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IRREVERSIBLE INACTIVATION OF CHICKEN LIVER FATTY ACID SYNTHETASE BY ITS SUBSTRATES ACETYL AND MALONYL CoA

Effect of Temperature and $NADP^+$ on Fatty Acid and Triacetic Acid Lactone Synthesis

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SUMMARY

Chicken liver fatty acid synthetase is irreversibly inactivated by malonyl CoA and by acetyl and malonyl CoA. Two active forms of the enzyme existing above and below 11.5° are inactivated at different rates. Activities for fatty acid and triacetic acid lactone synthesis are lost at about the same rate and $NADP^+$ protects the enzyme against inactivation. Inactivation results from the enhanced covalent binding of malonyl groups in addition to those required for fatty acid synthesis.

Fatty acid synthetases from avian and mammalian livers are multifunctional complexes of molecular weight approximetely 500,000 and catalyse the synthesis of long chain fatty acids (C_{16} and C_{18}) when incubated with acetyl and malonyl CoA and NADPH, whereas the enzyme complex from yeast forms acyl CoA thioesters as the final products (1,2). In the absence of NADPH but in the presence of acetyl and malonyl CoA, yeast and pigeon liver enzymes lead to the formation of product identified as triacetic acid lactone (TAL) (3,4). While carrying out studies on the substrate mediated conformational changes in the enzyme, we observed that chicken liver fatty acid synthetase is irreversibly inactivated by malonyl CoA or malonyl CoA plus acetyl CoA. In a previous publication, it was reported that malonyl CoA is an allosteric inhibitor of pigeon liver fatty acid synthetase and that fructose-1,6-diphosphate and other hexose phosphates relieve this inhibition acting as allosteric activators (5). However, we found no evidence for such allosteric in-

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hibition and the increase in activity in the presence of hexose phosphates could be partly accounted for by an increase in ionic strength (6). In this communication we report the results of studies on irreversible inactivation of chicken liver fatty acid synthetase by malonyl CoA. Substrate mediated inactivation results in the loss of activities for both fatty acid and TAL synthesis. The rate of inactivation is controlled by the conformational state of the enzyme. $NADP^+$ protects the enzyme against inactivation by acetyl and malonyl CoA.

MATERIALS AND METHODS

Acetyl CoA, malonyl CoA, NADPH and $NADP^+$ were purchased from P.L. Biochemicals, and TAL (4-hydroxy-6 methyl 1-2-pyrone) was a product of the Aldrich Chemical Co., [1-^{14}C] Acetyl CoA and [2-^{14}C] malonyl CoA were obtained from New England Nuclear, Boston. The [2-^{14}C] malonyl CoA was purified by high voltage electrophoresis (1 hr at 2500 volts) on 3MM paper using pyridine-acetic acid-H_2O (1:11:289) at pH 3.9 All other chemicals were of reagent quality.

Assay for fatty acid and triacetic acid lactone synthesis

Chicken liver fatty acid synthetase was prepared and assayed for fatty acid synthesis as described by Srinivasan and Kumar (7). Assays for TAL synthesis were carried out in 0.5 ml volume and contained 0.2 M K-phosphate, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, 100 μM [2-^{14}C] malonyl CoA (2665 dpm/nmole) and 100 μM acetyl CoA. The reaction was started by adding 20-40 μg enzyme and stopped after 2 min with 100 μl of glacial acetic acid (final pH 3.0). Unlabelled TAL (20 μg) was added and the reaction mixture was placed on 0.2 x 6 cm column of Biorad-1 X 1-(chloride)anion exchange resin. TAL was eluted from this column with 10 ml of 0.2 N acetic acid. Recovery of lactone was 90-95%. Native enzyme synthesized 26 nmoles of TAL per min per mg of protein.

