Acyl Carrier Protein. VI. Purification and Properties of β-Ketoacyl Acyl Carrier Protein Synthetase*

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ABSTRACT: The enzyme β-ketoacyl acyl carrier protein synthetase, which catalyzes the formation of acetoacetyl acyl carrier protein from acetyl acyl carrier protein and malonyl acyl carrier protein, has been purified 190-fold and resolved of acetyl and malonyl transacylase activity. This enzyme is specific for thioesters of the acyl carrier protein, but longer-chain fatty acyl thioesters of the acyl carrier protein will substitute for acetyl acyl carrier protein. The K_m for acetyl acyl carrier protein is 1.9×10^{-5} M and that for malonyl acyl carrier protein is 2.5×10^{-5} M. The synthetase is inhibited by low levels of iodoacetamide and *N*-ethylmaleimide but is not affected by potassium arsenite or $CdCl_2$. The inhibition by iodoacetamide is prevented by prior incubation of the enzyme with acetyl acyl carrier protein but not with acetyl-CoA or malonyl acyl carrier protein. β-Ketoacyl acyl carrier protein synthetase activity was detected in a mammalian fatty acid synthetase preparation. Experiments in which the acyl carrier protein was acylated with acetyl and with malonyl groups indicate that identical molecules of the acyl carrier protein can accept either of these acyl groups.

An acyl carrier protein (ACP)[1] (Majerus *et al.*, 1964) has been shown to be involved in fatty acid synthesizing systems from *Clostridium kluyveri* (Alberts and Vagelos, 1961; Goldman *et al.*, 1961, 1963), *Escherichia coli* (Lennarz *et al.*, 1962; Goldman and Vagelos, 1962; Goldman *et al.*, 1963; Wakil *et al.*, 1964), avocado mesocarp (Overath and Stumpf, 1964), lettuce chloroplasts (Brooks and Stumpf, 1965), and spinach chloroplasts (Brooks and Stumpf, 1965). The acyl compounds involved in fatty acid synthesis are bound to ACP through the sulfhydryl group of a prosthetic group, 4′-phosphopantetheine (Majerus *et al.*, 1964, 1965b). Thus ACP is similar to coenzyme A which also has 4′-phosphopantetheine as part of its structure.

The reactions of fatty acid synthesis are as follows:

$$\text{acetyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{acetyl-S-ACP} + \text{CoA-SH} \quad (1)$$

$$\text{malonyl-S-CoA} + \text{ACP-SH} \rightleftharpoons \text{malonyl-S-ACP} + \text{CoA-SH} \quad (2)$$

$$\text{acetyl-S-ACP} + \text{malonyl-S-ACP} \rightleftharpoons \text{acetoacetyl-S-ACP} + CO_2 + \text{ACP-SH} \quad (3)$$

$$\text{acetoacetyl-S-ACP} + \text{TPNH} + H^+ \rightleftharpoons \text{D-(−)-β-hydroxybutyryl-S-ACP} + \text{TPN}^+ \quad (4)$$

$$\text{D-(−)-β-hydroxybutyryl-S-ACP} \rightleftharpoons \text{crotonyl-S-ACP} + H_2O \quad (5)$$

$$\text{crotonyl-S-ACP} + \text{TPNH} + H^+ \rightleftharpoons \text{butyryl-S-ACP} + \text{TPN}^+ \quad (6)$$

The purification of acetyl transacylase, malonyl transacylase, and β-ketoacyl-ACP reductase, which catalyze reactions 1, 2, and 4, respectively, in *E. coli* (Alberts *et al.*, 1964), as well as enoyl-ACP hydrase catalyzing reaction 5 (Majerus *et al.*, 1965a), have been reported previously. In addition it has been shown that acetoacetyl-S-ACP is converted to butyryl-S-ACP by extracts of *E. coli* (Goldman, 1964), indicating the presence of the enzyme catalyzing reaction 6, enoyl-ACP reductase. The reaction between acetyl-ACP and malonyl-ACP to form acetoacetyl-ACP (reaction 3) also has been demonstrated (Alberts *et al.*, 1964); however, β-ketoacyl-ACP synthetase, the enzyme that catalyzes this reaction, was not separated from malonyl transacylase activity during preliminary purification of these enzymes.

The purification and properties of β-ketoacyl-ACP synthetase are reported in this paper. This enzyme has been resolved of malonyl transacylase activity and acetyl transacylase activity. In addition, experiments are described which indicate that only a single species of ACP functions in fatty acid synthesis. A preliminary report of this work has appeared (Alberts, 1965).

Methods

Preparation of β-Ketoacyl-ACP Synthetase. Frozen cells of *E. coli* (strain B) (100 g) were suspended in 100 ml of 0.01 M triethanolamine hydrochloride, pH 7.5, containing 0.01 M 2-mercaptoethanol. The cells were

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[1] Abbreviations used in this work: ACP, acyl carrier protein; CoA, coenzyme A; TPNH, reduced triphosphopyridine nucleotide; TPN^+, oxidized triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

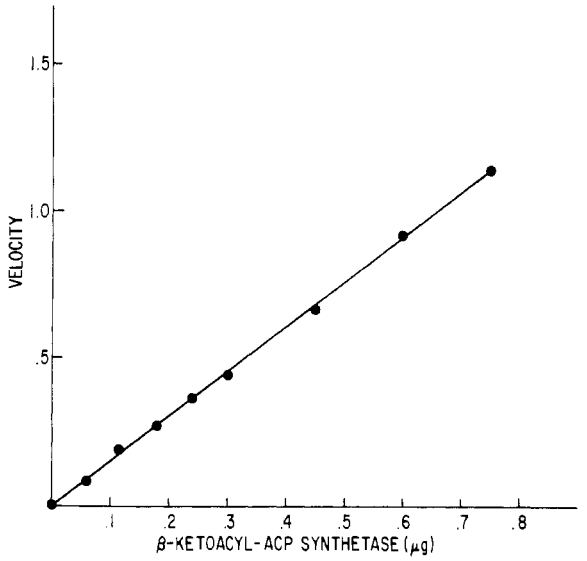

FIGURE 1: β-Ketoacyl-ACP synthetic activity as a function of protein concentration. Velocity is expressed as mμmoles of TPNH oxidized/min.

