

SPECIFIC BINDING OF β -LACTONE 1233A
TO 3-HYDROXY-3-METHYLGLUTARYL-
COENZYME A SYNTHASE

Sir:

A naturally occurring β -lactone, 1233A (F-244, L-659,699), the structure of which has been elucidated to be 2*R*-hydroxymethyl-3*R*-hydroxy-8*R*,10,12-trimethyl-(*E,E*)-10,12-tetradecadiendioic acid 1,3-lactone,^{1,2)} is a potent and specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase.^{3~5)} Several research groups have studied the mode of action of 1233A on HMG-CoA synthase, but the reversibility of 1233A inhibition was still unclear. We reported preliminarily that the inhibition of HMG-CoA synthase by 1233A is irreversible because the enzyme activity was not recovered by any degree of dilution when the enzyme was pretreated with 1233A.⁴⁾ However, by similar experiments, GREENSPAN *et al.*⁵⁾ reported the data suggesting reversible inhibition, while MAYER *et al.*⁶⁾ suggested irreversible inhibition. To clarify this discrepancy, we tried to use [¹⁴C]1233A with a highly specific radioactivity prepared biosynthetically.⁷⁾ In this communication, we provide the evidence showing that 1233A inhibits HMG-CoA synthase by binding to the enzyme specifically and irreversibly.

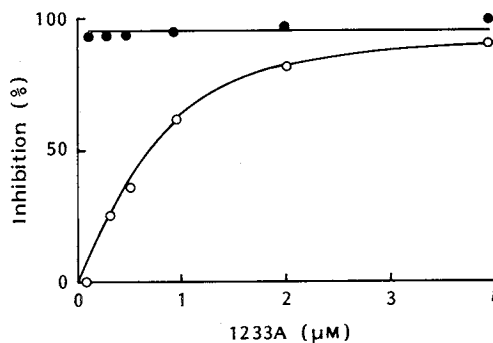
The preparation of 1233A and [¹⁴C]1233A (27.2 Ci/mol) was reported previously.^{7,8)} HMG-CoA synthase was prepared from livers of rats fed lovastatin (0.1%) and cholestyramine (5%) for 7 days. The enzyme was purified through the DEAE-cellulose step according to the similar method of MEHRABIAN *et al.*⁹⁾ HMG-CoA synthase activity was assayed by two methods. In Method A, the breakdown of acetoacetyl-CoA in the presence of acetyl-CoA or CoA was followed by monitoring spectrophotometrically at 303 nm of the enol form as described by LOWE and TUBBS.¹⁰⁾ The assay mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 μ M acetoacetyl-CoA and enzyme in a total volume of 950 μ l was preincubated at 30°C and the reaction was initiated by the addition of 50 μ l of 2 mM acetyl-CoA (final 100 μ M). In Method B, HMG-CoA synthase was assayed using [¹⁻¹⁴C]acetyl-CoA as described previously.⁴⁾

The result given in Fig. 1 showed the irreversible inhibition of HMG-CoA synthase by 1233A. The IC₅₀ value in the experiment without preincubation was calculated to be 0.65 μ M, supporting the previous observation (IC₅₀: 0.20 μ M).⁴⁾ However, when the

enzyme was preincubated with 0.5 μ M 1233A which produced only 35% inhibition in the assay without preincubation, no enzyme activity was observed regardless of the final concentration of 1233A (0.05~4.0 μ M). Furthermore, even after Sephadex G-25 gel filtration or dialysis of the pretreated enzyme, no HMG-CoA synthase activity was recovered (data not shown).

To examine whether 1233A reacts directly on HMG-CoA synthase, partially-purified HMG-CoA synthase was incubated with [¹⁴C]1233A at 37°C for 5 minutes, and the reaction was stopped by heating the mixture in the presence of SDS. The SDS-PAGE analysis revealed that only 53 kilodalton protein was labeled with [¹⁴C]1233A (Fig. 2), suggesting that 1233A reacts specifically with the subunit of HMG-CoA synthase.^{9,11)} The [¹⁴C]-1233A-bound protein was resistant to SDS-, heat-, and mercaptoethanol-treatments. The β -lactone ring of 1233A, responsible for HMG-CoA synthase inhibition, appears reactive with a cysteine SH-group, or a serine or threonine OH-group. In fact, HADVARY *et al.*¹²⁾ demonstrated that β -lactone tetrahydrolipstatin blocks pancreatic lipase by binding covalently to the active site serine. From the study on the reaction mechanism of HMG-CoA synthase, it is proposed that the active site cysteine SH-group is rapidly acetylated by acetyl-CoA to yield acetyl-S-enzyme and that further condensation reaction with acetoacetyl-CoA is proceeded on

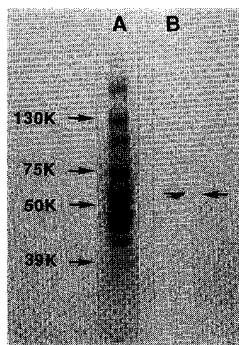
Fig. 1. Effect of dilution on HMG-CoA synthase pretreated with 1233A.



