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Kinetic Analysis of the Malonyl Coenzyme A Decarboxylation and the Condensation Reaction of Fatty Acid Synthesis. Application to the Study of Malonyl Coenzyme A Inactivated Chicken Liver Fatty Acid Synthetase†

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ABSTRACT: A kinetic analysis of the decarboxylation of malonyl-CoA and the condensation-CO_2 exchange reaction of fatty acid synthesis has been carried out. The analysis supported by experimental evidence defines conditions under which the decarboxylation of malonyl-CoA quantitatively reflects the activity for the condensation reaction between enzyme-bound acyl and malonyl groups. $NADP^+$ decreases the release of $^{14}CO_2$ from radiolabeled malonyl-CoA by lowering the rates of the processes leading to the formation of triacetic acid lactone. For accurate measurements, the enzyme concentration should be less than 200 μg/mL, and malonyl-CoA/enzyme ratios should be 200 or less. Short reaction periods (1 min or less) and inclusion of $NADP^+$ (100 μM) enhance the accuracy of measurements. These analyses have been used to explain the mechanism of malonyl-CoA mediated inactivation of chicken liver fatty acid synthetase and are appropriate for determining the functional condensing site of the polyfunctional polypeptide chains comprising the dimeric enzyme.

Animal fatty acid synthetases (M_r 500 000) consist of two polyfunctional polypeptide chains held together by noncovalent bonds (Arslanian et al., 1976). Dissociation of the dimer into monomers results in the loss of the critical rate-limiting condensation reaction between enzyme-bound acetyl and malonyl groups (Kumar et al., 1970). Additionally, the activity for the condensation reaction is inhibited during antigen-antibody complexation (Kumar et al., 1977), modification by chloroacetyl-CoA (Kumar et al., 1980), and irreversible inactivation by malonyl-CoA (Kumar & Srinivasan, 1981) and by less specific thiol inhibitors, iodoacetamide and 1,3-dibromopropanone (Wakil & Stoops, 1980). Thus, the stability of the condensing component is related to the overall functional integrity of the enzyme. Furthermore, there is growing evidence from recent studies (Arslanian et al., 1976; Wood et al., 1978; Wakil & Stoops, 1980; Kumar et al., 1980; Poulose et al., 1980) that the half molecular weight subunits of animal fatty acid synthetases are structurally and perhaps functionally identical. If this is true, then it will be of interest to determine how the two catalytically inactive subunits function cooperatively or in a coordinate manner to initiate and maintain fatty acid synthesis. Whether each of the subunits contains a minimally functional condensing site whose function is altered on reassociation of the subunits needs experimental demonstration.

For an estimation of the condensing activity, an accurate, fast, and reliable method of measurement is needed. The usual method is to monitor this reaction in the reversible direction, i.e., measure the incorporation of $^{14}CO_2$ from $H^{14}CO_3^-$ into the three position of malonyl-CoA (Kumar et al., 1970). This is a slow, cumbersome, and time-consuming method, and because of the complexity introduced by various side reactions (see Scheme I), accurate estimates are difficult to obtain.

In this article, we present kinetic analysis of the condensation-CO_2 exchange reaction and the decarboxylation of malonyl-CoA catalyzed by the chicken liver fatty acid synthetase. This analysis supported by experimental data defines conditions under which enzyme-catalyzed decarbox-