ruptured in a French pressure cell at 9000 psi, and 200 ml of the same buffer was added to the extract. The particulate matter was removed by centrifugation at 37,500 × *g* for 30 min. To the 320 ml of supernatant solution containing 8850 mg of protein was added 96 ml of 20% streptomycin sulfate with stirring. This suspension was immediately centrifuged for 15 min at 37,500 × *g* and the precipitate was discarded. The supernatant solution (380 ml) was diluted fourfold with distilled water and applied to a 3 × 60 cm DEAE-cellulose column. The column had been equilibrated with, and all subsequent solutions contained, 0.01 M potassium phosphate, pH 7.0, and 0.01 M 2-mercaptoethanol. After application of the protein solution the column was washed with 1000 ml of 0.075 M LiCl. The enzyme was eluted with a 3-l. linear gradient of LiCl between 0.075 and 0.25 M, and 200 fractions were collected. The tubes containing synthetase activity were pooled, and the solution was adjusted to 50% ammonium sulfate saturation by the addition of solid ammonium sulfate. The resulting precipitate was removed by centrifugation and discarded. The supernatant solution then was adjusted to 80% ammonium sulfate saturation. The precipitate was collected by centrifugation for 1 hr at 17,500 × *g* and dissolved in a minimal volume of 0.01 M potassium phosphate, pH 7.0, containing 0.01 M 2-mercaptoethanol. This solution (25 ml) was then applied to a Sephadex G-100 column (4.5 × 30 cm) that had previously been equilibrated with 0.01 M potassium phosphate, pH 7.0, containing 0.01 M 2-mercaptoethanol, and the column was eluted with the same buffer. The fractions containing synthetase activity were pooled, diluted twofold with distilled water, and applied to a calcium hydroxylapatite column (2 × 10 cm) that had previously been equilibrated with 0.005 M potassium phosphate, pH 6.8, containing 0.01 M 2-mercaptoethanol. This column was eluted by successive applications of 100 ml each of 0.01, 0.025, 0.050, and 0.075 M potassium phosphate, pH 7.0, containing 0.01 M 2-mercaptoethanol. β-Ketoacyl-ACP synthetase was found in the 0.075 M eluate. To concentrate the enzyme this solution was adjusted to 95% ammonium sulfate saturation by the addition of solid ammonium sulfate and the resulting precipitate was collected by centrifugation at 37,500 × *g* for 30 min. The precipitate was dissolved in a minimal volume of 0.05 M potassium phosphate containing 0.01 M 2-mercaptoethanol and stored at −20°.

Assay of β-Ketoacyl-ACP Synthetase. β-Ketoacyl-ACP synthetase was assayed by coupling it to β-ketoacyl-ACP reductase (reaction 4) and measuring the decrease in absorbancy at 340 mμ due to the oxidation of TPNH. Reaction mixtures contained 15 μmoles of potassium phosphate, pH 7.0, 8 μmoles of 2-mercaptoethanol, 0.1 μmole of EDTA, 0.015 μmole of acetyl-ACP, 0.015 μmole of malonyl-ACP, 0.02 μmole of TPNH, and 0.01 unit of β-ketoacyl-ACP reductase (Alberts *et al.*, 1964) in a volume of 0.15 ml. Reactions were started by the addition of 0.0002–0.001 unit of enzyme and absorbancy was recorded every 30 sec at 25°. Under these conditions the assay was linear for several minutes, and the formation of acetoacetyl-ACP was linear over a wide range of β-ketoacyl-ACP synthetase concentrations (Figure 1). Similar results were obtained using the assay previously described where acetoacetyl-ACP formation was measured directly (Alberts *et al.*, 1964). An enzyme unit is defined as the amount required to catalyze the oxidation of 1 μmole of TPNH/0.15 ml per min dependent upon acetyl-ACP, malonyl-ACP, and β-ketoacyl-ACP reductase.

Sulfhydryl determinations were by the method of Ellman (1959) and protein determinations by the method of Lowry *et al.* (1951). Thioesters were measured quantitatively by the hydroxamate method (Stadtman, 1957).

Materials

Acetyl-ACP and malonyl-ACP were synthesized chemically as previously described (Alberts *et al.*, 1964). Acetyl-CoA was prepared by the method of Simon and Shemin (1953) and malonyl-CoA by the method of Trams and Brady (1960). Malonyl transacylase, acetyl transacylase, and β-ketoacyl-ACP reductase were purified and assayed as previously described (Alberts *et al.*, 1964). TPNH and DPNH were purchased from P-L Laboratories; Sephadex G-100 from Pharmacia Corp.; DEAE-cellulose from Whatman; calcium hydroxylapatite from Clarkson Chemical Co.; and crystalline pig heart β-hydroxyacyl-CoA dehydrogenase from Calbiochem.

Results

Purification of β-Ketoacyl-ACP Synthetase. Utilizing the coupled assay the enzyme was purified 193-fold with an 8% recovery of activity (Table I). It has been

