Kinetic studies of the fatty acid synthetase multienzyme complex from *Euglena gracilis* variety *bacillaris*

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A fatty acid synthetase multienzyme complex was purified from *Euglena gracilis* variety *bacillaris*. The fatty acid synthetase activity is specifically inhibited by antibodies against *Escherichia coli* acyl-carrier protein. The *Euglena* enzyme system requires both NADPH and NADH for maximal activity. An analysis was done of the steady-state kinetics of the reaction catalysed by the fatty acid synthetase multienzyme complex. Initial-velocity studies were done in which the concentrations of the following pairs of substrates were varied: malonyl-CoA and acetyl-CoA, NADPH and acetyl-CoA, malonyl-CoA and NADPH. In all three cases patterns of the Ping Pong type were obtained. Product-inhibition studies were done with NADP⁺ and CoA. NADP⁺ is a competitive inhibitor with respect to NADPH, and uncompetitive with respect to malonyl-CoA and acetyl-CoA. CoA is uncompetitive with respect to NADPH and competitive with respect to malonyl-CoA and acetyl-CoA. When the concentrations of acetyl-CoA and malonyl-CoA were varied over a wide range, mutual competitive substrate inhibition was observed. When the fatty acid synthetase was incubated with radiolabelled acetyl-CoA or malonyl-CoA, labelled acyl-enzyme was isolated. The results are consistent with the idea that fatty acid synthesis proceeds by a multisite substituted-enzyme mechanism involving Ping Pong reactions at the following enzyme sites: acetyl transacylase, malonyl transacylase, β -oxo acyl-enzyme synthetase and fatty acyl transacylase.

Fatty acid synthetase (FAS) enzyme systems have been isolated from numerous sources, and a few representatives have been extensively characterized. In plants and in some bacteria, e.g. *Escherichia coli*, the reactions of fatty acid synthesis are catalysed by a group of non-aggregated enzymes; in advanced prokaryotes, fungi and animals, the reactions are catalysed by multienzyme complexes. The synthesis of fatty acids from malonyl-CoA is accomplished by a series of sequential reactions. In addition to malonyl-CoA, a 'primer' (often acetyl-CoA) and a reductant (NADPH and, sometimes, NADH) are also required. The overall fatty acid synthesis reaction involves

substrate binding and reactions at several active sites. A carrier, 4'-phosphopantetheine, serves to shuttle reaction intermediates from site to site. The steady-state kinetics of fatty acid synthesis by pigeon liver FAS have been analysed by Cleland's (1973) method for deriving rate equations for multisite Ping Pong reactions. The results supported a mechanism in which partial reactions at the acetyl transacylase, malonyl transacylase and β -oxo acyl synthetase sites are Ping Pong in nature (Katiyar *et al.*, 1975; Katiyar & Porter, 1975).

Euglena gracilis, an organism which adapts readily to heterotrophic or photoauxotrophic growth conditions, has two 'de novo' FAS systems (Delo *et al.*, 1971). In dark-grown achloroplastic cells, fatty acid synthesis is catalysed by a large multienzyme complex. *Euglena* grown in the light contain a non-aggregated system of separate enzymes in addition to the multienzyme complex (Ernst-Fonberg, 1973; Ernst-Fonberg *et al.*, 1974). The isolation of the *Euglena* FAS multienzyme complex

Abbreviations used: FAS, fatty acid synthetase; ACP, acyl-carrier protein.

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and an analysis of the steady-state kinetics of the reaction catalysed are described here.

Experimental

Materials

CoA, acetyl-CoA and malonyl-CoA were obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.). Radiolabelled substrates, [1- 14 C]acetyl-CoA, [3 H]acetyl-CoA and [2- 14 C]malonyl-CoA, were from New England Nuclear (Boston, MA, U.S.A.). NADPH, NADP $^+$, NADH, Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid) and calcium phosphate gel Type I were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were reagent grade.

Fatty acid synthetase preparation

Euglena gracilis variety *bacillaris* were grown in the dark for 6–7 days, harvested, and stored as described by Ernst-Fonberg (1973). Cultures were checked microscopically for contamination at the time of harvest.

Purification of the enzyme was done at 4°C. Protein was determined by the method of Bradford (1976) or by amino acid analysis of the enzyme after hydrolysis for 24 h at 105°C *in vacuo* (Walsh & Brown, 1962; Moore & Stein, 1963). Corrections were not made for the loss of tryptophan and cysteine.