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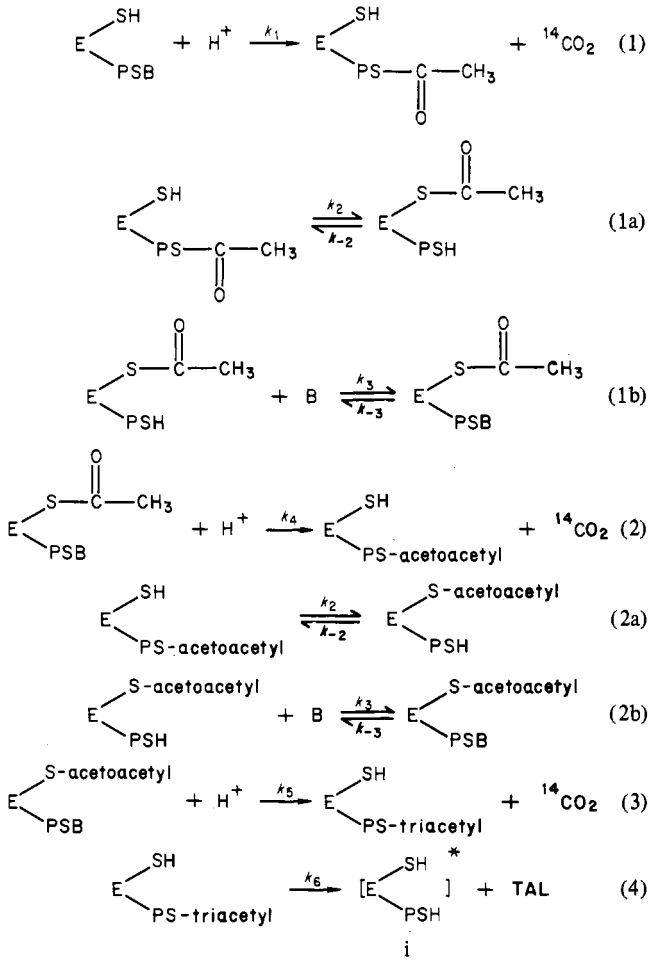
Scheme I: Proposed Sequence of Reactions Catalyzed by Fatty Acid Synthetase Leading to the Formation of Triacetic Acid Lactone



ylation of malonyl-CoA quantitatively reflects the activity of the condensation system. Such analysis has been carried out before. Additional kinetic analysis of the decarboxylation reaction permits us to explain the mechanism of malonyl-CoA mediated inactivation of the enzyme described in the preceding paper (Kumar & Srinivasan, 1981).

Experimental Procedures

Chicken liver fatty acid synthetase was purified and assayed according to our published procedure (Srinivasan & Kumar, 1976). Other details of the procedures for the inactivation of fatty acid synthetase by malonyl-CoA or malonyl-CoA plus acetyl-CoA and for the measurement of the rate of decarboxylation of [1,3-^{14}C]malonyl-CoA are given in the preceding paper (Kumar & Srinivasan, 1981). When the effect of acetyl-CoA or $NADP^+$ on the rate of decarboxylation of malonyl-CoA was to be determined, the enzyme was initially incubated with these substrates, and the reaction was initiated by injecting malonyl-CoA into the flask. Similar results were obtained if acetyl-CoA or the $NADP^+$ and malonyl-CoA mixture was injected into the flask containing the enzyme.

Condensation–CO_2 Exchange Reaction. The experimental procedure was similar to that of Kumar et al. (1970). The amount of enzyme was varied between 15 and 250 μg per 0.25 mL. The exchange reaction was carried out by two different methods, by measuring the loss of $^{14}CO_2$ from the labeled 3-position carboxyl group of malonyl-CoA (900 dpm/nmol of $^{14}CO_2$) in the presence of unlabeled $NaHCO_3$ or by determining the incorporation of $^{14}CO_2$ from $NaH^{14}CO_3$ into the 3-carboxyl position of malonyl-CoA. After the exchange

