Kinetic Analysis of the Individual Reductive Steps Catalyzed by β-Hydroxy-β-methylglutaryl-coenzyme A Reductase Obtained from Yeast†

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ABSTRACT: The mechanism of action of yeast β-hydroxy-β-methylglutaryl-coenzyme A reductase has been investigated through kinetic studies on the oxidation of mevaldate by nicotinamide adeninine dinucleotide phosphate (NADP) in the presence of coenzyme A (CoA) and on the reduction of mevaldate by reduced NADP (NADPH) in the absence or presence of CoA or acetyl-CoA. NADP and mevalonate were also used as product inhibitors of the reduction of mevaldate. In the reduction of mevaldate to mevalonate, coenzyme A and acetyl-CoA decreased the K_m for mevaldate 30- and 3-fold, respectively. Both compounds increased the V_{max} 1.5-fold. These results suggest that CoA is an allosteric activator for the second reductive step and that it acts by enhancing the binding of mevaldate. The intersecting patterns obtained from initial velocities and the patterns produced by product inhibitions suggest the following features of the mechanism. The binding of substrates and release of products proceeds sequentially in both reductive steps, and is ordered throughout or random with respect to the binding of the β-hydroxy-β-methylglutaryl-coenzymeA and the first NADPH. The binding of NADPH enhances the binding of the β-hydroxy-β-methylglutaryl portion of the CoA ester and the binding of free mevaldate, whereas the binding of NADP leads to an increased affinity of the enzyme for the hemithioacetal (of mevaldate and CoA) and for mevalonate. Thus, the replacement of NADP by NADPH after the first reductive step promotes the conversion of the hemithioacetal to the free carbonyl form, which is then rapidly reduced. The products, CoA and mevalonic acid, of the second reductive step leave the enzyme before the release of the second NADP. This release of the last product is probably the rate-limiting step for the overall process.

The present investigation was undertaken to determine the sequence of events that take place when substrates are added to, and products are released from, HMG-CoA[1] reductase during the reduction of HMG-CoA to mevalonic acid by yeast. This is a two-step reduction of an ester to an alcohol and might proceed by either of the alternate routes shown in reactions 1 or 2. It is also possible that hemithioacetal formed in the first step is converted to free aldehyde prior to the second reductive step.

The reaction utilizes NADPH as the reducing pyridine nucleotide and K_{eq} is very large (Durr and Rudney, 1960). The product of the first reductive step has not been isolated (Bensch and Rodwell, 1970; Durr and Rudney, 1960), suggesting that there is not a free intermediate. However, it has been shown that the β-hydroxy-β-methylglutaryl moiety is not covalently bound to the enzyme through acyl transfer from coenzyme to an enzyme sulfhydryl group (Retey et al., 1970). Coenzyme A accelerates the second reductive step (Retey et al., 1970) either by forming the hemithioacetal, or if the free aldehyde is the substrate for the second reductive step, by serving as an allosteric activator of the enzyme.

In a previous kinetic study, Kirtley and Rudney (1967) found a parallel initial velocity pattern for HMG-CoA and NADPH and concluded that the mechanism was ping-pong.

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[1] Abbreviations used are: HMG-CoA, β-hydroxy-β-methylglutaryl-coenzyme A; MVA, mevalonic acid; MVAL, mevaldic acid; AcCoA, acetyl-coenzyme A; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate; NADH, reduced NAD; NADPH, reduced NADP; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

However, a ping-pong mechanism is difficult to rationalize with the direct hydrogen transfer catalyzed by the enzyme (Dugan and Porter, 1971).

A kinetic analysis of the reduction of HMG-CoA by an enzyme induced by growing *Pseudomonas* on mevalonic acid was carried out by Bensch and Rodwell (1970). This enzyme differs from the reductase of yeast and mammalian liver in that it utilizes NADH instead of NADPH as the reducing coenzyme. Double-reciprocal plots of initial velocities for the two-step reduction of HMG-CoA to mevalonic acid yielded intersecting patterns, suggesting a sequential mechanism. Intersecting patterns were also obtained for the second reductive step, mevaldic acid to mevalonic acid.

In the present investigation,the two reductive steps of HMG-CoA to mevalonic acid have been studied separately. The second reductive step was studied by using mevaldic acid with and without coenzyme A as the substrate reduced by NADPH. The first reductive step was studied through its back reaction, i.e., the oxidation by NADP in the presence of coenzyme A of mevaldic acid to HMG-CoA. From the results of initial velocity and product inhibition studies, a mechanism has been developed for the sequence of interactions of HMG-CoA reductase with substrates and products.

Materials and Methods

Chemicals. Most of the chemicals in this study are described under Materials and Methods in the previous paper of this issue.