Measurement of the Covalent binding sites for acyl groups

Enzyme (100 μg/ml) was incubated with 100 μM acetyl and malonyl CoA. Separate and parallel incubations were made for [1-^{14}C] acetyl CoA or [2-^{14}C] malonyl CoA binding. At different time intervals, aliquots were withdrawn for the measurement of activity for fatty acid synthesis and stiochimetry of acyl binding. Enzyme (100 μg) was precipitated with an equal volume of cold 20% TCA to which 0.5 mg of unlabelled enzyme was added. The precipitates were filtered, thoroughly washed with 10% TCA, digested with 0.5 ml of the tissue solubiliser and counted for radioactivity. The calculation of acyl binding stoichiometry is based on a molecular weight of 500,000 for the dimeric enzyme.

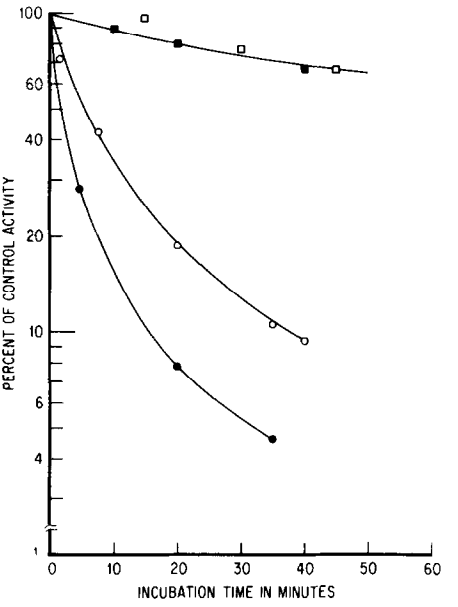

Figure 1

Rate of inactivation of chicken liver fatty acid synthetase (100 µg/ml) by 100 µM malonyl CoA (○), 100 µM malonyl CoA plus 100 µM acetyl CoA (●), 100 µM malonyl CoA plus 100 µM $NADP^+$ (□) and 100 µM malonyl CoA plus 100 µM acetyl CoA plus 100 µM $NADP^+$ (■). At different time intervals acetyl CoA (30 µM), malonyl CoA (100 µM) and NADPH (100 µM) were added to the enzyme to measure the activity for fatty acid synthesis.

RESULTS AND DISCUSSION

The loss of activity for fatty acid synthesis when 100 µg chicken liver enzyme is incubated with 100 µM malonyl CoA plus 100 µM acetyl CoA in the presence or absence of $NADP^+$ is shown as a semilogarithmic plot in Fig. 1. The substrate concentrations are those generally used in the assay of this enzyme for fatty acid and TAL synthesis. Fifty percent loss of activity occurs in about 5-6 min in the presence of malonyl CoA and in about 2 min in the presence of acetyl and malonyl CoA. At these time intervals, very little loss of activity is observed in the presence of 100 µM $NADP^+$ (Fig. 1). Removal of substrates by dialysis or further incubation of the inactive enzyme with CoA does not regenerate the active enzyme (data not shown). These data demonstrate that the inactivation of chicken liver fatty acid synthetase is not a reversible process.

Table 1

Effect of temperature on malonyl CoA and acetyl and malonyl CoA mediated inactivation of the chicken liver fatty acid synthetase

Enzyme (100 µg) was incubated with either 100 µM acetyl CoA or 100 µM malonyl CoA or with both the substrates in a total volume of 1 ml of 0.2 M K-phosphate, 1 mM EDTA, 1 mM dithiothreitol at different temperatures. Activity of the enzyme was measured by adding above concentrations of acetyl or malonyl CoA and 100 µM NADPH.

Temperature	Percent of control activity remaining after 5 min incubation with			Activity ratio Acetyl + malonyl CoA / Malonyl CoA
°C	Acetyl CoA	Malonyl CoA	Acetyl CoA + Malonyl CoA	
30	92	43	27	0.62
20	96	55	34	0.62
10	90	60	42	0.7
5	100	82	70	0.85

The rate of inactivation by malonyl CoA and malonyl and acetyl CoA depends upon temperature (Table 1). Interestingly, the ratio of loss of activity after 5 min incubation with malonyl CoA and acetyl CoA plus malonyl CoA is 0.62 at 30° and 20°, increases to 0.7 at 10° and to 0.85 at 5°. An active enzyme conformation with a single energy of activation is expected to give the same ratios of loss of activity at all temperatures, assuming the same mechanism of inactivation of the enzyme. Increase in ratio at lower temperatures suggests the presence of a second active conformation with a transition temperature around 10° or above, and presumably this conformation responds differently to inactivation by malonyl CoA and malonyl CoA plus acetyl CoA. The existence of two active conformations above and below 11.5° with activation energies of 8.4 and 22.1 Kcal/mole respectively above and below the transition temperature is shown by the data of Fig. 2 in which rate of fatty acid synthesis was determined at a number of tempertures between 40° and 0°.