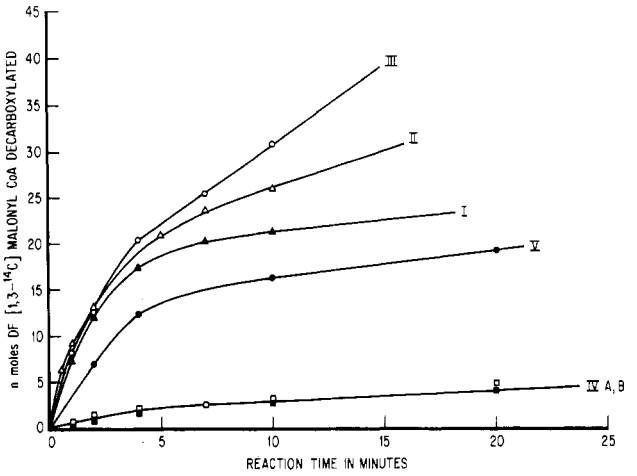


FIGURE 1: Time dependence of the rate of decarboxylation of [1,3-^{14}C]malonyl-CoA. The reactions were carried out in 1-mL volume and at an enzyme concentration of 100 μg/mL in all cases. Released $^{14}CO_2$ (950 dpm/nmol) was trapped in 0.5 M hyamine hydroxide and counted for radioactivity. When $NaH^{14}CO_3$ was used, the efficiency of the trapping procedure was found to be more than 95%. (Curve I) Malonyl-CoA/enzyme, 500:1; (curve II) malonyl-CoA/enzyme, 1000:1; (curve III) acetyl-CoA/malonyl-CoA/enzyme, 500:500:1; (curve IVA) acetyl-CoA/malonyl-CoA/$NADP^+$/enzyme, 500:500:500:1; (curve IVB) malonyl-CoA/$NADP^+$/enzyme, 500:500:1; (curve V) malonyl-CoA/enzyme, 500:1, in the presence of 10 mM acetoacetyl-*N*-acetylcysteamine.

reaction was stopped with HCl as described before (Kumar & Srinivasan, 1981), the contents of the reaction mixture were quantitatively transferred to the counting vials by using 0.2 N HCl and evaporated to dryness at 60 °C under vacuum provided by a water aspirator. The residue was dissolved in 0.5 mL of water and counted for radioactivity. When used, the concentration of $NADP^+$ was 100 μM.

Results and Discussions

Kinetic Analysis of the Decarboxylation Reaction. For an explanation of the mechanism of malonyl-CoA mediated inactivation of chicken liver fatty acid synthetase, an understanding of the fate of enzyme-bound malonyl group is essential. Scheme I lists possible reactions that the bound malonyl group undergoes. We chose to measure the release of $^{14}CO_2$ from radiolabeled malonyl-CoA since formation of CO_2 accompanies both the decarboxylation and the condensation reactions (reactions 1–3 of Scheme I). Figure 1 shows the rate of decarboxylation of [1,3-^{14}C]malonyl-CoA under conditions of enzyme inactivation listed in the preceding paper (Kumar & Srinivasan, 1981). To obtain an estimate of the contributions of reactions 1–3 of Scheme I to the released CO_2, equations of Scheme I can be written in the notation of enzyme kinetics (Cleland, 1963) (eq 5).

$$\mathrm{EB} \xrightarrow{k_1} \mathrm{EA} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E'A} + \mathrm{B} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E'AB} \xrightarrow{k_4} \mathrm{EAA'} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E'AA'} + \mathrm{B} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E'AA'B} \xrightarrow{k_5} \mathrm{EAA'A''} \xrightarrow{k_6} \mathrm{E^*} + \text{product} \quad (5)$$

B refers to malonyl-CoA. Enzyme states [EB], [E'AB], and [E'AA'B] relate to the release of CO_2. The rate of decarboxylation will be given by

$$-\frac{dB}{dt} = k_1[\mathrm{EB}] + k_4[\mathrm{E'AB}] + k_5[\mathrm{E'AA'B}] \quad (6)$$

where the enzyme concentrations refer to their respective instantaneous values rather than the steady-state values. From eq 5 and 6

$$-\frac{dB}{dt} = \frac{k_1}{K_3}[E][B_0 - B] \times \left[1 + \frac{K_2k_4}{K_3}[B_0 - B] + \frac{(K_2)^2k_4k_5}{(K_3)^2}[B_0 - B]^2\right] \quad (7)$$

where $[B_0 - B]$ is the instantaneous value of B, K_3 is the dissociation constant for the binding of the malonyl-CoA to the enzyme irrespective of its state, and K_2 is the equilibrium constant for the thiol–thioester exchange between the internal groups of the enzyme (derivation of eq 7 is given; see paragraph at end of paper regarding supplementary matrial). The value of [E] in eq 7 is the active enzyme, and if there was no inactivation, then [E] will be a constant independent of time. This will be true so long as the substrate concentration is far above the dissociation constant K_3 and the equilibrium represented by K_2 is quite rapid and dynamic.

