Malate Synthase: Proof of a Stepwise Claisen Condensation Using the Double-Isotope Fractionation Test†

James D. Clark, Stephen J. O'Keefe, and Jeremy R. Knowles*

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

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ABSTRACT: Although aldolase-catalyzed condensations proceed by stepwise mechanisms via the intermediacy of nucleophilic enol(ate)s or enamines, the mechanisms of those enzymes that catalyze Claisen-type condensations are unclear. The reaction pathway followed by an enzyme from this second group, malate synthase, has been studied by the double-isotope fractionation method to determine whether the reaction is stepwise or concerted. In agreement with earlier work, a deuterium kinetic isotope effect $^{D}(V/K)$ of 1.3 ± 0.1 has been found when [$^{2}H_3$]acetyl-CoA is the substrate. The ^{13}C isotope effect at the aldehydic carbon of glyoxylate has also been measured. For this determination, the malate product (containing the carbon of interest at C-2) was quantitatively transformed into a new sample of malate having the carbon of interest at C-4. This material was decarboxylated by malic enzyme to produce the appropriate CO_2 for isotope ratio mass spectrometric analysis. The ^{13}C isotope effect with [$^{1}H_3$]acetyl-CoA [that is, $^{13}(V/K)_H$] is 1.0037 ± 0.0004. By use of the known values of the intermolecular and intramolecular deuterium effects and of $^{13}(V/K)_H$, the value of the ^{13}C isotope effect when deuteriated [$^{2}H_3$]acetyl-CoA is the substrate [that is, $^{13}(V/K)_D$] can be predicted for three possible mechanisms. If $^{13}(V/K)_H$ is a *kinetic* isotope effect and the reaction is concerted, the value of the ^{13}C effect on deuteriation of acetyl-CoA will rise to 1.011; if $^{13}(V/K)_H$ is a *kinetic* isotope effect and the reaction is stepwise, the value of the ^{13}C effect will fall to 1.0025; and if the ^{13}C effect is an *equilibrium* isotope effect deriving from glyoxylate dehydration, the reaction is necessarily stepwise, and the value of $^{13}(V/K)_D$ will be 1.0037, unchanged from that of $^{13}(V/K)_H$. Experimentally, the value of $^{13}(V/K)_D$ is 1.0037 ± 0.0007, which requires that *malate synthase follow a stepwise path*. It is therefore clear that the two salient characteristics of enzymes that catalyze Claisen-like condensations, namely, the absence of enzyme-catalyzed proton exchange with solvent and the inversion of the configuration at the nucleophilic center, which had been suggestive of a concerted pathway, are not mechanistically diagnostic.

One of the most common ways of forming new carbon–carbon bonds, whether mediated by enzymes in vivo or by chemists in vitro, is by condensation reactions. In these processes, the nucleophilic carbon of an enol(ate) or enamine attacks the electrophilic carbonyl group of an aldehyde, ketone, or ester. Two classes of enzymes catalyze such condensations: aldolases, which catalyze aldol condensations with nucleophilic ketone enol(ate)s or enamines, and Claisen enzymes, which catalyze Claisen-like condensations where the nucleophilic carbon derives from acetyl-CoA. These two groups of enzymes have very different mechanistic characteristics, and while the pathway followed by the aldolases is well charted, the mechanism of Claisen enzymes is unclear. In this paper, we report the results of experiments designed to answer a fundamental question of an enzyme from the second group: is the reaction catalyzed by malate synthase a concerted or a stepwise process? The addition reactions of carbanions to carbonyl centers may in principle follow either concerted or stepwise pathways (see Figure 1). In a concerted reaction, carbon–carbon bond formation is concomitant with carbon–hydrogen bond cleavage, whereas in a stepwise process the carbon–hydrogen bond is first broken to form an enol(ate) intermediate prior to the carbon–carbon bond being formed. While for the nonenzymic reaction entropic considerations disfavor formation of the ternary complex necessary for a concerted reaction, binding interactions between the enzyme and its substrates might so stabilize the ternary complex that a concerted reaction would be preferred.

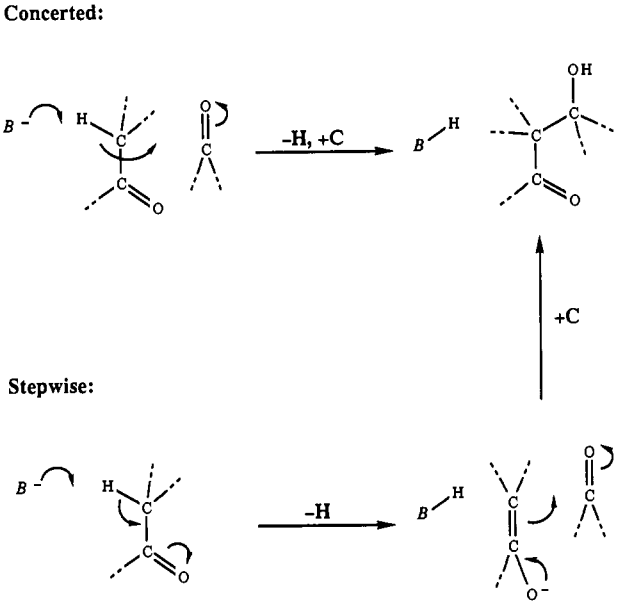

FIGURE 1: Concerted and stepwise condensations.

Aldolases proceed in two steps with the enolate (or its equivalent) as a reaction intermediate. Three properties of aldolases support this hypothesis. First, aldolases catalyze the exchange of the α-hydrogen of the nucleophilic substrate with

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solvent protons in the absence of the cosubstrate (Rose & Rieder, 1958; Richards & Rutter, 1961; Feingold & Hoffee, 1972; Wood, 1972; Grady & Dekker, 1979). Hydrogen exchange without bound cosubstrate proves that aldolases catalyze enolization, but before the enol(ate) can be accepted as a true intermediate, enolization must be shown to be at least as fast as the rate of the overall condensation. Gratifyingly, class I and class II fructose-1,6-bisphosphate aldolases (Rose & Rieder, 1958; Kadonaga & Knowles, 1983), 2-keto-4-hydroxyglutarate aldolase (Grady & Dekker, 1979), and 2-keto-3-deoxygluconate-6-phosphate aldolase (Wood, 1972) all catalyze proton exchange with solvent at rates surpassing the rates of the aldol condensations, thus demonstrating the kinetic competence of the respective enol(ate)s in these reactions. The second line of support for a stepwise mechanism for the aldolases comes from incubation of enzyme and enolizable substrate with tetranitromethane, which reacts with carbanions to produce nitroformate (Christen & Riordan, 1968; Riordan & Christen, 1969). For the fructose-1,6-bisphosphate aldolase reaction, the rate of nitroformate production parallels the rate of fructose 1,6-bisphosphate cleavage, and in the absence of cosubstrate the rate of formation of nitroformate parallels the rate of exchange of the 1-*pro-S* hydrogen of dihydroxyacetone phosphate (Christen & Riordan, 1968; Riordan & Christen, 1969). Finally, all aldolases proceed with retention of stereochemistry at the nucleophilic center (Hanson & Rose, 1975). This result is most easily accommodated by a stepwise mechanism, for if the reaction were to follow a concerted path, the enzymic base and the electrophilic carbonyl would have to be in close proximity on the same face of the nucleophilic carbon.

On the basis of nonenzymic precedent, a condensation where the nucleophilic enol(ate) derives from a thiol ester should be similar to one involving a ketone enol(ate). The pK_a of a hydrogen on the α-carbon of a thioester is close to that of the hydrogen of an analogous ketone (Lynen, 1953; Schwarzenbach & Felder, 1944), and we might expect both mechanistic and kinetic similarity between these reactions. Yet Claisen enzymes have decidedly different mechanistic properties from the aldolases: Claisen enzymes do *not* catalyze proton exchange with solvent in the absence of cosubstrate, and all Claisen enzymes proceed with *inversion* of stereochemistry at the nucleophilic center.

