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pH Properties and Chemical Mechanism of Action of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase†

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ABSTRACT: The pH variation of the kinetic parameters V and V/K for the oxidation of mevaldate by $NADP^+$ in the presence of CoA (reverse reaction) and for the reduction of mevaldate by NADPH in the presence or absence of CoA (forward reaction) for the reactions catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was examined. In the reverse reaction a group, X, on the enzyme with a pK of 7.9 must by unprotonated for $NADP^+$ binding and catalysis. The presence of $NADP^+$ shifts this pK to a value below 6. The V/K profile for mevaldate shows that deprotonation of a group, Y, with a pK of 6.7 decreased the reaction rate by a factor of 27. In the forward reaction, the pK of the X group was about 6.9 except when CoA and mevaldate were both present, in which case it was shifted to 7.8. CoA decreased the K_ms for mevaldate about 10-fold without changing the V_{max} at the optimum protonation state. The catalytic group, X, was identified as a cationic acid, probably histidine. A catalytic mechanism is proposed in which the protonated form of histidine induces hydride transfer from the A side of NADPH by donating a proton to the carbonyl of HMG-CoA or to the aldehyde form of mevaldate. The role of the Y group, which from its pK of 6.7 and the chemistry involved may be a carboxyl group, is presumably to catalyze conversion of mevaldate thiohemiacetal formed in the reduction of HMG-CoA to CoA and the free aldehyde form of mevaldate. Mevaldate was shown by ^1H NMR to contain 44% hydrate in D_2O and 39% in H_2O. When an enzymatic method was used, it was also determined that only one stereoisomer of mevaldate is used by HMG-CoA reductase.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] reductase catalyzes the two-step reduction of (4*R*,3*S*)-HMG-CoA to the isoprenoid precursor, (2*R*,3*R*)-mevalonate (Cornforth et al., 1974). This compound is a precursor in the synthesis of terpenes and steroids that are vital compounds in plants and animals. Because of the close correlation of mammalian HMG-CoA reductase activity with the rate of cholesterol biosynthesis, the physiological regulation of this enzyme in rat liver and human fibroblasts has been extensively studied [for reviews, see Rodwell et al. (1976) and Gibson & Ingebritsen (1978)]. Since the yeast enzyme presents some features similar to those of the mammalian enzyme (Kawaguchi, 1970; Hatanaka et al., 1970) and since its purification and physicochemical properties are well-defined (Qureshi et al., 1976a), the current study of the chemical mechanism of HMG-CoA reductase action was undertaken with this enzyme.

The kinetic mechanism and the probable rate-limiting step of the reaction catalyzed by yeast HMG-CoA reductase were determined with steady-state kinetics by Qureshi et al. (1976b) in the reverse and forward reactions by analyzing the two partial reductive steps:

$$\text{mevaldate} + NADP^+ + \text{CoA} \rightleftharpoons \text{HMG-CoA} + NADPH + H^+ \quad (1)$$

$$\text{mevaldate} + NADPH + H^+ \rightleftharpoons \text{mevalonate} + NADP^+ \quad (2)$$

Qualitative and quantitative initial velocity analysis of reactions

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[1] Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DMF, dimethylformamide; NMR, nuclear magnetic resonance; Me_4Si, tetramethylsilane.

1 and 2 showed the following: (a) the binding of substrates and release of products is sequential for both reactions; (b) CoA is necessary for mevaldate to bind to the enzyme in the reverse reaction to form the E–mevaldate–CoA complex; (c) $NADP^+$ can add randomly or in the third position in this reaction; (d) strongly bound E–mevaldate–CoA thiohemiacetal is formed by the first reduction; (e) $NADP^+$ and NADPH occupy the same site on the enzyme; (f) replacement of $NADP^+$ by NADPH after reduction of HMG-CoA leads to the thiohemiacetal cleavage into mevaldate, which is rapidly reduced by reaction 2, and free CoA, which remains bound to the enzyme during the second reductive step. These studies, together with the initial velocity patterns observed in the overall forward reaction of HMG-CoA and NADPH by Kirtley & Rudney (1967), led Qureshi et al. (1976b) to postulate that the reverse of reaction 1 has a random kinetic mechanism and that reaction 2 is the rate-limiting step in the overall reaction. These studies predicted that the chemical mechanism of this enzymatic reaction might follow that of the general chemical mechanism postulated for dehydrogenase catalysis in which a group, X, on the enzyme acts as an acid–base catalyst to assist in the direct transfer of a hydride ion between the nucleotide and substrate (Cleland, 1977). According to this general mechanism, E–NADPH would have a protonated X group and an increased affinity for the HMG moiety of HMG-CoA or for the free carbonyl of mevaldate, while E–$NADP^+$ would have an unprotonated X group and a strong affinity for mevalonate and the thiohemiacetal of mevaldate and CoA but a weak affinity for substrate carbonyl groups.

In order to provide further evidence related to the above hypothesis and to identify the X group of the enzyme, the pH variation of the kinetic parameters was studied for each of the substrates of reactions 1 and 2 in the directions shown. On the basis of the present work and on previous determinations of the kinetic mechanism and rate-limiting steps, a chemical mechanism of action for HMG-CoA reductase is postulated.

Materials and Methods

Source of Chemicals. Chemicals were obtained from the following sources: dibenzylethylenediamine salt of DL-mevaldic acid dimethyl acetal from NK Labs and Calbiochem; Good buffers, from Calbiochem; $NADP^+$ monosodium salt, NADPH tetrasodium salt, type X, 3-hydroxy-3-methylglutaric acid, deuterium oxide, and sodium deuterium oxide from Sigma; CoA, lithium salt, chromatapure, and agarose–hexane–CoA affinity gel, type V, from P-L Biochemicals; dimethylformamide and isobutyraldehyde from Aldrich; active dry baker's yeast from Fleischmann. All other chemicals were of analytical grade. Double-distilled water was used in the final purification step of HMG-CoA reductase and in all solutions used for kinetic studies.