Preparation of Substrates. To prepare mevaldic acid pure enough for kinetic studies, the dibenzylethylenediamine salt of DL-mevaldic dimethyl acetal (NK Labs) was precipitated three times from chloroform with ether, passed through Florisil in chloroform, evaporated under nitrogen to a small volume, and precipitated again with ether. It was then converted to the ammonium salt by solution of 0.5 g in 3 ml of 1 M ammonia, followed by extraction with four 5-ml portions of ether. It was stored at −20 °C as a 0.4 M solution after removal of excess ammonia with a stream of nitrogen.

Mevaldic acid was freshly prepared prior to use by acidifying the dimethyl acetal to pH 1-2 with 1 N hydrochloric acid. After 30 min at room temperature, the pH was adjusted to 6 with 1 N potassium hydroxide. Care must be exercised throughout the purification of the acetal and particularly during the preparation of the acid to minimize the formation of a degradation product which is oxidized by a dehydrogenase that copurifies with the reductase. Although NAD is the preferred coenzyme, NADP also functions with the contaminating dehydrogenase.

β-Hydroxy-β-methylglutaryl-coenzyme A was synthesized by reacting coenzyme A with HMG-anhydride prepared from the acid as described by Goldfarb and Pitot (1971). HMG-CoA and acetyl-CoA were purified by paper chromatography on Whatman 3 MM paper, in a solvent system of NH_4OH (28% NH_3)-0.1 M EDTA, pH 4.5-isobutyric acid-H_2O (2.3:1.0:60.2:36.5). The absence of free coenzyme A in acetyl-CoA preparations was established by paper chromatography and by testing for free sulfhydryl groups by the method of Ellman (1970). Concentrations of nucleotides were routinely determined from their absorbance at 260 and 340 nm.

Purification of Enzyme. Yeast HMG-CoA reductase was purified through the DEAE-cellulose column chromatographic step as described in the preceding paper of this issue (Qureshi et al., 1976). The enzyme was then stored at 4 °C in 0.15 M potassium phosphate buffer. pH 7.0, containing dithiothreitol, 1 mM, and EDTA, 1 mM.

Kinetic Experiments. The following reactions catalyzed by yeast HMG-CoA reductase were studied.

$$\text{HMG-CoA} + \text{NADPH} + 2\text{H}^+ \longrightarrow \text{MVA} + 2\,\text{NADP}^+ + \text{CoA} \quad (3)$$

$$\text{MVAL} + \text{NADPH} + \text{H}^+ \longrightarrow \text{MVA} + \text{NADP}^+ \quad (4)$$

$$\text{MVAL} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{CoA}} \text{MVA} + \text{NADP}^+ \quad (5)$$

$$\text{MVAL} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{AcCoA}} \text{MVA} + \text{NADP}^+ \quad (6)$$

$$\text{MVAL} + \text{NADP}^+ + \text{CoA} \longrightarrow \text{HMG-CoA} + \text{NADPH} + \text{H}^+ \quad (7)$$

Kinetic experiments were carried out in 1.0-ml reaction mixtures containing potassium phosphate, 0.1 M, pH 7.0, dithiothreitol, 5 mM, and appropriate concentrations of substrates, inhibitors, and enzyme. After preincubation at 30 °C for 5 min, the reaction was started with the addition of one of the substrates. The reaction compartment of the Gilford spectrophotometer was maintained at 30 °C with thermospacers and the reaction was followed spectrophotometrically at 340 nm (full scale on the recorder, 0.1 absorbance unit). The reaction velocities were corrected for blanks containing all of the components except HMG-CoA, mevaldate, or coenzyme A. A unit of enzyme activity is defined as the amount of enzyme required to catalyze the oxidation of 1 nmol of NADPH/min.

Data Processing. Reciprocal velocities were plotted against the reciprocal of substrate concentrations. Linear data were then fitted to eq 8 using a least-squares method and assuming equal variance for the velocities. In eq 8-13 v is the velocity, A and B represent substrate concentrations, V is the initial velocity, and the K's are equilibrium constants.

$$v = \frac{VA}{K + A} \quad (8)$$

Calculations were carried out with the Fortran programs of Cleland, which yield values for the kinetic constants and standard errors of the estimates. Data for the sequential initial velocity patterns were fitted to eq 9, for ping-pong initial velocity patterns to eq 10, for linear competitive inhibition to eq 11, for linear uncompetitive inhibition to eq 12, and for linear noncompetitive inhibition to eq 13.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (9)$$

$$v = \frac{VAB}{K_aB + K_bA + AB} \quad (10)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (11)$$

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \quad (12)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (13)$$

Results

Oxidation of Mevaldic Acid to HMG-CoA. In order to isolate and identify the product, the oxidation of mevaldic acid to HMG-CoA was carried out on a large scale in 50-ml reaction mixtures that contained potassium phosphate, 0.1 M, pH 7.0, dithiothreitol, 5 mM, enzyme, 5 mg of protein, mevaldic acid, 20 mM, NADP, 3.2 mM, and coenzyme A, 200 μM. A control without NADP was also run. When the absorbance at 340 nm became constant, the sample and control were each

TABLE I: Michaelis Constants from Initial Velocity Experiments.

