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*2-MERCAPTOETHYLAMINE AND β-ALANINE AS COMPONENTS OF ACYL CARRIER PROTEIN**

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The synthesis of long-chain fatty acids from acetyl CoA and malonyl CoA by soluble extracts from *E. coli* has been studied by Vagelos and co-workers[1, 2] and by Bloch and his group.[3] These workers found that the responsible enzyme system was separable into a heat-stable and a heat-labile fraction, both of which were required for the synthesis of palmitic and *cis*-vaccenic acids. Recently, Majerus *et al.*[4] and Wakil *et al.*,[5] independently, found that the activity of the heat-stable fraction resided in a protein with a molecular weight of about 9,000. During operation of the system, the heat-stable protein accepts the acyl groups from acetyl or malonyl CoA so as to form covalently linked acetyl and malonyl derivatives which have been found to be the immediate substrates for the fatty acid synthesizing enzymes. Thus, the heat-stable protein serves as a coenzyme rather than as an enzyme. The free form acts as an acyl acceptor while the acylated form can serve as acyl donor; all of the reactions in which the fatty acyl chain is elongated, reduced, and dehydrated appear to occur while the chain is in an acyl linkage to this protein. For this reason the heat-stable protein has been designated an acyl carrier protein, abbreviated herein as "ACP."

Vagelos and his group[4] as well as we[5] have presented evidence which suggests that fatty acid biosynthesis proceeds according to the following sequential steps:

$$CH_3COSCoA + ACPSH \rightleftharpoons CH_3COSACP + CoASH \quad (1)$$

$$HOOCCH_2COSCoA + ACPSH \rightleftharpoons HOOCCH_2COSACP + CoASH \quad (2)$$

$$CH_3COSACP + HOOCCH_2COSACP \rightarrow CH_3COCH_2COSACP + ACPSH + CO_2 \quad (3)$$

$$CH_3COCH_2COSACP + TPNH + H^+ \rightleftharpoons CH_3CHOHCH_2COSACP + TPN^+ \quad (4)$$

$$CH_3CHOHCH_2COSACP \rightleftharpoons CH_3CH{=}CHCOSACP + H_2O \quad (5)$$

$$CH_3CH{=}CHCOSACP + TPNH + H^+ \rightarrow CH_3CH_2CH_2COSACP + TPN^+. \quad (6)$$

The butyryl ACP formed in step 6 can then be elongated by malonyl ACP in a reaction analogous to step 3, to form β-ketohexanoyl ACP which is reduced by TPNH to form the saturated acyl ACP, etc. Thus, repetition of steps 3–6 would ultimately yield palmityl ACP which is then hydrolyzed by a specific deacylase to palmitic acid and ACP. This proposed reaction sequence was based on the following observations: (1) in the presence of the appropriate enzyme, acetyl ACP and malonyl ACP condense to form acetoacetyl ACP; (2) acetoacetyl ACP is reduced by TPNH to yield butyryl ACP; (3) crotonyl ACP can be hydrated in the absence of TPNH to β-hydroxybutyryl ACP, reduced in the presence of TPNH to butyryl ACP and incorporated in the presence of TPNH and malonyl ACP into palmitate or *cis*-vaccenate;[6] (4) C^{14}-labeled butyryl ACP and octanoyl ACP (prepared chemically) were incorporated into long-chain fatty acids in the presence of TPNH and malonyl ACP; (5) in the absence of the "β-hydroxy C_{10} dehydrase,"[7] β-hydroxydecanoyl, β-hydroxylauryl, and β-hydroxymyristyl ACP were isolated;[5] and (6) the product of fatty acid synthesis when stoichiometric amounts of ACP were added, has been identified as palmityl ACP.[5, 6]

The amino acid composition of ACP was reported recently by Majerus *et al.*[4] Of the 89 residues in the molecule, one was reported to be cysteine. Because one mole of thiol group per molecule was titratable with *p*-mercuribenzoate and the thiol group disappeared after enzymatic acylation, these workers concluded that the acyl group forms a thioester bond with the thiol group of the apparent single cysteine of the molecule. We have determined the amino acid composition of six different preparations of ACP and have obtained results in essential agreement with those of Majerus *et al.*,[4] with two significant exceptions. First, the "half-cystine" content of ACP preparations varied from 0.09 to 0.47 residues per mole. Those preparations with the lowest half-cystine content had a higher specific activity than preparations with the highest half-cystine content. Second, all ACP preparations contained one residue each of 2-mercaptoethylamine and β-alanine. In contrast to the variable half-cystine content of different preparations, stoichiometric amounts of β-alanine and 2-mercaptoethylamine were always observed.

