dures. Along these lines, several investigators (Harmon et al., 1980; Maturo & Hollenberg, 1978; Katzen, 1979) using different experimental approaches have proposed the existence of associated components capable of modifying the affinity of the insulin binding site. Further studies are being performed in an attempt to directly identify such an affinity regulator.

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Inactivation of Chicken Liver Fatty Acid Synthetase by Malonyl Coenzyme A. Effects of Acetyl Coenzyme A and Nicotinamide Adenine Dinucleotide Phosphate†

Suriender Kumar* and K. R. Srinivasan

ABSTRACT: Chicken liver fatty acid synthetase complex is irreversibly inactivated by one of the substrates, malonyl-CoA. Acetyl-CoA has a dual role. At concentrations less than or comparable to those of malonyl-CoA, the rate of inactivation is enhanced, whereas at acetyl-CoA/malonyl-CoA ratios greater than 2, the rate of inactivation is slowed down. $NADP^+$ at low concentrations (25 μM) affords considerable protection against malonyl-CoA mediated inactivation whereas NAD^+ even at 1.0 mM concentration has no effect. The inactivation process does not lead to the dissociation of the enzyme complex and is accompanied by subtle conformational changes as measured by circular dichroism measurements. Of all the model partial reactions, decarboxylation of malonyl-CoA and the condensation–CO_2 exchange are the only reactions which are not catalyzed by the modified species. The process of inactivation is accompanied by enhanced covalent binding of malonyl groups such that approximately 6 mol of the acyl group is bound per mol of the enzyme at complete inactivation. The available evidence suggests that the inactivation of the enzyme results from the binding of malonyl group(s) at or near the condensing site of the enzyme.

Hepatic fatty acid synthesizing enzymes from avian and mammalian sources have been isolated as homogeneous multifunctional units and in a sequential series of reactions catalyze the addition of "2C" units to the priming substrate acetyl-CoA, resulting in the formation of mainly C_{16} saturated fatty acids: acetyl-CoA + 7malonyl-CoA + 14NADPH + $14H^+$ → palmitic acid + 8CoA + $7CO_2$ + $6H_2O$ + $14NADP^+$.

A number of different approaches are being followed to study the mechanism of action of this complex enzyme. In earlier studies, it was of interest to determine if the enzyme complex of approximately 500 000 molecular weight is constituted from monofunctional enzymes like the *Escherichia*

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coli system (Vagelos et al., 1966) or from polyfunctional polypeptide subunits. We were able to show that the pigeon liver enzyme dissociates into subunits of nearly equal molecular weight and that the resulting subunits catalyze all the partial reactions of fatty acid synthesis except the condensation–CO_2 exchange (Kumar et al., 1970a,b). These data suggested that the condensing site requires the coordinated interaction of both the subunits and that therefore the two subunits could not be functionally completely identical. This is in accord with the identification of two gene loci for the two polypeptide chains of yeast enzyme (Dietlein & Schweizer, 1975) and the reported separation of the subunits of avian and mammalian enzymes by affinity chromatography (Lornitzo et al., 1974). As an alternative handle for delineating the structural complexity and for elucidating the kinetic details of the function of this enzyme, we have resorted to mild chemical perturbation of the enzyme structure. Thus, specific and irreversible inactivation of fatty acid synthetase by phenylmethanesulfonyl fluoride led to the identification of two fatty acyl-CoA deacylase functions on the pigeon liver enzyme (Kumar, 1975). During the course of these studies, we observed that, whereas acetyl-CoA slowed down phenylmethanesulfonyl fluoride mediated inactivation of pigeon liver enzyme, malonyl-CoA actually promoted it (Kumar, 1975). Since there appeared to be no evidence supporting the promotion of phenylmethanesulfonyl fluoride binding to fatty acid synthetase by malonyl-CoA, the inactivating role of the latter was suspected.

The present communication describes the irreversible inactivation of chicken liver enzyme by malonyl-CoA and the effect of acetyl-CoA, $NADP^+$, and NAD^+ on the rate of inactivation and on the moles of malonyl group bound per mole of enzyme. Physical and kinetic properties of the inactivated enzyme have been measured to determine the role of mild perturbations on the structure–activity relationship.

Experimental Procedures

Materials

Acetyl-CoA, malonyl-CoA, NADPH, $NADP^+$, β-NAD^+, and dithiothreitol were obtained from Sigma. Radioactive substrates were obtained from New England Nuclear. The purity of radioactive compounds was checked by chromatography. A small contamination of acetyl-CoA in malonyl-CoA was removed by high-voltage electrophoresis.

Methods

Preparation and Assay of Fatty Acid Synthetase Activity. Chicken liver fatty acid synthetase was purified and assayed according to procedures outlined before (Kumar, 1975).

Inactivation by Malonyl-CoA. The enzyme solution stored at −20 °C in 0.2 M potassium phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM dithiothreitol was thawed and incubated at 30 °C for 2 h. Dithiothreitol was removed by Sephadex G-25 filtration. Suitable dilutions were made, and the enzyme was added to a buffer system consisting of 0.2 M potassium phosphate and 1 mM EDTA, pH 7.0, which contained either malonyl-CoA or acetyl- and malonyl-CoA mixture. Dithiothreitol when present was added to the preincubation mixture just before the addition of enzyme to minimize acyl transfer from CoA derivatives to dithiothreitol (Stokes & Stumpf, 1974). A control sample was treated in an identical manner except that the substrates were absent. Unless otherwise mentioned, the enzyme concentration was 100 μg/mL.