Purification and Stability of HMG-CoA Reductase. HMG-CoA reductase was purified from baker's yeast as reported by Qureshi et al. (1976a). The final purification step in this procedure, which employs CoA affinity chromatography, was necessary to remove a contaminant dehydrogenase that copurifies with HMG-CoA reductase. This contaminant catalyzes the reduction of $NADP^+$ in the absence of CoA with a high activity at pH 8–9. The specific activity of HMG-CoA reductase was calculated for protein determined by the method of Lowry et al. (1951) after precipitation of the protein with trichloroacetic acid (Heller & Gould, 1973). By use of this procedure, protein concentrations were about 2-fold higher than those determined with dialyzed protein as described by Qureshi et al. (1976a), which gives a value for HMG-CoA reductase specific activity of 8–11 μmol of NADPH oxidized min^{-1} (mg of protein)$^{-1}$. The purified enzyme was found to be stable at −60 °C for at least 4 months and at −20 °C for 3 weeks.

Preparation and Standardization of Substrates. HMG-CoA was synthesized according to Goldfarb & Pitot (1971) by using a 15% excess of 3-hydroxy-3-methylglutaric acid over CoA. The yield of DL-HMG-CoA was determined by the following method to be 92–97% of the CoA used for its preparation. The disappearance of NADPH was followed to completion spectrophotometrically in a system containing 120 mM potassium phosphate buffer (pH 7.0), 4 mM dithiothreitol, 0.2 mM NADPH, and DL-HMG-CoA in concentrations from 0.03 to 0.2 mM. The reaction was started with 0.055 unit of HMG-CoA reductase. (A unit is 1.0 μmol of NADPH oxidized min^{-1}.)

Mevaldic acid, purchased as the stable dibenzylethylenediamine salt of DL-mevaldic acid dimethyl acetal, was separated from dibenzylethylenediamine and purified as reported previously (Qureshi et al., 1976b) and then stored as DL-mevaldate dimethyl acetal. The mevaldate used was freshly prepared before every experiment by hydrolysis of the mevaldate derivative with HCl (final pH 1.7) for 30 min followed by neutralization with KOH to pH 5.5–6.5. Analyses of the mevaldate dimethyl acetal and of its hydrolysates were carried out with proton NMR spectroscopy in D_2O (270 MHz) for a determination of the purity of these compounds and of the proportion of mevaldate in aldehyde and hydrate forms. For these assays, NaOD was used for alkalinization of the dibenzylethylenediamine salt of the mevaldic acid dimethyl acetal, and DCl and NaOD were used for acid hydrolysis and neutralization. Total concentrations of aldehyde in the mevaldate solutions were determined colorimetrically after phenylhydrazone formation by reaction with 2,4-dinitrophenylhydrazine (2,4-DNP). The assay using isobutyraldehyde as the standard was carried out in a cuvette containing 1.8 mL of 0.5 N HCl in 50% methanol, 0.2 mL of 2,4-DNP in methanol, and the sample or standard and water in a final volume of 2.2 mL. This mixture was read at 430 nm after 20 min of incubation at room temperature. The methanol used was freed of carbonyl by treatment with $NaHSO_3$. The concentration of the stereoisomeric form of mevaldate that served as substrate in the HMG-CoA reductase reaction was determined enzymatically in a mixture containing 100 mM potassium phosphate, pH 7.5, 5 mM dithiothreitol, 0.15 mM NADPH, and 0.55 unit of HMG-CoA reductase. The reaction was started with the addition of 40 μL of 20 mM CoA (a final concentration of 1.1 mM).

The concentrations of $NADP^+$, NADPH, and CoA were determined routinely by the methods of Lowry & Passonneau (1972a,b) and of Garland (1964), respectively.

Kinetic Assays. Kinetic reactions were followed at 340 nm with either a Gilford–Honeywell 6040 monochromator and recorder or a Gilford 2400S monochromator and a Gilford–Honeywell recorder with adjustable zero and multispeed drive. The full-scale sensitivity was 0.1 absorbance, and the chart speed was 0.5–6.0 in./min. Cuvettes (1-cm path length) were used with or without 0.5-cm thick spacers. The temperature was maintained with a circulating water bath. The pH values were measured with a Radiometer 51 pH meter standardized at the given temperature to 0.01 pH unit with the Radiometer precision buffer types 51500 and 51510, and buffers from Beckman, calibrated from 0 to 95 °C. HMG-CoA reductase activity was measured before the kinetic assays in a cuvette containing 100 mM potassium phosphate, pH 7.0, 5 mM dithiothreitol, 0.17 mM NADPH, and enzyme, unless oth-

erwise specified. After incubation of this mixture for 5 min at 30 °C, the reaction was started by the addition of 15 μL of 20 mM DL-HMG-CoA. Enzyme activity is expressed in units (micromoles of NADPH oxidized per minute) or in micromoles of NADPH oxidized per minute per milligram of protein. Enzyme activity was normalized to an arbitrary value of 20 μmol of NADPH oxidized per min per mg of protein.

The pH profiles of reactions 1 and 2 were carried out in a mixed-acid buffer that was 35 mM in acetic acid, 2-(*N*-morpholino)ethanesulfonic acid (Mes), *N*-tris(hydroxymethyl)methylglycine (Tricine), and (cyclohexylamino)-ethanesulfonic acid (Ches) and contained 5 mM dithiothreitol, enzyme, and $NADP^+$ or NADPH (in the concentrations indicated in the Results section). The reaction mixture was incubated for 4 min at 30 °C. Mevaldate was then added (in the concentrations indicated in the Results section), and absorbances were recorded for reaction 2. The HMG-CoA reductase reactions (reactions 1 and 2) carried out in the presence of CoA were initiated by the addition of 40 μL of 23 mM CoA. In this way, inactivation of the enzyme by incubation with CoA (Kirtley & Rudney, 1967) was avoided. The pH was recorded after each assay. The buffer mixtures in concentrations 3-fold those used in the kinetic analyses did not inhibit the enzyme. Also, HMG-CoA reductase was found to be stable at all of the pH ranges studied.