The proton exchange activity of Claisen enzymes is insignificant. Citrate synthase does not catalyze tritium incorporation into acetyl-CoA in the absence of oxalacetate (Eggerer, 1965), and 3-hydroxy-3-methylglutaryl-CoA lyase catalyzes proton exchange into acetyl-CoA at only one thousandth the rate of the overall reaction (Kramer & Miziorko, 1983). This exchange rate is far too slow for a kinetically competent enol, and (substrate synergism notwithstanding) the relevance of the exchange to the condensation reaction must be questioned. Analogous experiments cannot be performed with the enzyme thiolase, since both the nucleophilic substrate and the electrophilic cosubstrate are acetyl-CoA. In this case, however, Davis et al. (1987) have used a carba analogue of acetyl-CoA in which the sulfur of the thioester is replaced by a methylene group to give a ketone that can serve only as the nucleophilic component. These workers have shown that there is no proton exchange with the solvent here either, even though the corresponding methylene analogue of acetoacetyl-CoA is lysed by thiolase. In a complementary experiment, thiolase inactivated by blocking of the essential cysteine residue has been shown not to catalyze exchange of tritium into acetyl-CoA (Davis et al., 1987). Consistent with all these observations,

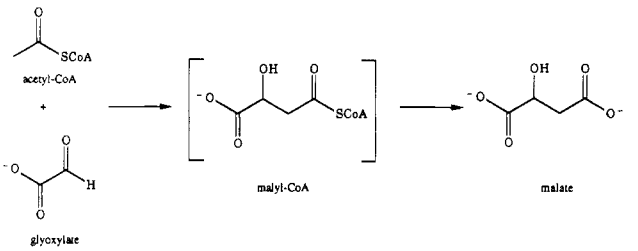

FIGURE 2: Reaction catalyzed by malate synthase.

malate synthase, which mediates the condensation of glyoxylate and acetyl-CoA to form L-malate (Figure 2), catalyzes the exchange of solvent tritium into acetyl-CoA at only 0.1% the rate of the overall reaction (Eggerer & Klette, 1967). The view that this slow tritium exchange is not on the path of the normal reaction is supported by the incongruous metal requirements for substrate binding, tritium exchange, and condensation. Thus the tritium exchange process requires more than 25 mM Mg^{2+} for an optimum rate, whereas only 4 mM Mg^{2+} is necessary to maximize the rate of the synthase reaction (Eggerer & Klette, 1967). This disparity is even more surprising since Mg^{2+} is required for the binding of glyoxylate, but the binding of acetyl-CoA is independent of the Mg^{2+} level (Durchschlag et al., 1981). To evaluate the importance of substrate synergism, Eggerer and Klette (1967) investigated several glyoxylate analogues. It was found that oxalate and glycolate do not accelerate the proton exchange rate even though these molecules bind about as tightly as glyoxylate to the enzyme. In contrast, α-keto acids such as pyruvate and α-ketobutyrate do activate the tritium exchange activity of the enzyme. Pyruvate increases the exchange rate by 1000-fold, making exchange as fast as the enzyme-catalyzed condensation of glyoxylate and acetyl-CoA. This exchange could, however, derive from an enzyme-catalyzed condensation between acetyl-CoA and pyruvate which would result in 2-methylmalyl-CoA. If the condensation reaction does occur in this experiment, the 2-methylmalyl-CoA is not hydrolyzed (Eggerer and Klette measured the total CoA thioester concentration, and a decrease was not detected). Exogenous 2-methylmalyl-CoA is not hydrolyzed by malate synthase, whereas malyl-CoA is, albeit much more slowly than the overall reaction. In summary, the lack of significant proton exchange reactions catalyzed by malate synthase or by other Claisen enzymes has cast doubt on the intermediacy of a nucleophilic enol(ate) in these reactions.

The inversion of stereochemistry seen for all Claisen enzymes (Hanson & Rose, 1975) is also consistent with a concerted mechanism. A concerted reaction is most likely to proceed with inversion; otherwise, both the catalytic base and the electrophilic substrate carbonyl must be simultaneously confined to the same face of the nucleophilic carbon. We must stress, however, that the stereochemical course of these reactions can only be suggestive of mechanism.

In summary, the proton exchange activity, the tetranitromethane trapping of the carbanion intermediate, and the retention of stereochemistry make it clear that aldolases follow a stepwise mechanism. In contrast, the lack of exchange catalyzed by malate synthase in particular and by Claisen enzymes in general and the inversion of stereochemistry make a concerted reaction an intriguing possibility for enzyme-catalyzed Claisen condensations. We have therefore applied the method of double-isotope fractionation (Hermes et al., 1982; Belasco et al., 1983) to the reaction catalyzed by malate synthase by measuring the change in the ^{13}C isotope effect at the aldehydic carbon of glyoxylate when deuteriated [$^2H_3$]-

acetyl-CoA is substituted for unlabeled [$^1H_3$]acetyl-CoA in the condensation reaction.

Materials and Methods

Chemicals. AMP (sodium salt), coenzyme A (trilithium salt), dithioerythritol, 5,5′-dithiobis(2-nitrobenzoic acid), EDTA (disodium salt), glyoxylic acid, HEPES, NAD^+, NADH (disodium salt), $NADP^+$ (sodium salt), phenylmethanesulfonyl fluoride, triethanolamine hydrochloride, and Tris were from Sigma Chemical Co. (St. Louis, MO). Glyoxylic acid was converted to the sodium salt with NaOH and recrystallized following the procedure of Metzler et al. (1954). L-Carnitine hydrochloride, 2-ketoglutarate (disodium salt), and thiamin pyrophosphate hydrochloride were from Boehringer Mannheim Biochemicals (Indianapolis, IN). D_2O (99.8 atom %) was from Merck and Co. (Rahway, NJ). [$^2H_6$]Acetic anhydride was from Aldrich Chemical Co. (Metuchen, NJ). All other chemicals were of commercial reagent grade or better.

Chromatography. Dowex 50 (H^+ form, 100–200 mesh, 8% cross-linked) Sephadex G-100 (120 mesh) were from Sigma. AG 1-X8 (acetate form, 200–400 mesh) was from Bio-Rad Corp. (Rockville Center, NY).

Enzymes. Glutathione reductase (yeast) and malic enzyme (chicken liver) were from Sigma. Carnitine acetyltransferase (pigeon breast muscle), citrate synthase (porcine heart), glutamate dehydrogenase (beef liver) in 50% glycerol, lactate dehydrogenase (beef heart), and malate dehydrogenase (porcine heart) in 50% glycerol were from Boehringer. For the second series of steps in the rearrangement, the $(NH_4)_2SO_4$ suspension of carnitine acetyltransferase was converted to a 50% glycerol solution via repeated concentration by ultrafiltration and dilution into 50% glycerol. Unless otherwise stated, the enzymes are stored as $(NH_4)_2SO_4$ suspensions. Pyruvate dehydrogenase was a generous gift from Scott Yang and Douglas Slournoy.

Malate synthase was isolated by the method of Durchschlag et al. (1981), except that the Sephadex G-50 desalting column was sometimes replaced by dialysis against the same buffer. The malate synthase had a specific activity of 11 units/mg and was free of fumarase and other enzyme impurities that could interfere with either the partial or complete reactions that form malate or the subsequent analysis of the product malate. The enzyme was stored at −78 °C without loss of activity over at least 6 months.

Assays. A unit of enzyme activity is defined as that amount necessary to convert 1 μmol of substrate to product per minute. Malate synthase was assayed by the method of Durchschlag et al. (1981). The carnitine acetyltransferase assay was from Biochemica Information II (distributed by Boehringer Mannheim). Glutathione reductase was assayed by following the loss of NADPH at 340 nm (Bergmeyer, 1974). Pyruvate dehydrogenase was assayed according to Maldonado et al. (1972).

L-Malate concentrations were determined according to Hermes et al. (1982), except that $NADP^+$ was substituted for 3-acetylpyridine adenine dinucleotide phosphate. Acetyl-CoA and coenzyme A were assayed according to Tubbs and Garland (1969). Mixtures of acetyl-CoA and acetylcarnitine were assayed by a modification of the acetate kinase assay for acetate (Bergmeyer, 1984). Carnitine acetyltransferase was substituted for acetate kinase, and ATP, phosphotransacetylase, and lactate dehydrogenase were omitted from the assay mixture. Glyoxylate (Rendina et al., 1984) and pyruvate (Bergmeyer, 1974) were assayed with lactate dehydrogenase and NADH.

Methods. Ultraviolet–visible absorption measurements were made at 25 °C on either a Perkin-Elmer 554 spectrophotometer or a Hewlett-Packard 8452A diode array spectrophotometer. pH measurements were made with a Radiometer PHM62 standard pH meter.

Acetyl-CoA and [$^2H_3$]Acetyl-CoA. The isotopically labeled and unlabeled materials were prepared following the procedure of Simon and Shemin (1953) for the synthesis of succinyl-CoA. Coenzyme A (300 μmol) and $KHCO_3$ (2.5 mmol) were dissolved in H_2O (10 mL) at 0 °C. Acetic anhydride (530 μmol) was added with vigorous stirring. Reaction was complete in under 10 min as judged by a nitroprusside assay for free thiol. An additional portion of acetic anhydride (100 μmol) was added as a precautionary measure. The pH was lowered to pH 2.5 with Dowex 50 (H^+ form), and the Dowex 50 was then removed by filtration. The solution was freeze-dried to give a white powder that was redissolved in water. The pH of this solution was ~2.0. The ratio of acetyl-CoA to total free thiol (assayed as described above) was greater than 100 to 1. The material was stored at −78 °C. D_2O and [$^2H_6$]acetic anhydride were substituted for H_2O and acetic anhydride in the synthesis and storage of the deuteriated compound.

Deuterium Isotope Effect. [$^2H_3$]Acetyl-CoA was stored frozen in D_2O until use. The reactions were run in 27 mM sodium pyrophosphate buffer, pH 8.1, containing glyoxylate (11 mM), $MgCl_2$ (3.6 mM), and acetyl-CoA or [$^2H_3$]-acetyl-CoA (44–890 μM) at 25 °C. The reaction was initiated by addition of malate synthase. The initial reaction velocities were determined by monitoring the decrease in A_{232nm} caused by the hydrolysis of the thioester bond. The V/K isotope effect was determined by comparing the slopes of the derived double-reciprocal plots.