In the presence of acetyl-CoA, the rate equation (eq 7) is modified in the following way

$$-\frac{dB}{dt} = \frac{k_4}{K_3K_3'}[E]\left[A_0 - \frac{B}{2}\right][B_0 - B]\left[1 + \frac{k_5K_2}{K_3}[B_0 - B]\right] \quad (7a)$$

corresponding to

$$E + A \underset{k_{-3}'}{\overset{k_3'}{\rightleftharpoons}} EA + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EAB \xrightarrow{k_4} EAA' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'AA' + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E'AA'B \xrightarrow{k_5} EAA'A'' \xrightarrow{k_6} E^* + \text{product} \quad (8)$$

where K_3' represents the enzyme–acetyl-CoA dissociation constant and [A] stands for the instantaneous concentration of acetyl-CoA. The other terms are the same as in eq 7.

The difference between the rates of inactivation of the enzyme in the presence and absence of acetyl-CoA can be appreciated from the time derivative of eq 7 and 7a. To a first approximation, (7) gives (9a) and (7a) gives (9b).

$$\frac{d}{dt}\frac{(dB)}{dt} \simeq \frac{d}{dt}(k_1, k_4, k_5, \text{and } [E]) \quad (9a)$$

$$\frac{d}{dt}\frac{(dB)}{dt} \simeq \frac{d}{dt}(k_4, k_5, \text{and } [E]) \quad (9b)$$

If the common terms from eq 9a and 9b are eliminated, the differences in the rates of decarboxylation of malonyl-CoA or the rates of inactivation of the enzyme in the presence of acetyl-CoA can be related to k_1, the rate constant for the decarboxylation of the enzyme-bound malonyl group (Scheme I). The results of Figure 1 can be explained on the basis of eq 7, 7a, 9a, and 9b. In curves I and II (corresponding to malonyl-CoA/enzyme ratios of 500 and 1000, respectively), the decarboxylation of malonyl-CoA increases with time, but very little further increase occurs after 15 min. According to eq 7, this can happen if [E], $[B_0 - B]$, or k_1 approximates zero. Since $[B_0 - B]$, the concentration of malonyl-CoA remaining, is not zero (total malonyl-CoA in the reaction mixture in 100 μmol) and since [E], the concentration of active enzyme, is still about 30% of the control at this time (Kumar & Srinivasan, 1981), it stands to reason that the rate constant for the first decarboxylation step is encountering an energy barrier and that this step becomes rate limiting in the overall decarboxylation scheme. Species i of Scheme I (see eq 4) is inactive because it cannot efficiently catalyze the initial decarboxylation. These data suggest that the decarboxylation of the enzyme-bound malonyl group is a prerequisite for the malonyl-CoA mediated inactivation of fatty acid synthetase

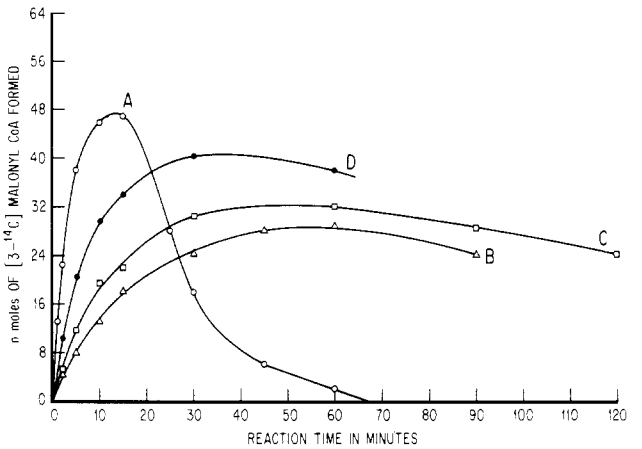


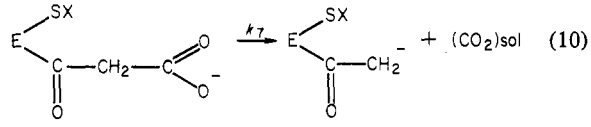
FIGURE 2: Attainment of condensation–CO_2 exchange equilibrium: effect of time-dependent inactivation and of $NADP^+$ (0.1 mM). The extent of CO_2 exchange has been plotted as a function of time. The data plotted include a few of the points of column four of Table I. Curves A, B, and C represent experimental conditions of parts A, B, and C of Table I. Curve D corresponds to 250 μg of malonyl-CoA inactivated enzyme having 35% of the control activity. Therefore, the effective concentration of active enzyme is 87.5 μg/250 μL.

