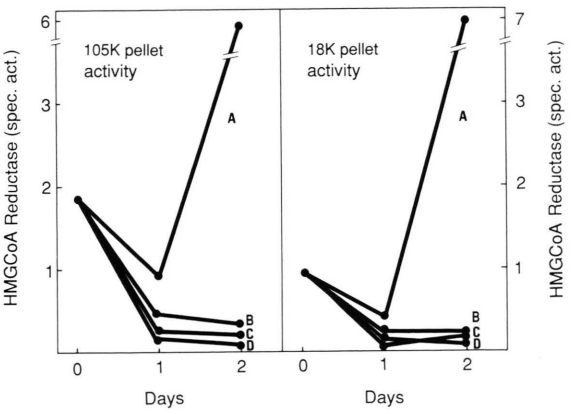

Fig. 3. Induction of HMGCoA reductase in needles. Needles (0.5–0.7 cm) were cut twice, suspended in medium (3 g fresh weight/150 ml) and incubated. Incubations A–D were the same as in Fig. 2.

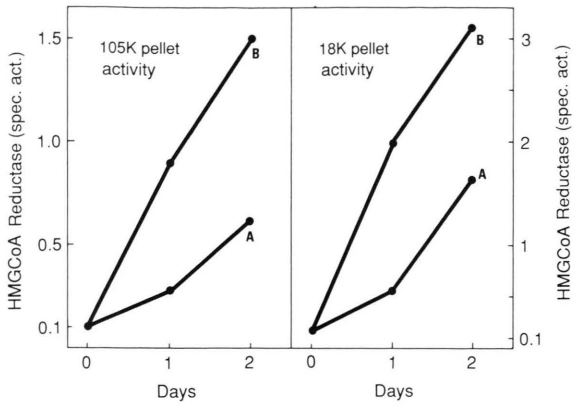

Fig. 4. Induction of HMGCoA reductase in intact and in cut seedlings. Seedlings grown for 14 days in the light were used. They were incubated (3 g fresh weight/150 ml) in medium, containing 0.5% (w/v) sucrose.
A Seedlings were carefully suspended in the medium to minimize damage of tissue;
B seedlings were cut to small pieces and immediately suspended in medium.

of oxygen and it was completely prevented by cycloheximide. This is also depicted in Fig. 2. There was only a small rise in the absence of a carbohydrate (B), this probably due to endogenous material still available. No increase occurred when oxygen was replaced by nitrogen (C). There was also no induction when 15 µg/ml of cycloheximide was added to the complete system (D). When the system containing carbohydrate was kept unter nitrogen for 48 h and then oxygen was given, a maximal induction still occurred (not shown).

When needles were aerated in a carbohydrate containing medium a rapid increase of HMGCoA reductase activity was found. This is shown in Fig. 3. However, as can be seen activity of both fractions first declined considerably before it began to increase (A). From the data presented in this figure it can be seen that in the complete system which contained sucrose and oxygen (A), this decline of activity was smaller than in a system lacking either carbohydrate or oxygen (B and C). The decline might represent an inactivation of enzyme, resulting from the action of intracellular components such as phenols. This inactivation is counteracted by the induction, which can take place in the complete system only, gradually leading to the final strong increase of activity. The enzyme becomes further inactivated (B and C) when carbon source or oxygen are lacking.

HMGCoA reductase activity also increased in seedlings when these were aerated in medium containing a carbon source. This is shown in Fig. 4 (A). When cotyledons and hypocotyls, which have different levels of activity (see Table I) were induced separately, both velocity and maximum levels of induction were identical. When induction was compared in intact seedlings and in seedlings, which had been cut to small pieces, large differences were observed (compare A and B in Fig. 4). It can be seen that the increase was much more intense in the cut (wounded) seedlings than in the intact ones.

The reason for this difference might be a greater sensitivity of the treated seedlings to the inductory stimulus. However, it is known from potato tubers, that wounding of tissue increases HMGCoA reductase activity, which is followed by an accumulation of phytoalexin (rishitin) [34] (see also [35]). It might well be that wounding of the cells in spruce also induces HMGCoA reductase. This would then become apparent in a greater increase of enzyme activity as seen in Fig. 4. However, experiments using cell suspension cultures made it rather unlikely that in spruce terpenes do have a function as phytoalexins in the pathogen-challenged system. When cell suspension cultures, which contained very small amounts of mono- and sesquiterpenes (unpublished data) were infected with the fungus *Rhizosphaera kalkhoffii,* an important needle pathogen (intact fungus or cell wall fractions), no effect on HMGCoA reductase was found (unpublished data). Thus, in contrast to phenylalanine ammonia-lyase, which was strongly induced under these conditions [36], HMGCoA reductase obviously was not inducible by the fungal elicitors. As reported, phytoalexins of *Picea abies* are stilbens (see [37]), which are biosynthetically derived from cinnamic acid, the regulatory enzyme of this pathway being phenylalanine ammonia-lyase.

The induction of HMGCoA reductase required the presence of a carbohydrate (see Fig. 2 and 3). Enzyme activity increased identically when the plant material was suspended in either sterile water or in medium, indicating that only the carbohydrate is essential for an induction to occur (unpublished data). A number of compounds was tested for their ability to induce HMGCoA reductase. Sucrose, fructose, glucose, galactose, mannose,

maltose, ribose, glycerol and pyruvate induced the enzyme. The first three compounds had an equivalent inductory capacity and were most effective. The others are listed in sequence of decreasing inductory ability. 6-deoxyglucose, lactose, xylose, arabinose, mannitol, citrate, acetate, malate and succinate were without effect. Determination of the effective concentration for an induction of HMGCoA reductase by sucrose is shown in Table IV: 0.02 percent sucrose could already induce the enzyme slightly and with 0.15 percent maximal induction was obtained.

From a more detailed investigation of the regulation of phenylalanine ammonia-lyase in cell suspension cultures of *Petroselinum hortense,* a model was proposed, in which the transient changes of the activity would arise from an interplay of potentially large variations in the rate of synthesis and an approximately constant rate of degradation of the enzyme [38]. This might also be valid for the HMGCoA reductase from *Picea abies.*

Table IV. Effect of sucrose concentration on the induction of HMGCoA reductase in spruce.

Percent sucrose in medium	Fold increase of activity
–	no increase
0.02	1.2
0.04	4.8
0.08	8.7
0.15	11.2
0.5	12.0

Callus cultures were suspended in medium (3 g fresh weight/150 ml) containing different concentrations of sucrose and were aerated for 24 h. 105 K pellet activity is shown as representative activity.

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