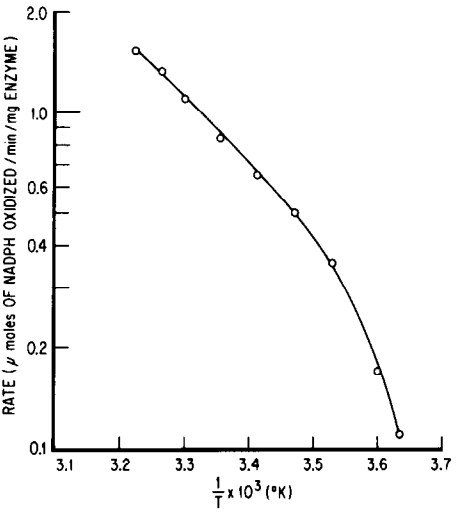

Figure 2
Arrhenius plot of the rate of fatty acid synthesis at different temperatures. Substrates (30 μM acetyl CoA, 100 μM malonyl CoA, 100 μM NADPH) and buffer (0.2M K-phosphate, 1 mM EDTA, 1 mM dithiothreitol) were incubated at different temperatures in a total volume of 1 ml and the reaction was started by adding 50 μl of 200 μg/ml enzyme.

Since modification by acetyl and malonyl CoA results in the loss of activity for fatty acid synthesis and since TAL and fatty acid synthesis reportedly use the same sites on the enzyme for the transacylation and the codensation sequence (2-4), it was of interest to see if TAL synthesis is also inhibited in the modified enzyme. The data of Table II show that the loss of activity for fatty acid and TAL synthesis follow identical patterns (Fig 1 and Table II) and $NADP^+$ shows a protective slowing down effect on both these reactions equally. These data suggest that the condensing site of the enzyme is susceptible to inactivation by acetyl and malonyl CoA. To further understand the mechanism of inactivation by the substrates, the number of covalent binding sites for acetyl and malonyl groups were determined as the enzyme is being inactivated (Table III). As shown in table I, acetyl CoA by itself does not inactivate the enzyme but it promotes the inactivating effect of malonyl CoA (Fig 1). Consistent with these observations, we find that during different

Table II

Effect of $NADP^+$ on acetyl and malonyl CoA mediated inhibition of TAL synthesis by chicken liver fatty acid synthetase

Enzyme (100 µg/ml) was incubated with 500 fold molar excess of acetyl CoA and malonyl CoA. At different time intervals, aliquots were withdrawn and assayed for TAL synthesis. $NADP^+$ was added at a concentratin of 50 µM.

Incubation time min	Percent of Control Activity	
	$-NADP^+$	$+NADP^+$
0	100	100
5	38	90
20	20	85
40	13.6	80
60	7.7	80

stages of inactivation, approximately 1.5 moles of acetyl group are bound per mole of the enzyme whereas the number of bound malonyl groups increase as the extent of inactivation of the enzyme increases (Table III). The increase is approximately two moles per mole of the enzyme.

Table III

Covalent binding of [1-^{14}C] acetyl or [2-^{14}C] malonyl groups of CoA thioesters during enzyme inactivation

Reactions were carried out in 0.2 M K-phosphate, pH 7.0, 1 mM EDTA at 30°. Other experimental details are given in the text.