Reaction 1 was also carried out in a cationic acid buffer system, 58 mM in Mes, Tricine, and Ches, or in a neutral acid buffer system, 58 mM in acetic acid, phosphate, and pyrophosphate, in the presence or absence of 21% (v/v) dimethylformamide (DMF). The pH of the buffers was adjusted with HCl or KOH. $NADP^+$ was the variable substrate in these assays. When the kinetic assays were carried out in the presence of DMF, the pH values were measured before the addition of the perturbing solvent. The effect of the organic solvent was then analyzed according to Findlay et al. (1962a).

The kinetic parameters V and V/K were determined at various pH values for reactions 1 and 2 by varying each of the substrates of these reactions at high fixed levels of the others. Each plot of reciprocal velocity vs. reciprocal substrate was obtained with at least four different concentrations of substrate.[2] The plots were linear in all the cases, and the data were fitted to

$$v = \frac{VA}{K + A} \tag{3}$$

The pKs were determined by fitting the calculated data obtained from eq 3 to

$$\log y = \log \frac{C}{1 + (H/K_A) + (K_B/H)} \tag{4}$$

$$\log y = \log \frac{C}{1 + K_B/H} \tag{5}$$

$$\log y = \log \frac{C}{1 + H/K_A} \tag{6}$$

$$\log y = \log \frac{YL + YH(K/H)}{1 + K/H} \tag{7}$$

In eq 4–7, y is V or V/K and H is the hydrogen ion concentration; C (eq 4–6) is the value of y at the optimum state of protonation; K_A and K_B are acid dissociation constants of groups that, for activity, must be unprotonated and protonated, respectively; in eq 7, K is the dissociation constant of the ionizable group, and YL and YH are the values of V/K at low and high pH, respectively.

[2] The plots of reciprocal velocities vs. reciprocal substrates are published in the Ph.D. Thesis of Dulce Veloso, George Washington University, 1979.

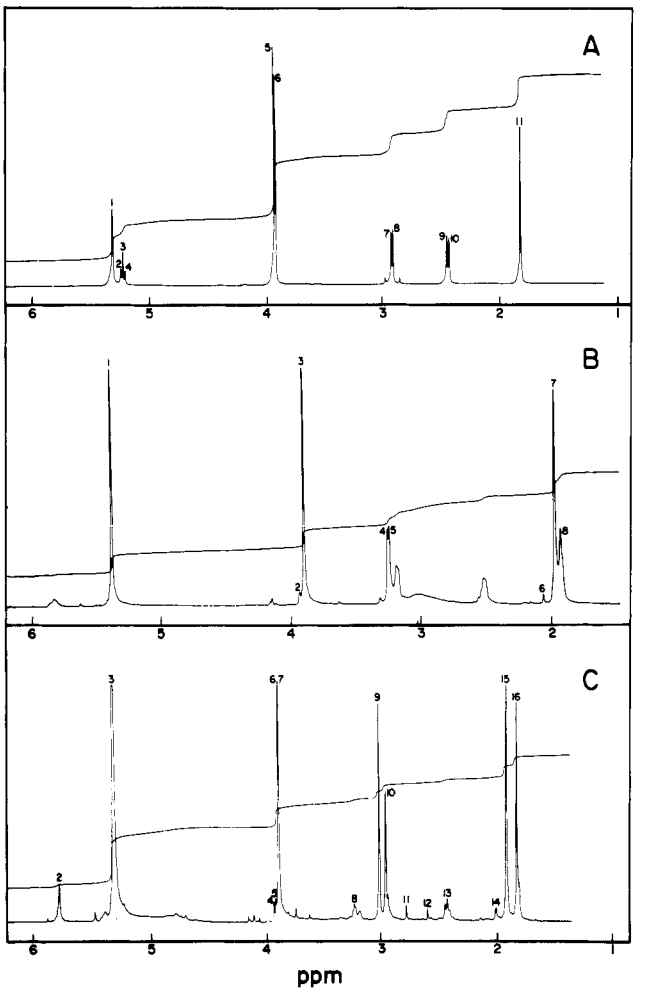


FIGURE 1: Proton NMR resonance (270 MHz, D_2O) of DL-mevaldate dimethyl acetal (A), DL-mevaldic acid, H_2O, and methanol formed by acid hydrolysis (B), and DL-mevaldic acid, after neutralization with base (C), over the region shifted 1–6 ppm downfield from Me_4Si.

The nomenclature used is that of Cleland (1963). Experimental data were fitted to eq 3–7 by the least-squares method assuming equal variances for v or log y (Wilkinson, 1961) and with a digital computer and the Fortran programs of Cleland (1979) which also gives the standard error of the mean of the V, K_m, and pK values. The points in all plots were experimentally determined. The solid line curves were drawn from the calculated data, and the dash line curves were visually drawn through the experimental points.

Results

Analyses of DL-*Mevaldic Acid Solutions.* Figure 1 shows the spectra obtained by ^1H NMR spectroscopy of DL-mevaldate dimethyl acetal (A), of DL-mevaldic acid and methanol formed by acid hydrolysis (B), and of DL-mevaldate after neutralization (C) (for details, see Materials and Methods). Integration of the peaks of the spectrum in (A) shows that all the peaks can be assigned to the DL-mevaldate dimethyl acetal. Impurities were therefore not detectable by ^1H NMR spectroscopy. Acid hydrolysis showed the disappearance of the peak assigned to the methylene adjacent to the aldehyde group which was expected to be between peaks 5 and 6 (Figure 1B). This disappearance is explained by a rapid exchange of these

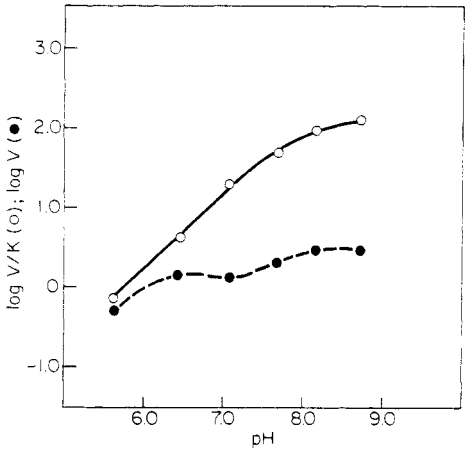


FIGURE 2: pH variation of V/K for $NADP^+$ (○) fitted to eq 6 (pK = 7.88) and of V (●) for the reverse reaction.