During the periods of inactivation with malonyl-CoA or with acetyl- and malonyl-CoA or with $NADP^+$ and malonyl-CoA, an aliquot of the incubation mixture was added to the assay mixture, and this increased the concentration of the substrates in the assay medium. Corrections for this were applied by measuring the activity of the control enzyme under identical conditions. The activity of the control enzyme was monitored before, during, and after each set of experiments and was found to be invariant for at least 24 h.

Conditions for the Measurement of the Partial Reactions of Fatty Acid Synthesis. The conditions for all the partial reactions were essentially similar to those described by Kumar et al. (1970b). Malonyl-CoA inactivated enzyme was extensively dialyzed to remove excess substrates(s) after which the overall activity for fatty acid synthesis and activities for other partial reactions were measured. For the acyl-CoA-pantetheine transacylase assay, the acylpantetheines were isolated by using anion-exchange chromatography as described by Kumar (1975).

Decarboxylation of Malonyl-CoA. The reaction was carried out in metabolic flasks (Kontes Glass Co.) by using [1,3-^{14}C]malonyl-CoA as the substrate (0.9 μCi/μmol). The flask contained 0.2 M potassium phosphate, 1 mM EDTA, pH 7.0, and enzyme. The reaction was initiated by the addition of malonyl-CoA through a double-seal septum and terminated by injecting 100 μL of 6 N HCl. The released $^{14}CO_2$ was trapped in 0.5 mL of 1 M hyamine hydroxide contained in a polypropylene well hung off-center in the flask. The efficiency of the trapping procedure was tested by using a known quantity of $NaH^{14}CO_3$ and was found to be greater than 95%.

Circular Dichroism Measurements. Circular dichroic spectra of the native and malonyl-CoA modified enzymes were recorded on a Cary 61 spectropolarimeter. The conditions were 0.2 M potassium phosphate and 1 mM EDTA, pH 7.0. For the sake of comparison, the enzyme was also dissociated in low ionic strength buffer as described previously (Kumar et al., 1970a). All measurements were made at room temperature (25 °C) with cells of 0.1- or 1.0-cm path length and at enzyme concentration of 0.25 or 0.5 mg/mL.

Measurement of Released CoA. The enzyme at a concentration of 100 μg/mL in 0.2 M potassium phosphate and 1 mM EDTA, pH 7.0, was incubated at 30 °C with a 500 molar excess of malonyl-CoA or malonyl-CoA plus acetyl-CoA (each at 500 molar excess). At different time intervals, aliquots were withdrawn and assayed for activity for fatty acid synthesis. At the same time, 0.5-mL aliquots of the incubation mixture were acidified with 50 μL of 6 N HCl; 100 μg of the carrier fatty acid synthetase was added, and the precipitate was removed by centrifugation. The amount of CoA in the supernatant was estimated by 5,5′-dithiobis(2-nitrobenzoic acid) as described elsewhere (Kumar, 1975). Corrections for liberation of the thiol group during spontaneous hydrolysis of acyl-CoA esters were made by separate and parallel incubations lacking the enzyme.

High-Voltage Electrophoresis of Malonyl Peptides of Chicken Liver Fatty Acid Synthetase. Fatty acid synthetase (1.3 mg) in 0.2 M potassium phosphate and 1 mM EDTA, pH 7.0, was treated at room temperature (25 °C) with a 30-fold molar excess of [2-^{14}C]malonyl-CoA (about 5000 dpm/nmol), and an identical amount was treated with a 500-fold molar excess of the same substrate (about 3000 dpm/nmol). The reaction was stopped with 5% $HClO_4$ after 1-min incubation with 30-fold molar excess substrate and after 4-h incubation with 500-fold molar excess substrate. Unlabeled enzyme (2 mg) added to each of the treated enzyme preparations. The procedure for the preparation of peptic peptides and high-voltage electrophoresis of these peptides has been described before (Kumar et al., 1970b).

Table I: Effect of Acetyl-CoA on Malonyl-CoA Mediated Inactivation of Chicken Liver Fatty Acid Synthetase[a]

sets	acetyl-CoA (μM)	malonyl-CoA (μM)	molar ratio	$t_{25\%}$ (min)	$t_{50\%}$ (min)
A		100	0:500:1	8	20
	10	100	50:500:1	4	8
	50	100	250:500:1	2	5
	100	100	500:500:1	2	5
B	0	75	0:375:1	18	114
	100	75	500:375:1	7	12
	200	75	1000:375:1	28	140
C		40	0:200:1	21	150
	100	40	500:200:1	60	>200

[a] The studies were carried out at 30 °C at an enzyme concentration of 100 μg/mL. The numbers in the column under molar ratio represent the ratio of acetyl-CoA and malonyl-CoA, respectively, to the enzyme. The $t_{25\%}$ and $t_{50\%}$ represent values of time for 25% and 50% loss of activity as measured from a graph of activity remaining against time.

Results

Effect of Acetyl-CoA on Malonyl-CoA Mediated Inactivation of Fatty Acid Synthetase. In a previous publication, we showed that chicken liver fatty acid synthetase is inactivated by malonyl-CoA (Kumar & Srinivasan, 1979). The rate of inactivation was found to be biphasic and was dependent upon the ratio of the concentration of malonyl-CoA to enzyme. Because of the complexity of the inactivation process, comparative rates were presented in the form of $t_{25\%}$ and $t_{50\%}$ values which represent the time required for 25% and 50% inactivation of the enzyme complex. To further understand the role of substrates in controlling the activity of this enzyme, we studied the effect of acetyl-CoA on malonyl-CoA mediated inactivation, and the results are shown in Table I. As long as the acetyl-CoA to malonyl-CoA ratio is near 1 or less than 1, the rate of inactivation of fatty acid synthetase by malonyl-CoA is enhanced by acetyl-CoA, whereas at acetyl CoA/malonyl-CoA ratios of greater than 2, there is significant slowing down of the inactivation process. These data appear to suggest that critical sites (site) on fatty acid synthetase preferentially accept(s) malonyl-CoA, and this binding ultimately leads to inactivation of the enzyme. Acetyl-CoA can effectively compete for these sites only when its concentration increases significantly over that of malonyl-CoA.

