SHORT COMMUNICATIONS

Acta Cryst. (1995). D**51**, 386–389

Crystallization of HMG-CoA reductase from *Pseudomonas mevalonii.**

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(*Received* 3 *January* 1994; *accepted* 23 *August* 1994)

Abstract

Crystals of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase from *Pseudomonas mevalonii* have been grown by vapor diffusion in hanging drops at pH 6.7 using ammonium sulfate as the precipitant. Serial dilution seeding and manipulation of glycerol concentration were both used to obtain crystals larger than 1.0 mm. The crystals are cubic, space group $I4_132$, with $a = 229.4$ Å. A V_m value of 2.71 Å^3 Da^{-1} indicates 96 molecules per unit cell with two molecules in the asymmetric unit. These crystals diffract to 2.8 Å with conventional X-ray sources, and beyond 2.4 Å with synchrotron radiation.

Introduction

HMG-CoA reductase is an enzyme which catalyzes the interconversion of HMG-CoA and mevalonate. Mevalonate serves as the precursor of a wide variety of isoprenoids which include dolichol, heme A, isopentenyl adenine and ubiquinone as well as cholesterol and its derivatives: bile salts, steroid hormones and vitamin D (Goldstein & Brown, 1990; Rodwell, Nordstrom & Mitschelen, 1976). In mammals, the bulk product of mevalonate metabolism is cholesterol, and the rate-limiting step in its synthesis is the production of mevalonate by HMG-CoA reductase. Complex regulatory mechanisms in these systems balance the diverse needs for mevalonate against the damaging effects of overproduction of cholesterol (Goldstein & Brown, 1990). In mammalian cells, HMG-CoA reductase is regulated by feedback control of transcription and translation (Goldstein & Brown, 1990), by enzyme degradation (Chin *et al.*, 1985; Chun & Simoni, 1992; Roitelman, Olender, Bar-Nun, Dunn & Simoni, 1992), and by reversible phosphorylation of a serine residue (Clarke & Hardie, 1990; Gibson & Parke, 1985; Kennelley & Rodwell, 1985).

The interconversion of (*S*)-HMG-CoA and (*R*)-mevalonate catalyzed by HMG-CoA reductase is a four-electron oxidoreduction which in eukaryotes uses NADPH as the reductant. The reaction is believed to occur through two successive reductive stages and to involve the putative enzyme-bound intermediate mevaldehyde (Veloso, Cleland & Porter, 1981; Qureshi, Dugan, Cleland & Porter, 1976). While in physiologic terms the conversion of HMG-CoA to mevalonate is effectively irreversible, the isolated enzyme catalyzes the reaction in either direction. Both rat liver (Qureshi *et al.*, 1976) and yeast (Sherban, Kennelly, Brandt & Rodwell, 1985) HMG-CoA reductases will catalyze (*a*) reduction of HMG-CoA to mevalonate, (*b*) the oxidation of mevalonate to HMG-CoA, as well as the two putative half reactions (*c*) reduction of mevaldehyde to mevalonate and (*d*) the oxidation of mevaldehyde to HMG-CoA.

$$(a)\ \text{HMG-CoA} + 2\text{NADPH} + 2\text{H}^+ \rightarrow \text{Mevalonate} + 2\text{NADP}^+ + \text{CoASH};$$

$$(b)\ \text{Mevalonate} + 2\text{NADP}^+ + \text{CoASH} \rightarrow \text{HMG-CoA} + 2\text{NADPH} + 2\text{H}^+;$$

$$(c)\ \text{Mevaldehyde} + \text{NADPH} + \text{H}^+ \rightarrow \text{Mevalonate} + \text{NADP}^+;$$

$$(d)\ \text{Mevaldehyde} + \text{NADP}^+ + \text{CoASH} \rightarrow \text{HMG-CoA} + \text{NADPH} + \text{H}^+.$$

While coenzyme A is not required for reaction (*c*), it stimulates reduction of mevaldehyde to mevalonate by all known forms of HMG-CoA reductase (Qureshi *et al.*, 1976; Sherban *et al.*, 1985; Jordan-Starck & Rodwell, 1989*a*).

Eukaryotic HMG-CoA reductases are membrane-bound molecules with an approximate molecular mass of 100 kDa (Goldstein & Brown, 1990; Olender & Simoni, 1992; Roitelman *et al.*, 1992). Sequence analysis indicates the enzyme consists of two approximately equally sized domains. The hydrophobic N-terminal domain anchors the protein to the endoplasmic reticulum, while the cytosolic C-terminal domain contains the catalytic center.