^{13}C Isotope Effect. (A) Partial Conversion. The ^{13}C isotope effect at C-2 of glyoxylate was determined with natural abundance glyoxylate. For the partial conversion of glyoxylate to malate, glyoxylate (680 μmol), $MgCl_2$ (200 μmol), and malate synthase (4 units) were equilibrated at 25 °C in 27 mM sodium pyrophosphate buffer, pH 8.1 (50 mL). The reaction was initiated by the addition of acetyl-CoA (68 μmol, 0.1 equiv) added in 16 equal portions over 2 h. After the final addition of acetyl-CoA, the mixture was incubated for an additional 2 h. The extent of reaction was determined by measuring the concentration of glyoxylate and of malate at the end of the reaction.

Malate was isolated from the reaction mixture according to O'Leary et al. (1981). The reaction mixture was diluted 8-fold with water and loaded directly onto a column (1.5 × 20 cm) of AG 1-X8 (formate form). After the column was washed with water (100 mL), the malate was eluted with a linear gradient of formic acid (300 mL + 300 mL, 0–6 M), and fractions (20 mL) were collected. Each fraction was evaporated to dryness under reduced pressure, the residue was dissolved in water (3 mL), and a portion (10–50 μL) was assayed for malate. Fractions containing malate were pooled, concentrated to dryness under reduced pressure, and stored at −78 °C. After one ion exchange column, the malate was generally still contaminated with glyoxylate. The chromatographic purification was therefore repeated twice more to remove all the glyoxylate, which otherwise interfered with the subsequent analysis.

(B) Complete Conversion. In a typical conversion of glyoxylate to malate, glyoxylate (74 μmol), $MgCl_2$ (80 μmol), and acetyl-CoA (96 μmol, 1.3 equiv) were incubated at 25 °C in 26 mM sodium pyrophosphate buffer (15 mL), pH 8.1. A portion (250 μL) was removed for assay of the initial gly-

oxylate and acetyl-CoA concentrations. The reaction was initiated by the addition of malate synthase (5 units). Assays of the final concentrations of glyoxylate, malate, and acetyl-CoA confirmed that all of the glyoxylate (±2%) had been converted to malate. The malate was then isolated by the method described above.

The partial and complete conversion samples were each dissolved in 15 mM HEPES buffer (10 mL), pH 7.8, divided into two equal portions, and treated with charcoal before the rearrangement described below.

Rearrangement of Malate. The volume of the mixture was adjusted to keep the initial malate concentration the same for all rearrangement reactions. The concentrations of all reactants and enzymes were also kept constant. A typical rearrangement reaction mixture contained malate (30 μmol), CoA (3.12 μmol), NAD^+ (6.25 μmol), $NADP^+$ (6.25 μmol), thiamin pyrophosphate (5 μmol), $MgCl_2$ (50 μmol), carnitine (54 μmol), α-ketoglutarate (312 μmol), AMP (12.5 μmol), carnitine acetyltransferase (33 units), glutamate dehydrogenase (400 units), and pyruvate dehydrogenase (62.5 units) in 50 mM potassium phosphate (12.5 mL), pH 7.0. The ammonia necessary for the conversion of α-ketoglutarate to glutamate was derived solely from the $(NH_4)_2SO_4$ (~240 μmol) in the enzyme suspensions. Before the reaction was initiated, a portion (400 μL) of the rearrangement mixture was filtered through a Centricon-10 microconcentrator to remove the enzymes, and the filtrate was then assayed to determine the initial malate concentration. Malic enzyme (2.75 units) was added to the rearrangement mixture to initiate the conversion of malate to acetyl-CoA and acetylcarnitine. After 2.5 h, a portion (600 μL) was filtered through a Centricon-10 microconcentrator and assayed for acetyl-CoA and acetylcarnitine. Once it was determined that the conversion of malate to acetylcarnitine and acetyl-CoA was complete, the mixture was passed through an Amicon PM-10 filter to remove the enzymes. The filter was rinsed twice with $MgCl_2$ (25 μmol) in 50 mM potassium phosphate buffer (6.25 mL), pH 7.0. The eluate was assayed to ensure that all enzyme activity had been removed. Coenzyme A (62.5 μmol), carnitine acetyltransferase (50 units) in 50% (v/v) glycerol, and malate synthase (15 units) were added to the combined filtrates. A portion (1 mL) was filtered through a Centricon-10 membrane and then assayed for acetyl-CoA and acetylcarnitine. The reaction was initiated by adding glyoxylate (45 μmol, 1.5 equiv) in 20 equal portions over an 80-min period. Thirty minutes after addition of the last portion of glyoxylate, the malate concentration was determined. The solution was diluted to 200 mL with water, and the malate was then purified on a column of AG 1-X8 (formate) as described earlier. These samples were treated with charcoal and decarboxylated.

Decarboxylation. The purified, rearranged malate was decarboxylated by the method of Hermes et al. (1982). The malate sample (30 μmol) was dissolved in 75 mM HEPES buffer (8 mL), pH 7.8, and transferred to a 50-mL reaction vessel of the type described by O'Leary (1980). Oxidized glutathione (135 μmol) and $MgCl_2$ (110 μmol) were added to the solution, which was then sparged overnight with CO_2-free N_2. NADH (48 μmol), $NADP^+$ (30 μmol), and dithioerythritol (6.5 μmol) were added, and the solution was then sparged for an additional 3.5 h. The vacuum line connector with stopcock was attached, the flask was flushed with N_2, and the stopcock was closed before the needle attached to the nitrogen line was removed from the septum. Glutathione reductase (30 units), lactate dehydrogenase (17 units), and malate enzyme (3 units) were added (in 50–75 μL) to the solution through the septum. After 18 h, the reaction was quenched with concentrated H_2SO_4 (0.5 mL). The resulting CO_2 was isolated by the method of O'Keefe and Knowles (1986). Assay of the neutralized reaction mixture showed that all decarboxylations were more than 99.6% complete.

The ^{13}C:^{12}C ratio in the isolated CO_2 was determined by Krueger Enterprises, Cambridge, MA, on an Isogas 903 isotope ratio mass spectrometer.

Theory. The data from the determination of the deuterium isotope effect were first plotted to check the linearity of the double-reciprocal plots and then were fitted to eq 1 and 2 by

$$v = \frac{VA}{(K + A)(1 + F_1E_{V/K})} \quad (1)$$

$$v = \frac{VA}{K(1 + F_1E_{V/K}) + A(1 + F_1E_V)} \quad (2)$$

the least-squares method, with a BASIC translation of the appropriate Fortran program of Cleland (1979). Equation 1 assumes equal isotope effects on V and V/K, while eq 2 treats the isotope effects independently. A is the concentration of acetyl-CoA, F_1 is the fraction of deuterium label in this substrate, and $E_{V/K}$ and E_V are the isotope effects minus 1 for V/K and V, respectively.

The ^{13}C isotope effect is given by

$$^{13}V/K = \frac{\log (1 - f)}{\log [1 - f(R_f)/(R_\infty)]} \quad (3)$$

where f is the fraction of reaction, R_f is the ^{13}C:^{12}C ratio at C-2 of malate after a fraction of reaction f, and R_∞ is the ^{13}C:^{12}C ratio at the same center after complete reaction.

RESULTS

^{13}C Isotope Effect. The ^{13}C isotope effect at the glyoxylate carbonyl was determined by measuring the ^{13}C:^{12}C ratio of the C-2 carbon of the malate product, after partial and complete reaction. For the generation of the partial conversion samples, glyoxylate and acetyl-CoA (in 10:1 molar ratio) were incubated with malate synthase to give approximately 10% conversion of glyoxylate to malate. The precise extent of conversion was determined by assaying the concentrations of glyoxylate and malate at the end of the incubation. Although it is known that the substrates bind in sequential random order (Durchschlag et al., 1981), acetyl-CoA was added to the reaction vessel slowly over 2 h to avoid any increase in the forward commitment of glyoxylate induced by the presence of the acetyl-CoA. The concentration of acetyl-CoA was kept at or below its K_m at all times. For the generation of the complete conversion samples, excess acetyl-CoA (1.3 equiv) was incubated with glyoxylate and malate synthase. The complete conversion of glyoxylate to malate was confirmed by assays of glyoxylate, malate, acetyl-CoA, and CoA.

To determine the $^{13}V/K$ isotope effect, the ^{13}C:^{12}C ratio at C-2 of the product malate (from both partial and complete conversion samples) had to be measured. The most precise way to determine a ^{13}C:^{12}C ratio is to convert the carbon of interest to CO_2 which is then analyzed by an isotope ratio mass spectrometer. All the reactions used in the conversion of the carbon of interest to CO_2 must be quantitative, in order to avoid any isotopic fractionation in these transformations. At the start of this project, no reliable method was available for the quantitative conversion of the C-2 carbon of malate to CO_2, though a simple method for quantitative decarboxylation of the C-4 position was known (Hermes et al., 1982; O'Keefe & Knowles, 1986). If, therefore, our product malate could be

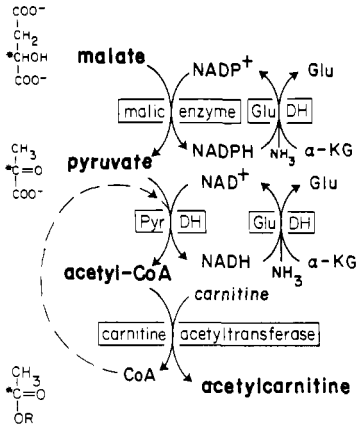

FIGURE 3: Production of labeled acetylcarnitine from malate. The carbon atom of interest is labeled with an asterisk.