Effect of Dithiothreitol and Coenzyme A on Inactivation of Chicken Enzyme by Malonyl-CoA. We studied the effect of dithiothreitol and CoA hoping that the enzyme-bound malonyl group might be transacylated to these compounds through enzymatic or nonenzymatic transfer and thereby lead to reactivation. The effect of dithiothreitol (Figure 1) in slowing down the inactivating process was rather striking in that at a malonyl-CoA to enzyme ratio of 1000 the $t_{25\%}$ and $t_{50\%}$ values were nearly doubled (from 4 to 7 min and from 11 to 18 min, respectively) in the presence of 1 or 10 mM dithiothreitol. Similar observations were made at a malonyl-CoA/enzyme ratio of 500, whereas at a higher malonyl-CoA to enzyme ratio (5000) the effect of dithiothreitol was not very noticeable (Figure 1). The rate of inactivation at the highest malonyl-CoA concentration is so rapid that the effect of dithiothreitol is difficult to measure. Slowing down of the rate of inactivation was not observed when 1 mM CoA replaced dithiothreitol. As reported by Stokes & Stumpf (1974), we found that the malonyl group of malonyl-CoA could be transacylated to dithiothreitol to the extent of 20–25% in 30 min at pH 7.0. Therefore, the protective effect of dithiothreitol could simply be due to enzymatic or nonenzymatic depletion of malonyl-CoA. At a higher malonyl-CoA/enzyme ratio

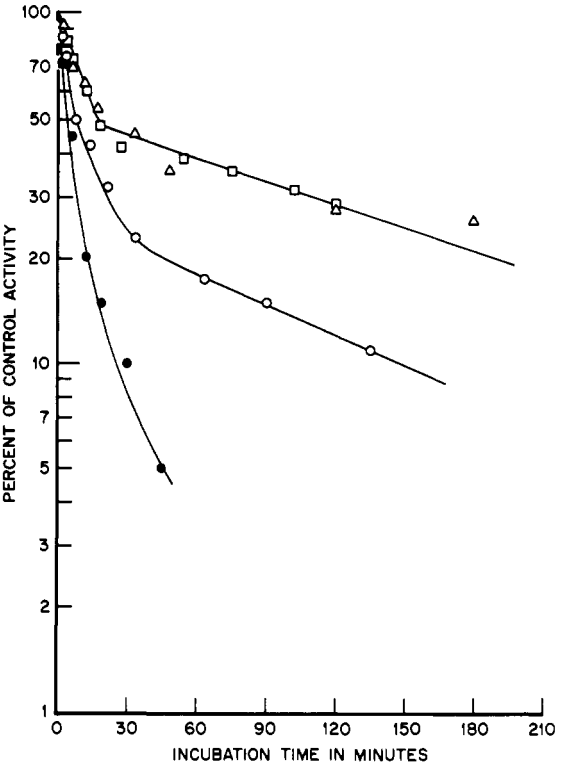

FIGURE 1: Effect of dithiothreitol on malonyl-CoA mediated inactivation of chicken liver fatty acid synthetase. Figure shows the rates of inactivation at malonyl-CoA to enzyme ratio of 1000 in the absence or presence of 1 mM CoA (○) and in the presence of 1 mM (△) or 10 mM (□) dithiothreitol. Data at malonyl-CoA to enzyme ratio of 5000 (●) are in the presence or absence of 1 or 10 mM dithiothreitol.

Table II: Effect of $NADP^+$ and β-NAD^+ on Malonyl-CoA or Malonyl- and Acetyl-CoA Mediated Inactivation of Chicken Liver Fatty Acid Synthetase[a]

effector	molar ratio of effector to enzyme	$t_{25\%}$ (min)	$t_{50\%}$ (min)
	A		
control (no effector)		2	5
$NADP^+$ (75 μM)	375:1	28	85
β-NAD^+ (500 μM)	2500:1	1–2	5
	B		
control (no effector)		4	11
$NADP^+$ (25 μM)	125:1	16	44
$NADP^+$ (75 μM)	375:1	26	70
β-NAD^+ (500 μM)	2500:1	2	8

[a] The studies were carried out at 30 °C at an enzyme concentration of 100 μg/mL. In (A), acetyl- and malonyl-CoA were present in equal concentration (100 μM) to give a molar (acyl-CoA/enzyme) ratio of 500:1. In (B), no acetyl-CoA was added, and the molar ratio of malonyl-CoA to the enzyme was 1000:1.

(5000), the amount of substrate depleted is low enough, and no apparent slowing of the rate of inactivation is observed.