A bacterial HMG-CoA reductase has been identified in *Pseudomonas mevalonii*, a Pseudomonad capable of growth on mevalonate as its sole carbon source (Gill, Beach & Rodwell, 1985). When *P. mevalonii* is grown on mevalonate, expression of HMG-CoA reductase is induced up to 800-fold. Under these conditions the enzyme serves a catabolic role, converting mevalonate into HMG-CoA, which is further catabolized to acetoacetate and acetyl-CoA. Consistent with its catabolic role, the *P. mevalonii* enzyme utilizes NADH as a reductant. In further contrast to the eukaryotic enzymes, HMG-CoA reductase from *P. mevalonii* lacks a membrane anchor domain, and is a soluble protein of molecular mass 45 kDa which is easily purified to homogeneity (Gill *et al.*, 1985). *P. mevalonii* HMG-CoA reductase has been cloned, sequenced and overexpressed in *Escherichia coli* (Beach & Rodwell, 1989).

P. mevalonii HMG-CoA reductase has been shown to be a catalytic analog of the mammalian enzymes (Jordan-Starck & Rodwell, 1989*a*,*b*). This enzyme has 22% sequence identity with a consensus sequence of the catalytic domain of eukaryotic HMG-CoA reductases, while an additional 35% of the residues

* The abbreviations used in this paper are: HMG, 3-hydroxy-3-methylglutaryl; ADA, *N*-(2-acetamido)-2-iminodiacetic acid.

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are conservative substitutions. Conserved residues include residues known to be critical for catalysis (Wang, Darnay & Rodwell, 1990; Darnay, Wang & Rodwell, 1992). Like the mammalian enzymes, the bacterial enzyme catalyzes both the forward and reverse reactions as well as both half reactions. In the bacterial enzyme, coenzyme A also stimulates the conversion of mevaldehyde to mevalonate. These similarities to the mammalian HMG-CoA reductases, in conjunction with the availability of large quanties of the enzyme, make *P. mevalonii* HMG-CoA reductase an attractive subject for X-ray crystallographic studies. We report here the crystallization of HMG-CoA reductase from *P. mevalonii*.

Crystallization

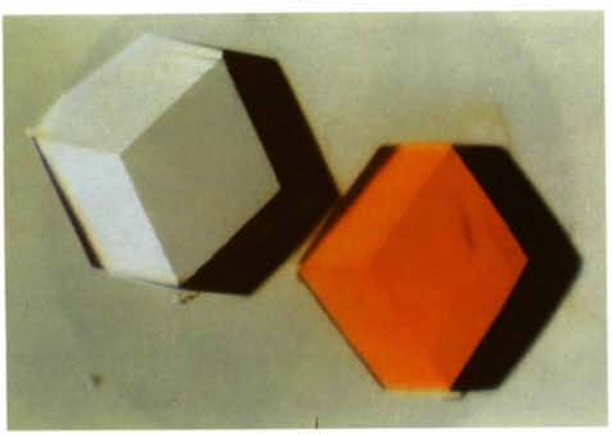
Recombinant *P. mevalonii* HMG-CoA reductase was purified to homogeneity as described previously (Beach & Rodwell, 1989). Purified enzyme in 10 m*M* Tris, pH 8.2, 400 m*M* KCl and 10% glycerol (added to stabilize the enzyme activity and prevent the formation of aggregates upon freezing) was filtered and concentrated by ultrafiltration. Crystallization attempts were made using the hanging-drop vapor-diffusion method (McPherson, 1982). Hanging drops which contained 5 µl of enzyme solution and 5µl of reservoir solution were hung over wells containing 1.0 ml of reservoir solution. Despite the use of a wide variety of buffers, precipitants, counter-ions, protein concentrations and temperatures, initial crystallization trials were unsuccessful. With the belief that glyerol was interfering with nucleation, we then removed glycerol by passing the enzyme over a Sephadex G-25 column equilibrated with 400 m*M* KCl, 10 m*M* Tris, pH 8.2. Enzyme treated in this fashion was stable by the criterion of a high specific activity as long as it was not refrozen. Subsequent attempts at crystallization of the glycerol-free HMG-CoA reductase occasionally produced crystals under a variety of conditions, all of which included ammonium sulfate. Most of the crystals produced in the experiments were too small or badly twinned. However, those grown over 1.2 *M* ammonium sulfate, 75 m*M* sodium citrate, 100 m*M* sodium ADA, pH 6.5 as the reservoir solution (synthetic mother liquor) gave clusters of crystals which could be separated into single crystals (Fig. 1*a*). These clusters appeared in one to six months and were difficult to reproduce.