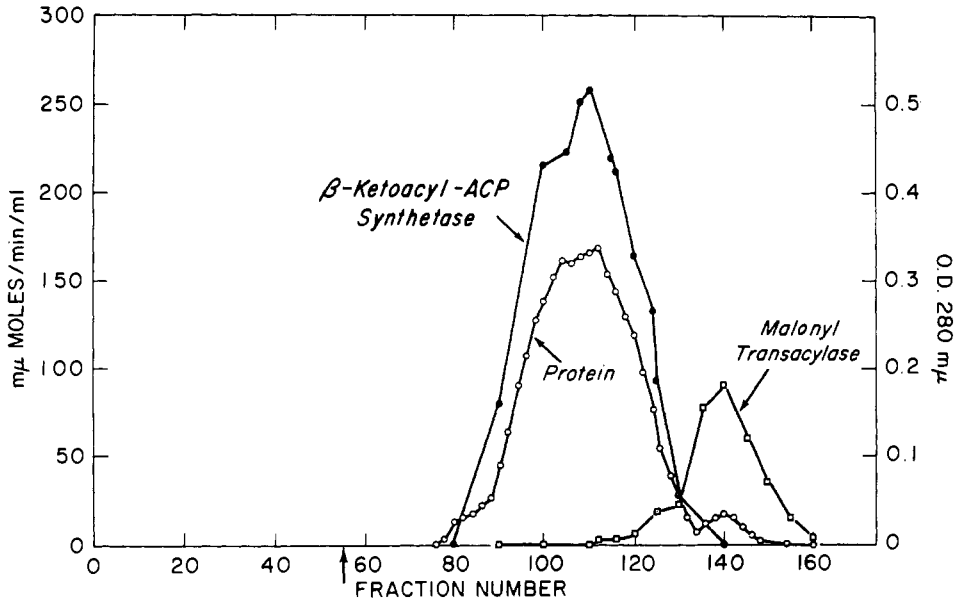

FIGURE 2: Gel filtration of β-ketoacyl-ACP synthetase. β-Ketoacyl-ACP synthetase from the hydroxylapatite step was adjusted to 95% ammonium sulfate saturation. The resulting precipitate was collected by centrifugation and dissolved in 2 ml of 0.01 M potassium phosphate, pH 7.0, containing 0.01 M 2-mercaptoethanol and applied to a 4.5 × 10 cm Sephadex G-100 column which had been equilibrated with 0.01 M potassium phosphate, pH 7.0, containing 0.01 M 2-mercaptoethanol. The protein was then eluted with the latter buffer. Fractions (170 2-ml) were collected and assayed for synthetase and malonyl transacylase as described under Methods.

TABLE I: Purification of β-Ketoacyl-ACP Synthetase.

	Specific Activity (U/mg)[a]	Recovery (%)	Purification
Extract	0.0138	100	1
Streptomycin sulfate	0.0145	100	1.05
DEAE-cellulose	0.368	61	26.6
Ammonium sulfate I	1.045	25	75.6
Sephadex G-100	1.930	21	140
Calcium hydroxylapatite	2.660	8	193

[a] One unit = 1 μmole of TPNH oxidized/0.15 ml per min.

reported previously (Alberts *et al.*, 1964) that β-ketoacyl-ACP synthetase activity did not separate from malonyl transacylase activity during partial purification of the latter enzyme, and it was found that the 193-fold purified synthetase still contained considerable malonyl transacylase activity. However, the two activities were readily resolved by subjecting the purified enzyme to a second filtration on Sephadex G-100. Figure 2 shows that this procedure yielded β-ketoacyl-ACP synthetase (tubes 90–120) virtually free of malonyl transacylase (tubes 130–160). No significant increase in enzyme specific activity was obtained by this last step. Acetyl transacylase was absent from this preparation. Following the final gel filtration the enzyme was stored as an ammonium sulfate suspension at −20°; no loss of activity was observed after 4 months under these conditions.

Requirements of the Reaction. Requirements for each of the components of the coupled assay are demonstrated in Table II. It is noted that the reaction is

TABLE II: Requirements for the β-Ketoacyl-ACP Synthetase Reaction.[a]

Omissions	Activity (mμmoles/min)
None	1.1
Acetyl-ACP	0
Malonyl-ACP	0
β-Ketoacyl-ACP Synthetase	0
β-Ketoacyl-ACP Reductase	0
2-Mercaptoethanol	0.10
EDTA	0.68

[a] The complete system contained in 0.15 ml: 15 mμmoles of acetyl-ACP, 15 mμmoles of malonyl-ACP, 8 μmoles of 2-mercaptoethanol, 0.1 μmole of EDTA, 0.0011 unit of β-ketoacyl-ACP synthetase, 0.01 unit of β-ketoacyl-ACP reductase, 20 mμmoles of TPNH, and 15 μmoles of potassium phosphate, pH 7.0. Decrease in optical density at 340 mμ was recorded every 30 sec, and the activity was calculated from the initial velocities.

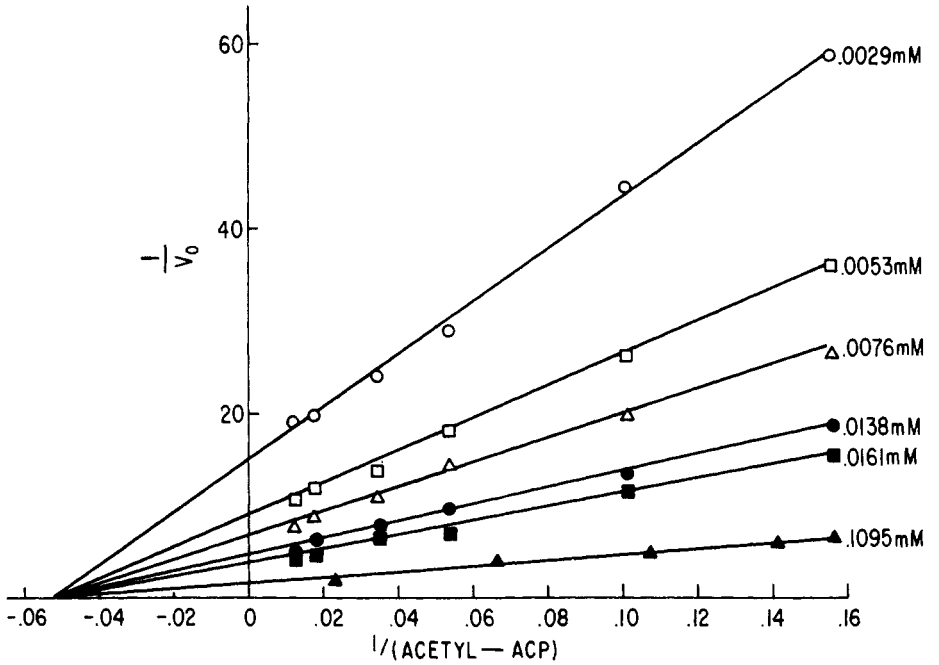

FIGURE 3: Lineweaver–Burk plots of β-ketoacyl-ACP synthetase activity as functions of acetyl-ACP concentration and a series of fixed malonyl-ACP concentrations. The other conditions were the same as those described in Methods for the standard assay. Acetyl-ACP concentration is expressed as mμmoles/ml. V_0, the initial velocity, is expressed as mμmoles of TPNH oxidized/0.15 ml min.