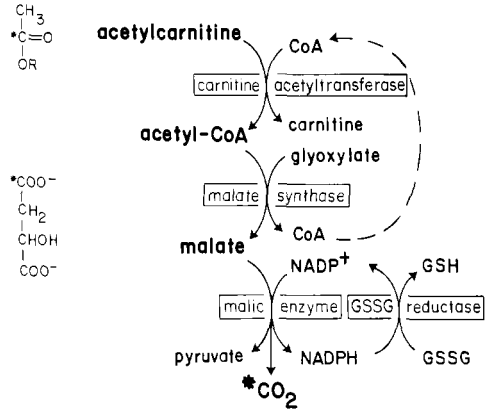

FIGURE 4: Production of labeled carbon dioxide from acetylcarnitine. The isotopically labeled atom is marked with an asterisk.

quantitatively rearranged such that the C-2 carbon (the carbon of interest) became the C-4 carbon of a new molecule of malate, the latter material could be quantitatively decarboxylated. The enzymic system illustrated in Figures 3 and 4 was developed to effect this rearrangement.

In the first sequence of reactions, malate was converted to acetylcarnitine (in equilibrium with a small proportion of acetyl-CoA) as illustrated in Figure 3. Malic enzyme produces pyruvate with the concomitant reduction of $NADP^+$, thus converting the C-2 carbon of malate to the C-2 carbon of pyruvate. The resulting pyruvate is oxidized in situ by the pyruvate dehydrogenase complex to give acetyl-CoA in which the carbon of interest is now the thioester carbonyl carbon. NAD^+ is concomitantly reduced to NADH in the pyruvate dehydrogenase reaction, and both NADPH and NADH are recycled in situ by glutamate dehydrogenase. Carnitine acetyltransferase catalyzes the in situ transfer of the acetyl group from acetyl-CoA to carnitine to give an equilibrium mixture of acetylcarnitine and acetyl-CoA. A large excess of carnitine ensures that less than 5% of the acetyl groups exist as acetyl-CoA at equilibrium. This sequence of reactions was stopped by filtering the solution through an Amicon PM-10 membrane to remove all the enzymes. The extent of the conversion of malate to acetylcarnitine plus acetyl-CoA was determined by assaying the initial malate concentration just prior to the addition of malic enzyme and by assaying the sum of the acetyl-CoA and the acetylcarnitine concentrations in the quenched, enzyme-free, reaction mixture. The yield of acetyl ester was at least 97% in every case: residual malate and pyruvate could not be detected.

Several aspects of this first sequence must be noted. High levels of conversion are only achieved when the pyruvate concentration is kept low by use of large amounts of the pyruvate dehydrogenase complex relative to malic enzyme. Although pyruvate is stable under the reaction conditions in the absence of the pyruvate dehydrogenase complex, unacceptably low yields (≤95%) in acetylcarnitine plus acetyl-CoA were obtained when low levels of pyruvate dehydrogenase complex were used. We believe that some pyruvate loss may occur by the carbo-ligase activity of the pyruvate dehydrogenase complex that is more pronounced at high pyruvate concentrations. A second problem derives from the ability of the pyruvate dehydrogenase complex to catalyze acetyl-CoA hydrolysis in the presence of NADH (CaJacob et al., 1985). Accordingly, the NADH concentration was kept low by recycling the NADH to NAD^+ with glutamate dehydrogenase. Lastly, transfer of the acetyl group to carnitine has two benefits. The oxygen ester of acetylcarnitine is less susceptible to hydrolysis or ammonolysis than is the thioester of acetyl-CoA, and product inhibition of the pyruvate dehydrogenase complex by acetyl-CoA is minimized. As an additional precaution, AMP, which reduces product inhibition by acetyl-CoA (Reed & Cox, 1970), was added to the reaction mixture.

The carbonyl carbons of acetyl-CoA and of acetylcarnitine were transformed into the C-4 position of malate in the second series of reactions, illustrated in Figure 4. Coenzyme A was added to the ultrafiltrate from the first sequence so that the ratio of coenzyme A to carnitine was now 1:1. Carnitine acetyltransferase and malate synthase were added, and a portion of the mixture was assayed to determine the concentrations of acetyl-CoA and acetylcarnitine. Slow addition of glyoxylate to this equilibrated mixture of acetyl-CoA and acetylcarnitine produced malate having the carbon of interest at C-4 (see Figure 4). Glyoxylate inhibition of the second series of steps prohibited the addition of all the glyoxylate at one time. The extent of the reaction was determined by enzymatic assay of the final malate concentration. The yields of malate were always greater than 97%.

The rearranged malate was isolated and then decarboxylated as described by O'Keefe and Knowles (1986) with malic enzyme and $NADP^+$ (see Figure 4) in a sealed vacuum flask. The NADPH produced was reoxidized in situ with glutathione reductase and oxidized glutathione. The pyruvate produced in the decarboxylation was reduced in situ with NADH and lactate dehydrogenase. The reaction solution was first sparged with CO_2-free N_2 to rid the solution and vacuum flask of exogenous CO_2, and the reaction was initiated by the addition of the enzymes mentioned above in a small volume. The CO_2 produced in the decarboxylation was isolated on a vacuum line and analyzed on an isotope ratio mass spectrometer. When the spent reaction mixture was assayed after the isolation of CO_2, no malate could be detected.

The precision of the ^{13}C:^{12}C ratio analysis at C-2 of malate is demonstrated by the constancy of the ^{13}C:^{12}C ratios determined for all the complete conversion samples. Thus samples of malate were generated from glyoxylate and an excess of either [$^1H_3$]acetyl-CoA (two samples) or [$^2H_3$]acetyl-CoA (four samples) in the presence of malate synthase. Each of these six samples was then analyzed as described above. The mean value for the ^{13}C:^{12}C ratios in the derived CO_2 was $(1106.1 \pm 0.45) \times 10^{-5}$. The $^{13}(V/K)_H$ and $^{13}(V/K)_D$ isotope effects were determined by a comparison of the ^{13}C:^{12}C ratios of CO_2 from the partial and complete conversion samples according to eq 3. The results are summarized in Table I.

Deuterium Isotope Effect. The deuterium isotope effect on malate synthase was determined under conditions similar to those used for the ^{13}C isotope effect. The glyoxylate con-

Table I: ^{13}C:^{12}C Isotope Effects on the Condensation of Glyoxylate with Acetyl-CoA Catalyzed by Malate Synthase

cosubstrate	extent of reaction (%)[a]	^{13}C:^{12}C ratio ($\times 10^5$) in carbon dioxide[b]		$^{13}(V/K)$[c]
[$^1H_3$]acetyl-CoA	9.46	1102.2		1.0037
	10.2	1102.6		1.0034
	8.81	1101.8		1.0041
			mean of $^{13}(V/K)_H$:	1.0037 ± 0.00035[d]
[$^2H_3$]acetyl-CoA	9.77	1101.7		1.0042
	10.8	1103.1		1.0029
	8.32	1102.2		1.0039
			mean of $^{13}(V/K)_D$:	1.0037 ± 0.0007[d]

[a] Defined as 100 × [malate]/([malate] + [glyoxylate]). [b] The ^{13}C:^{12}C ratio of C-2 of malate after reaction to the extent shown in the previous column. The ratios were adjusted for the contribution of ^{17}O (Craig, 1957). [c] Calculated according to eq 3 with the ^{13}C:^{12}C ratio for complete conversion of (1106.1 ± 0.45) × 10^{-5} (see the text). [d] The error quoted is 1 SD.

centration was more than 10 mM, which is 100 times the K_m for this substrate. The value for $^DV/K$ was found to be 1.3 ± 0.1, and ^DK_m was 1.0 ± 0.1. The K_m for acetyl-CoA was 6 × 10^{-5} M. Durchschlag et al. (1981) have previously determined the deuterium isotope effect under similar conditions, (except that the glyoxylate concentration used by these workers was only 6.7 times K_m) and found $^DV/K$ to be 1.36, ^DK_m to be 1.0, and K_m for acetyl-CoA to be 6.3 × 10^{-5} M. Our results are thus in close agreement with the earlier data. The fact that the deuterium isotope effect is independent of glyoxylate concentration shows that glyoxylate does not increase the forward commitment of acetyl-CoA.

Discussion

The fact that malate synthase does not catalyze the exchange of solvent protons with acetyl-CoA in the absence of glyoxylate (Eggerer & Klette, 1967) and the observation of inversion of stereochemistry at the nucleophilic carbon (Lenz & Eggerer, 1976) are suggestive of a concerted mechanism, particularly when these results are compared with the behavior of the unambiguously stepwise reaction catalyzed by the aldolases. To resolve the question of concertedness for malate synthase (and, by analogy, for other enzymes that mediate Claisen-type condensations), we have applied the method of double-isotope fractionation (Hermes et al., 1982; Belasco et al., 1983).