Effect of $NADP^+$ and β-NAD^+. NADPH and its oxidized form $NADP^+$ are both catalytically and structurally important in their interaction with the enzyme (Kumar & Porter, 1971; Srinivasan & Kumar, 1976). However, the mechanism of interaction and the nature of the structural role that these coenzymes play are not well understood. We felt that if malonyl-CoA binding brought conformational distortion during inactivation, then $NADP^+$ and NADPH might oppose it and thereby slow down inactivation. The results of such studies are shown in Table II. $NADP^+$ (25 or 75 μM) affords considerable protection against inactivation by malonyl-CoA or a mixture of acetyl- and malonyl-CoA whereas β-NAD^+, which does not protect chicken or pigeon liver enzymes against

Table III: Specific Activities for the Model Partial Reactions of Fatty Acid Synthesis Catalyzed by the Native and Malonyl-CoA Modified Chicken Liver Fatty Acid Synthetases[a]

partial reaction	sp act. (nmol min^{-1} mg^{-1}) control	modified enzyme
acetyl-CoA-pantetheine transacylase[b]	450	440 ± 15
malonyl-CoA-pantetheine transacylase[c]	520 ± 33	500 ± 30
S-acetoacetyl-*N*-acetylcysteamine reductase[d]	2.6 ± 0.1 × 10^3	2.7 ± 0.1 × 10^3
S-β-hydroxybutyryl-*N*-acetylcysteamine reductase[e]	11.0 ± 1.0	10.2 ± 1.0
S-crotonyl-CoA reductase[f]	8.4 ± 0.6	8.0 ± 0.2
palmityl-CoA deacylase[g]	50 ± 3	51 ± 2.5
malonyl-CoA decarboxylase[h]	30	6.6

[a] All reactions were carried out 30 °C in 0.2 M potassium phosphate, pH 7.0, containing 1 mM EDTA. The native enzyme was modified with malonyl-CoA or acetyl- and malonyl-CoA. Unless otherwise mentioned, the fatty acid synthetase activity of the modified enzyme was about 25%. [b] [1-^{14}C]Acetyl-CoA, 100 μM (3800 cpm/nmol); pantetheine, 4 mM; enzyme concentration 0.5–1.0 μg/mL. [c] [2-^{14}C]Malonyl-CoA, 100 μM (6000 cpm/nmol); pantetheine, 4 mM; enzyme concentration 0.5–1.0 μg/mL. [d] NADPH, 100 μM; *S*-acetoacetyl-*N*-acetylcysteamine, 10 mM; enzyme concentration 10 μg/mL. Oxidation of NADPH at 340 nm was followed as a function to time. [e] DL-*S*-(β-Hydroxybutyryl)-*N*-acetylcysteamine, 5 mM; enzyme concentration 50–100 μg/mL. Formation of crotonyl-*N*-acetylcysteamine was followed at 270 nm. [f] NADPH, 100 μM; crotonyl-CoA, 0.4 mM; enzyme concentration 100 μg/mL. Oxidation of NADPH was measured at 340 nm. [g] [1-^{14}C]Palmitoyl-CoA, 5 μM (12 000 cpm/nmol); enzyme concentration 10 μg/mL. [h] [1,3-^{14}C$_2$]Malonyl-CoA, 50 μM (1900 cpm/nmol); enzyme concentration 9 μg/mL. Fatty acid synthetase specific activity of the modified enzyme was 23% of the control, and the reaction time was 2 min.

dissociation and inactivation at low ionic strength (Kumar & Porter, 1971; Srinivasan & Kumar, 1976), also appears to have no effect on the rate of malonyl-CoA mediated inactivation. It therefore appears that the initial distortions brought about during inactivation by malonyl-CoA are akin to those associated with the dissociation of the enzyme which is also slowed down by NADP$^+$.

Catalysis of the Partial Reactions of Fatty Acid Synthesis. The above results on the effects of NADP$^+$ indicated that the reaction of the enzyme with malonyl-CoA might involve distortions like those produced during dissociation of fatty acid synthetase to the half molecular weight subunits. In the studies reported in Table III, the enzyme was modified with malonyl-CoA till it reached 20–30% of the control fatty acid synthetase activity, after which the treated enzyme was rapidly dialyzed to remove excess substrates. The activity for fatty acid synthesis in the dialyzed samples was again measured. As seen in Table III, the activities for acetyl- and malonyl-CoA-pantetheine transacylases, *S*-β-ketoacyl-*N*-acetylcysteamine and crotonyl-CoA reductases, the dehydrase, and the palmityl-CoA deacylase were nearly identical for the native or malonyl-CoA inactivated enzyme even though the modified enzyme has only about 25% of the activity of the native enzyme for fatty acid synthesis. These studies led us to believe that no gross conformational changes are associated with malonyl-CoA mediated inhibition of the enzyme since the accessibility of the substrates to their catalytic sites is not appreciably altered. The reaction that is altered to a significant extent (5–6-fold) in the inactivated enzyme is the decarboxylation of malonyl-CoA and the condensation–$^{14}CO_2$ exchange reaction (Srinivasan & Kumar, 1981). This value corresponds well with the partial activity remaining for fatty acid synthesis

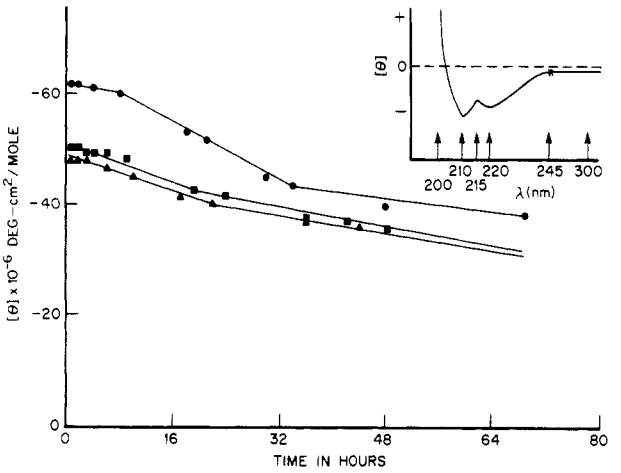

FIGURE 2: The inset shows the circular dichroic spectrum of native chicken liver fatty acid synthetase with minima at 220 and 210 nm. The figure shows the changes in 220-nm minimum with time for the native enzyme (●), for the enzyme incubated with 500-fold molar excess of malonyl-CoA (■), and for the enzyme in 5 mM Tris–35 mM glycine, pH 8.3 (▲). Other reaction conditions are described under Experimental Procedures.