When peptic digests of enzymically prepared acetoacetyl-C^{14}-ACP[5] were fractionated by ion-exchange chromatography, a radioactive peptide was isolated which contained the acetoacetate-C^{14}, 9 amino acid residues, and stoichiometric amounts of 2-mercaptoethylamine and β-alanine. Although the exact nature of the linkages among acetoacetate, 2-mercaptoethylamine, and β-alanine remain to be established, it appears that each is present in covalent linkage in ACP. These findings offer strong support to the view that acylation of ACP involves formation of a thioester with the single thiol group of 2-mercaptoethylamine.

Preparation of Acyl Carrier Protein (ACP).—The acyl carrier protein was assayed enzymatically by measuring the amount of C^{14}-acetyl CoA converted to long-chain fatty acids in the presence of TPNH, malonyl CoA, and the enzyme fractions previously designated[5] as E_{II}, E_{III}, and E_{IV}. A unit of enzyme activity was defined as that which catalyzes the conversion of one mμmole of acetyl CoA to fatty acids in 60 min; specific activity was defined as units per mg protein.

The acyl carrier protein was prepared from *E. coli* cells by the following procedure: two kg of wet frozen *E. coli* were suspended in 5 l of 0.01 *M* potassium phosphate pH 7.4 and the mixture maintained at 90° for 15 min, with constant stirring. The suspension was cooled immediately, centrifuged at 12,000 × *g* for 10 min, and the precipitate was discarded. Sufficient 5 *N* H_2SO_4 was added to the supernatant solution so as to give a final concentration of 0.1 *N* H_2SO_4 while the mixture was maintained at 5°. The precipitate which formed settled rapidly to the bottom of the flask. The supernatant solution was removed with the aid of a siphon, and the precipitate was resuspended in 4 l of 0.1 *N* H_2SO_4. After the precipitate had again settled, the supernatant solution was removed by siphoning; the precipitate was washed three additional times with the same volume of 0.1 *N* H_2SO_4. The washed precipitate was collected by centrifugation, suspended in 200 ml of H_2O, and neutralized with 2 *N* KOH. The solution was dialyzed overnight against 0.01 *M* potassium phosphate, pH 7.4, containing 5 × 10^{-3} *M* 2-mercaptoethanol. At this stage, 2–4 gm of protein were obtained which exhibited an activity of 4–10 mμmoles acetyl CoA converted to fatty acids per hour per mg of protein. The dialyzed protein preparation was adsorbed on a DEAE-cellulose column (5 × 40 cm) which was then washed with 2–3 l of a solution containing 0.01 *M* potassium phosphate (pH 7.4), 5 × 10^{-3} *M* 2-mercaptoethanol, and 0.25 *M* NaCl until no more UV-absorbing material was eluted. The active component was then eluted with 0.33 *M* NaCl, 0.01 *M* phosphate (pH 7.4), and 5 × 10^{-3} *M* mercaptoethanol, and the fractions containing ACP were lyophilized. The solid material was dissolved in a minimal amount of H_2O, dialyzed overnight against 0.01 *M* phosphate buffer (pH 7.4), and filtered through Sephadex G-75 (4 × 40 cm) in 0.05 *M* potassium phosphate (pH 7.4). The ACP thus obtained had a specific activity of 70–100.

Properties of ACP.—Preparations of ACP of highest specific activity were colorless to the eye, but at 275 mμ exhibited a molar extinction coefficient of 1.8 × 10^3. Analyses for ribose and organic phosphate revealed the presence of 0.1 and 0.4 moles per mole of ACP, respectively. The essential absence of phosphate, ribose, and of any component with specific absorption at 260 mμ suggested that purine nucleotides, such as AMP, are not present in ACP. Although microbiological assays[8] for pantothenic acid were negative, it is not certain that pantoic acid is absent from ACP. The inability to detect pantoic acid by microbiological procedure[8] might have been due to the lack of enzymic hydrolysis of ACP by the pigeon liver enzymes which are used to liberate pantothenic acid from pantotheine or CoA.