Double-Isotope Fractionation. The magnitude of an observed isotope effect depends upon the size of the intrinsic isotope effect and the degree to which the isotopically sensitive transition state limits the rate of the overall reaction. If this transition state is cleanly rate limiting, the observed isotope effect will equal the intrinsic isotope effect. However, if the isotopically sensitive transition state is not cleanly rate limiting, the observed kinetic isotope effect will be modulated by a factor that depends on the contribution of that transition state to the overall reaction rate. To the extent that enzymes have evolved so that several transition states are of roughly equal free energy and that no single elementary step is cleanly rate limiting, observed kinetic isotope effects are often smaller than the intrinsic effects.

In the reaction catalyzed by malate synthase, a carbon–hydrogen bond is broken and a carbon–carbon bond is formed. In a concerted reaction involving a single isotopically sensitive transition state (Figure 5), the carbon–hydrogen bond cleavage occurs simultaneously with carbon–carbon bond formation; if this transition state is partially rate limiting, both a deuterium and a ^{13}C isotope effect will be observed, but if this transition state is not partially rate limiting, neither isotope effect will be seen. In a stepwise mechanism, carbon–hydrogen bond cleavage and carbon–carbon bond formation occur in consecutive steps,[1] and if only one of the two isotopically

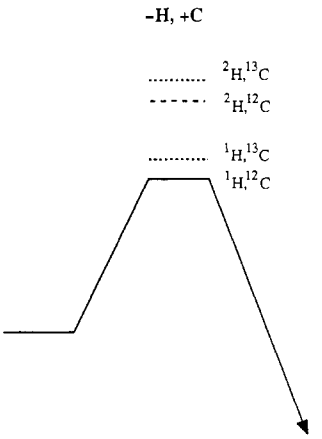

FIGURE 5: Free-energy profile for a concerted condensation reaction in which a carbon–hydrogen bond is broken and a carbon–carbon bond is formed. Free energy differences deriving from isotopic substitution are, of course, predominantly ground-state effects: these changes are here shown in the transition state *only* for the purposes of illustration. Upon deuteriation of the acetyl-CoA, the activation free energy increases, but the intrinsic ^{13}C isotope effect is unaltered.

sensitive transition states is partially rate limiting, only one isotope effect will be observable (see Figure 6a,b). However, if both transition states are partially rate limiting, both deuterium and ^{13}C isotope effects will be observed (Figure 6c). A stepwise mechanism in which both transition states are kinetically significant is called a balanced stepwise mechanism.

It is obvious from the above that if only one isotope effect is observed, the reaction mechanism is stepwise. However, if both ^2H and ^{13}C isotope effects are observed, the reaction may be either concerted or balanced stepwise. A distinction between these possibilities may be made by measuring the consequence of isotopic substitution at one site on the kinetic isotope effect at the other site. In the case of malate synthase, we measure the perturbation of the ^{13}C isotope effect at the glyoxylate carbonyl group by substitution of deuterium for hydrogen in the acetyl-CoA. Deuterium substitution in acetyl-CoA cannot affect the *size* of the intrinsic ^{13}C isotope effect, but deuterium substitution in acetyl-CoA will alter the *kinetic significance* of the ^{13}C-sensitive step. For a concerted condensation (Figure 5), if the isotopically sensitive step is cleanly rate limiting, deuterium substitution will have no effect on the size of the ^{13}C effect: the intrinsic effect will be measured in each case (that is, using [$^1H_3$]- or [$^2H_3$]acetyl-CoA). If the isotopically sensitive transition state is not cleanly rate limiting, deuterium substitution will increase the kinetic significance

[1] In the present case, the dictates of chemistry require that carbon–hydrogen bond cleavage occurs *before* carbon–carbon bond formation. In the general case, the order of events can be determined (Hermes et al., 1982).

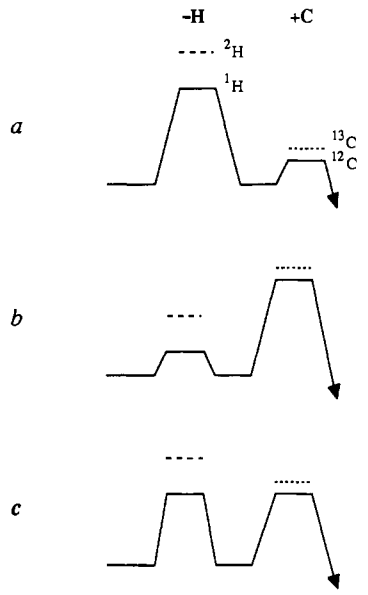

FIGURE 6: Free-energy profile for a stepwise condensation reaction in which carbon–hydrogen bond cleavage precedes reaction of the resulting carbanion with the electrophile. Free energy differences deriving from isotopic substitution are shown in the transition state *only* for purposes of illustration. (Case a) Proton abstraction is cleanly rate limiting, and the carbanionic intermediate partitions forward to product. Only a deuterium isotope effect is observed. (Case b) Carbon–carbon bond formation is cleanly rate limiting, and the carbanionic intermediate is in preequilibrium with the substrate. Only a ^{13}C kinetic isotope effect is observed. (Case c) Proton abstraction and condensation are each partially rate limiting, and the partitioning of the intermediate carbanion is balanced. Upon deuteriation of the substrate, the partitioning of the intermediate forward to product increases. This change in the partitioning results in a decrease in the observed ^{13}C isotope effect.

of that transition state, and the observed ^{13}C isotope effect will rise toward the intrinsic value. For a balanced stepwise condensation (Figure 6c), deuterium substitution will slow the first step, and thus decrease the kinetic significance of the second, ^{13}C-sensitive, step. In this case, then, deuterium substitution in acetyl-CoA will result in a smaller observed ^{13}C isotope effect.

Deuterium Isotope Effects. Before applying the double-isotope fractionation test to malate synthase, let us examine what is known from the values of the deuterium isotope effects for this enzyme. We have observed a deuterium isotope effect ($^DV/K$) of 1.3 ± 0.1 [in good agreement with the value of 1.36 reported by Durchschlag et al. (1981)]. This is an intermolecular isotope effect and compares the relative rates of reaction of [$^1H_3$]acetyl-CoA and of [$^2H_3$]acetyl-CoA. Malate synthase also shows an *intra*molecular deuterium isotope effect, where the enzyme discriminates among the three protons of a partially deuteriated methyl group. The intramolecular isotope effect ($^Dk_{intra}$) for malate synthase is 3.8 (Lenz & Eggerer, 1976).

Although the intermolecular and intramolecular isotope effects result from the same carbon–hydrogen bond cleavage and therefore stem from the same intrinsic isotope effect, the intermolecular isotope effect is smaller than the intramolecular effect. $^DV/K$ intermolecular isotope effects compare the rate of reaction of labeled and unlabeled substrates from the substrate and enzyme free in solution through the transition state of the first irreversible step. In contrast, an intramolecular isotope effect reflects the competition between deuterium and hydrogen in the same molecule, from the carbon–hydrogen bond cleavage step through the first irreversible step. The magnitude of the intermolecular isotope effect is modulated by all partially rate limiting steps up to and including the first irreversible step, whereas the magnitude of the intramolecular isotope effect is affected only by partially rate limiting steps *after* the carbon–hydrogen bond cleavage step up to and including the first irreversible step. Since in the case of malate synthase the intermolecular isotope effect (1.3) is smaller than the intramolecular isotope effect (3.8), the carbon–hydrogen bond breaking step in the malate synthase mechanism *must be preceded* by a partially rate limiting transition state that is isotopically insensitive.

^{13}C Isotope Effect. In any reaction in which a carbon–hydrogen bond is broken and a carbon–carbon bond is formed, the existence of a ^{2}H kinetic isotope effect but not a ^{13}C kinetic isotope effect (or the converse) demands a stepwise path in which the bonds are made and broken in different transition states. The mechanistic ambiguity between concerted and stepwise paths only occurs when both ^{2}H and ^{13}C isotope effects are seen. The discussion in the previous section relates to the existence of a ^{2}H effect in the reaction catalyzed by malate synthase, and we report in this paper the existence of a ^{13}C effect. With [$^1H_3$]acetyl-CoA as substrate, we have found a $^{13}(V/K)_H$ effect of 1.0037 ± 0.0004 (Table I). To resolve the mechanistic ambiguity thus posed, we have determined the effect of deuteriation of the acetyl-CoA on the observed ^{13}C effect. There are three mechanistic possibilities: (i) the ^{13}C effect is a kinetic isotope effect, and the reaction of malate synthase is concerted; (ii) the ^{13}C effect is a kinetic isotope effect, and the reaction of malate synthase is stepwise; (iii) the ^{13}C effect is an equilibrium isotope effect and the enzyme reaction is stepwise. Each of these possibilities is discussed below.