Reaction	NADPH (μM)	Mevaldate (mM)	CoA (μM)	NADP (μM)
Mevaldate + NADP + CoA		0.23 ± 0.07	7 ± 2	(fixed; 8000)
		0.07 ± 0.04	(fixed; 200)	0.33 ± 0.05
		(fixed; 10)	22 ± 3	1.0 ± 0.2
		(fixed; 0.5)	13 ± 4	0.25 ± 0.08
Mevaldate + NADPH	24 ± 10	8.0 ± 2.0		
Mevaldate + NADPH + CoA	(fixed; 160)	0.4 ± 0.1	7.0 ± 1.6	
	16 ± 4	0.5 ± 0.1	(fixed; 200)	

TABLE II: Kinetic Constants for the Reduction of Mevaldic Acid in the Absence or Presence of CoA Obtained from Product Inhibition Experiments.

Status of CoA	Inhibitor	Variable Substrate	Fixed Substrate (mM)	Type of Inhibition[a]	K_{is}	K_{ii}
Absent	NADP	NADPH	Mevaldate, 20	C	0.11 ± 0.02	
Absent	NADP	Mevaldate	NADPH, 0.16	NC	0.57 ± 0.10	1.25 ± 0.26
Absent	Mevalonate	Mevaldate	NADPH, 0.16	NC	25.5 ± 3.7	53.7 ± 11.3
Absent	Mevalonate	NADPH	Mevaldate, 20	NC	34.6 ± 4.3	35.5 ± 3.6
Present	NADP	NADPH	Mevaldate, 20, CoA, 0.2	C	0.21 ± 0.03	
Present	Mevalonate	NADPH	Mevaldate, 0.5, CoA, 0.2	NC	2.2 ± 1.2	0.9 ± 0.7
Present	Mevalonate	NADPH	Mevaldate, 20, CoA, 0.2	UC		34.6 ± 3.0

[a] C, competitive; NC, noncompetitive; UC, uncompetitive.

lyophilized and extracted with 99% methanol. HMG-CoA was isolated by paper chromatography (Methods) and converted enzymatically into mevalonic acid. The reaction was carried out until the rate of decline of 340 absorbance of the sample decreased to the slow steady rate of the control. The enzyme was denatured with hydrochloric acid under conditions which lactonized mevalonic acid, and the sample was lyophilized and an acetone extract was subjected to gas–liquid chromatography as described by Dugan and Porter (1971). The main product exhibited the same elution pattern as authentic mevalonolactone, whereas the control showed no mevalonolactone.

Initial velocity studies of the back reaction of the first reduction step catalyzed by HMG-CoA reductase (eq 7) gave intersecting initial velocity patterns[2] when mevaldic acid and either CoA or NADP were the variable substrates. With NADP and CoA as the variable substrates, however, the pattern was parallel at saturating and intersecting at nonsaturating levels of mevaldate (Figure 1). The Michaelis constants from fits of these data to eq 9 or 10 are listed in Table I.

Reduction of Mevaldate to Mvalonate in the Absence of CoA. The initial velocity pattern for this reaction (eq 4) was intersecting.[2] The Michaelis constants are given in Table I. NADP was a competitive inhibitor of NADPH,[2] but a noncompetitive inhibitor of mevaldate.[2] Mevalonate was noncompetitive vs. either mevaldate[2] or NADPH.[2] The product inhibition data are summarized in Table II.

Reduction of Mevaldate to Mevalonate in the Presence of CoA. Initial velocities for this reaction (eq 5) were measured