Thawed cells were suspended in 2 vol. of 0.01 M-Tes (pH 7.0)/0.002 M-dithiothreitol/2.2 M-glycerol (buffer A) and broken by sonication or by passage through a Parr Nitrogen Bomb. The broken cell suspension was centrifuged at 100 000 g for 1 h. The supernatant was collected, and the pellet was washed with buffer A and centrifuged a second time. The supernatants were combined, diluted 10-fold with 0.001 M-potassium phosphate buffer (pH 7.0)/0.002 M-dithiothreitol/2.2 M-glycerol and applied to calcium phosphate gel (3–5 mg of protein/ml of gel), which had been washed three times in the same buffer. The gel was washed three times with 0.05 M-potassium phosphate buffer (pH 7.0)/0.002 M-dithiothreitol/2.2 M-glycerol. Each wash consisted of 2–2.5 vol. of buffer/vol. of gel. FAS activity was then eluted by washing twice with 0.30 M-potassium phosphate buffer (pH 7.0)/0.002 M-dithiothreitol/2.2 M-glycerol (1.5–2 vol./vol. of gel). The eluate was adjusted to 45% saturation with (NH $_4$) $_2$ SO $_4$. After 30 min, the precipitated protein was collected by centrifugation (48 000 g for 20 min), dissolved in buffer A, and dialysed against 200 vol. of buffer A. The dialysed material was centrifuged (48 000 g for 20 min), and the clarified solution was applied to a Bio-Gel A-15m column (2.5 cm $\times$ 32 cm) equilibrated and eluted with buffer

A. Fractions comprising the coinciding peaks of protein and FAS activity were pooled.

Enzyme purified through calcium phosphate gel and (NH $_4$) $_2$ SO $_4$ concentration was used in kinetics experiments. Immediately on isolation, the protein solution was divided into 50 μ l batches and stored at –46°C. After thawing, enzyme activity was stable at 0.5°C for 1–1.5 h. Only freshly thawed enzyme was used to obtain kinetic data.

Fatty acid synthesis assays

The enzyme-catalysed incorporation of radioactivity from [2- 14 C]malonyl-CoA into long-chain fatty acids was measured (Delo *et al.*, 1971). The reaction mixture (0.5 ml total volume) contained 30 μ M-acetyl-CoA, 60 μ M-[2- 14 C]malonyl-CoA (0.5 μ Ci/ μ mol), 0.80 mM-NADPH, 2 mM-dithiothreitol and 100 mM-Tes, pH 7.0. Reactions were started with enzyme and incubated at 35°C for 10 min unless specified otherwise. After saponification, fatty acids were extracted with 2 $\times$ 5 ml of pentane. The pentane extracts were poured into liquid-scintillation counting vials and the pentane was evaporated over a warm water bath. The radio-labelled fatty acids were dissolved in 5 ml of toluene containing 4.0 g of 2,5-diphenyloxazole/litre, and samples were counted for radioactivity at 94% efficiency.

For steady-state kinetic analysis, malonyl-CoA-dependent oxidation of NADPH was measured. Reactions were carried out in a Cary model 118C spectrophotometer equipped with a jacketed cell holder. Temperature was maintained at 35°C by a Haake circulating bath, and reaction temperature was measured with a Markson model 5650 digital thermometer with a YSI model 729 thermistor probe. Assay volume was 0.80 ml and contained, in addition to substrates and enzyme as indicated, 100 mM-Tes buffer, pH 7.0, and 2 mM-dithiothreitol. Purity of acetyl-CoA and malonyl-CoA was checked by t.l.c. in three systems. Concentrations were determined by measuring the A_{260} (ϵ 15 400). NADPH solutions were freshly prepared, and concentrations were determined from the A_{340} (ϵ 6200).

Assay components, except NADPH and enzyme, were assembled and incubated for 7 min at 35°C. NADPH (in a volume of 20 μ l) was added, and within 1 min, the reaction was initiated by the addition of 10–20 μ l of FAS solution (30–40 μ g of protein). The specific activity is expressed in units of nmol of NADPH oxidized/min per mg of protein. Protein used in all kinetic studies was measured by the Bradford procedure, which gives an inflated (by a factor of about 8) estimate of protein with *Euglena* FAS. Thus the velocity units in kinetics experiments are lower than the specific activity reported in the purification.

Analysis of kinetic data

Kinetic data were analysed by the Direct Linear method (Cornish-Bowden & Eisenthal, 1974, 1978; Eisenthal & Cornish-Bowden, 1974). For comparisons between different experimental conditions (for example, in initial-velocity studies, between different concentrations of 'fixed' substrate, or in product-inhibition studies, between different concentrations of inhibitor), a median test was used to determine how well separated the Direct Linear best estimates were. Data were also analysed graphically by using the three linear transformations of the Michaelis-Menten equation. Results obtained by the four methods were similar. Numerical values used are the Direct Linear best estimates. For illustrative purposes, Hanes plots, s/v versus s , in which ordinate intercepts are estimates of $(K_m/V)^{app}$ and slopes are estimates of $(1/V^{app})$, are used in most cases. Analyses were done with the aid of programs written by Ignacy Fonberg (Jonesboro, TN, U.S.A.) for use on a Wang 600 programmable calculator.