The spontaneous crystals were successfully utilized for microseeding to give single seeded crystals. To produce a seeding solution, a crystal was placed in 10 µl of synthetic mother liquor and crushed. Tenfold serial dilutions of the crushed crystal were then made in synthetic mother liquor. Hanging drops were assembled as described above with the addition of 2 µl of diluted seeding solution. Approximately 10^5-fold dilutions of seeds produced a single crystal per hanging drop, while 10^2-fold dilutions produced a shower of microcrystals. Showers of microcrystals produced in this way were substituted for the crushed crystal in subsequent seeding experiments. Small single crystals from these seeding experiments appeared overnight as rhombic dodecahedra, but rapidly grew into an irregular morphology. Since nucleation of crystals was now provided by seeding, 10% glycerol was reintroduced in an attempt to slow crystal growth. This resulted in large single crystals with rhombic dodecahedral morphology that grew in two weeks time to a diameter greater than 1 mm (Fig. 1*b*). It is now apparent that glycerol both inhibits nucleation and slows crystal growth, and that manipulation of glycerol concentrations can be exploited in the crystallization of *P. mevalonii* HMG-CoA reductase.

X-ray diffraction data

Characterization of the HMG-CoA reductase crystals employed precession photography using a Supper precession camera at a crystal-to-film distance of 100 mm and Ni-filtered Cu $K\alpha$ radiation generated by an Elliot GX-20 rotating-anode X-ray generator operated at 35 kV and 40 mA. Analysis of the diffraction patterns showed that these crystals belong to the cubic space group $I4_132$ with $a = 229.4$ Å. Assuming a partial specific volume for protein of 0.75 $cm^3\ g^{-1}$, two monomers in each of the 48 asymmetric units of this space group gives a V_m of 2.71 $Å^3\ Da^{-1}$, consistent with average protein crystal densities (Matthews, 1968). Precession photographs ($\mu = 12°$, maximum resolution 3.7 Å) down the fourfold [100] and threefold [111] axes are shown in Fig. 2. Both photographs

(*a*)



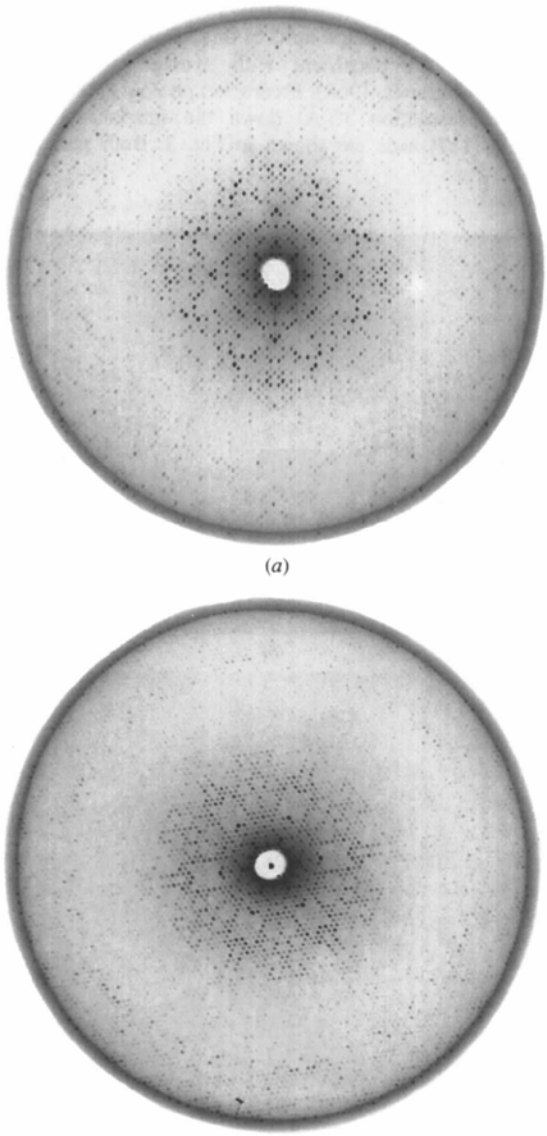
(*b*)

Fig. 1. Crystals of *P. mevalonii* HMG-CoA reductase. (*a*) Spontaneous crystals of HMG-CoA reductase. Crystal clusters such as these appear infrequently and take one to six months to grow. (*b*) Crystals of HMG-CoA reductase produced by seeding in the presence of 10% glycerol. The largest dimension of these crystals is approximately 1.5 mm. The crystal on the right has been soaked in the presence of fluorescein mercuric acetate; the yellow color demonstrates the uptake of this heavy-atom compound by the crystal.

clearly show the strong falloff of the diffraction data with resolution which is characteristic of these crystals.