totally dependent upon acetyl-ACP, malonyl-ACP, β-ketoacyl-ACP synthetase, and β-ketoacyl-ACP reductase. In addition the reaction is partially dependent upon both 2-mercaptoethanol and EDTA since omission of either of these decreased the rate of the reaction. Glutathione is about 50% as effective as 2-mercaptoethanol in stimulating the reaction. The function of 2-mercaptoethanol is further delineated in Table III. Here it is seen that a relatively high concentration of 2-mercaptoethanol (5.3×10^{-2} M) is required in the assay mixture for maximal activity. 2-Mercaptoethanol at 3.3×10^{-4} M allowed only 12% of the maximal rate. However, when enzyme was subjected to prior incubation with 5×10^{-2} M 2-mercaptoethanol for 90 min and then diluted, the initial rate of the reaction was 60% of maximum even though the concentration of 2-mercaptoethanol in the assay mixture was only 3.3×10^{-4} M. Maximal reaction rate after a prior incubation with 2-mercaptoethanol was achieved only when the high concentration of 2-mercaptoethanol was also included in the assay mixture. 2-Mercaptoethanol does not affect the rate of β-ketoacyl-ACP reductase which is not a sulfhydryl enzyme (Alberts *et al.*, 1964); therefore, these experiments suggested that the effect of 2-mercaptoethanol is attributable to β-ketoacyl-ACP synthetase and indicate that it must be reduced for maximal activity.

TABLE III: Effect of Prior Incubation with 2-Mercaptoethanol on β-Ketoacyl-ACP Synthetase.[a]

2-Mercaptoethanol in Prior Incubation (M)	2-Mercaptoethanol in Assay (M)	Relative Activity
0	5.3×10^{-2}	100
0	0	8
0	3.3×10^{-4}	12
5×10^{-2} for 5 min	3.3×10^{-4}	18
5×10^{-2} for 90 min	3.3×10^{-4}	60
5×10^{-2} for 90 min	5.3×10^{-2}	102

[a] In the prior incubation, 0.150 unit of β-ketoacyl-ACP synthetase was incubated in 0.1 ml of 0.01 M potassium phosphate, pH 7.5, with 2-mercaptoethanol as indicated. At the times shown, 0.001-ml aliquots were assayed as described in Methods except that each assay contained the concentration of 2-mercaptoethanol shown.

Michaelis Constants for Acetyl-ACP and Malonyl-ACP. The initial reaction rates were determined over a range of acetyl- and malonyl-ACP concentrations. Reciprocal plots according to the method of Lineweaver and Burk (1934) are based on these data (Figures 3 and 4). From these plots the K_m value for acetyl-ACP was determined to be approximately 1.9×10^{-5} M and for malonyl-ACP to be approximately 2.5×10^{-5} M. The K_m for either substrate was not affected by the concentration of the other substrate, indicating non-

TABLE IV: Substitution of CoA Derivatives for ACP Derivatives.[a]

	System	Activity (mμmoles/min)
A	Complete	0.87
	−Malonyl-CoA	0
	−Malonyl transacylase	0.05
	−ACP	0.08
	−Acetyl-ACP	0
B	Complete	0.57
	−Acetyl-CoA	0
	−Acetyl transacylase	0
	−ACP	0.13
	−Malonyl-ACP	0

[a] A: The complete system contained in 0.15 ml: 15 μmoles of potassium phosphate, pH 7.0, 15 mμmoles of malonyl-CoA, 1.0 unit of malonyl transacylase, 10 mμmoles of ACP, 10 mμmoles of acetyl-ACP, 0.001 unit of β-ketoacyl-ACP synthetase, 8 μmoles of 2-mercaptoethanol, 0.1 μmole of EDTA, 0.01 unit of β-ketoacyl-ACP reductase, and 0.02 μmole of TPNH. TPNH oxidation was measured spectrophotometrically at 340 mμ. B: The complete system contained in 0.15 ml: 15 μmoles of potassium phosphate, pH 7.0, 15 mμmoles of acetyl-CoA, 0.92 unit of acetyl transacylase, 10 mμmoles of malonyl-ACP, 0.001 unit of β-ketoacyl-ACP synthetase, 8 μmoles of 2-mercaptoethanol, 0.1 μmole of EDTA, 0.01 unit of β-ketoacyl-ACP reductase, and 0.02 μmole of TPNH. TPNH oxidation was measured spectrophotometrically at 340 mμ.

consecutive binding of both substrates at independent binding sites (Frieden, 1957).

Substrate Specificity. β-Ketoacyl-ACP synthetase, purified according to the procedure outlined above, is essentially free of both acetyl and malonyl transacylase. Thus when acetyl-CoA and malonyl-CoA were substituted in the reaction for acetyl-ACP and malonyl-ACP, no activity was detected even at elevated substrate and enzyme concentrations. However, when acetyl-CoA or malonyl-CoA was supplemented with ACP and the appropriate transacylase, the acyl-ACP was formed (Alberts *et al.*, 1964) which then reacted in the β-ketoacyl-ACP synthetase reaction. In experiment A of Table IV it is seen that β-ketoacyl-ACP synthetase was active when malonyl-CoA, ACP, and malonyl transacylase were substituted for malonyl-ACP, and the reaction was dependent upon each of these components. Experiment B demonstrates that acetyl-CoA, ACP, and acetyl transacylase similarly substituted for acetyl-ACP. Thus the acyl-CoA compounds were active in the reaction only when they could undergo acyl transfer to ACP. It was not possible to demonstrate total dependence of the reactions upon free ACP due to the contamination of the acyl-ACP substrates by small amounts of ACP (Vagelos, unpublished experiments). A longer-chain fatty acyl-ACP, hexanoyl-ACP, could replace acetyl-ACP in the synthetase reaction. Thus the enzyme does not appear to be specific for the chain length of the acyl group, but it is specific for acyl thioesters of ACP.

Stoichiometry of the Reaction. Study of the stoichiometry of the coupled reaction suggested that the β-ketoacyl-ACP synthetase and reductase were relatively free from contamination by enoyl-ACP hydrase and enoyl-ACP reductase. Thus when varying amounts of acetyl-ACP were treated with a constant amount of malonyl-ACP, β-ketoacyl-ACP synthetase, β-ketoacyl-ACP reductase, and TPNH, and the reactions allowed to go to completion, the amount of TPNH oxidized was stoichiometric with the amount of acetyl-ACP added (Table V). Conversely, similar results were obtained when the variable substrate was malonyl-ACP. If the enzyme preparations were contaminated by enoyl-ACP hydrase alone similar results would have been obtained. However, if both enoyl-ACP hydrase and enoyl-ACP reductase were present, more than 1 equiv of TPNH would have been oxidized per mole of acyl-ACP added.