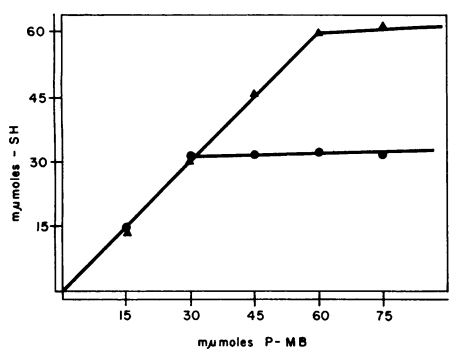

FIG. 1.—Titration of ACP with *p*-mercuribenzoate (*p*-MB). A sample of acyl carrier protein was reduced with 0.5% sodium amalgam at pH 6–7, precipitated in 0.1 *N* HCl and redissolved in 0.01 *M* potassium phosphate pH 7.4 under helium. Thirty and sixty mμmoles, respectively, of the reduced ACP were titrated with *p*-mercuribenzoate (*p*-MB) with a simultaneous measurement of optical density-at 252 mμ until no further increase in absorbancy was observed. Assuming the same extinction coefficient for the mercuri-ACP complex as that reported by Boyer[10] for other thiol groups, it was possible to calculate the —SH content of ACP as shown above.

Preparations of ACP with the highest specific activity were homogeneous as judged by ultracentrifugation and by electrophoresis on gelatinized cellulose acetate strips at pH 8.9. The average molecular weight of ACP was estimated to be 9,400 by sedimentation equilibrium analysis.[9]

To determine the sulfhydryl content of ACP it was first reduced with 0.5 per cent sodium amalgam and then titrated with *p*-mercuribenzoate. A typical titration is shown in Figure 1. These results as well as those reported by Majerus *et al.*[4] indicate the presence of one —SH group per mole of ACP.