Native data from the *P. mevalonii* HMG-CoA reductase crystals were collected on a San Diego Multiwire Systems (SDMS) two-chamber area detector mounted on a Rigaku 12 kW high-brilliance X-ray generator run at 50 kV and 100 mA with monochromator-selected radiation. The crystal-to-detector distances were 980 and 910 mm for the two detectors. Two min frames were collected with an oscillation angle of 0.1°. Integrated intensities corrected for Lorentz and polarization effects were obtained using the *SDMS* software (Xuong, Sullivan, Nielsen & Hamlin, 1985; Xuong, Nielsen, Hamlin & Anderson, 1985). These data were then scaled together using a locally modified version of the program *CORRECT* (Kabsch, 1988). A total of 180000 observations of 25001 unique reflections to 2.8 Å resolution were collected from a single crystal wet mounted in synthetic mother liquor and held in place with pipe cleaner fibers. The resulting data set is 98.8% complete with an overall R_{sym} on intensity of 5.6%. The highest resolution shell (3.16 to 2.83 Å) is 98.4% complete with $I > 3\sigma(I)$ for 75% of the unique reflections.

High-resolution oscillation data have been collected from native crystals of HMG-CoA reductase on station F1 at the Cornell High Energy Synchrotron Source (CHESS). The data were collected on image plates at a wavelength of 0.96 Å and a crystal-to-film distance of 75 mm. The oscillation angle was 0.5° and the exposure time was 5 s. The crystals diffracted well to 2.4 Å with some data to 2.2 Å, however, the crystals experience severe decay problems such that only the first two images contain this high-resolution data. Therefore, images were collected from 60 different crystals shot in random orientations. In the high-symmetry space group $I4_132$ this amount of data should suffice for a complete high-resolution native data set with considerable redundancy.



(*a*)

(*b*)

Fig. 2. Precession photographs ($\mu = 12°$) from crystals of *P. mevalonii* HMG-CoA reductase. (*a*) Precession photograph showing the diffraction pattern taken along the fourfold [100] axis of crystals of HMG-CoA reductase. (*b*) Precession photograph showing the diffraction pattern taken along the threefold [111] axis of crystals of HMG-CoA reductase.

Discussion

The effects of using glycerol as a cosolvent in protein crystallization have been studied in the crystallization of T7 RNA polymerase where the inclusion of glycerol was found to be essential for crystallization (Sousa & Lafer, 1990; Sousa, Lafer & Wang, 1991). In contrast, the results described here clearly indicate that 10% glycerol acts to inhibit nucleation of *P. mevalonii* HMG-CoA reductase crystals. This study suggests that glycerol should be removed for initial crystallization trials if crystal growth is nucleation limited. In addition, when crystal growth is not nucleation limited, glycerol may be introduced as an additive to influence crystal morphology, or to reduce the numbers of nucleation centers. When either micro- or macroseeding protocols are being developed, glycerol may be used to eliminate spontaneous nucleation. Addition of glycerol as a cosolvent may at the same time have positive effects on crystal morphology. The effect of glycerol on the growth rate and morphology of HMG-CoA reductase crystals does not begin to appear until glycerol concentrations are greater than 5%. This suggests that glycerol concentrations of 5% or greater should be considered for micro- and macroseeding when there is a need to reduce or eliminate spontaneous nucleation events.

The determination of the crystal structure of HMG-CoA reductase from *P. mevalonii* will provide greater understanding of the structure–function relationships of four-electron oxidoreductases for which there is a lack of crystallographic information, and more specifically, will provide further insights into the mechanism of action of HMG-CoA reductase, a key enzyme in cholesterol biosynthesis.

We thank Tujuanda Jordan-Starck and Jack Green for many useful discussions and helpful suggestions. CML gratefully acknowledges support from an American Heart Association, Indiana Affilate, Predoctoral Fellowship. This work was also supported by Grant-In-Aid No. 91014190 from the American Heart Association to CVS and, in part, by a National Institutes of Health Grant No. HL 4713 to VWR.

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