TABLE V: Stoichiometry of the β-Ketoacyl-ACP Synthetase.[a]

Acetyl-ACP (mμmoles)	Malonyl-ACP (mμmoles)	TPNH Oxidized (mμmoles)
1	10	1.01
2	10	1.95
3	10	2.74
5	10	4.40
10	1	0.98
10	2	2.02
10	3	2.85
10	5	4.68
10	10	8.56

[a] Assays were carried out as detailed in Methods except that concentrations of thioesters were varied as indicated and reactions were allowed to continue until TPNH oxidation ceased. Total TPNH oxidized is indicated.

pH Optimum. β-Ketoacyl-ACP synthetase has a broad pH optimum between pH 7 and 7.8. The rate of the reaction falls off sharply below pH 6.5 and above pH 8.0.

Effect of Sulfhydryl Inhibitors. The finding that β-ketoacyl-ACP synthetase requires 2-mercaptoethanol for maximal activity suggested that the enzyme has a functionally significant sulfhydryl group (or groups). This was confirmed by the use of sulfhydryl inhibitors. The enzyme was inhibited by low levels of inhibitors, but in order to obtain maximal inhibition by iodo-

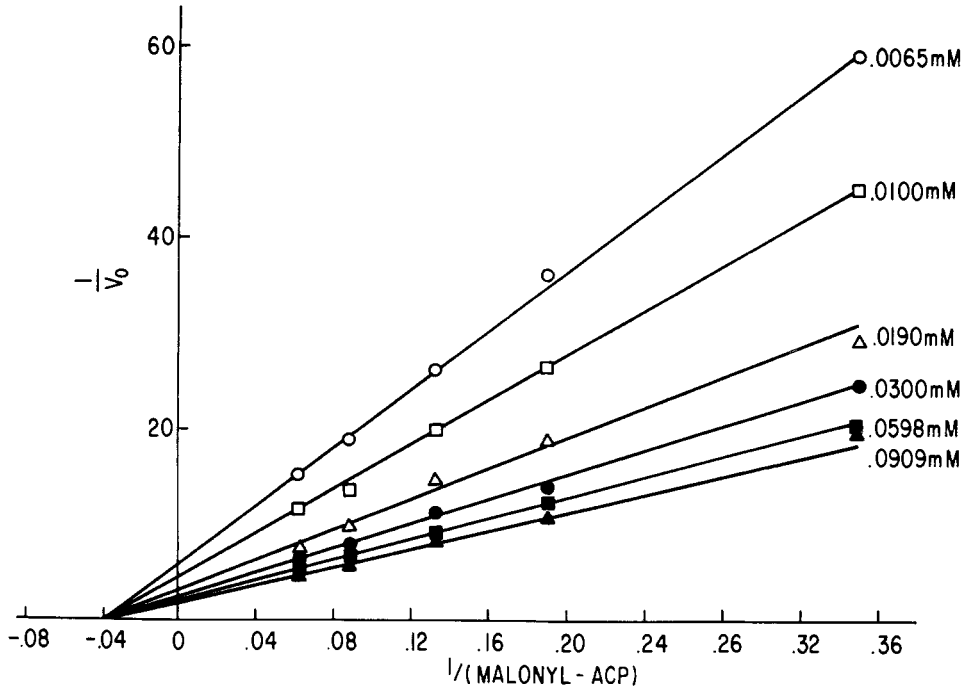

FIGURE 4: Lineweaver–Burk plots of β-ketoacyl-ACP synthetase activity as functions of malonyl-ACP concentration and a series of fixed acetyl-ACP concentrations. The other conditions were the same as those described in Methods for the standard assay. Malonyl-ACP concentration is expressed as mμmoles/ml. V_0, the initial velocity, is expressed as mμmoles of TPNH oxidized/0.15 ml per min.

acetamide or *N*-ethylmaleimide it was necessary to reduce the enzyme with 2-mercaptoethanol prior to the addition of alkylating agent (Table VI). Iodoacetamide at 5.3×10^{-5} M caused 59.7% inhibition, while 2.7×10^{-4} M caused 90.5% inhibition. Similar results were obtained with another alkylating agent, *N*-ethylmaleimide. In order to limit the effect of the inhibitors to the β-ketoacyl-ACP synthetase excess 2-mercaptoethanol was added after the incubation of enzyme with inhibitor. To be sure that iodoacetamide was completely eliminated by this procedure other experiments were performed in which excess 2-mercaptoethanol was added to the enzyme before the iodoacetamide. Enzyme treated in this way was not inhibited. In other experiments untreated enzyme was added to a preparation that had been completely inhibited by iodoacetamide and then treated with excess 2-mercaptoethanol; the added enzyme was not inhibited. These experiments show that the inhibition was due to an effect on the β-ketoacyl-ACP synthetase alone. Although alkylating agents severely inhibited the enzyme, potassium arsenite and $CdCl_2$, two inhibitors of vicinal dithiol groups, had little or no effect on the synthetase reaction (Table VI).

In order to determine whether the sulfhydryl group susceptible to alkylation by iodoacetamide is involved in the binding of either acetyl-ACP or malonyl-ACP, the ability of these substrates to protect the enzyme against inhibition by iodoacetamide was tested. As can be seen in Table VII incubation of β-ketoacyl-ACP synthetase with 6 mμmoles of acetyl-ACP for 1 or 10 min prior to addition of iodoacetamide completely prevented inhibition. Neither malonyl-ACP, the other substrate of the reaction, nor acetyl-CoA protected the enzyme. Thus such protection was specific both for the acyl and the ACP moieties of the acyl thioester. Protection against inhibition was dependent upon the concentration of acetyl-ACP as seen in Figure 5. With increasing concentrations of acetyl-ACP the per cent inhibition of the enzyme by iodoacetamide decreased. Also shown in Figure 5 is the effect of the concentration of enzyme on the protection by acetyl-ACP. It is noted that with the higher enzyme concentrations there was less protection afforded by any one concentration of acetyl-ACP. It is of interest that the lowest enzyme concentration (0.27 μl) was 50% protected by 3.1×10^{-6} M acetyl-ACP, a concentration well below the K_m value for acetyl-ACP. This fact and the observation that higher concentrations of enzyme require higher concentrations of acetyl-ACP for equivalent protection remain without adequate explanation. Further study of the interaction of acetyl-ACP and enzyme awaits the availability of substrate quantities of this enzyme.