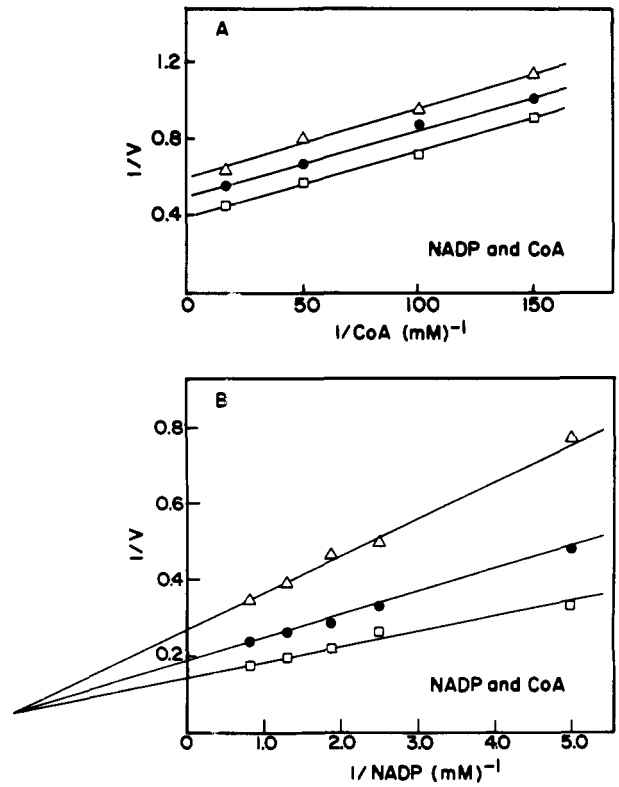


FIGURE 1: Initial velocity pattern for the oxidation of mevaldic acid to HMG-CoA with NADP and CoA as the varied substrates. (A) Mevaldic acid was 10 mM. Fixed concentrations of NADP used were 0.4 mM (△—△), 0.53 mM (●—●), and 0.8 mM (□—□). (B) Mevaldic acid was 0.5 mM. Fixed concentrations of CoA were 10 μM (△—△), 20 μM (●—●), and 50 μM (□—□). Units on the ordinate are nmol of NADP reduced per min per ml.

[2] The plots obtained for initial velocity and product inhibition studies are not presented here because of space limitations. The interested reader is directed to the Ph.D. thesis of Nilofer Qureshi, University of Wisconsin, Madison, 1975, for these plots.

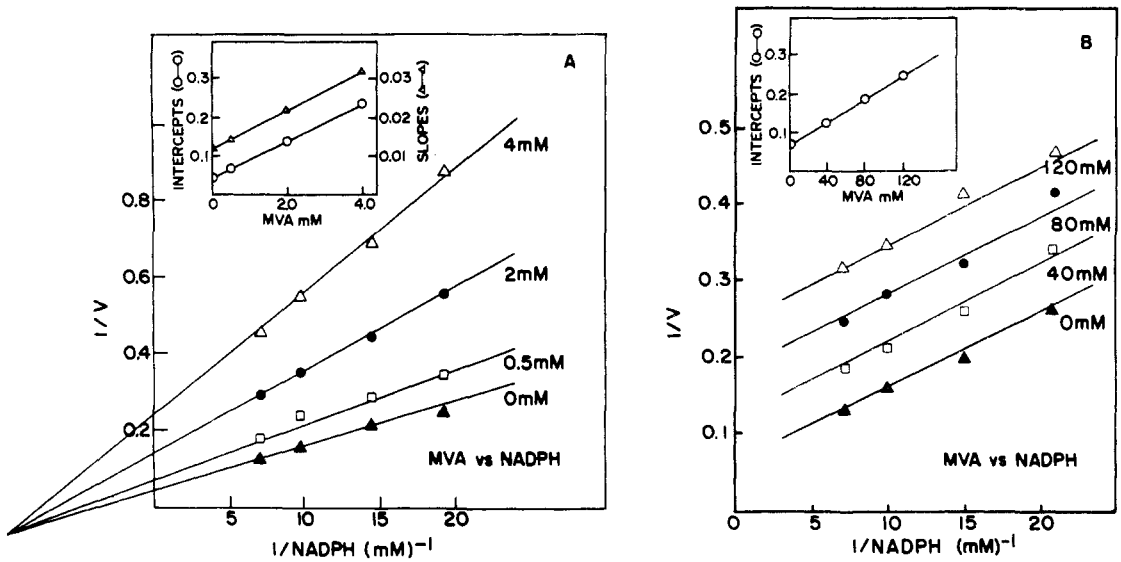


FIGURE 2: Inhibition by mevalonic acid of the reduction of mevaldic acid to mevalonic acid in the presence of 200 μM CoA. (A) Mevaldate, 0.5 mM; (B) mevaldate, 20 mM. The units on the ordinate are nmol of NADPH oxidized per min per ml.

TABLE III: Kinetic Constants for Reactions Catalyzed by HMG-CoA Reductase.[a]

Reaction	V_{max}[b]	Mevaldate K_m app (mM)	CoA or AcCoA K_m app (μM)	V/K Mevaldate ratio
HMG-CoA + NADPH	2331 ± 40			
MVAL + NADPH	833 ± 50	11.0 ± 1.6		76
MVAL + NADPH + CoA	1147 ± 23	0.42 ± 0.03	7.0 ± 1.0	2731
MVAL + NADPH + AcCoA	1106 ± 45	4.0 ± 0.9	15.6 ± 2.0	277
MVAL + NADP + CoA	236 ± 5			

[a] Data processing as described under Methods. [b] nmol of pyridine nucleotide oxidized or reduced per min per mg protein.

with the substrates mevaldate and NADPH, and the activator CoA varied in pairs, while the third component was held constant. Intersecting patterns were obtained in each case,[2] and the Michaelis constants are given in Table I. NADP gave competitive inhibition[2] vs. NADPH, while mevalonate was a noncompetitive inhibitor of NADPH at nonsaturating mevaldate (Figure 2A), and an uncompetitive inhibitor at saturating mevaldate (Figure 2B). The product inhibition data are summarized in Table II. Coenzyme A increased the V_{max} approximately 1.5-fold and decreased the K_m for mevaldic acid 30-fold in the reduction of the latter compound to mevalonic acid. However, when acetyl-CoA was substituted for coenzyme A (eq 6) it increased the V_{max} to the same extent and decreased the K_m to a lesser extent, Table III. In the oxidative reaction to form HMG-CoA, acetyl-CoA did not increase the rate of oxidation of mevaldic acid by NADP.