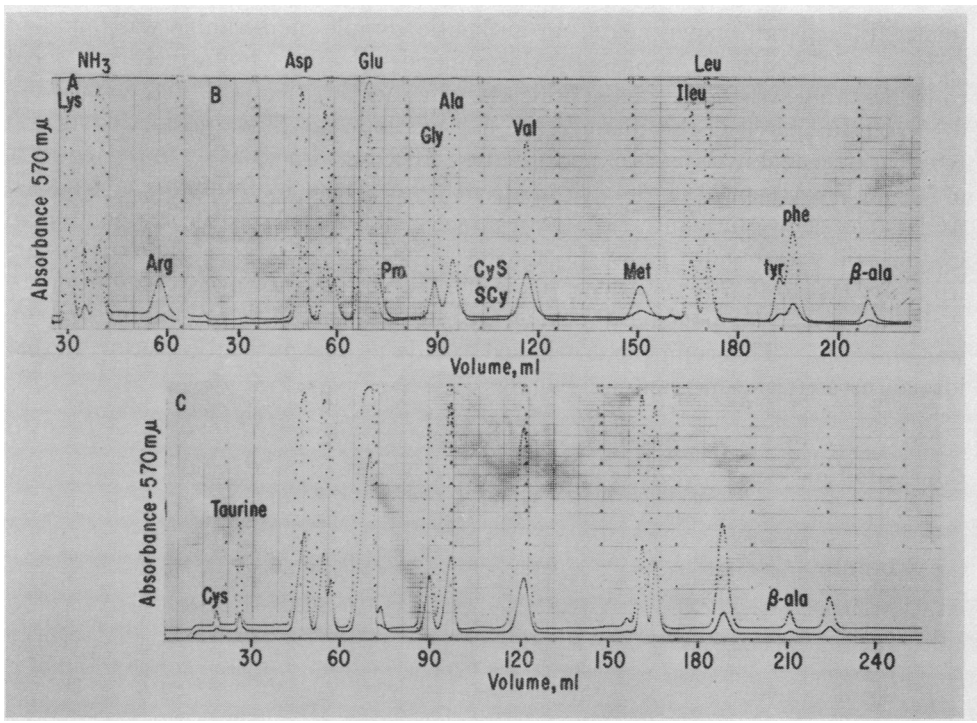

FIG. 2.—(*A*) Basic amino acids from 0.5 mg of ACP developed on a 5.5 × 0.9-cm column Dowex 50 × 8 (300–400 mesh). Flow rate = 45 ml per hour. (*B*) Acidic, neutral, and aromatic amino acids from 0.5 mg of ACP developed on a 45 × 0.9-cm column Dowex 50 × 8 (300–400 mesh). Flow rate = 45 ml/hr. Proline was not resolved from glutamic acid because of the high glutamic content of the hydrolysate. (*C*) Acidic, neutral, and aromatic amino acids from 0.6 mg ACP developed in a 48 × 0.9 cm-column of Dowex 50 × 8 spherical resin. Flow rate = 68 ml/hr.

Amino Acid Composition.—Two to five mg (0.2–0.5 μmoles) of ACP were hydrolyzed, *in vacuo*, at 110° for 24 hr with 6 *N* HCl according to the procedures of Moore and Stein.[11] The HCl was then removed by rotary evaporation at reduced pressure. The dried hydrolysate was dissolved in a carefully measured volume of water, and aliquots were analyzed chromatographically with an automatic amino acid analyzer (model 120B, Spinco, Inc., Palo Alto, California). A chromatographic pattern, typical of those obtained, is shown in Figure 2. It is noteworthy that ninhydrin-reactive material was not observed at the point of emergence of cystine. However, a ninhydrin-reactive component was found which emerged 24 min (27.2 ml) after phenylalanine. This position does not correspond to any of the conventional α-amino acids of pro-

TABLE 1

AMINO ACID COMPOSITION OF ACYL CARRIER PROTEIN

	Residues/Mole	
	Non-oxidized	Oxidized
Lysine	4	—
Histidine	1	—
Arginine	1	—
Aspartic acid	10	12
Threonine	6	6
Serine	3	3
Glutamic acid	20	20
Proline	1	1
Glycine	4	4
Alanine	8	8
Valine	7	7
Methionine	1	—
Isoleucine	6	6
Leucine	6	6
Tyrosine	1	—
Phenylalanine	2	2
β-alanine	1	1
Mercaptoethylamine*	—	1
Cysteine†	—	0.1

* Measured as taurine.
† Measured as cysteic acid.

teins. In separate experiments, an authentic sample of β-alanine was found to emerge at this position. Accordingly, this component has been tentatively considered to be β-alanine. Because of the difficulty of measuring "half-cystine" in acid hydrolysates prepared in this manner, the half-cystine content was estimated as cysteic acid after acid hydrolysis of performate-oxidized ACP according to the methods of Moore.[12] A typical chromatographic pattern of acid hydrolysates of oxidized ACP is also shown in Figure 2. It can be seen that a small peak corresponding to cysteic acid was found. In six different preparations the cysteic acid content varied from 0.09 to 0.47 residues per mole (cf. Table 2). However, in addition to β-alanine, a new component, which emerged after 21 min at a volume of 23.8 ml, was observed. This component behaved exactly as does authentic taurine, the oxidation product of 2-mercaptoethylamine.

TABLE 2

COMPARISON OF VARIOUS ACP COMPOSITIONS WITH VARIATION OF CYSTEINE CONTENT AND SPECIFIC ACTIVITY IN SOME ACP PREPARATIONS

Amino acids	Residues/Mole in Preparation 1	2	3	4	5	6
Leucine	6.0	6.0	6.0	6.0	6.0	6.0
Glycine	4.3	3.8	4.0	4.3	4.4	5.2
Alanine	7.6	7.6	7.5	8.1	7.7	8.0
β-Alanine	1.0	1.0	1.1	0.8	0.9	1.0
Mercaptoethylamine*	1.0	1.2	1.0	1.0	0.9	1.0
Cysteine†	0.09	0.14	0.13	0.09	0.09	0.47
Specific activity	57	80	70	80	80	36

* Measured as taurine.
† Measured as cysteic acid.