methylene hydrogens with deuterium from the medium as a result of enolization of the aldehyde at low pH. Confirmation of this conclusion is the disappearance of the triplet assigned to the hydrogen bound to the dimethyl acetal carbon atom (peaks 2, 3, and 4 in Figure 1A and peak 2 in Figure 1C). Integration of the peaks of spectrum C shows that the contaminants formed during neutralization amounted to less than 10%. The aldehyde peak, as expected, appeared downfield (9.3 ppm). The hydration equilibrium of mevaldate in D_2O was determined to be 56% aldehyde and 44% hydrate by integration of peaks 15 and 16 (methyl groups) and 57% aldehyde and 43% hydrate by integration of peaks 9 and 10 (methylene groups adjacent to the carboxyl groups) of Figure 1C. Since one expects an inverse solvent isotope effect on the hydration equilibrium of 0.84 (Gruen & McTigue, 1963), mevaldate should be 39% hydrated in H_2O.

The concentrations of mevaldate determined enzymatically after acid hydrolysis of mevaldate dimethyl acetal at pH 1.7 for 30–120 min or at pH 0.7 for 30 min (results not shown) were 45% of the values determined with a colorimetric method (see Materials and Methods). These results show that one of the stereoisomers, presumably the D-mevaldate, was the enzymatically reactive substrate.

pH Profiles for the Substrates of the Reverse Reaction, Equation 1. The pH profiles for $NADP^+$ presented in Figure 2 were obtained with 1.1 mM CoA and saturating concentrations of mevaldate (8.7 mM at pH 5.63–7.69 and 34.8 mM at pH 8.16–8.72). The V/K profile when fitted to eq 6 gives a pK value of 7.88 ± 0.02. Since this pK does not correspond to those of $NADP^+$ (3.9, 6.1, and 11.3; Apps, 1973), it must reflect the protonation of a group on the enzyme–CoA–mevaldate complex. Qureshi et al. (1976b) showed that binding of $NADP^+$ to HMG-CoA reductase is random, and the presence of CoA and mevaldate is not necessary for its binding. However, the presence of saturating concentrations of CoA and mevaldate implies that $NADP^+$ is bound to E–CoA–mevaldate. Because of the complexity of the V profile at saturating concentrations of $NADP^+$, a calculated pK could not be determined, but visual observation suggests a partial effect produced by a pK around 8 and a further drop below a pK below 6.

Figure 3 shows the pH profiles for CoA in the presence of mevaldate at 3.5 mM (pH 5.62, 6.48, and 7.05), 5.9 mM (pH 7.68), 11.8 mM (pH 8.36), and 23.6 mM (pH 8.83). the concentrations of $NADP^+$ used were 9.3 mM (pH 5.62), 3.1 mM (pH 6.48 and 7.05), and 0.93 mM (pH 7.68, 8.36, and 8.83). The V/K profile shows a drop to a new value with a pK around 7 and a decrease with a slope of −1 above a pK

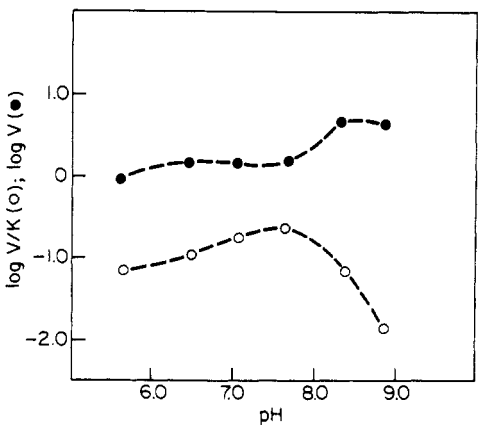


FIGURE 3: pH variation of V/K for CoA (○) and of V (●) for the reverse reaction.

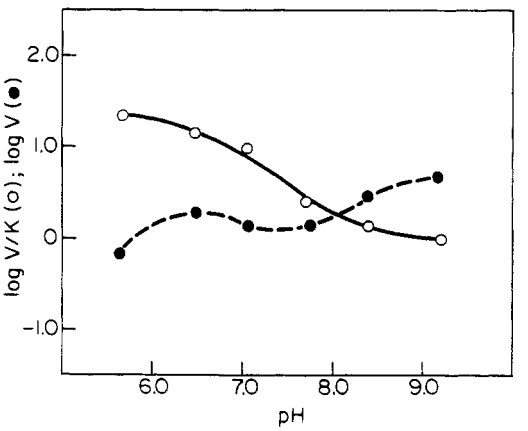


FIGURE 4: pH variation of V/K for mevaldate (○) fitted to eq 7 (pK = 6.67) and of V (●) for the reverse reaction.

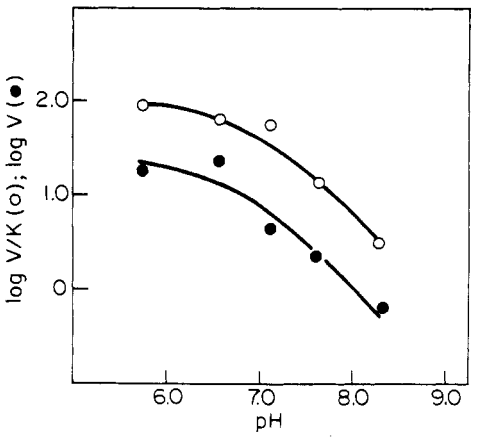


FIGURE 5: pH variation of V/K for NADPH (○) and of V (●) fitted to eq 5 (pK = 6.89 and 6.66, respectively). CoA was not present.

around 8. The V profile is similar to that for $NADP^+$ (Figure 2).