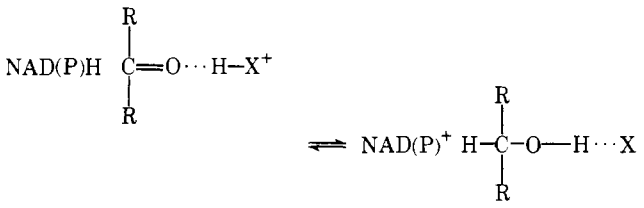
Discussion

The intersecting initial velocity patterns for mevaldic acid and coenzyme A, NADP and mevaldic acid, and NADP and coenzyme A at nonsaturating levels of mevaldic acid indicate that the three substrates in the oxidation of mevaldic acid to HMG-CoA bind to HMG-CoA reductase before any release of product. However, a parallel initial velocity pattern was obtained when NADP and coenzyme A were varied at saturating mevaldate (Figure 1A). These kinds of results have usually been interpreted to mean ordered addition of all three substrates (with mevaldate as the second molecule to add in this case). However, analysis of slope effects according to Cleland (1970) shows that it really means only that two substrates add in obligatory order, with the third combining either randomly or after the other two. Since in the overall reduction of HMG-CoA NADP is released and NADPH is added, while mevaldate and CoA remain in place on the enzyme, NADP can not be necessary for mevaldate to bind. Thus, the initial velocity patterns in the back reaction of the first reduction step suggest that CoA adds to the enzyme first, followed by mevaldate, with NADP able to add randomly or in third position.

This interpretation is challenged by the fact that the reduction of mevaldate does not require CoA, but is only stimulated by it, so that CoA clearly is not necessary for mevaldate to bind, at least in the presence of NADPH. A consistent explanation of these results is obtained by considering the probable state of protonation of the enzyme in the presence of NADP and NADPH, and the nature of the binding of the various reactants. The overall reaction in which NADP must leave and be replaced by NADPH between the two reduction steps argues for nucleotide binding independent of the occupancy of the other binding sites. The large size of a pyridine nucleotide also argues for the existence of a nucleotide binding site at all times. The enzyme must also have an extensive site that binds CoA or the CoA portion of HMG-CoA. Because of their size and the resulting extensive contact made with the enzyme, the CoA and pyridine nucleotides might be expected to be "sticky", and show relatively low rates of dissociation

from the enzyme. This view is supported by the parallel initial velocity pattern obtained by Kirtley and Rudney (1967) for NADPH and HMG-CoA in the overall reaction. Although these authors suggested that the mechanism was ping-pong, Bar-Tana and Cleland (1974) have shown for phosphofructokinase that this pattern is obtained in a random sequential mechanism when both substrates dissociate more slowly than V_{max}. The failure of the enzyme to catalyze exchange between free CoA and HMG-CoA in the absence of NADPH (Retey et al., 1970) rules out the ping-pong mechanism and supports this interpretation of the initial velocity pattern.

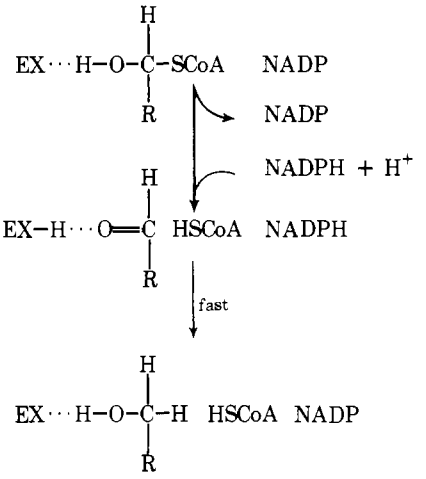
The binding site for the HMG part of the HMG-CoA, or for mevaldate or mevalonate, will be much smaller than the CoA or pyridine nucleotide sites, and thus the binding of mevaldate and mevalonate may be sensitive to occupancy of the other sites. Reductions by pyridine nucleotides proceed by a catalytic mechanism in which a protonated group hydrogen bonded to the carbonyl oxygen of the substrate causes the development of sufficient carbonium character on the carbonyl carbon to induce hydride transfer from the reduced nucleotide.