The amino acid compositions of ACP and ACP oxidized by performate are listed in Table 1. With the exception of the cysteine, 2-mercaptoethylamine, and β-alanine content, the compositions obtained from these analyses are in good agreement with those of Majerus *et al.*[4] On the other hand, it is clear that the most pure preparations of ACP, which possess the highest specific activity, are devoid of cysteine, cystine, and their oxidation products. The only component containing a thiol group in acid hydrolysates is 2-mercaptoethylamine, which was identified as its oxidation product, taurine. Furthermore, since taurine and β-alanine were present in stoichiometric amounts when compared to the remaining amino acid constituents, it is highly likely that these compounds are an integral part of ACP.

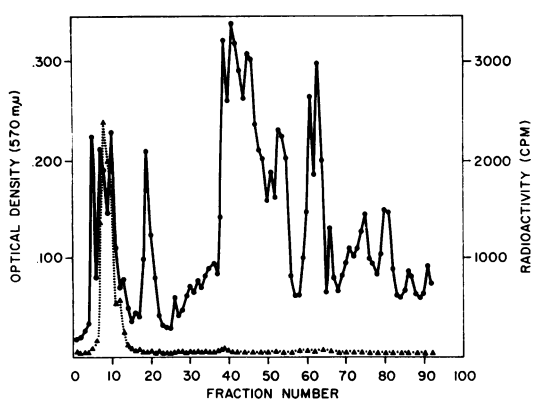

FIG. 3.—Chromatography of peptides in a peptic hydrolysate of acetoacetyl-C^{14} ACP. The circles represent the peptides as detected by the ninhydrin procedure, and the triangles represent the radioactivity in cpm. See text for experimental details.

Fractionation of Peptic Hydrolysates of Acetoacetyl-C^{14} ACP.—Acetoacetyl-C^{14} ACP was prepared by the following procedure: 10 mg ACP was added to a reaction mixture containing 500 μmoles potassium phosphate, pH 7.4, 40 μmoles 2-

mercaptoethanol, 2 μmoles acetyl-1-C^{14} CoA (10^7 cpm), 6 μmoles malonyl CoA, 2 mg of the previously described[5] enzyme fraction E_{II}, and water to a final volume of 6 ml. The reaction mixture was incubated for 15 min at 38° and was then filtered through Sephadex G-75 column (4 × 40 cm) using 0.05 *M* potassium phosphate, pH 7.4. The acetoacetyl-C^{14} ACP was recovered from the filtrate by lyophilization. Fifty mg of acetoacetyl-C^{14} ACP were hydrolyzed with pepsin as follows: the acetoacetyl-C^{14} ACP was dissolved in 2.5 ml of water and adjusted to pH 3.5 with dilute HCl. One half mg of pepsin (3 × crystallized, Worthington Biochem. Corp.) was added and the mixture was allowed to stand at 40° for 16 hr. The solution was then applied to a 0.9 × 140-cm column of Dowex 50, X 2 (200–300 mesh) which had been equilibrated with 0.1 *N* pyridine acetate buffer, pH 3.1. The column was developed with two separate linear gradients[13] at 50°. The first gradient was formed from 100 ml of 0.1 *N* pyridine acetate buffer, pH 3.1, in the first chamber and 100 ml of 0.2 *N* pyridine acetate buffer, pH 3.1, in the second chamber. After 90 ml of gradient were consumed, the chambers were emptied, and a new gradient was formed with 200 ml of 0.2 *N* pyridine acetate buffer, pH 3.1, in the first chamber and 200 ml of 2 *N* pyridine acetate, pH 5.0, in the second chamber. After 270 ml of this gradient had been consumed, the column was developed with 180 ml of 2 *N* pyridine acetate, pH 5. The flow rate was maintained at 45 ml per hr, and 6-ml fractions were collected. Aliquots were scanned for radioactivity or treated with alkali[14] before development with ninhydrin. The resultant pattern is shown in Figure 3. A portion of the radioactive peak was separated by ionophoresis-chromatography as described by Kimmel *et al.*,[15] except that pyridine-butanol-acetic acid-water (100:150:30:120 v/v) was used as solvent for the chromatography. Radioautography and development with ninhydrin indicated that only radioactive peptides (one major and four minor) were present in the sample. The amino acid composition of the major radioactive peak is presented in Table 3.