(23%) (Table III). These data support our proposition that the irreversible inactivation of native structure alters the coordinates of the decarboxylation site of fatty acid synthetase complex.

Circular Dichroism Spectra of the Native and Malonyl-CoA Modified Enzymes. The circular dichroic spectrum of the native enzyme resembles a typical α helix with negative extrema at 220 and 210 nm, respectively, and a positive peak at 195 nm (Figure 2, inset). On comparing the 220-nm peak heights of the control and the enzyme modified with malonyl-CoA (500:1 molar ratio of malonyl-CoA/enzyme) or undergoing dissociation in the presence of low ionic strength buffer, the molar ellipticity of 220-nm absorption is reduced by about 20% in the modified species within 20 min after transfer to the modifying environments (Figure 2). During this time, the loss of fatty acid synthetase activity is between 40% and 45% for both types of enzymes. In the next 36 h, the absolute value of ellipticity for the native enzyme (6.1 × 10^6 deg cm^{-1} dmol^{-1}) drops by about 25%, as do the values for the modified and dissociated enzymes; however, the differences in molar ellipticity between the native and the modified enzymes are maintained. These data tend to indicate that during malonyl-CoA modification and in low ionic strength buffer, there is a subtle rapid change in the secondary and/or tertiary structure of the enzyme which is followed by slow irreversible changes.

Though the rapid changes in secondary or tertiary structures during the modification and dissociation are similar, disc electrophoresis of the modified enzyme shows that malonyl-CoA treatment does not lead to the dissociation of the enzyme (Kumar & Srinivasan, 1979). Thus, the integrity of the enzyme complex is maintained in the inactivated state.

Release of CoA and Binding of Malonyl Groups during Inactivation. The studies using circular dichroic measurements indicated an initial rapid change in the secondary and tertiary structure of enzyme when treated with malonyl-CoA. Therefore, we measured the formation of CoA as a function of loss of fatty acid synthetase activity. Treatment of the enzyme either with malonyl-CoA or with malonyl-CoA and acetyl-CoA mixtures results in rapid liberation of CoA (Figure 3). With malonyl-CoA, the extent of CoA liberated (nmol/mL) levels off after about 30 min. This leveling off corresponds to a rapid change in the circular dichroic spectrum

Table IV: Covalent Binding of [2-^{14}C]Malonyl Groups to Chicken Liver Fatty Acid Synthetase in the Presence and Absence of Acetyl-CoA and NADP$^+$[a]

sets	incubation time (min)	molar ratio of malonyl-CoA to enzyme	acetyl-CoA	NADP$^+$	% of control activity for fatty acid synthesis	mol of malonyl groups bound per mol of enzyme
A	1.5	500	−	−	90	3.5 ± 0.1
	26	500	−	−	30	4.1 ± 0.1
	44	500	−	−	17	5.7 ± 0.1
	240	500	−	−	<5	6.4 ± 0.1
B	0.8	500	+	−	87	2.6 ± 0.2
	12	500	+	−	16	4.5 ± 0.1
	120	500	+	−	<5	5.2 ± 0.3
C	1.5	500	+	+	97	2.5 ± 0.2
	15	500	+	+	84	2.9 ± 0.1
	46	500	+	+	77	3.7 ± 0.1
	99	500	+	+	63	3.8 ± 0.1
D	1.5	1000	−	+	100	3.1 ± 0.1
	30	1000	−	+	62	3.9 ± 0.1
	63	1000	−	+	50	4.8 ± 0.2

[a] Native enzyme was incubated with 500 or 1000 molar excess of malonyl-CoA in the presence or absence of acetyl-CoA or NADP$^+$. At different time intervals, activity for fatty acid synthesis was measured, and aliquots (100 μg) were precipitated with 60% $HClO_4$. Carrier fatty acid synthetase (1.0 mg) was added and the precipitate allowed to stand at 0 °C overnight. The precipitate was filtered, washed successively with 12 mL of cold 10% trichloroacetic acid and 5 mL of ethanol, solubilized in protosol, and assayed for radioactivity. The values of bound substrate were calculated by using 500 000 as the molecular weight of the enzyme. When present, the molar ratio of acetyl-CoA and/or NADP$^+$ to the enzyme was 500:1.

and to about 45% retention of enzyme activity. With acetyl- and malonyl-CoA, the amount of CoA liberated at 30 min is considerably greater than that with malonyl-CoA alone (Figure 3), and there is a further slow increase after 30 min. In this case, CoA released up to 30 min corresponds to about 30% retention of enzyme activity. These data indicate that the inactivation of enzyme complex is related to the depletion of acyl-CoA thioesters and possible binding of acyl groups to the enzyme complex.