The nature of this X group varies, but usually its pK is greatly perturbed by the nature of the nucleotide present so that it is unprotonated in enzyme (E)-NAD(P) and protonated in E-NAD(P)H complexes. In alcohol dehydrogenases, for example, the X group is water coordinated to Zn (Sloan et al., 1975) and the presence of NADH raises the pK so that this water is always protonated, while the presence of NAD lowers the pK from 8.5–8.8 to below 7 (Theorell and McKinley-McKee, 1961). If a similar situation exists with HMG-CoA reductase, one would expect E-NADPH to have a protonated X group and thus to show affinity for the HMG part of HMG-CoA or the free carbonyl form of mevaldate,[3] but not to adsorb strongly either mevalonate or mevaldate in its *gem*-diol form or as a hemithioacetal with CoA. Conversely, E-NADP should have an unprotonated X group with weak affinity for the carbonyl-containing substrates, and show relatively tight binding of mevalonate and the hemithioacetal form of mevaldate.

If the above analysis is correct, mevaldate will have little affinity for E-NADP or (if the pK of the X group is 6 or below) for free enzyme. Under these circumstances the presence of CoA on the enzyme would be a prerequisite for the addition of mevaldate with subsequent formation of a strongly-bound (and oxidizable) hemithioacetal. The parallel initial velocity pattern between CoA and NADP in the presence of 10 mM mevaldate is thus explained. The formation of the hemithioacetal on the enzyme also considerably tightens the binding of CoA. Thus, its dissociation constant drops from 63 ± 21 μM in the absence of mevaldate but presence of 8 mM NADP (a similar value of 75 ± 22 μM is obtained in the presence of 160 μM NADPH), to 24 ± 12 μM in the absence of NADP and presence of 0.5 mM mevaldate, and to near zero under the same conditions except for a higher (10 mM) mevaldate concentration.

If the above mechanism is accepted, the reduction of HMG-CoA by NADPH would lead to an enzyme complex with unprotonated X, NADP, and the hemithioacetal of mevaldate and CoA. NADP could freely dissociate, but it is harder to see why mevaldate could not then dissociate to give E-CoA. The explanation for the observed failure of mevaldate to accumulate as an intermediate in the overall reaction may be the following. If NADPH adds in place of NADP and causes the uptake of a proton along with the splitting of the hemithioacetal, then an E-NADPH-CoA-mevaldate complex would result. This complex would react rapidly to give mevalonate and NADP:



This postulated mechanism is in full agreement with the observed kinetics of the reduction of mevaldate by NADPH. First, it is clear why mevaldate will combine and react in the absence of CoA, since NADPH maintains the enzyme in the protonated E-X-H form which is capable of binding the carbonyl form of mevaldate. CoA, and to some extent acetyl-CoA, acts as an activator, presumably by altering the conformation of the enzyme to promote binding of mevaldate, since the major effect is on the K_m rather than on V_{max}. The ability of acetyl-CoA to activate supports this interpretation, since it is not capable of forming a hemithioacetal. Thus, in the overall reaction, the splitting of the hemithioacetal after NADP is replaced by NADPH leaves CoA in place as an activator so as to prevent appreciable mevaldate release at this point in the mechanism.

The product inhibition studies give further information about the second reductive step. First, the competitive inhibition of NADP vs. NADPH and noncompetitive inhibition vs. mevaldate are in agreement with the postulated independent binding of NADP and NADPH at a pyridine nucleotide site. Second, the noncompetitive inhibition of mevalonate vs. either NADPH or mevaldate suggests that (1) mevalonate dissociates more rapidly than NADP so that considerable E-NADP is present in the steady state for combination with mevalonate, and (2) mevaldate has little affinity for E-NADP in the absence of CoA because the X group is not protonated in this complex. Thus, mevaldate, even at infinite concentration, does not prevent mevalonate inhibition by competing for E-NADP as might be expected in a random mechanism.

When CoA is present, mevalonate binds tighter just as does mevaldate, which is further evidence that the activation by CoA results from a conformational change rather than from hemithioacetal formation. The inhibition vs. NADPH is still noncompetitive, but the inhibition constants are now 20- to

[3] The proportion of mevaldate at pH 7 that is in the free carbonyl form as opposed to the gem-diol is not known, but by analogy with acetaldehyde and 3-hydroxypropionaldehyde phosphate it should be about one-third.