Reciprocal plots for mevaldate were obtained in the presence of 1.1 mM CoA. The concentrations of $NADP^+$ were 7.9 mM at pH 5.65, 2.6 mM at pH 6.45 and 7.04, and 0.79 mM at pH 7.72, 8.44, and 9.20. The values of V/K were fitted to eq 7, giving a pK of 6.67 ± 0.07 (Figure 4). The V profile in Figure 4 is similar to those observed for $NADP^+$ and CoA (Figures 2 and 3).

pH Profiles for the Substrates of the Forward Reaction. The initial velocity patterns for reaction 2 were carried out in the presence and absence of CoA. The V/K profile in the absence of CoA with NADPH as the variable substrate and mevaldate at 16.5 mM (pH 5.74–7.63) and 33.0 mM (pH

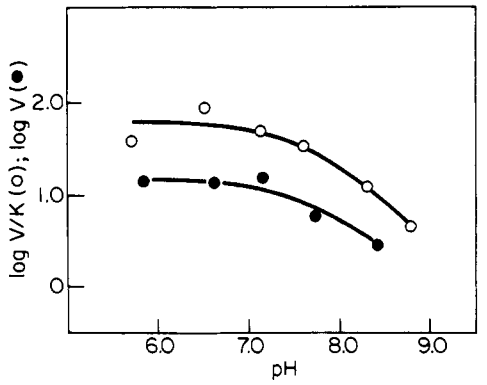


FIGURE 6: pH variation of V/K for NADPH (○) and of V (●) fitted to eq 5 (pK = 7.76 and 7.78, respectively). CoA was present.

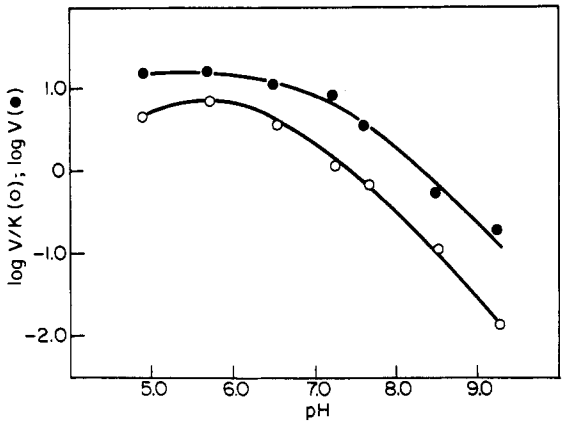


FIGURE 7: pH variation of V/K for mevaldate (○) fitted to eq 4 (pK_A = 4.88 and pK_B = 6.52) and of V (●) fitted to eq 5 (pK = 7.2) in the forward reaction. CoA was not present.

Table I: Values of V/K and of V at Optimum States of Protonation

variable substrate	reaction direction	CoA	V/K[a]	V[a]
$NADP^+$	reverse	+	130 ± 5	
mevaldate	reverse	+	24 ± 2	
NADPH	forward	−	102 ± 9	25 ± 5
NADPH	forward	+	61 ± 5	15.2 ± 1.3
mevaldate	forward	−	8.4 ± 1.0	14.6 ± 1.0
mevaldate	forward	+	85 ± 2	18.0 ± 0.9

[a] These values were calculated from eq 4–7 and from data obtained with mixed-acid buffer at 30 °C. The values of V are expressed in micromoles of pyridine nucleotide oxidized or reduced per minute per milligram of protein. V/K represents a ratio calculated from the V values as above expressed and K_m values expressed as micromoles of the binding substrate per milliliter of reaction mixture. For details see Materials and Methods.

8.32) was fitted to eq 5, giving a pK of 6.89 ± 0.06 (Figure 5). A similar pK of 6.66 ± 0.12 was obtained from the V profile. With CoA present, the pH profiles for both V/K and V (Figure 6) were also fitted to eq 5, giving pK values of 7.76 ± 0.07 and 7.71 ± 0.08, respectively.[3] The pK values, in the presence of CoA, were shifted about one pH unit toward a higher pH, although the values of V/K and of V at an optimum state of protonation were similar in the presence and absence of CoA (Table I).

The initial-velocity patterns for mevaldate as the variable substrate were also determined in the presence and absence

[3] When the profiles in Figures 5 and 6 were fitted to eq 4, pK_A was 5.4–5.8, and pK_B was 0.1–0.2 pH unit less than that obtained from a fit to eq 5. The standard errors on the pK_A values, however, make it questionable whether a true pK is being seen.

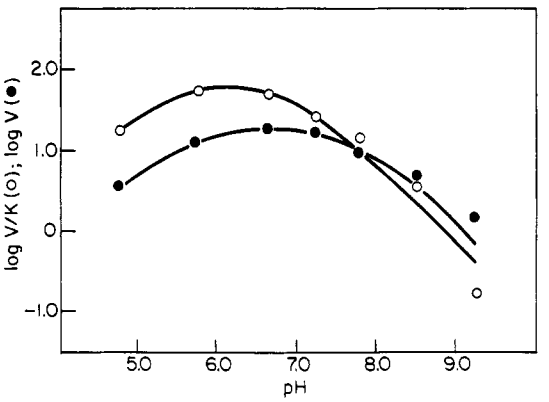


FIGURE 8: pH variation of V/K for mevaldate (○) fitted to eq 4 (pK_A = 5.32 and pK_B = 6.92) and of V (●) also fitted to eq 4 (pK_A = 5.32 and pK_B = 8.18) in the forward reaction. CoA was present.

Table II: Effect of DMF on the V/K Profile for $NADP^+$

buffer type	apparent pK values[a] −DMF	+DMF
neutral	7.96 ± 0.05	7.53 ± 0.01
cationic	8.08 ± 0.08	7.94 ± 0.05

[a] The pK values were obtained from computer fits of the data to eq 6. The pH were measured before addition of DMF.

of CoA. The V/K and V profiles in the absence of CoA were carried out at 0.40 mM NADPH. In the presence of CoA (1.0 mM), the concentration of NADPH was 0.46 mM. In the absence of CoA, the V/K profile was fitted to eq 4, giving pKs of 4.88 ± 0.16 and 6.52 ± 0.06 (Figure 7). The V profile was fitted to eq 5, giving a pK of 7.2 ± 0.05.[4] The presence of CoA decreased the K_ms for mevaldate about 10-fold at all pH values studied except at pH 8.50 and 9.25 where decreases of 3-fold and 1.5-fold, respectively, were observed. From fits to eq 4, pKs of 5.32 ± 0.23 and 6.92 ± 0.16 were shown for the V/K profile and 5.32 ± 0.05 and 8.18 ± 0.04 for the V profile (Figure 8).