(*i*) *The Concerted Pathway.* For a concerted mechanism where the isotopically sensitive step is preceded by a kinetically significant but isotopically *in*sensitive step (see Figure 7a), the observed ^{2}H and ^{13}C isotope effects can be expressed in terms of fractionation factors[2] as $\phi_{gs}/\phi_{1,2}$ (Albery & Knowles, 1976), where ϕ_{gs} is the ground-state fractionation factor and $\phi_{1,2}$ is the mixed transition state fractionation factor. The mixed transition state fractionation factor (Belasco et al., 1983) is the weighted average of the individual fractionation factors for transition state 1 (the isotopically insensitive step) and for transition state 2 (the isotopically sensitive step). On the basis that the isotopes fractionate independently (Kresge, 1964; Hegarty & Jencks, 1975), four fractionation factors are required to describe the transition states in Figure 7a. The fractionation factors $^{13}\phi_1$, $^{13}\phi_2$, $^D\phi_1$, and $^D\phi_2$ describe the fractionation of carbon and of hydrogen in transition states 1 and 2, respectively. The mixed transition state fractionation factor for deuterium is

$$^D\phi_{1,2} = \frac{1 + \kappa}{^D\phi_1^{-1} + \kappa\,^D\phi_2^{-1}} \quad (4)$$

where $\kappa = k_{-1}/k_2$ and the corresponding mixed transition state fractionation factor for carbon with a protio cosubstrate (i.e., [$^1H_3$]acetyl-CoA) is

$$^{13}(\phi_{1,2})_H = \frac{1 + \kappa}{^{13}\phi_1^{-1} + \kappa\,^{13}\phi_2^{-1}} \quad (5)$$

[2] The fractionation factor, ϕ, for substitution of deuterium in place of protium at site X is the equilibium constant for the transfer of deuterium from a 50:50 mixture of H_2O and D_2O to X (Albery & Knowles, 1976), and as such, ϕ is a measure of the relative Gibbs free energy difference between XD and XH. The fractionation factor for substitution of isotopes of carbon is the equilibrium constant for the hypothetical transfer of ^{13}C from CO_2 to the compound of interest.

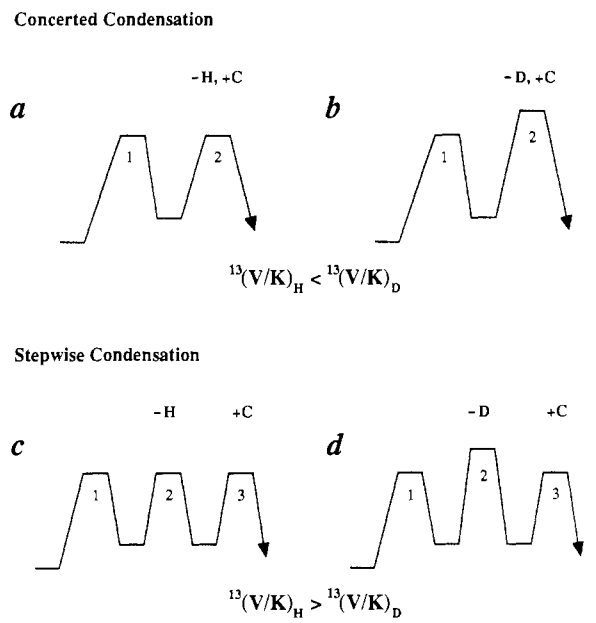

FIGURE 7: Free-energy profiles for a concerted condensation (cases a and b) and a balanced stepwise condensation (cases c and d). Free energy differences deriving from isotopic substitution are shown in the transition state *only* for purposes of illustration. (Case a) A kinetically significant but isotopically insensitive transition state (transition state 1) precedes the single transition state in which the carbon–hydrogen bond is cleaved and the carbon–carbon bond is formed (transition state 2). (Case b) When [$^1H_3$]acetyl-CoA is replaced by [$^2H_3$]acetyl-CoA, the kinetic significance of the second transition state (transition state 2) increases, and the observed ^{13}C isotope effect increases. (Case c) An isotopically insensitive step (transition state 1) is followed by a stepwise reaction in which the carbon–hydrogen bond is first cleaved (transition state 2) and then the carbon–carbon bond is formed (transition state 3). (Case d) Upon deuteriation of the acetyl-CoA, the kinetic significance of the second transition state increases, and that of the ^{13}C-sensitive step decreases.

Since transition state 1 is isotopically insensitive, $^D\phi_1 \approx {}^D\phi_{gs}$ and $^{13}\phi_1 \approx {}^{13}\phi_{gs}$. [While it is possible that the carbonyl group of the thioester is polarized on binding at the active site, the consequence for the value of $^D\phi_1$ will be negligible. Even complete protonation of a carbonyl group only lowers the fractionation factor for an α-hydrogen by a factor of 1.09 (Arnett et al., 1961).] The value of $^D\phi_{gs}$ for acetyl-CoA is approximately 0.89.[3] Transition state 2 is isotopically sensitive with both the hydrogen and carbon undergoing covalency changes in the transition state, making $^D\phi_2$ less than $^D\phi_{gs}$ and $^{13}\phi_2$ less than $^{13}\phi_{gs}$. Equations 4 and 5 show how the observed isotope effects are dependent on the kinetic significance of the isotopically sensitive step. If the isotopically sensitive transition state is cleanly rate limiting, $\kappa \gg 1$, the mixed transition state fractionation factor $\phi_{1,2}$ equals ϕ_2, and the observed isotope effect is equal to the intrinsic effect. Conversely, if the first step is cleanly rate limiting, $\kappa \ll 1$, and $\phi_{1,2}$ equals ϕ_1. Since ϕ_1 for the isotopically insensitive step is close to ϕ_{gs}, the observed isotope effect will be near to 1.0. Finally, if the two transition states are of roughly equal free energy as illustrated in Figure 7a, κ is near 1, and both the observed isotope effects lie between 1.0 and the values of the full intrinsic effects.

With this background, we can now compare the value of the ^{13}C isotope effect when protonated acetyl-CoA is used (Figure 7a) with that when the acetyl-CoA is deuteriated (Figure 7b). When [$^2H_3$]acetyl-CoA replaces [$^1H_3$]acetyl-CoA, the fractionation factors remain the same, but the partition ratio increases by a factor of $^D\phi_1/{}^D\phi_2$.[4] The expression for the mixed transition state fractionation factor then becomes

$$^{13}(\phi_{1,2})_D = \frac{1 + (^D\phi_1/{}^D\phi_2)\kappa}{^{13}\phi_1^{-1} + (^D\phi_1/{}^D\phi_2)\kappa\,{}^{13}\phi_2^{-1}} \quad (6)$$

Since only transition state 2 is isotopically sensitive, the increase in the kinetic significance of this transition state when deuteriated acetyl-CoA is used leads to an *increase* in the observed ^{13}C isotope effect. In summary, if the reaction catalyzed by malate synthase is concerted, the ^{13}C isotope effect at the carbonyl group of glyoxylate will rise toward the intrinsic value, when [$^1H_3$]acetyl-CoA is replaced by [$^2H_3$]-acetyl-CoA as the cosubstrate.

(*ii*) *The Stepwise Pathway.* If the malate synthase reaction follows a stepwise path, we have the situation illustrated in Figure 7c where the deuterium-sensitive transition state is followed by the ^{13}C-sensitive transition state and (from the discussion above) is preceded by a partially rate limiting isotopically insensitive transition state. Here, the mixed fractionation factor $\phi_{1,2,3}$ (Albery & Knowles, 1987) must be used to describe the isotope effect, which is $\phi_{gs}/\phi_{1,2,3}$. The mixed transition state fractionation factor $^D\phi_{1,2,3}$ is given by

$$^D\phi_{1,2,3} = \frac{1 + \kappa(1 + \alpha)}{^D\phi_1^{-1} + \kappa(^D\phi_3 + \alpha\,{}^D\phi_2)\,{}^D\phi_2^{-1}\,{}^D\phi_3^{-1}} \quad (7)$$

and the corresponding mixed transition state fractionation factor for carbon with protio acetyl-CoA as cosubstrate is

$$^{13}(\phi_{1,2,3})_H = \frac{1 + \kappa(1 + \alpha)}{^{13}\phi_1^{-1} + \kappa(^{13}\phi_3 + \alpha\,{}^{13}\phi_2)\,{}^{13}\phi_2^{-1}\,{}^{13}\phi_3^{-1}} \quad (8)$$

where $\kappa = k_{-1}/k_2$ and $\alpha = k_{-2}/k_3$. Of the six fractionation factors required fully to describe the transition states for the stepwise mechanism in Figure 7c, $^{13}\phi_1$, $^{13}\phi_2$, and $^{13}\phi_3$ describe the fractionation of carbon in transition states 1, 2, and 3, and the fractionation of hydrogen in these transition states is described by $^D\phi_1$, $^D\phi_2$, and $^D\phi_3$. Since transition state 1 is isotopically insensitive, $^{13}\phi_1 \approx {}^{13}\phi_{gs}$ and $^D\phi_1 \approx {}^D\phi_{gs}$, and $^{13}\phi_2 \approx {}^{13}\phi_{gs}$ since transition state 2 involves only carbon–hydrogen bond cleavage. Transition state 3 involves carbon–carbon bond formation, and $^{13}\phi_3$ will therefore be less than $^{13}\phi_{gs}$. Since hydrogen is in flight in the second transition state, $^D\phi_2$ will be less than $^D\phi_{gs}$, and since this hydrogen is at rest on the enzymic base in transition state 3, $^D\phi_3$ will be near to 1 if the enzyme uses an oxygen or a nitrogen base or approximately 0.5 if the enzymic base is a thiol (Schowen, 1977; Szawelski et al., 1982).