Covalent Binding of Acyl Groups to Fatty Acid Synthetase. A series of studies were designed to probe the relationship between the covalent binding of acyl groups and the loss of enzyme activity (Table IV). In the absence of NADP$^+$, addition of 500-fold molar excess of malonyl-CoA results in progressive inactivation of the enzyme which is accompanied by increased binding of the malonyl groups (Table IV, set A). Approximately 3.5 mol of the malonyl group is bound per mol of the enzyme when the reaction is stopped after 1.5 min, which corresponds to 10% loss of activity for fatty acid synthesis. After 44-min incubation, 83% loss of activity corresponds to the binding of approximately 5.7 mol of malonyl group per mol of enzyme (Table IV, set B). At longer time intervals when the enzyme is nearly completely inactivated, 6.4 mol of the malonyl group is bound per mol of enzyme. In the presence of equimolar concentrations of acetyl-CoA, the binding of the malonyl group is reduced by approximately 1 mol/mol. Thus, at short incubation times, 2.6 mol of malonyl-CoA is bound in the presence of acetyl-CoA against 3.5 mol/mol in its absence (Table IV, set B). At longer periods, the corresponding values are about 6.4 and 5.2, respectively. These data suggest that on the average acetyl-CoA competes only for one of the malonyl binding sites of chicken liver fatty acid synthetase.

In the presence of NADP$^+$, similar differences in the number of moles of malonyl group bound per mole of the enzyme are observed in the presence and absence of acetyl-CoA (Table IV, sets B–D). When NADP$^+$ and acetyl-CoA are present (Table IV, set C), 2.5 nmol of the malonyl group is bound similar to the value obtained in Table IV (set B). Since the presence of NADP$^+$ lowers the rate of inactivation of the enzyme by malonyl-CoA, one finds that the number of moles of malonyl group bound is also less. Table IV (set D) shows

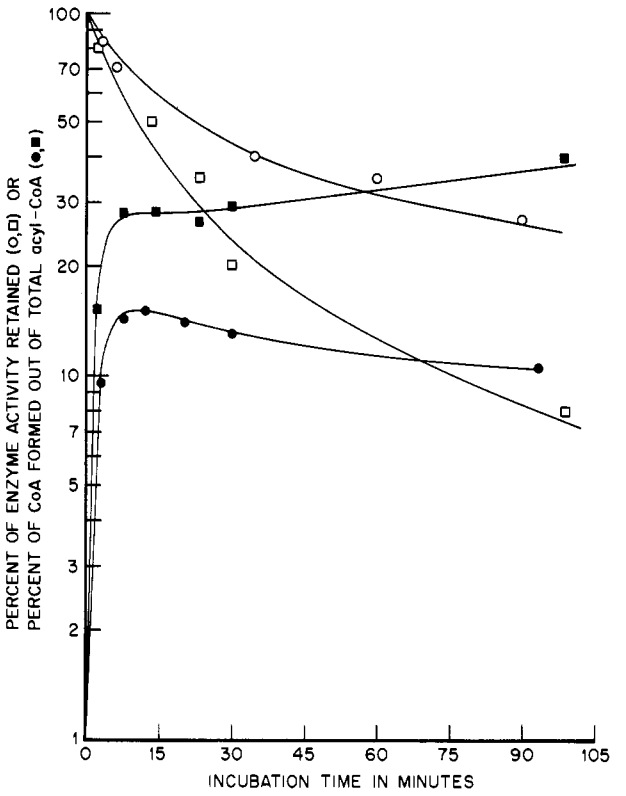

FIGURE 3: Measurements of the release of CoA and the rate of inactivation when chicken liver fatty acid synthetase is incubated with 500-fold molar excess of malonyl-CoA (○, ●) or 500-fold molar excess of acetyl- and malonyl-CoA (□, ■).

the data obtained for the binding of malonyl groups in the presence of NADP$^+$ but in the absence of acetyl-CoA. Under conditions where the enzyme is not inhibited, 3 mol of malonyl group is bound per mol of the enzyme compared with approximately 2.5 mol bound in the presence of acetyl-CoA (Table IV, set C). These data suggest that a relationship exists between the moles of malonyl group bound and the loss of activity for fatty acid synthesis.

Electrophoretic Pattern of Malonyl Peptides. High-voltage electrophoretic patterns of peptic digests of chicken liver fatty acid synthetase containing covalently bound malonyl group

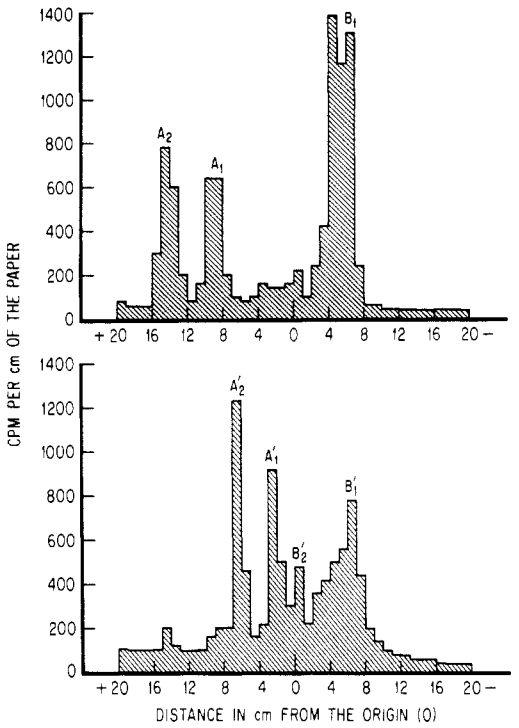

FIGURE 4: Electrophoretic patterns of [2-^{14}C]malonyl peptides obtained by peptic digestion of the chicken liver fatty acid synthetase treated with 30-fold molar excess of [2-^{14}C]malonyl-CoA (top) and 500-fold molar excess of [2-^{14}C]malonyl-CoA (bottom). Symbols B_1, A_1 and A_2 (top) refer to the peptide fragments obtained from the region of serine hydroxyl and the 4'-phosphopantetheine SH sites. Other details are given under Experimental Procedures.