Time of incubation min	Percent of control activity	moles of acyl group bound per mole of the enzyme	
		Acetyl	Malonyl
0.66	86.3	1.61 ± 0.1	2.5 ± 0.2
1.50	73.0	1.57 ± 0.1	-
2.50	58.3	1.42 ± 0.1	3.5 ± 0.1
6	29	1.49 ± 0.1	-
10	19.1	1.52 ± 0.1	4.4 ± 0.1
18	12	1.47 ± 0.1	4.5 ± 0.1

From these data, it is clear that the inactivation of fatty acid synthetase by malonyl CoA or malonyl CoA plus acetyl CoA results from the binding of the malonyl group at multiple binding sites on the enzyme. Two catalytically functional malonyl binding sites which have been identified on the enzyme are: (a) the serine hydroxyl transacylase site associated with the transfer of the malonyl group from CoA ester to the enzyme and (b) the pantetheine-SH site (8). If the subunits of chicken liver fatty acid synthetase were to be non-identical as is apparently the case with the yeast enzyme (9,10) one would expect a stoichiometry of two malonyl groups bound per 500,000 gm of enzyme. On the other hand, in structurally and functionally identical subunits, four malonyl groups would be bound per 500,000 gm protein. Under non-inactivating conditions we find a stoichiometry of approximately 2.5. A value greater than two could result from partial decarboxylation of enzyme bound malonyl group to acetyl group. If the subunits were to be structually identical, it will be possible to have more than two malonyl binding sites per enzyme dimer. Recent evidence from our (11,12) and other laboratories (13,14) suggests that the subuntis of dimeric fatty acid synthetase are strucually and perhaps functionally identical. Based on the structural identity of monomers and their synmmetrical association within the dimer, we have proposed a model for the mechanism of action of animal fatty acid synthetases (15). This model proposes that the substrates bind to one of the subunits to initiate fatty acid synthesis and that the second subunit acts as a coordinator in the cycle of reactions. The model implies functional interdependence between the catalytic and the coordinating subunit. The binding of the malonyl group to the coordinating subunit would, according to our model, destroy functional interdependence resulting in a non-functional dimer. Whether the inactivation of the dimer due to the increased binding of the malonyl group results from such a transformation or is simply a result of the binding of malonyl groups at sites other the cataytic sites will be of immense future interest. We have previously shown that $NADP^+$ and NADPH stabilise the active conformation of fatty acid synthetase (7,15). The

present studies demonstrate an interesting reciprocal relationship among substrates of the enzyme in the control of its active conformation.

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REFERENCES

1. Hsu, R. Y., Wasson, G., and Porter, J. W. (1965) J. Biol. Chem., 240, 3730-3746.
2. Lynen, F. (1967) Biochem, J., 102, 381-400.
3. Yalpani, M., Willecke, K., and Lynen, F. (1969) Eur. J. Biochem., 8 495-502.
4. Nixon, J. E., Putz, G. R., and Porter., J. W. (1968) J. Biol. Chem., 243, 5471-5478.
5. Plate, C. A., Joshi, V. C., Sedgwick, B., and Wakil, S. J. (1968). J. Biol. Chem. 243, 5439-5445.
6. Porter, J. W., Kumar, S., and Dugan, R. E. (1971) Progr. Biochem. Pharmacol 6, 1-101.
7. Srinivasan, K. R. and Kumar, S. (1976) J. Biol. Chem., 251, 5352-5360.
8. Joshi, V. C., Plate, C. A., and Wakil, S. J. (1970) J. Biol. Chem. 245, 2857-2867.
9. Kresze, G. B., Oesterhelt, D., and Lynen, F. (1976) Biochem. Biophys. Res. Commun., 69, 893-899.
10. Engeser, H., Wieland, E., and Lynen, F. (1977) FEBS Lett., 82, 139-142.
11. Kumar, S., Opas, E., Alli, P., and Lee, S. (1980) Fed. Proc. 39, 1642.
12. Kumar, S., Opas, E., and Alli, P. (1980) Biochem. Biophys. Res. Commun. 95, 1642-1649.
13. Wakil, S. J. and Stoops, J. K. (1980) Fed. Proc. 39, 1642.
14. Wood, W. I., Peterson, D. O., and Bloch, K. (1978) J. Biol. Chem. 253, 2650-2656.
15. Kumar, S. (1981), submitted to Science.
16. Kumar, S., and Porter, J. W. (1971) J. Biol. Chem., 246, 7780-7789.