As can be seen by inspection of Figure 7c, if a ^{13}C kinetic isotope effect is observed in the overall reaction, then transition state 3 must be kinetically significant. When [$^1H_3$]acetyl-CoA (Figure 7c) is replaced by [$^2H_3$]acetyl-CoA (Figure 7d) as cosubstrate, transition state 3 becomes kinetically less significant, and the observed ^{13}C kinetic isotope effect will *fall*. The algebraic equivalent of this statement can be derived from eq 8 to obtain the expression for $^{13}(\phi_{1,2,3})_D$, in the same way that eq 6 was derived from eq 5, to give

[3] $^D\phi_{gs}$ for the hydrogen at C-3 of malate is 0.98 (Cleland, 1980). Schimerlik et al. (1975) have shown that the ratio of the fractionation factors of secondary hydrogens to those of primary hydrogens is about 1.10 and substituents at the β-carbon are unimportant except when the β-carbon is the carbon of a carbonyl group. $^D\phi_{gs}$ for acetyl-CoA is therefore taken to be 0.89.

[4] The intrinsic isotope effect for the reaction of the intermediate ES over transition state 1 equals $^D\phi_{ES}/{}^D\phi_1$ and that for reaction over transition state 2 equals $^D\phi_{ES}/{}^D\phi_2$. Therefore, when [$^2H_3$]acetyl-CoA is the cosubstrate, the partition ratio can be expressed in terms of κ and fractionation factors as $(^D\phi_1/{}^D\phi_2)\kappa$.

$$^{13}(\phi_{1,2,3})_D = \frac{1 + \frac{^D\phi_1}{^D\phi_2}\kappa\left(1 + \frac{^D\phi_2}{^D\phi_3}\alpha\right)}{^{13}\phi_1^{-1} + \frac{^D\phi_1}{^D\phi_2}\kappa\left(^{13}\phi_3 + \frac{^D\phi_2}{^D\phi_3}\alpha\,^{13}\phi_2\right)\,^{13}\phi_2^{-1}\,^{13}\phi_3^{-1}} \quad (9)$$

(*iii*) *Preequilibrium Dehydration.* In sections i and ii above, it has been presumed that the observed ^{13}C effect is a kinetic isotope effect. Yet the value is small, 0.37%, and the possibility must be entertained that this effect is an *equilibrium* ^{13}C isotope effect relating to the hydration of glyoxylate. More than 99% of glyoxylate is hydrated in aqueous solution, and the rate of aldehyde–hydrate interconversion is very much faster than the rate of glyoxylate consumption during the isotope effect determinations (Rendina et al., 1984), so the aldehyde and hydrate remain at equilibrium throughout the malate synthase reaction. Since the heavier isotope concentrates where the potential well is steeper, the unhydrated form of glyoxylate, which we can presume is the form that condenses with acetyl-CoA in the enzymic reaction, will have a lower ^{13}C content than the hydrate. Therefore, even if malate synthase shows no measureable *kinetic* ^{13}C isotope effect, the malate formed from the ^{13}C-depleted pool of the aldehydo form of glyoxylate will have a low ^{13}C:^{12}C ratio at C-2, exactly as we have observed. While the value of the ^{13}C equilibrium isotope effect for glyoxylate hydration is not known, P. Canellas and W. W. Cleland (private communication) have determined the analogous equilibrium ^{13}C effect for the hydration of glyceraldehyde 3-phosphate and have found a value of 1.0028. Since the replacement of [$^1H_3$]acetyl-CoA by [$^2H_3$]acetyl-CoA will not effect the equilibrium hydration of the other substrate, glyoxylate, we predict in this case that there will be *no change* in the observed ^{13}C isotope effect when the cosubstrate is deuteriated. It should be noted that if the ^{13}C isotope effect is indeed an equilibrium isotope effect, then there is no ^{13}C *kinetic* effect, which, with the existence of a deuterium kinetic isotope effect, requires a stepwise mechanism.

The predicted isotope effects can now be summarized. If deuteriation of the acetyl-CoA leads to an *increase* in the observed ^{13}C isotope effect at C-2 of glyoxylate, then the malate synthase reaction is a concerted process; if deuteriation of the acetyl-CoA leads to a *decrease* in the observed ^{13}C isotope effect, then the reaction is stepwise; and if the deuteriation of the acetyl-CoA has *no effect* on the ^{13}C isotope effect, then the ^{13}C perturbation is an equilibrium effect and the enzymatic reaction is stepwise. With the values of the intermolecular and intramolecular deuterium isotope effects, a value for κ can be calculated for the concerted mechanism, and if the mechanism is stepwise, values can be calculated for α and κ. With these parameters and the value of $^{13}(V/K)_H$, the predicted value for the ^{13}C isotope effect with deuteriated acetyl-CoA as the substrate [i.e., $^{13}(V/K)_D$] can be calculated for both the concerted and stepwise mechanisms as described in the Appendix. Upon deuteriation of acetyl-CoA, the ^{13}C isotope effect will increase from 1.0037 to 1.011 if the reaction is concerted, decrease to 1.0025 if the isotope effect is a kinetic isotope effect from a stepwise mechanism,[5] or remain unchanged at 1.0037 if the ^{13}C effect is an equilibrium effect preceding a stepwise mechanism in which the carbon–carbon bond forming transition state is kinetically insignificant.

When the ^{13}C isotope effect was measured with deuteriated acetyl-CoA as the cosubstrate, the isotope effect was unchanged, at 1.0037 ± 0.0007. *Thus malate synthase catalyzes a stepwise condensation reaction.* The fact that the ^{13}C isotope effect does not change on deuteriation of acetyl-CoA requires a stepwise path and strongly suggests that the observed ^{13}C effect relates to the equilibrium dehydration of glyoxylate.[6]

Since the condensation catalyzed by malate synthase follows a stepwise path, we may ask why malate synthase does not catalyze proton exchange between acetyl-CoA and solvent in the absence of the cosubstrate. There are two obvious possibilities. Either the conjugate acid of the enzymic base is sequestered from solvent thus preventing proton exchange with solvent, or the enzyme may not initiate proton abstraction until both substrates are bound. This second hypothesis of substrate synergism puts other constraints on the reaction mechanism, since it is known that solvent tritium does not appear either in product malate or in the remaining acetyl-CoA when all substrates are present (Eggerer & Klette, 1967). Substrate synergism is only consistent with this result if the enol(ate) intermediate always partitions forward to product. Such a partition ratio (i.e., $\alpha \ll 1$) in turn requires a kinetically insignificant transition state for carbon–carbon bond formation, which is in agreement with the isotope effects we have determined.

As discussed earlier, an isotopically insensitive but kinetically significant transition state precedes carbon–hydrogen bond cleavage, and we may speculate on the nature of this early step in the reaction. Three possibilities are diffusion-limited binding of substrates, formation of an acetyl enzyme intermediate, and a conformational change. We can eliminate the first possibility, for even allowing for the small fraction of glyoxylate that exists as the free aldehyde (which we presume to be the substrate), the value of k_{cat}/K_m for glyoxylate is far below that expected for a diffusion-limited reaction (Hammes & Schimmel, 1970). Furthermore, Durchschlag et al. (1981) have shown (and we have confirmed) that the $^DV_{max}$ and $^DV/K$ isotope effects are equal, which cannot easily be accommodated by a partially rate limiting diffusive transition state. The second possibility is that the isotopically insensitive step involves the transfer of the acetyl group of acetyl-CoA to a residue on the enzyme prior to carbon–hydrogen bond cleavage. The existence of acetyl enzyme intermediates is well precedented among other Claisen enzymes. Thus 3-hydroxy-3-methylglutaryl-CoA synthase (Miziorko et al., 1975) and bacterial citrate lyase (Srere, 1975) both proceed via acetyl enzyme intermediates. Thiolase also catalyzes the formation of an acetyl enzyme [though this ester acts as the electrophilic rather than the nucleophilic partner in the reaction (Gilbert et al., 1981)]. In the case of malate synthase, Dixon et al. (1960) investigated the possibility of an acetyl enzyme intermediate by looking for enzyme-catalyzed exchange between [^{35}S]coenzyme A and acetyl-CoA in the absence of glyoxylate. No significant exchange was detected, and the existence of an acetyl enzyme intermediate in the malate synthase reaction seems unlikely. The third possibility for the isotopically insensitive step is a conformational change that occurs after both substrates have bound. In support of this

[5] If the enzymic base were a thiolate, $^D\phi_3$ would be near 0.5, and the predicted $^{13}(V/K)_D$ isotope effect for a stepwise mechanism would be approximately 1.0051.

[6] Within the accuracy limits of our experiments and the uncertainty in the estimates of the values of $^D\phi_{gs}$ (0.89) and $^D\phi_3$ (1.0), the predicted value of $^{13}(V/K)_D$ for the stepwise reaction (case ii) could be somewhat higher than 1.0025 and lie within the experimental error of the measured $^{13}(V/K)_D$ isotope effect. This uncertainty does not effect our mechanistic conclusions: in either case the reaction proceeds by a stepwise pathway.

possibility, low-angle X-ray studies have suggested that the formation of the ternary complex is accompanied by a 4% decrease in enzyme volume relative to that of the binary complex with acetyl-CoA (Zipper & Durchschlag, 1978). Such a change could account for both the existence of the isotopically insensitive step and the fact that carbon–hydrogen bond cleavage does not occur until both acetyl-CoA and the acceptor cosubstrate have bound.