The residual pyridine salt was removed by passing a solution of the peptide through a column of Sephadex G-25 equilibrated with water. Almost all of the radioactivity was associated with the major peptic-peptide which will be referred to as acetoacetyl-C^{14}-peptic-peptide. Solutions of the latter exhibited no significant absorption over the range 240–300 mμ, indicating the absence of tyrosine, phenylalanine, tryptophan as well as such other absorbing compounds as purines or pyrimidines.

Conversion of the Acetoacetyl-C^{14} Peptic-Peptide to Fatty Acids.—When acetoacetyl-C^{14}-peptic-peptide was incubated with malonyl CoA, ACP, TPNH, and the usual three enzyme fractions, significant amounts of radioactivity were recovered in long-chain fatty acids. A sample of acetoacetyl-C^{14} peptic peptide (3,000 cpm) was incubated with 30 μmoles potassium phosphate, pH 7.0, 94 mμmoles malonyl CoA, 15 mμmoles ACP, 100 mμmoles TPNH, 200 mμmoles 2-mercaptoethanol, 140 μg E_{II}, 120 μg E_{III}, and 50 μg E_{IV}, and water to a final volume of 0.5 ml. After

TABLE 3

AMINO ACID COMPOSITION OF ACETOACETYL-C^{14}-PEPTIC-PEPTIDE

Amino acid	Number of residues/mole
Aspartic acid	3
Threonine	1
Serine	1
Glutamic acid	0.5
Glycine	1
Alanine	1
Leucine	2
β-Alanine	1
Mercaptoethylamine*	1

* Measured as taurine.

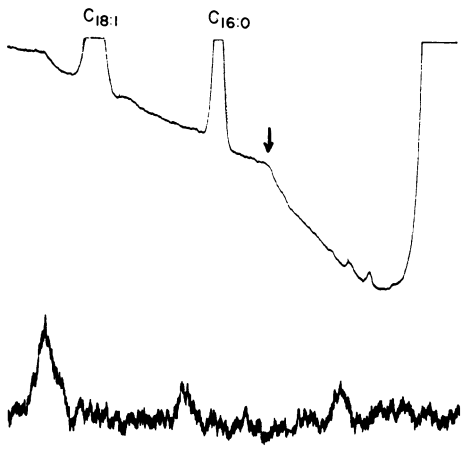

FIG. 4.—Gas-liquid chromatographic analysis of the methyl esters of fatty acids synthesized from acetoacetyl-C^{14} peptic-peptide. A sample containing 600 cpm of C^{14}-labeled methyl esters (palmitate and *cis*-vaccenate were added as carrier, cf. text) was injected into a gas-liquid chromatograph equipped with diethyleneglycol succinate column and attached to a Nuclear-Chicago proportional detector with a recorder. Immediately after injection of the sample the temperature was increased linearly from 110 to 180° and then held at 180° where indicated by the arrow. The upper tracing is from the mass detector, and lower tracing is from the radioactivity detector. The obvious lag between mass and the corresponding radioactivity peak was due to the fact that the two detectors were operating consecutively. Out of the 600 cpm of C^{14} methyl esters injected, 400 cpm were recovered in the *cis*-vaccenate peak, 100 cpm in the palmitate peak, and 100 cpm in an unidentified short chain ester.

30 min at 38°, carrier palmitic and *cis*-vaccenic acids were added and the total mixture was hydrolyzed with NaOH. Fatty acids were extracted with pentane, methylated with diazomethane, and analyzed on a gas-liquid chromatogram attached to a Nuclear-Chicago proportional detector. As shown in Figure 4, methyl palmitate and *cis*-vaccenate exhibited radioactivity indicating about 10 per cent conversion of the acetoacetyl-C^{14} peptide to these long-chain fatty acids. In a parallel experiment with undegraded acetoacetyl-C^{14} ACP incorporation of radioactivity in the long-chain fatty acids occurred to the same extent as the peptide derivative.

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