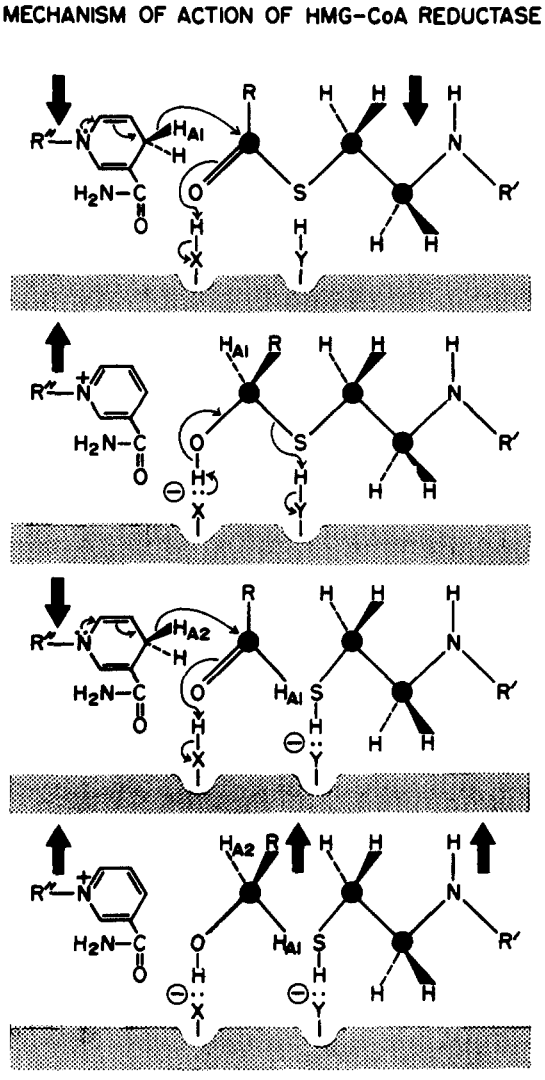


FIGURE 3: Stereochemistry and proposed sequence of events in the two-step reduction of carbon 5 of the HMG moiety in HMG-CoA. The bound pyridine nucleotide molecules are located in a plane behind the plane of the other bound reactants.

40-fold lower. This pattern, however, becomes uncompetitive at 20 mM mevaldate, showing that saturating mevaldate prevents the partial reversal of the reaction that causes the inhibition at low NADPH levels. The conversion of a non-competitive to an uncompetitive product inhibition pattern by saturation with a substrate is usually taken to imply an ordered reaction, but will also occur in random mechanisms where one set of reactants (the nucleotides here) dissociates more slowly than the other.

Still further corroboration of the model presented here comes from a quantitative analysis of the effect of mevaldate on mevalonate inhibiton vs. NADPH in the presence of CoA. The K_{ii} for mevalonate is 40-fold higher at 20 mM than at 0.5 mM mevaldate, which suggests that in contrast to the situation in the absence of CoA, mevaldate can combine with E-NADP-CoA and prevent mevalonate inhibition. This is fully consistent with our previous conclusion that mevaldate can combine with E-NADP (as the hemithioacetal) only when CoA is present. The 40-fold increase in mevaldate has apparently raised the K_{is} value *more* than a factor of 40, since the inhibition has become uncompetitive. This result is expected because mevaldate overcomes mevalonate inhibition at low NADPH in two ways that will reinforce each other. First, the high mevaldate level prevents rapid release of mevaldate from the E-NADPH-mevaldate-Coa central complex that is formed from E-NADP-CoA-mevalonate, and thus the partial reversal responsible for the slope effect. However, since this central complex is piled up by combination of mevalonate with E-NADP-CoA which has undergone a 40-fold reduction in level due to competition between mevaldate and mevalonate, the net result is essentially a complete elimination of the slope effect.

Two points not covered in the above discussion are the relative rates of release of CoA and NADP after the second reduction and the relative rates of the first and second reduction steps. Some information on these points can be obtained from the relative V_{max} values in Table III. Since these values are expressed in terms of NADPH oxidized per min per mg, the value for the reduction of HMG-CoA must be divided by two for comparison with that for reduction of mevaldate in the presence of CoA. When this is done the values are nearly identical, which suggests that (1) the second reduction step is the slower of the two and limits the rate of the reduction of HMG-CoA, (2) CoA release (which need not occur during the reduction of mevaldate, since CoA is only an activator and not a product) can not be rate-limiting for the second reduction and thus for the reduction of HMG-CoA, and (3), as a result, the NADP release step of the second reduction is probably rate-limiting for both the reduction of mevaldate and HMG-CoA.

One must be cautious about these conclusions, however, since in the reduction of HMG-CoA the release of NADP after the second reduction step may be stimulated by the addition of HMG-CoA, which would favor protonation of the X group and thus looser binding of NADP; while in the reduction of mevaldate, the addition of mevaldate to the E-NADP-CoA complex remaining after rapid release of mevalonate would lead to some oxidation to form HMG-CoA and thus give rise to a lower apparent V_{max} for mevaldate reduction. The amount of HMG-CoA formed by dismutation of mevaldate under these conditions has not been measured and as a result the above conclusions must be considered tentative.