In summary, from the identity of the values of the ^{13}C isotope effects at the aldehydic carbon of glyoxylate when either [$^1H_3$]- or [$^2H_3$]acetyl-CoA is the cosubstrate, we conclude that the condensation catalyzed by malate synthase follows a stepwise path. The mechanistic similarity among the enzymes catalyzing other condensations of the Claisen type suggests that these enzymes, too, follow stepwise pathways.

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APPENDIX

The equations presented in the text describe the mixed transition state fractionation factors for the concerted and stepwise mechanisms in which the steps for carbon–hydrogen bond cleavage and carbon–carbon bond formation are preceded by a kinetically significant but isotopically insensitive transition state. In the text, we have shown qualitatively how the ^{13}C isotope effect at the aldehydic carbon of glyoxylate will change (for both concerted and stepwise mechanisms) when deuteriated acetyl-CoA is substituted for protioacetyl-CoA. Here, we use these equations, in conjunction with the values of the intermolecular and intramolecular deuterium isotope effects, to predict in each case the value of the ^{13}C isotope effect when deuteriated acetyl-CoA is the substrate.

(*i*) *The Concerted Path.* Before we can calculate $^{13}(\phi_{1,2})_D$ from eq 6, we require values for κ, $^{13}\phi_2$, and $^D\phi_1/^D\phi_2$. (We already know that since transition state 1 is isotopically insensitive, $^D\phi_1 \approx {}^D\phi_{gs}$ and $^{13}\phi_1 \approx {}^{13}\phi_{gs}$.) The value of κ is calculated from the magnitude of the intermolecular ($^DV/K$) and intramolecular ($^Dk_{intra}$) deuterium isotope effects. Assuming that $^Dk_{intra}$, the intramolecular isotope effect, is equal to the intrinsic deuterium isotope effect,[7] we may write $^Dk_{intra} = {}^D\phi_{ES}/{}^D\phi_2$, where $^D\phi_{ES}$ is the fractionation factor for the intermediate between transition states 1 and 2 in Figure 7a, and this fractionation factor may be approximated by $^D\phi_{gs}$. Since $^D\phi_1 \approx {}^D\phi_{gs}$ and $^DV/K$ (the intermolecular isotope effect) $= {}^D\phi_{gs}/{}^D\phi_{1,2}$, we rearrange eq 4 and write

$$\kappa = \frac{^DV/K - 1}{^Dk_{intra} - {}^DV/K} \quad \text{(A1)}$$

Equation A1 shows how the partition ratio for the unlabeled reactants, κ, is determined from the fraction of the intramolecular isotope effect that is manifested in the value of $^DV/K$. As the kinetic significance of transition state 2 (Figure 7a) increases, κ rises, and the magnitude of the intermolecular isotope effect approaches that of the intramolecular effect.

Knowing the value of κ, we then calculate the value of $^{13}\phi_2$ from eq 5, $^{13}(V/K)_H$, and the fact that $^{13}(V/K)_H = {}^{13}\phi_{gs}/{}^{13}(\phi_{1,2})_H$.

[7] If there are transition states following the deuterium- and ^{13}C-sensitive transition states which are partially rate limiting, $^Dk_{intra}$ will not equal $^Dk_{intrin}$. Nevertheless, isotopically insensitive transition states following the last isotopically sensitive transition state can be combined with this transition state to be represented as one composite transition state without affecting the value of $^{13}(V/K)_D$ that is calculated.

The magnitude of the ^{13}C isotope effect when [$^2H_3$]-acetyl-CoA is the substrate can now be estimated. We know κ, $^{13}\phi_2$, and $^{13}\phi_1$, and that $^Dk_{intra} = {}^D\phi_1/{}^D\phi_2$. Substituting these values into eq 6 yields $^{13}(\phi_{1,2})_D$. The predicted value of the ^{13}C isotope effect for deuterioacetyl-CoA as cosubstrate is then obtained from $^{13}(V/K)_D = {}^{13}\phi_{gs}/{}^{13}(\phi_{1,2})_D$. Equation A2 expresses this ^{13}C isotope effect in terms of κ (from eq A1) and of other measured quantities:

$$^{13}(V/K)_D = \frac{1 + {}^Dk_{intra}[{}^{13}(V/K)_H(\kappa + 1) - 1]}{1 + {}^Dk_{intra}\kappa} \quad \text{(A2)}$$

(*ii*) *The Stepwise Path.* To define the stepwise mechanism illustrated in Figure 7c, we require both α and κ and the fractionation factor for the ^{13}C-sensitive transition state, $^{13}\phi_3$. From the measured quantities, there is not a unique solution for α, κ, and $^{13}\phi_3$, but we can calculate sets of values for these parameters. Fortunately, the quantities are interrelated, and a unique value of $^{13}(V/K)_D$ can be obtained from the measured values of $^{13}(V/K)_H$ and the deuterium isotope effects.

In the stepwise pathway, the deuterium-sensitive transition state is followed by the ^{13}C-sensitive transition state, and the magnitude of the deuterium isotope effect is modulated by transition state 3 as well as by transition state 1. The *intra*-molecular isotope effect, $^Dk_{intra} = {}^D\phi_{ES}/{}^D\phi_{2,3}$ (where $^D\phi_{ES}$ is the fractionation factor for the first intermediate in Figure 7c and $^D\phi_{2,3}$ is the mixed transition state fractionation factor for transition states 2 and 3), is in this case less than the intrinsic isotope effect, $^Dk_{intrin} = {}^D\phi_{ES}/{}^D\phi_2$. The equation for $^D\phi_{2,3}$ is

$$^D\phi_{2,3} = \frac{1 + \alpha}{^D\phi_2{}^{-1} + \alpha{}^D\phi_3{}^{-1}} \quad \text{(A3)}$$

Substituting $^Dk_{intra}$ and $^Dk_{intrin}$ and taking $^D\phi_{ES} = {}^D\phi_{gs}$ give, from eq A3

$$\alpha = \frac{^Dk_{intrin} - {}^Dk_{intra}}{^Dk_{intra} - {}^D\phi_{gs}/{}^D\phi_3} \quad \text{(A4)}$$

While we know the value of $^Dk_{intra}$ and can estimate the values of $^D\phi_{gs}$ and $^D\phi_3$, the value of $^Dk_{intrin}$ is unknown. Since $^D\phi_1 \approx {}^D\phi_{gs}$ and $^DV/K = {}^D\phi_{gs}/{}^D\phi_{1,2,3}$, rearranging eq 7 gives κ as

$$\kappa = \frac{^DV/K - 1}{^Dk_{intrin} + \alpha{}^D\phi_{gs}/{}^D\phi_3 - {}^DV/K(1 + \alpha)} \quad \text{(A5)}$$

Once again the unknown quantity is the value of the intrinsic isotope effect. Since $^{13}(V/K)_H = {}^{13}\phi_{gs}/{}^{13}(\phi_{1,2,3})_H$, we can solve eq 8 for $^{13}\phi_3$ and obtain

$$^{13}\phi_3 = \frac{\alpha\kappa{}^{13}\phi_{gs}}{^{13}(V/K)_H[1 + \kappa(1 + \alpha)] - (1 + \kappa)} \quad \text{(A6)}$$

From eq A4, A5, and A6, we see that the values for α, κ, and $^{13}\phi_3$ are not independent of each other, and for every estimated value of $^Dk_{intrin}$, the same value of $^{13}(V/K)_D$ is predicted.

Substitution into eq 9 of any set of values of α, κ, and $^{13}\phi_3$ that derive from the measured isotope effects and the corresponding estimate of $^Dk_{intrin}$ gives the calculated value for $^{13}(\phi_{1,2,3})_D$. Since $^{13}(V/K)_D = {}^{13}\phi_{gs}/{}^{13}(\phi_{1,2,3})_D$, the expression for the ^{13}C isotope effect is readily obtained and is

$$^{13}(V/K)_D = \frac{1 + \kappa\left(^Dk_{intrin} + \frac{^D\phi_{gs}}{^D\phi_3}\frac{^{13}\phi_{gs}}{^{13}\phi_3}\alpha\right)}{1 + \kappa\left(^Dk_{intrin} + \frac{^D\phi_{gs}}{^D\phi_3}\alpha\right)} \quad \text{(A7)}$$

The value of the ^{13}C isotope effect with deuteriated cosubstrate may thus be predicted from the observed values of $^{13}(V/K)_H$ and the two deuterium isotope effects, for either the concerted or the stepwise mechanism. The predicted value of $^{13}(V/K)_D$ for the concerted mechanism is dependent on $^{13}(V/K)_H$ and the deuterium isotope effects. Substituting $^D(V/K) = 1.3$, $^Dk_{intra} = 3.8$, and $^{13}(V/K)_H = 1.0037$ into eq A2 gives a value of 1.011 for the predicted ^{13}C isotope effect with [$^2H_3$]acetyl-CoA as cosubstrate. The predicted value of $^{13}(V/K)_D$ for a stepwise mechanism is also dependent on $^D\phi_{gs}$ (0.89) and $^D\phi_3$ (1.0). Using these estimates for $^D\phi_{gs}$ and $^D\phi_3$, and eq A7, $^{13}(V/K)_D$ in the stepwise case is predicted to be 1.0025.

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