HMG-CoA synthase (65 μ g) was incubated in a volume of 100 μ l containing either no (○) or 0.5 μ M (●) 1233A at 37°C for 10 minutes. At the end of this period of time, 20 μ l of the enzyme solution was added to 180 μ l of the standard reaction mixture (Method B) containing various concentrations (0~3.95 μ M) of 1233A and the mixture was incubated at 37°C for 30 minutes to determine HMG-CoA synthase activity.

Fig. 2. SDS-PAGE of partially-purified HMG-CoA synthase incubated with [^{14}C]1233A.

A. Coomassie-blue staining. B. Autoradiography.

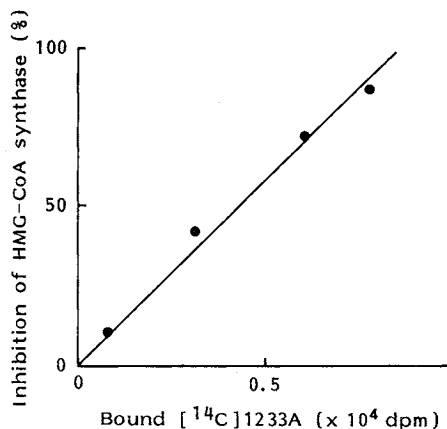


HMG-CoA synthase (500 μg) was incubated with [^{14}C]1233A (4 nmol, 0.11 μCi) at 37°C for 5 minutes in a total volume of 100 μl . Then, 100 μl of a buffer containing 6.25 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% BPB was added and the mixture was heated at 100°C for 3 minutes. Twenty microliters of the sample was applied for electrophoresis.

the enzyme to form HMG-CoA.^{13,14} Therefore, it is possible to imagine that the carbonyl moiety of the β -lactone ring attacks the active site cysteine to form an enzyme bound thioester. MIZIORKO *et al.*¹⁵ reported that 3-chloropropionyl-CoA inhibits irreversibly HMG-CoA synthase by alkylating the active site cysteine and that the synthase activity was protected against the inactivation by the inhibitor when it was preincubated with the substrate, acetyl-CoA or acetoacetyl-CoA. However, the inhibition caused by 1233A was not prevented even when the synthase was preincubated with 100 μM acetyl-CoA or acetoacetyl-CoA. It might be that the reactivity of 1233A to the cysteine is much higher than that of the substrates or that 1233A alkylates an amino acid of unknown active sites other than the active site cysteine.

As shown in Fig. 3, a linear correlation between [^{14}C]1233A bound to the enzyme and the enzyme inhibition was shown. In this experiment, about 1×10^4 dpm of [^{14}C]1233A (170 pmol) bound to the enzyme produced the complete inhibition of HMG-CoA synthase. Bases on the purity of the synthase estimated from the specific enzyme activity in comparison with that of the homogenous synthase, it is speculated that one to two moles of 1233A are bound to one mole of the enzyme with complete loss of the enzyme activity. This appears reasonable because HMG-CoA synthase comprises

Fig. 3. Correlation between the amount of [^{14}C]1233A bound to HMG-CoA synthase and the inhibition of HMG-CoA synthase activity.



HMG-CoA synthase (980 μg) was incubated with various concentrations of [^{14}C]1233A (0~1.5 nmol = 40 nCi) in a total volume of 200 μl at 37°C. After 10 minutes, 50 μl of the [^{14}C]1233A-treated enzyme solution removed was subjected to the assay for HMG-CoA synthase according to Method A, and at the same time 100 μl of the enzyme solution removed was added to 500 μl of 10% trichloroacetic acid (TCA) solution. The acid-denatured protein was cooled on ice for 30 minutes, centrifuged and washed twice with 10% TCA solution. The radioactivity of [^{14}C]1233A-bound precipitate was counted with a liquid scintillation spectrometer.

homo dimers of the 53 kilodalton subunit.^{9,11} However, the binding site and the binding mode of 1233A remain to be investigated.

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