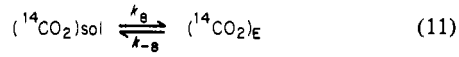
and accounts for the biphasic nature of malonyl-CoA mediated inactivation (Kumar & Srinivasan, 1979, 1981). A fast phase corresponding to rapid decarboxylation of the enzyme-bound malonyl group is followed by slow changes. In the presence of acetyl-CoA, decarboxylation of malonyl-CoA also has a fast phase (curve III, Figure 1), but in contrast to curve I, leveling of CO_2 evolution is not observed. This is consistent with a rapid, nearly monophasic, inactivation of the enzyme in the presence of acetyl- and malonyl-CoA (unpublished data).

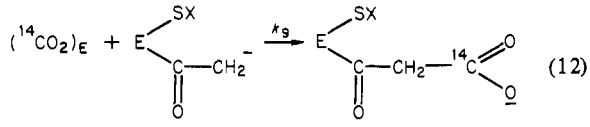
Curve IV (A, B) and curve V represent decarboxylation of malonyl-CoA in the presence of $NADP^+$ (100 μM) and acetoacetyl-*N*-acetylcysteamine (10 mM). $NADP^+$ slows down the decarboxylation of malonyl-CoA in the presence or absence of acetyl-CoA. This observation suggests that $NADP^+$ does not affect k_1, the rate constant for the decarboxylation of enzyme-bound malonyl group. The reduced level of CO_2 release must therefore represent slowing down of the condensation steps 2 and 3 of Scheme I. Our studies of the effect of $NADP^+$ on the condensation–CO_2 exchange reaction (see later) and the reported inhibition of triacetic acid lactone synthesis by $NADP^+$ (Nixon et al., 1968) are consistent with this suggestion. Therefore, the protective effect of $NADP^+$ on the inactivation of enzyme by malonyl-CoA is in slowing down the formation of species i (eq 4, Scheme I). Because of the low concentration of acetoacetyl-*N*-acetylcysteamine used [10 mM compared to its K_m value of 30 mM (Kumar et al., 1970)], its effect on the decarboxylation of malonyl-CoA is not as pronounced as $NADP^+$ (concentration/$K_D \simeq 10$). From these studies it can be concluded that (i) the inactivation of enzyme by malonyl-CoA requires the participation of the enzyme in the condensation reactions represented by reactions 2 and 3 of Scheme I, (ii) the inactive species produced do not catalyze the decarboxylation of malonyl-CoA, and (iii) $NADP^+$ and acetoacetyl-*N*-acetylcysteamine lower the rate of reactions 2 and 3 of Scheme I catalyzed by the enzyme. Therefore, $NADP^+$ must be included in the reaction mixture to get an accurate estimate of the malonyl-CoA decarboxylase activity of fatty acid synthetase. The last result is important for demonstrating the functional condensation–decarboxylation site either on the identical subunits or on the reconstituted complex. In the next section, we define conditions of true equilibrium for the condensation–CO_2 exchange reaction such that decarboxylation of malonyl-CoA quantitatively reflects

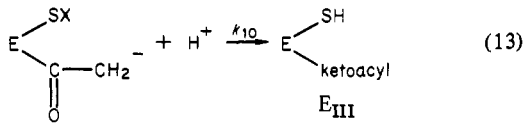
the activity for the condensation reaction.

Kinetic Analysis of the Condensation–CO_2 Exchange Reaction. Experimentally, the extent of incorporation of $^{14}CO_2$ into the malonyl moiety and its time dependence were measured (Figure 2). In order to explain these data, we will derive the conditions of equilibrium for the above reaction and predict how it can be attained and maintained. The overall condensation–CO_2 exchange reaction catalyzed by the enzyme results in the exchange of $^{14}CO_2$ from $H^{14}CO_3^-$ to the 3-carboxyl position of nonradioactive malonyl-CoA. This process can be represented by eq 10–13. In these equations, X = *n*-hexanoyl.