β-Ketoacyl-ACP Synthetase in Mammalian Fatty Acid Synthetase. The mammalian enzyme system which catalyzes fatty acid synthesis, called the fatty acid synthetase, is a multienzyme complex which has not been fractionated into enzymatically active components (Martin and Vagelos, 1965). In addition mammalian ACP has not been isolated, and therefore

TABLE VI: Effect of Sulfhydryl Inhibitors on β-Ketoacyl-ACP Synthetase.[a]

Inhibitor	Concn (M)	Inhibited (%)
Iodoacetamide	5.3×10^{-5}	59.7
	1.6×10^{-4}	81.4
	2.7×10^{-4}	90.5
N-Ethylmaleimide	1.3×10^{-4}	42.4
	1.3×10^{-3}	82.6
Potassium arsenite	1×10^{-3}	8.2
$CdCl_2$	1×10^{-3}	0

[a] Synthetase (0.150 unit) was incubated in 0.1 ml of 2×10^{-2} M 2-mercaptoethanol containing 0.1 M potassium phosphate, pH 7.5, for 3 hr. For the experiments with iodoacetamide and *N*-ethylmaleimide, 0.0003-ml aliquots were incubated in a volume of 0.075 ml containing 15 μmoles of potassium phosphate, pH 7.0, and the concentration of inhibitor indicated for 5 min. The concentrations of inhibitors shown have been corrected for the 2-mercaptoethanol added with the enzyme. At the end of this time, an excess of 2-mercaptoethanol (8 μmoles) was added and the mixture was incubated for an additional 5 min to eliminate the remaining alkylating agents. This was then assayed as described in Methods and compared to a control enzyme that was treated in a similar manner except that the inhibitor was omitted. For the experiments with potassium arsenite and $CdCl_2$ these inhibitors were added directly to the reaction mixtures using enzyme that had undergone prior incubation with 2-mercaptoethanol as above. Assays were carried out as described in Methods except that 2-mercaptoethanol was omitted from both experiments and EDTA was omitted from the $CdCl_2$ experiment. The results were compared to controls assayed in the same manner but without inhibitor.

TABLE VII: Protection against Iodoacetamide Inhibition of the β-Ketoacyl-ACP Synthetase.[a]

Prior Incubation	Inhibition (%)
None	91.0
Acetyl-CoA (10 mμmoles), 10 min	100
Acetyl-ACP (6 mμmoles), 1 min	0.8
Acetyl-ACP (6 mμmoles), 10 min	0.6
Malonyl-ACP (6 mμmoles), 10 min	94.0

[a] β-Ketoacyl-ACP synthetase (0.001 unit) that had been reduced with 2-mercaptoethanol as described in Table VI was incubated with appropriate thioester for the times indicated in a volume of 0.065 ml. Then iodoacetamide was added in a final concentration of 2.7×10^{-4} M and the assay mixture was treated as in Table VI. Additional acetyl or malonyl-ACP was added so that each assay contained 10 mμmoles of each substrate.

all experiments demonstrating the presence of the individual enzymatic reactions of fatty acid synthesis in mammalian systems have utilized model compounds with the native enzyme complex (Brady, 1960; Robinson *et al.*, 1963a,b; Martin and Vagelos, 1965). To demonstrate mammalian β-ketoacyl-ACP synthetase activity, *Escherichia coli* acetyl-ACP and malonyl-ACP were used as model substrates, and the fatty acid synthetase purified from rat epididymal adipose tissue as described previously (Martin *et al.*, 1961) was used as the enzyme source. β-Ketoacyl-ACP synthetase was coupled with pig heart β-hydroxyacyl-CoA dehydrogenase which has been shown to catalyze the DPNH-dependent reduction of acetoacetyl-ACP to form L-(+)-β-hydroxybutyryl-ACP (Majerus *et al.*, 1965a). Thus the enzymatic synthesis of acetoacetyl-ACP could be followed spectrophotometrically by observing the oxidation of DPNH. β-Hydroxybutyryl-CoA dehydrogenase was utilized in these experiments because the mammalian fatty acid synthetase does not metabolize L-(+)-β-hydroxybutyryl acyl thioesters (Robinson *et al.*, 1963a,b). As noted (Table VIII) in the complete system,

TABLE VIII: β-Ketoacyl-ACP Synthetase in Rat Fatty Acid Synthetase.[a]

Omissions	DPNH Oxidized (mμmoles/min)
None	0.252
Acetyl-ACP	0.003
Malonyl-ACP	0.072
Fatty Acid Synthetase	0
β-Hydroxyacyl-CoA dehydrogenase	0

[a] The complete system contained in 0.15 ml: 15 μmoles of potassium phosphate, pH 7.0, 0.1 μmole of EDTA, 0.38 unit of rat epididymal adipose tissue fatty acid synthetase, 10 mμmoles of acetyl-ACP, 10 mμmoles of malonyl-ACP, 20 mμmoles of DPNH, and 0.09 unit of β-hydroxyacyl-CoA dehydrogenase. Decrease in optical density at 340 mμ was recorded every 30, sec, and the activity was calculated from the initial velocities.

which included acetyl-ACP, malonyl-ACP, purified fatty acid synthetase, β-hydroxyacyl-CoA dehydrogenase, and DPNH, 0.252 mμmole of DPNH was oxidized/min. That this in fact represented β-ketoacyl-ACP synthetase activity was supported by the finding that DPNH oxidation was largely dependent upon each of the assay components. The product of the mammalian β-ketoacyl-ACP synthetase was further identified as acetoacetyl-ACP in experiments where β-hydroxyacyl-CoA dehydrogenase and DPNH were omitted and