Effect of DMF on the V/K Profiles for $NADP^+$ in Cationic and Neutral Acid Buffers. The nature (cationic or neutral acid) of the group on the enzyme involved in $NADP^+$ binding was investigated in the reverse reaction by analysis of the effect of DMF on the pKs. DMF shifts the pKs of neutral acids to higher pH and gives no change or slightly lowers the pK of cationic acids (Findley et al., 1962a). The highest concentration of DMF that would not appreciably inactivate the enzyme was determined to be 21% (v/v); this concentration of DMF gave an enzyme inactivation of only 14%.

The V/K and V pH profiles for $NADP^+$ were obtained in cationic or neutral acid buffers in the presence or absence of DMF (see Materials and Methods for details). The data in Table II show that DMF had no effect on the pK in a cationic acid buffer, but, in neutral acid buffer, DMF shifted the V/K profile to the left and clearly lowered the pK for $NADP^+$ binding from 7.96 to 7.53. This behavior is predicted only for a cationic group (Findley et al., 1962a).

[4] The pK of 7.21 in the V profile presumably represents the same group as that giving the pK of 6.52 in the V/K profile, except that the presence of mevaldate has perturbed the pK slightly, or the values are different because of experimental error. The pK of 4.9 in the V/K profile is presumably shifted to too low a value to be observed in the V profile. Such pK shifts to the outside of a pH profile are expected when a non-pH-dependent step such as release of the second product partially limits V or when the ionizing group forms a hydrogen bond during binding of the substrate (Cleland, 1977).

Effect of Temperature on the V/K Profile for NADP+. The heat of ionization of the group on the enzyme involved in $NADP^+$ binding was calculated from pK values in V/K profiles determined at 15.5, 30, and 39 °C in mixed-acid buffer. The enthalpy of ionization calculated with the Arrhenius plot was 7400 ± 4900 cal/mol.

Discussion

Interpretation of V/K and V Profiles of NADP+, CoA, and Mevaldate in the Reverse Reaction. The V/K profile for $NADP^+$ drops below a pK of 7.9 with a slope of 1 (Figure 2). Thus, $NADP^+$ binds to the unprotonated group on the enzyme and not to its protonated form. In the V profile, this group is displaced to below pH 6. The above data suggest that this pK belongs to the assisting catalytic group (X) which accepts the proton from the hydroxyl of the mevaldate–CoA thiohemiacetal during hydride transfer. In the presence of $NADP^+$, the enzyme apparently shifts the pK of the X group toward lower values. Similarly, in liver alcohol dehydrogenase, NAD^+ shifts the pK from 8.8 in free enzyme to 7.0 in E–NAD^+ while NADH raises the pK to above 10 (Coleman et al., 1972). This phenomenon ensures specific binding of oxidized or reduced substrate when reduced or oxidized nucleotide is present. It was concluded recently by Danenberg et al. (1978) that the positive charge at N-1 is responsible for the different effects of NADH and NAD^+, and identical conclusions are expected for $NADP^+$ and NADPH binding.

The V/K profile for CoA (Figure 3) shows a drop above pK around 8. Since this pK is lower than that of the SH group of CoA (pK = 9.6; Jaenicke & Lynen, 1959), it must represent a group on the enzyme. It is not clear, though, what the group is or what its role is in catalysis. Binding of CoA prevents deprotonation of this group since its pK is not seen in the V profile.

The V/K profile for mevaldate shows a pK of 6.7 (Figure 4), with mevaldate preferring the protonated form of the enzyme, but being capable of reacting more slowly with the unprotonated form. Although this pK of 6.7 has a value similar to the pKs of 6.5 and 6.9 in the absence and presence of CoA, respectively, seen in the V/K profiles for mevaldate in the forward reaction (Figures 7 and 8), these pKs are for the X group, whose pK in the present experiment is perturbed to low pH by the presence of saturating $NADP^+$ (see above). We thus think that this pK of 6.7 corresponds to a group on the enzyme that assists in the reaction of mevaldate with CoA to give the thiohemiacetal necessary for reaction with $NADP^+$, and we discuss the role of this group further below.

The V profiles seen in Figures 2–4 are all similar, as they should be. It is clear that all pKs seen in V/K profiles are displaced by substrate binding so that there is no pH variation between 6.5 and 9 except for a slight change caused by a component with a pK around 8–8.5. Whatever group is ionizing at this pH in the E–$NADP^+$–CoA–mevaldate complex is clearly not acting as an acid–base catalyst in the reaction.

Interpretation of V/K and V Profiles of NADPH and Mevaldate in the Presence and Absence of CoA in the Forward Reaction. CoA is not necessary for the forward reaction since the aldehyde form of mevaldate is the active species in the presence of NADPH. Its presence has an effect on the kinetics of the reaction, however. The pKs for the V/K and V profiles for NADPH (CoA absent) were 6.9 and 6.7, respectively (Figure 5). It is clear that the group with these pKs must be protonated for the reaction to occur. The similarity of the pK values indicates that the presence of NADPH does not affect the pK. The presence of CoA shifted the pKs to 7.8 for V/K and 7.7 for V (Figure 6). These pK values are close to the pK of 7.9 determined for the X group in the reverse reaction. Since this group has to be in the protonated form for NADPH binding and catalysis, as opposed to the need of deprotonation for $NADP^+$ binding and catalysis (compare Figures 2 and 6), it has the properties required for the X group. Similar groups are shown by the pH profiles of enzymes such as alcohol dehydrogenase (Theorell & McKinley-McKee, 1961; Coleman et al., 1972), lactic dehydrogenase (Winer & Schwert, 1958), and malic enzyme (Schimerlik & Cleland, 1977). The conformational change induced by the binding of reduced coenzymes in other dehydrogenases which results in a shift in the pK of the X group toward high pH values appears to be elicited by CoA in the second reductive step catalyzed by HMG-CoA reductase rather than by NADPH.