are shown in Figure 4. The pattern shown at the top corresponds to malonyl groups bound at the hydroxyl loading site (B_1) and the pantetheine SH site (A_1, A_2). Similar peptic peptide patterns have been observed for many of the purified fatty acid synthetase (Qureshi et al., 1976). Figure 4 (bottom) shows the pattern of malonyl peptides under conditions where the enzyme is completely inactivated by malonyl-CoA. A significant difference is seen in two peptide maps. Malonyl-CoA inhibited enzyme gives five peptides. B'_1 (bottom) has the same mobility as B_1 (top). A new peak (B'_2) appears at the origin, and peaks A'_1 and A'_2 have lower mobility than the A_1 and A_2 peptides obtained in the absence of inactivation by malonyl-CoA. These peptide maps show qualitative differences between the sites that bind malonyl groups normally and under inactivation conditions.

Reversibility of the Binding of Malonyl Groups to Fatty Acid Synthetase and Reactivation of the Inactive Enzyme. We attempted to reactivate the inactivated enzyme by treating it with reagents which lead to thioester exchange or to the cleavage of the thioester bond. Treatment with CoA does not lead to reactivation. This is expected since CoA does not slow down the rate of inactivation due to malonyl-CoA. Dithiothreitol (10 mM) treatment of the malonyl-CoA or acetyl- and malonyl-CoA inactivated enzyme leads to partial reactivation (about 10–12%). This action could be related to the exchange of enzyme-bound acyl groups to dithiothreitol. Treatment with hydroxylamine (0.2 M) leads to optimum reactivation to the extent of about 40%. Higher concentrations of hydroxylamine (0.5 M) give somewhat lesser reactivation because of nonspecific denaturation of the enzyme caused by this reagent. From these data, it is clear that the type of linkage which is at least partially responsible for the inactivation of the enzyme is the thioester linkage which can be cleaved by hydroxylamine. The binding of malonyl groups in the presence of hydroxylamine and performic acid supports these data. As shown in Table IV, complete inactivation results in the binding of approximately 6.4 mol of malonyl group per mol of the enzyme. Treatment with CoA reduces this number by approximately 0.8 mol/mol. However, other treatments (hydroxylamine, pH 11.0, or performic acid) result in the release of approximately 3 mol of bound acyl groups per mol of the enzyme. Since neutral hydroxylamine (0.2 M) has been shown by Ruch & Vagelos (1973) to lead to thioester bond cleavage, it can be suggested that the type of linkage responsible for the formation of inactive enzyme is the thioester linkage. (These data are given as Tables V and VI; see paragraph at end of paper regarding supplementary material).

Stoichiometry of Product Formation in the Native and Partially Inactive Enzymes. The purpose of these experiments was to determine if the stoichiometry of the end product of fatty acid synthesis changes when the enzyme is modified with malonyl-CoA. Thus, the rate of product formation was compared by using acetyl-, hexanoyl-, and octanoyl-CoA as primers and comparing the rate of NADPH oxidation to the incorporation of [2-^{14}C]malonyl-CoA into fatty acids. The rates of NADPH oxidation in the presence of identical concentrations of hexanoyl-CoA and octanoyl-CoA are lower than that in the presence of acetyl-CoA. The lower rates most probably reflect lower catalytic rate constants. Furthermore, when the rates of NADPH oxidation are compared to the incorporation of [2-^{14}C]malonyl groups into products, the amount of malonyl-CoA incorporated in the presence of higher homologues of acetyl-CoA is proportionately less. A comparison between the native and modified enzyme species shows that the rates of NADPH oxidation and malonyl-CoA incorporation reflect a comparative loss of the enzymatic activity for fatty acid synthesis. These data indicate that the reaction of the enzyme with malonyl-CoA results in the process of "all or none" inactivation (Ray & Koshland, 1961); the molecules that are inactivated have lost their capacity to synthesize fatty acids, and the remaining activity is due to native unaltered molecules. (The data are given as Table VII in the supplementary material.)

Discussion

Earlier communications concerning the inhibitory effects of high concentrations of malonyl-CoA dealt mainly with the initial rate of fatty acid synthesis as measured by conventional radiometric or optical assays. Thus, Plate et al. (1968) showed that malonyl-CoA was inhibitory at high concentrations and that this inhibition was relieved by fructose 1,6-bisphosphate. Malonyl-CoA was considered to be an allosteric inhibitor and fructose 1,6-bisphosphate as the allosteric activator of fatty acid synthetase. Lynen and co-workers (1967) observed that preincubation of yeast fatty acid synthetase with malonyl-CoA led to a lag period and a slowing down of the rate of oxidation of NADPH. Since the K_m value for acetyl-CoA is higher than that for malonyl-CoA for the yeast enzyme, it was assumed that malonyl-CoA blocked the binding of acetyl-CoA to the transacylase site and that this inhibition could be partially relieved by excess acetyl-CoA. In all these studies, time-dependent irreversible loss of fatty acid synthetase activity in the presence of malonyl-CoA was not measured.