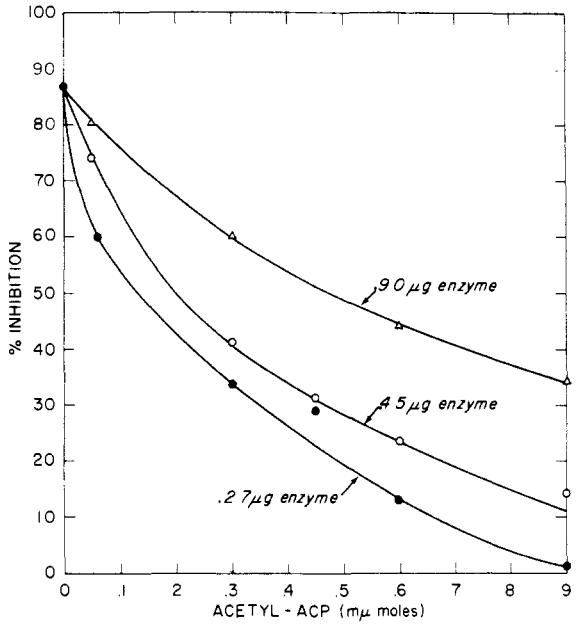

FIGURE 5: Relationship of per cent inhibition of the β-ketoacyl-ACP synthetase by iodoacetamide to concentration of acetyl-ACP at different fixed levels of synthetase. Conditions were the same as described in Table VII except for the varied levels of acetyl-ACP and synthetase (0.0011 unit/μg).

the formation of acetoacetyl thioester was measured directly (Alberts *et al.*, 1964).

Nonspecificity of ACP Accepting Acetyl or Malonyl Groups. In the first two reactions of fatty acid synthesis acetyl transacylase catalyzes the transfer of the acetyl group from CoA to ACP (reaction 1) and malonyl transacylase catalyzes the transfer of the malonyl group from CoA to ACP (reaction 2). ACP contains a single sulfhydryl group/mole of protein, and it is to this group that the acyl groups are transferred (Majerus *et al.*, 1964). Thus the acylation reactions may be followed by measuring the disappearance of the free sulfhydryl group of ACP. Previous experiments had indicated that under optimal conditions only 30–50% of ACP was converted to acetyl-ACP when ACP was treated with acetyl transacylase and an excess of acetyl-CoA. Similarly, only 50–70% of ACP was converted to malonyl-ACP when ACP was treated with malonyl transacylase and an excess of malonyl-CoA. After acylation of ACP with either acetyl or malonyl groups incubation with the alternative acyl-CoA and transacylase did not result in quantitative acylation of the ACP (Alberts, unpublished experiments). Because of these results experiments were performed to determine whether identical molecules of ACP were acylated by the acetyl and malonyl groups, respectively, or whether there were two types of ACP molecules, one which carries malonyl groups and the other acetyl groups. Table IX, A, indicates the results of an experiment in which ACP was allowed to react with acetyl-CoA and acetyl transacylase, resulting in the conversion of 44% of the ACP to acetyl-ACP. Although only 46% of the total ACP could be accounted for by direct sulfhydryl group analysis, it is clear that 56% of the ACP must have remained free, 10% having been oxidized during the prolonged transacylation reaction. The reduced free ACP was alkylated with *N*-ethylmaleimide in order to irreversibly inactivate that ACP which had not been acylated. Only 5% of the total ACP remained in the sulfhydryl form after alkylation. This mixture was then treated with neutral hydroxylamine to convert acetyl-ACP to acetyl hydroxamate and free ACP. Free ACP after hydroxylamine treatment included primarily ACP recovered from acetyl-ACP (44%), but also ACP which had been oxidized during transacylation but was now reduced (10%) and ACP which had not reacted with *N*-ethylmaleimide (5%). When the ACP mixture was allowed to react with malonyl-CoA and malonyl transacylase 42% of the total ACP was acylated. Since only 66% of the total ACP was present in the sulfhydryl form at this point, 63.6% (0.42/0.66) of the available ACP was acylated during the transacylation with malonyl-CoA. Thus the extent of reaction between malonyl-CoA and ACP which had been derived largely from acetyl-ACP was very similar to that expected between malonyl-CoA and ACP which had not undergone any selective reactions (50–70%).

The reverse experiment was also performed, acylating first with malonyl-CoA and then with acetyl-CoA (Table IX, B). The amount of ACP acylated with acetyl-CoA after ACP had undergone prior acylation with malonyl-CoA and the remaining free ACP had been alkylated with *N*-ethylmaleimide was 34%, and this represents acylation of 40.5% (0.34/0.84) of the available ACP (sulfhydryl form). This is similar to the yield of acetyl-ACP anticipated from acetyl-CoA and untreated ACP (30–50%). These experiments suggest that the ACP moieties in acetyl- and malonyl-ACP are identical.

Discussion

Purification of *E. coli* β-ketoacyl-ACP synthetase has made possible several observations which are pertinent to the elucidation of the mechanism of this reaction. Although the protein is not yet homogeneous, it has been resolved of all measurable transacylase activity, and with this preparation experiments have shown that this enzyme shows strict specificity toward acyl thioesters of ACP. Thus acetyl-CoA and malonyl-CoA cannot substitute for acetyl-ACP and malonyl-ACP in this enzymatic reaction. This is the second *E. coli* enzyme which is involved in fatty acid synthesis which shows absolute specificity toward ACP derivatives; the first one studied was enoyl-ACP hydrase (Majerus *et al.*, 1965a). Although acyl derivatives of both ACP and CoA are bound through thioester linkage to the 4′-phosphopantetheine moiety of the respective compounds, both β-ketoacyl-ACP synthetase and enoyl-ACP hydrase require the protein moiety of ACP for catalytic activity. What part of this protein

TABLE IX: Acylation of ACP with Acetyl-CoA and Malonyl-CoA.[a]

	Consecutive Treatment	Sulfhydryl Groups Remaining (moles/mole protein)	[^{14}C]Acyl-ACP Formed (moles/mole protein)
A	ACP-untreated	1.0	
	[1-^{14}C]Acetyl-CoA	0.46	0.44
	N-Ethylmaleimide	0.05	
	Hydroxylamine	0.66	0
	[2-^{14}C]Malonyl-CoA	0.27	0.42
B	ACP-untreated	1.0	
	[2-^{14}C]Malonyl-CoA	0.21	0.60
	N-Ethylmaleimide	0.05	
	Hydroxylamine	0.84	0
	[1-^{14}C]Acetyl-CoA	0.45	0.34