The V/K profile for mevaldate in the absence of CoA (Figure 7) shows that the binding of this substrate to the enzyme is dependent on two pKs, with values of 4.9 for pK_A and 6.5 for pK_B. The pK_A could be that of mevaldate (determined to be 4.4 by titration) or of a group on the enzyme, but the pK_B must belong to a group on the enzyme and presumably corresponds to the same X group seen in the V/K profile for NADPH since the presence of NADPH does not perturb this pK. The V profile showed a pK of 7.2 which, as expected, had to be in the protonated form for the reaction to occur. In the presence of CoA (Figure 8), both the V/K and V profiles for mevaldate showed a pK_A of 5.3, but the pK_B values were 6.9 and 8.2 for the V/K and V profiles, respectively.

It is interesting to compare the various effects of NADPH, mevaldate, and CoA on the pK of the X group in these pH profiles in the forward direction of reaction 2. In the presence of mevaldate or NADPH alone, or in the presence of both, the pK is in the range 6.5–7.2.[5] This value (6.9) is also seen in the presence of NADPH and CoA. However, the value in the presence of mevaldate and CoA, or of all three molecules, is 7.7 to 8.2,[5] and a similar value of 7.9 was seen in the V/K profile for $NADP^+$ in reaction 1, where mevaldate and CoA were present together. We ascribe this elevation of a full pH unit in the pK of the X group to the formation of an appreciable amount of thiohemiacetal when CoA and mevaldate are both present on the enzyme. Although it is the free aldehyde that is the substrate for reaction 2, Qureshi et al. (1976b) have concluded that the rate of reaction of aldehyde and NADPH is not the slow step for reaction 2, so that the nonproductive binding of CoA and mevaldate as the thiohemiacetal does not limit the rate of reaction 2. In the forward reaction, CoA increased the V/K for mevaldate at the optimum state of protonation about 10-fold (Table I) due to a decrease of K_m values rather than to changes in V_{max}. The values of V_{max} for NADPH and mevaldate in the forward reaction with or without CoA, at an optimum state of protonation, which vary from 15 to 25 μmol min^{-1} (mg of protein)$^{-1}$ (Table I), are probably not significantly different.

Identification of the X Group. DMF did not affect significantly the pK of the X group in cationic acid buffer but clearly decreased it from 8.0 to 7.5 in neutral acid buffer. [Since the pH was read before the addition of DMF, this is an apparent decrease caused by elevation of the pK of the neutral acid buffer by DMF (Findley et al., 1962b).] Thus the X group is a cationic acid, and the values of the pK under various conditions (6.5–8.2) suggest that histidine is the most

[5] The pKs in the V profiles when mevaldate was varied are about 0.5 pH unit higher than the values when NADPH was varied, although they should, of course, be the same. Presumably the deviations result from experimental error.

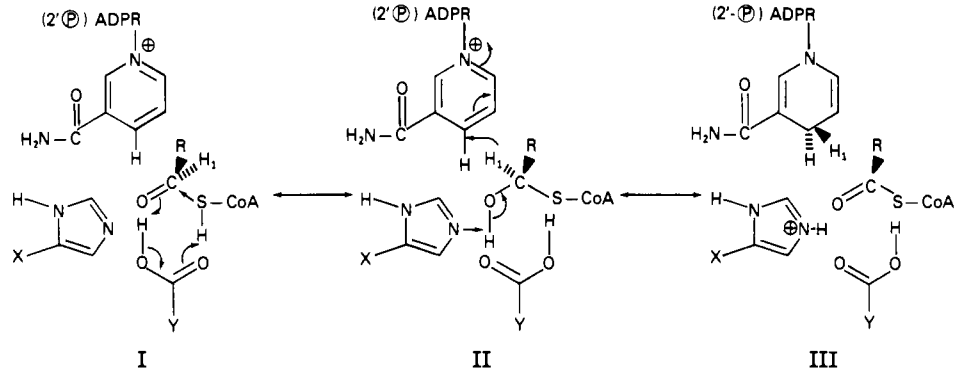
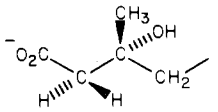


FIGURE 9: A possible mechanism for the oxidation of mevaldate and CoA to HMG-CoA that is catalyzed by HMG-CoA reductase. The formation of mevaldate–thiohemiacetal is induced by the transfer of a proton from the carboxyl of the Y group on the enzyme to the carbonyl of the mevaldate. The thiol anion of CoA formed by transfer of the proton of the CoA sulfhydryl to the carbonyl of the Y group then attacks the carbonyl carbon of mevaldate (I) after formation of a hydrogen bond from the hydroxyl of the thiohemiacetal to X (shown here as histidine). A hydride ion is transferred from the thiohemiacetal to $NADP^+$ and a proton to the histidine residue on the enzyme (II). HMG-CoA and NADPH are then released from the enzyme (III). The bound pyridine nucleotide molecules are located in a plane behind the plane of the other bound reactants. The R of mevaldate and HMG-CoA represents



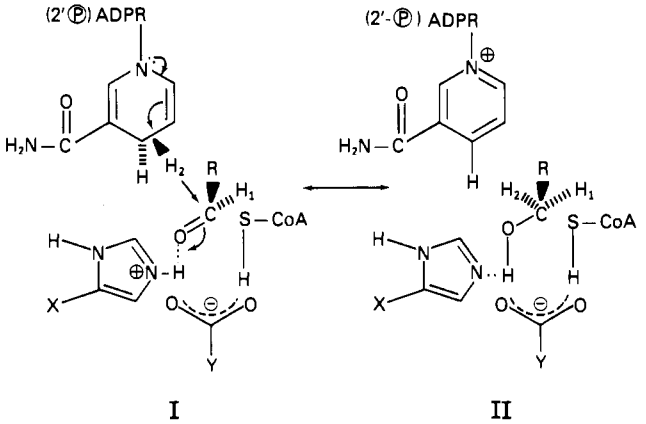


FIGURE 10: A possible mechanism for the reduction of mevaldate to mevalonate by HMG-CoA reductase. The protonated form of the catalytic histidine group donates a proton to the carbonyl of mevaldate with subsequent hydride transfer from the A side of NADPH (I). Mevalonate, CoA, and $NADP^+$ are then released from the enzyme (II). The role of CoA in this portion of the reaction is that of allosteric activator rather than substrate, and the reaction will proceed in its absence, although with a lower V/K for mevaldate (see Table I). The bound pyridine nucleotide molecules are located in a plane behind the plane of the other reactants. For the definition of R in mevaldate and mevalonate, see the legend to Figure 9.