Initiation of fatty acid synthesis by animal fatty acid synthetases have been postulated to occur when the acetyl group bound at the cysteine SH site of the condensing enzyme condenses with the malonyl group bound at the pantetheine site (Kumar et al., 1972). In the absence of NADPH, the normal process of fatty acid synthesis is circumvented, and the reaction product is triacetic acid lactone, which is presumably formed as a result of condensation between the

acetoacetyl moiety bound at the cysteine SH condensing site and the malonyl group bound at the pantetheine site (Nixon et al., 1968). The formation of lactone which presumably occurs through the nucleophilic attack of the enolate group at carbon atom 5 of enzyme-bound triacetic acid on the thiol-bound carboxyl group leading to cyclization could produce distortion of the coordinates of the reactive vicinal thiol groups, cysteine SH and pantetheine SH. The details of the mechanistic steps are given elsewhere (Srinivasan & Kumar, 1981); however, the following evidence suggests that inactivation of the enzyme by malonyl-CoA results from a selective and specific process as a result of the binding of the malonyl group at or near the condensing site of the enzyme.

(i) The inactivation is not due to nonenzymatic transesterification of the enzyme by malonyl-CoA because incubation of the enzyme for extended periods with acetyl-CoA or malonyldithiothreitol or succinyl-CoA or with malonyl-CoA containing 1 mM $NADP^+$ (under conditions where there is no inactivation) does not result in the binding of acyl groups or inactivation of the enzyme (unpublished data). Dithiothreitol, in fact, protects the enzyme against malonyl-CoA mediated inactivation (Figure 1).

(ii) $NADP^+$ but not NAD^+ affords protection against inactivation by malonyl-CoA. Since $NADP^+$ has been shown to slow down the dissociation of pigeon and chicken liver enzymes (Kumar & Porter, 1971; Srinivasan & Kumar, 1976) and as dissociation results only in the loss of the condensing activity of fatty acid synthesis, inactivation by malonyl-CoA which results in the loss of condensing activity appears to be related to the binding of the malonyl group at or near the condensing site of the enzyme.

(iii) Addition of malonyl- or malonyl- plus acetyl-CoA to the enzyme results in initial release of CoA followed by much less significant changes. This initial release corresponds to 50% or 70% loss of activity in the presence of malonyl-CoA or malonyl- plus acetyl-CoA, respectively (Figure 3). These data are consistent with the selective modification of enzyme by malonyl-CoA.

(iv) The initial instantaneous decrease in molar ellipticity of the enzyme at 220 nm is followed by further slow irreversible changes (Figure 2). The changes in circular dichroic measurements are qualitatively similar to the low ionic strength mediated dissociation of the enzyme complex. Since dissociation of the enzyme results in the alteration of the coordinates of the condensing site, malonyl-CoA mediated inactivation could have similar effects. Further slow changes in the circular dichroic spectra are irreversible, which is in accord with the slow and irreversible inactivation caused by malonyl-CoA.

(v) The decrease in the rate of condensation–CO_2 exchange and the decarboxylation of malonyl-CoA follow very closely the loss of activity for fatty acid synthesis. Since lactone synthesis is equally inhibited in the malonyl-CoA modified enzyme,[1] it is apparent that all these processes are related to the changes at the condensing site of fatty acid synthetase complex as a result of binding of the malonyl group at or near the condensing site.

(vi) A comparison of the peptic peptide maps of the enzyme treated under inactivating and noninactivating conditions with malonyl-CoA shows significant differences (Figure 4). One of the new peptides that appears under inactivating conditions maps in the same region as the previously identified malonyl peptide belonging to the cysteine SH condensing site (Nixon et al., 1970). Therefore, our data are consistent with the proposition that malonyl-CoA mediated inactivation results from the covalent modification of the enzyme. Complete inactivation results in the binding of approximately 6 mol of malonyl group per mol of the enzyme. In the absence of inactivation, three malonyl binding sites are saturated. This is probably a high estimated since the enzyme-bound malonyl group can be partially decarboxylated to the acetyl group which will occupy acetyl binding sites. The nature of additional malonyl binding sites responsible for inactivation is not known. In future studies, we plan to obtain two-dimensional peptide maps of the enzyme containing covalently bound malonyl groups. New peptides appearing under inactivating conditions will be isolated, purified, and sequenced to obtain information about the site or sites responsible for inactivation.

All the available experimental evidence obtained by Kumar et al. (1970b), Kumar & Porter (1971), and Srinivasan & Kumar (1976) on the avian fatty acid synthetase complexes indicates that the 250 000 molecular weight subcomplexes do not catalyze fatty acid synthesis from acetyl- and malonyl-CoA and NADPH. This results from the inability of subunits to transfer the acetyl group from the pantetheine SH site of the prosthetic group to the cysteine SH site of the condensing enzyme. Therefore, the condensation reaction appears to require the coordinated action of both the subunits. Though the genetic studies of Dietlein & Schweizer (1975) and the recent chemical modification studies by Wieland et al. (1979) point to the nonidentical nature of the two subunits of yeast fatty acid synthetase, there is growing evidence from recent studies (Arslanian et al., 1976; Wakil & Stoops, 1980; Kumar et al., 1980a,b; Wood et al., 1978) that the half molecular weight subunits of avian and mammalian fatty acid synthetase are structurally and perhaps functionally identical. Since there is evidence that fatty acid synthetases show half the site reactivity (Clements et al., 1979), another possible explanation of the inactivation process may be suggested. Substrate concentrations which result in saturation of all the malonyl binding sites on both the subunits may alter the intersubunit interaction necessary for catalysis, thereby rendering the enzyme incapable of fatty acid synthesis. We intend to pursue these studies in the future.

Supplementary Material Available

Tables giving data on reactivation of inactive enzyme and catalytic activity of the partially inactivated species (3 pages). Ordering information is given on any current masthead page.

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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†

K. R. Srinivasan and Suriender Kumar*

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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