[a] A: ACP (0.5 μmole in 0.5 ml) was incubated in 0.02 M Tris-HCl, pH 8.2, containing 0.02 M 2-mercaptoethanol for 10 min at 30°. Following this the pH was adjusted to 1.0 with HCl to precipitate the ACP and after centrifugation the precipitate was washed until the supernatant solution was free of 2-mercaptoethanol (4 washes). The precipitate was dissolved in 0.05 M imidazole-HCl, pH 6.8, and the sulfhydryl group content and protein concentration were measured. The solution was incubated with 5 μmoles of [1-C^{14}]acetyl-CoA (0.4 μcurie/μmole) and 0.16 mg of purified acetyl transacylase for 1 hr at 30° in a final volume of 1.5 ml. The reaction was stopped by adjusting the pH to 1.0 with HCl, and after centrifugation the precipitate was washed with 0.1 N HCl until the supernatant solution was free of radioactivity. The precipitate was then dissolved in 0.05 M imidazole-HCl, pH 6.8, and aliquots were counted in a liquid scintillation counter and the sulfhydryl group content and protein concentration were again determined. The solution then was incubated with 0.5 μmole of *N*-ethylmaleimide in a volume of 0.5 ml at 30° for 2 min. The reaction mixture was then adjusted to pH 1 with HCl and after centrifugation the precipitate was washed until the supernatant solution was free of *N*-ethylmaleimide. The precipitate was then dissolved in 0.05 M Tris-HCl, pH 8.0, and the sulfhydryl group content and protein concentration were measured. Following this 100 μmoles of neutral hydroxylamine and 20 μmoles of 2-mercaptoethanol were added in a final volume of 0.7 ml, and the reaction mixture was incubated at 30° for 30 min. The pH was then adjusted to 1.0 with HCl and after centrifugation the precipitate was washed with 0.1 N HCl until the supernatant solution was free of radioactivity and 2-mercaptoethanol. The precipitate was then dissolved in 0.05 M imidazole-HCl, pH 6.8, and aliquots were measured for radioactivity, sulfhydryl group content, and protein concentration. The reaction mixture was then incubated with 8 μmoles of [2-C^{14}]malonyl-CoA (0.4 μcurie/μmole) and 0.03 mg of purified malonyl transacylase in a final volume of 0.5 ml at 30° for 30 min. The reaction was stopped by adjusting to pH 1.0 with HCl, and after centrifugation the precipitate was washed with 0.1 N HCl until the supernatant solution was free of radioactivity. The precipitate was then dissolved in 0.5 M imidazole-HCl, pH 6.8, and the sulfhydryl content, protein concentration, and radioactivity were determined. B: The same protocol was followed as in (A) except that the protein was first acylated with [2-C^{14}]-malonyl-CoA and then acylated with [1-C^{14}]acetyl-CoA after the *N*-ethylmaleimide and hydroxylamine treatments.

endows this increased reactivity remains to be investigated.

The findings that the enzyme was stimulated by 2-mercaptoethanol and was inhibited by alkylating agents such as iodoacetamide and *N*-ethylmaleimide suggest that β-ketoacyl-ACP synthetase contains a functionally significant sulfhydryl group. Protection of this sulfhydryl group against iodoacetamide inhibition by acetyl-ACP but not by acetyl-CoA further demonstrates the specificity of this enzyme and also indicates that this sulfhydryl group is in some manner involved in the acetyl-ACP site of β-ketoacyl-ACP synthetase. Since, in the course of the synthetase reaction, the acetyl group of acetyl-ACP must be transferred to the methylene carbon of malonyl-ACP, it is possible that the acetyl group is initially transacylated from ACP to the sulfhydryl group of the enzyme. Experiments to elucidate the mechanism of this reaction are in progress. It should be pointed out that involvement of a sulfhydryl group which can be protected against sulfhydryl inhibitors by acetyl-CoA had been implicated in fatty acid synthesis in several laboratories (Bressler and Wakil, 1962; Lynen, 1962; Alberts *et al.*, 1963). However, localization of this sulfhydryl group to the β-ketoacyl-ACP synthetase and demonstration that the protecting substrate is acetyl-ACP rather than acetyl-CoA awaited the present resolution of the enzyme system.

The demonstration that a mammalian fatty acid synthetase catalyzed β-ketoacyl-ACP synthetase activity when *E. coli* acetyl- and malonyl-ACP were utilized as substrates extends the list of reactions that have been shown with the mammalian enzyme system and *E. coli* acyl-ACP derivatives (Alberts *et al.*, 1964; Vagelos *et al.*, 1965). The finding that the mammalian enzyme system catalyzed the metabolism of *E. coli* ACP derivatives previously prompted the suggestion that *E. coli* ACP was acting as a model compound for mammalian ACP, which has not yet been demonstrated. The recent report by Larrabee *et al.* (1965) that the partially purified mammalian enzyme system contains protein-bound 4′-phosphopantetheine strongly supports the existence of mammalian ACP, although it has

not been ruled out that the protein-bound 4′-phosphopantetheine is not associated with other proteins of the fatty acid synthetase.

Previous study of the transacylase reactions, in which acyl groups are transferred from CoA to ACP (reactions 1 and 2), has suggested the existence of two types of ACP molecules, one specific for acetyl and the other for malonyl groups. This was based on the finding that under optimal conditions only 30–50% of the ACP could be acylated by acetyl-CoA and 50–70% by malonyl-CoA. A direct investigation of this problem now supports the theory that there is only one type of ACP which accepts either acyl group indiscriminantly. Thus when ACP was acylated maximally with acetyl-CoA and acetyl transacylase to form acetyl-ACP, the ACP recovered from this acetyl-ACP reacted to the same extent with malonyl-CoA and malonyl transacylase as ACP that had not undergone previous reaction. Other evidence that favors a single type of ACP molecule comes from experiments in which ACP was acylated chemically, so that 90–98% of the ACP was converted to acetyl-ACP, malonyl-ACP, or acetoacetyl-ACP. The acetyl-ACP and malonyl-ACP formed in this manner were stoichiometrically converted to acetoacetyl-ACP (Table V) and the chemically synthesized acetoacetyl-ACP was stoichiometrically converted to D-(−)-β-hydroxybutyryl-ACP by the respective enzymes which catalyze these reactions (Alberts *et al.*, 1964). Therefore neither the β-ketoacyl-ACP synthetase nor the β-ketoacyl-ACP reductase could differentiate between different molecules of ACP. Although all the present evidence indicates a single type of ACP, the reason that ACP cannot be totally acylated in the transacylase reactions is not yet understood.

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