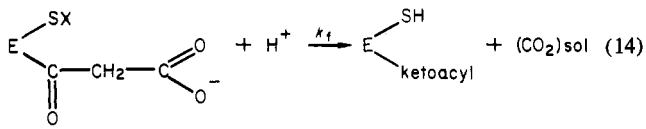




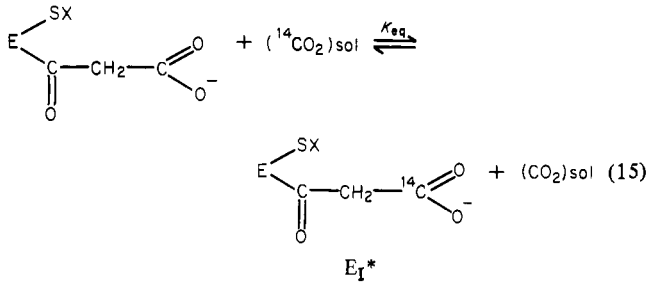




The roman numerals refer to the external or internal enzyme states. E_{II} refers to the metastable internal state. The asterisk refers to the moiety with radioactivity. The forward reaction is given by eq 10 plus eq 13.



The equilibrium reaction is a sum of reactions 10, 11, and 13 and is represented by



with a $\Delta G = 0$. It should be pointed out that E_{II} is an intermediate for both the forward and the equilibrium reactions.

Conditions for Equilibrium. The observed equilibrium is represented by eq 15. Therefore

$$K_{eq}(\text{exptl}) = \frac{[E_I^*][CO_2]\text{sol}}{[E_I][^{14}CO_2]\text{sol}} \quad (16)$$

$[E_I^*]$ and $[E_I]$ are respectively the concentrations of enzyme states E_I^* and E_I, and K_{eq}(exptl) is the experimentally obtained equilibrium constant. In practice, these states represent radioactive and nonradioactive malonyl-CoA in the medium because the reactions leading to their formation are in a reversible dynamic equilibrium and experimental conditions are chosen such that malonyl-CoA and CoA concentrations are much greater than the enzyme concentration. Equation 16 can be simplified to

$$K_{eq}(\text{exptl}) = \frac{[E_I^*]}{[E_I]}R \quad (16a)$$

where R is a constant as long as HCO_3^- concentrations are far in excess of the required amount, thereby maintaining constant specific radioactivity in solution. In all experiments reported here, this is the case. Therefore, the condition for equilibrium is that the $[E_I^*]/[E_I]$ ratio must be a constant of time. By use of distribution functions at equilibrium, it can be shown that $[E_I^*]/[E_I] = 1$ at equilibrium (the derivation is given as part of the supplementary material).

Table I: Condensation–CO_2 Exchange Reaction Catalyzed by Chicken Liver Fatty Acid Synthetase: Effect of Enzyme and $NADP^+$ Concentrations[a]

incubation time (min)	% of control activity	$^{14}CO_2$ released (nmol) (x)	$^{14}CO_2$ incorporated (nmol) (y)	$x-y$
		A		
2	>95	27.6	22.5	+5.1
5	90	47.1	38.0	+9.1
10	86	58.0	46.0	+12
15	82	65.0	47.0	+18
		B		
2	100	4.4	4.2	+0.2
5	90	7.9	8.0	−0.1
15	86	18.0	18.3	−0.3
30	82	30.0	24.0	+6.0
60	76	45.0	29.0	+16
		C		
2	90	4.5	4.9	−0.4
5	80	10.5	11.8	−1.3
10	76	18.8	19.4	−0.6
15	69	20.9	21.8	−0.9
30	53	30.8	30.5	+0.3
60	45	48.5	32.0	+16.5

[a] The data shown above were obtained as described under Experimental Procedures. The loss of activity for fatty acid synthesis was determined on aliquots taken from the condensation–CO_2 exchange reaction mixture and was related to the control activity measured on aliquots taken from a similar reaction mixture containing all the substrates except malonyl-CoA. The extent of $^{14}CO_2$ lost from the 3-carboxyl position of malonyl-CoA is given in column three whereas column four represents the incorporation of $^{14}CO_2$ into the 3-carboxyl position of malonyl-CoA. In parts A, B, and C, enzyme concentrations were 250 and 50 μg per 250 μL, respectively. In part B, the reactions were carried out in the presence of 100 μM $NADP^+$.