The proposed mechanism is shown in diagrammatic form in Figure 3. After HMG-CoA and NADPH bind randomly to the enzyme, hydride transfer from the A side of NADPH (Dugan and Porter, 1971) forms a hemithioacetal of mevaldate and CoA. After NADPH replaces NADP, the hemithioacetal reverts to its component aldehyde and thiol with mevaldate and CoA remaining bound. To facilitate this step, a proton is shown coming from Y-H. However, it is also possible that the proton comes from the OH of the hemithioacetal and that proton addition to X occurs upon the binding of NADPH. The correct mechanism could be distinguished from among these possibilities by determining the point of proton uptake during the reaction by rapid reaction techniques.

During the second reduction step hydride transfer again from the A side of NADPH reduces mevaldate to mevalonate. The hydrogen added at carbon 5 in this reaction takes on a *pro-S* orientation (Blattman and Retey, 1971), as shown in the last step of the diagram. The nature of the enzyme X and Y groups is unknown, but probably could be determined by a careful study of the pH variation of the kinetic parameters for the reaction.

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Kinetics and Equilibria of the Electron Transfer between Azurin and the Hexacyanoiron (II/III) Couple†

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ABSTRACT: The electron transfer reaction between the "blue" single copper protein azurin (from *Pseudomonas aeruginosa*) and the hexacyanoiron (II/III) couple has been studied. Equilibrium constants for the reduction of azurin were measured spectrophotometrically in the temperature range 5–33 °C ($K = 1.1 \times 10^{-2}$ at 25 °C, $\Delta H° = 10.9$ kcal/mol, 0.1 M potassium phosphate, pH 7.0, $I = 0.22$). The enthalpy change was also determined by microcalorimetry and from the analysis of chemical relaxation amplitudes. Following a temperature-jump perturbation of this equilibrium, only a single relaxation was observed. The reciprocal of the relaxation time increased linearly as oxidized azurin was reacted with increasing amounts of ferrocyanide, yet reached saturation when reduced azurin was titrated with ferricyanide. This behavior as well as the analysis of the relaxation amplitudes led to the following scheme for this system:

$$\mathrm{Az(II)} + \mathrm{Fe(II)} \overset{K_1}{\rightleftharpoons} \mathrm{Az(II)} \cdot \mathrm{Fe(II)} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{Az(I)} \cdot \mathrm{Fe(III)} \overset{K_2}{\rightleftharpoons} \mathrm{Az(I)} + \mathrm{Fe(III)}$$

At 25 °C the rate constants for the electron transfer were $k_{+3} = 6.4\ s^{-1}$ and $k_{-3} = 45\ s^{-1}$, the association constants $K_1 = 54\ M^{-1}$ and $K_2^{-1} = 610\ M^{-1}$. The activation and overall thermodynamic parameters as well as the individual thermodynamic values for the different steps were combined to construct a self-consistent energy profile for the reaction.

The azurins, "blue" single-copper proteins, being sequenced (Ambler and Brown, 1967) and readily available, have been used in recent years to study the electron transfer mechanism (Antonini et al., 1970; Pecht and Rosen, 1973), the function of specific amino acid residues (Finazzi-Agrò et al., 1970; McMillin et al., 1974), and the role of conformation (Grinvald et al., 1975) in this unique group of proteins. The exceptional spectroscopic characteristics of azurin from *Pseudomonas aeruginosa* are due to its copper chromophore which gives rise to an intense blue absorption band and to its single tryptophan which exhibits a well-resolved fine structure in the absorption and CD spectra (Tang et al., 1968) and an unusually blue-shifted emission band (Finazzi-Agrò et al., 1970). Though no structural information for azurin is yet available from x-ray crystallography, spectroscopic, magnetic resonance, and chemical studies indicate that the copper is bound at a relatively inaccessible site inside the protein (Finazzi-Agrò et al., 1970; Boden et al., 1974). No direct interaction between the copper ion and external ligands can be found. NMR[1] measurements of water proton relaxation establish a minimum distance of ~5 Å between the protein surface and the metal site (Koenig and Brown, 1973). Still, in terms of electron transfer, an efficient pathway to and from this site must exist as illustrated by the very fast electron exchange of azurin with its biological partner cytochrome-*c* P551 and the ease of its reduction by hydrated electrons (Faraggi and Pecht, 1971).

The electron transfer between azurin and cytochrome-*c* P551 has been investigated by stopped-flow and chemical relaxation methods (Wilson et al., 1975; Rosen and Pecht, 1976). Conformational equilibria of both proteins were found to affect this process. The actual electron-exchange step between the two proteins was evaluated to be some hundred to thousand times faster than observed for the electron transfer between azurin and simple redox reagents. This, together with the zero

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[1] Abbreviations used: Az(II), oxidized azurin; Az(I), reduced azurin; Fe(III), ferricyanide; Fe(II), ferrocyanide; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; phen, 1,10-phenanthroline.