likely candidate. Histidine has also been shown to be the X group in lactate dehydrogenase (Adams et al., 1973), glyceraldehyde dehydrogenase (Moras et al., 1975), malate dehydrogenase (Holbrook et al., 1974), and alcohol dehydrogenase (Sloan et al., 1975). The data for the temperature effect on the pK for $NADP^+$ binding were not very accurate, but the value of 7400 cal/mol observed for ΔH_{ion} is consistent with histidine as the X group.

Chemical Mechanisms. Figures 9 and 10 show proposed chemical mechanisms for the oxidation of mevaldate and CoA by $NADP^+$ and the reduction of mevaldate by NADPH which are consistent with data from the present work, that of Qureshi et al. (1976b), and the known stereochemistry of the reaction (Dugan & Porter, 1971). In Figure 9, Y is shown as a carboxyl group which assists in thiohemiacetal formation by donating a proton to the carbonyl oxygen on mevaldate at the same time as it accepts one from the SH group of CoA. When this carboxyl is ionized, formation of the thiohemiacetal would have to be a two-step process in which CoA donated a proton to X or Y before attacking the carbonyl carbon of mevaldate and X or Y donated the proton back during this attack, or possibly thiohemiacetal formation (which occurs rapidly in solution) could be uncatalyzed on the enzyme.[6] The state of protonation of Y does not appear to affect the reaction of mevaldate and NADPH, and it is tempting to postulate that after reduction of HMG-CoA to mevaldate, Y may donate its proton (if it has one) to X to produce the correct state of protonation for the subsequent reduction of mevaldate to mevalonate as shown in Figure 10. When Y is not originally protonated as well as X, a proton will have to be taken up from the medium by X so that reaction 2 can occur to complete the overall reduction of HMG-CoA.

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[6] The proposal that Y is a carboxyl group is not based on information other than the pK of 6.7 from the V/K profile for mevaldate in reaction 1 and the observations that the reaction is more rapid when this group is protonated and that a carboxyl group can accept a proton at the same time as it donates one. Y and X are apparently two separate groups since the pK of X in the V profile is below 6, while that of Y under similar conditions of $NADP^+$ saturation is 6.7. If Y were a histidine or if X and Y were the same group and a histidine, one would expect the fastest thiohemiacetal formation when CoA was ionized and Y was protonated, but this state of protonation occurs above, not below, the pK of Y. Only with a bifunctional catalyst such as a carboxyl group can a more rapid reaction occur with both CoA and Y protonated, as shown in Figure 9.

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Interaction of Isocitrate Dehydrogenase with (*RS*)-3-Bromo-2-ketoglutarate. A Potential Affinity Label for α-Ketoglutarate Binding Sites†

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ABSTRACT: The interaction of oxidized nicotine adenine dinucleotide phosphate dependent isocitrate dehydrogenase (from pig heart) with (*RS*)-3-bromo-2-ketoglutarate was investigated in an effort to evaluate the reagent's potential as a selective reagent for α-ketoglutarate binding sites. The enzyme is rapidly inactivated by 0.1 mM bromoketoglutarate at pH 7.4. With increasing concentrations of reagent, the reaction shows a rate saturation; the minimum inactivation half-time is 3 min and K_{inact} for bromoketoglutarate is 250 μM. Isocitrate and $NADP^+$ protect against inactivation, while ketoglutarate does not. When tested in the assay that monitors isocitrate oxidation, bromoketoglutarate is a competitive inhibitor (K_i = 100 μM) of the dehydrogenase. As judged by oxidation of NADPH, bromoketoglutarate is also a substrate for isocitrate dehydrogenase, exhibiting a K_m of 250 μM and a V_{max} comparable to that for isocitrate oxidation. The reduction of bromoketoglutarate is competitively inhibited by isocitrate (K_i = 3 μM) and ketoglutarate (K_i = 50 μM). Like the enzyme-catalyzed oxidation of isocitrate, the reduction of bromoketoglutarate is stereospecific, requires divalent metal ions, and shows absolute specificity for NADPH. However, since CO_2 is not required for catalytic turnover of bromoketoglutarate, its reduction is likely comparable to that of oxalosuccinate rather than the reductive carboxylation of ketoglutarate. Although bromoketoglutarate, as a substrate for isocitrate dehydrogenase, clearly has affinity for the active site, the irreversible inactivation of the enzyme by the reagent may result from modification outside the active-site region, since inactivation during catalytic turnover of bromoketoglutarate is not observed. Commercial isocitrate dehydrogenase is purified 12-fold by affinity chromatography on thiol–agarose alkylated by bromoketoglutarate.

α-Ketoglutarate is an unusually diverse metabolite: a Krebs cycle intermediate, a critical link between carbohydrate and amino acid metabolism as the amino group acceptor in numerous transaminations, a component of the malate–aspartate shuttle which accomplishes net transport of NADH into mitochondria, and a biosynthetic precursor of vitamin K, lysine (in fungi), and porphyrins (in plants). Because of the large number of enzymes which thus possess binding sites for α-ketoglutarate, the design of reactive analogues of α-ketoglutarate as potential chemical probes of these sites appears warranted. One such reagent, (*RS*)-3-bromo-2-ketoglutarate, was prepared and shown to inactivate glutamate synthase (Mäntsälä & Zalkin, 1976). For further exploration of its

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