Values of E_I and E_I^* or unlabeled and [3-^{14}C]malonyl-CoA are given as parts A, B, and C of Table I corresponding to different sets of conditions. The concentration of E_I was obtained by using radioactive [3-^{14}C]malonyl-CoA and cold HCO_3^-, while in the case of E_I^* cold malonyl-CoA and $H^{14}CO_3^-$ were used. The values of activity for fatty acid synthesis given in Table I were measured for the enzyme equilibrated with the condensation exchange mixture by the usual procedure and were suitably corrected for the presence of extra substrates like $NADP^+$, free CoA, etc. In normal fatty acid synthesis, reversal of condensation does not occur; therefore, y of Table I will be very small and $x - y \simeq x$. In part A, $x > y$ (the rate of decarboxylation is greater than condensation exchange), and the equilibrium is never reached, whereas in part C, $x = y$ (rate of decarboxylation is equal to condensation exchange), and an apparent equilibrium is attained. The conditions used in part A are the ones generally used to measure the condensation–CO_2 exchange reaction, and it is obvious from these data that these conditions give erroneous results of the true rate of the reaction. The only dif-

ference in the reaction conditions between parts A and B is the presence of $NADP^+$ in part B of Table I. As expected, the rate of decarboxylation (x) is lower in the presence of $NADP^+$ (Table IB and Figure 1), but it is equal to the rates of exchange up to about 15 min. Therefore, studies carried out for short time periods (less than 2 min) under conditions of parts B and C quantitatively reflect equivalence of decarboxylation and exchange rates. The data of Table I, analysis of which is based on eq 16a, show that a pseudoequilibrium will be attained in two cases (250 μg of enzyme/250 μL and 0.1 mM $NADP^+$, part B; 50 μg of enzyme/250 μL, part C). However, at 250 μg of enzyme/250 μL (part A) without any $NADP^+$, only a transient maximum will be seen, which will continuously decline as the time progresses. It is possible to predict the approximate time taken for the equilibrium (or, more correctly, the pseudoequilibrium) to set in. In the case of part A, only a transient maximum is expected, and the equilibrium is never reached. The data of parts B and C predict that a pseudoequilibrium of limited duration will be attained in 30–60 min. Finally, in all cases, there will be continual loss of [3-^{14}C]malonyl-CoA formed, leading to an eventual irreversible loss of equilibrium.

The predictions of Table I are supported by the data of Figure 2 in which formation of [3-^{14}C]malonyl-CoA from cold malonyl-CoA and $H^{14}CO_3^-$ is plotted as a function of time at various enzyme concentrations. A pseudoequilibrium is discernible in parts B and C while in curve A only a transient maximum can be observed. Curve D represents the condensation–CO_2 exchange catalyzed by the enzyme partially inactivated by malonyl-CoA. Though the total enzyme concentration is 250 μg/250 μL, the concentration of active enzyme in this case is 87.5 μg/250 μL of reaction mixture. These data fall between curves A and C (corresponding to enzyme concentrations of 250 and 50 μg per 250 μL, respectively). Therefore, these results are in accord with the proposition that the malonyl-CoA inactivated species are not active in the decarboxylation and condensation reactions (reactions 1–3 of Scheme I). The effect of $NADP^+$ on the decarboxylation and the exchange reactions appears to suggest a certain degree of interaction between the condensing and β-ketoacyl reductase sites. This suggestion is supported by the observed 2–3-fold decrease in the affinity of NADPH for the enzyme in the presence of acetyl- and malonyl-CoA (Srinivasan & Kumar, 1976). The experimental evidence in support of the kinetic analysis shows that the usual conditions of the condensation–CO_2 exchange (1 mg/mL enzyme; 15-min reaction time) do not accurately reflect the activity of this reaction. Furthermore, the presence of $NADP^+$ in the reaction mixture is essential for preventing the loss of malonyl-CoA due to the decarboxylation of the enzyme-bound malonyl group followed by the deacylation of the acetyl group (Kumar, 1975). For accurate measurements of this reaction, $NADP^+$ should be included in the reaction mixture, and reaction periods should be less than 1 min. Under these conditions, decarboxylation of malonyl-CoA quantitatively reflects the activity of the condensation reaction. We are currently using these conditions to monitor the minimal decarboxylase activity of polyfunctional monomers and dimers of fatty acid synthetase by using fast-flow kinetics and appropriate indicators.

Supplementary Material Available

Kinetic analysis of eq 7 and 16a (3 pages). Ordering information is given on any current masthead page.

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