Acyl-enzyme intermediates

FAS (0.3 mg in 0.45 ml) was incubated at 0.5°C with 40 μ l of 1.0 mM-[1-¹⁴C]acetyl-CoA (10 μ Ci/ μ mol) or with 40 μ l of 1.0 mM-[2-¹⁴C]malonyl-CoA (5 μ Ci/ μ mol). After 4 min, the protein was precipitated with 1.0 ml of cold 10% (w/v) trichloroacetic acid. The precipitated protein was collected by centrifugation and washed with 2 ml portions of cold 5% trichloroacetic acid till the wash was free of [¹⁴C]acyl-CoA (about five washes). The protein was dissolved in 1 M-NaOH, and a portion was neutralized and then mixed with Bray's (1960) solution and counted for radioactivity. A similar experiment was done to compare the amount of [¹⁴C]malonate bound to protein in the presence and absence of [³H]acetyl-CoA. Enzyme was incubated with [2-¹⁴C]malonyl-CoA alone or with a combination of [³H]acetyl-CoA and [2-¹⁴C]malonyl-CoA.

Form of the product of FAS

The proportions of free and esterified fatty acids

produced by the FAS reaction were determined by measuring the amount of radioactivity from [¹⁴C]-malonyl-CoA incorporated into fatty acids extracted before and after saponification. After 2 min at 35°C, the enzyme reaction was stopped by addition of 0.1 ml of 12 M-HCl. Free fatty acids were extracted with 3 $\times$ 5 ml of pentane. Next, 0.2 ml of 45% (w/v) KOH was added to each reaction mixture, and tubes were placed in a 100°C bath for 30 min. After addition of 0.4 ml of 12 M-HCl, fatty acids were extracted with 3 $\times$ 5 ml of pentane. Pentane extracts were handled as usual.

Results

Enzyme preparation

Purification of FAS is summarized in Table 1. The final specific activity of the enzyme was comparable with those reported for some other fatty acid synthetases (e.g., Smith & Abraham, 1970; Kinsella *et al.*, 1975; Buckner & Kolattukudy, 1976). At -20°C to -46°C the enzyme was stable, showing no loss of activity over several months.

Gel-filtration chromatography, the last step in the isolation, gave a single major peak of protein with constant specific activity across the six fractions comprising the protein peak. Gel filtration on an analytical column calibrated with proteins of known molecular weight and bacteriophage T₇ indicated that the molecular weight of the multienzyme complex was several million. The purified FAS gave a single peak of protein, with constant specific enzyme activity, on DEAE-cellulose DE-52 ion-exchange chromatography. The purified enzyme gave a single band on discontinuous poly-acrylamide-gel electrophoresis in several systems.

On Ouchterlony plates, rabbit antibodies against freshly purified FAS gave a single sharp precipitin band with the purified multienzyme complex. Antibodies against the synthetase markedly inhibited its activity (Ernst-Fonberg *et al.*, 1974). Antibodies generated against *E. coli* ACP (Ernst-Fonberg *et al.*, 1977) specifically inhibited FAS activity by more

Table 1. Isolation of fatty acid synthetase from *E. gracilis* variety *bacillaris*

Fatty acid synthetase was prepared, as described in the text, from 19 g (wet wt.) of cells. Protein in the crude extract was measured by the Bradford (1976) procedure; in subsequent steps protein concentration was measured by amino acid analysis.

	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Recovery of protein (%)	Recovery of activity (%)
Crude extract	504	4718	9.4		
Calcium phosphate eluate; 0-45%-satd. (NH ₄) ₂ SO ₄ fraction	5.8	1787	308.1	1.2	37.9
Bio-Gel A-15m peak	1.4	530	379.6	0.3	11.2

* 1 unit = 1 nmol of NADPH oxidized/min, or 0.5 nmol of [2-¹⁴C]malonate incorporated/min, at 35°C.

than 60%, whereas normal immunoglobulin did not. The specific inhibition by anti-*(E. coli)* ACP antibodies indicates that this FAS contains its own acyl-carrier protein or acyl-carrier domain, sharing at least some structural features of the *E. coli* ACP.

The FAS required acetyl-CoA, malonyl-CoA, and a reduced nicotinamide nucleotide as substrates and was not dependent on added ACP (from *E. coli*) for activity at any stage of purification. The velocity of the enzyme-catalysed reaction was linear with respect to enzyme protein and with respect to time for 20 min. The fatty acids synthesized by the multienzyme complex from *Euglena* are mainly palmitate (69%), with some stearate (21%) and myristate (11%) (Ernst-Fonberg *et al.*, 1974). Some 11% of the product was isolated as unesterified fatty acid; the other 89% was esterified, probably in the form of acyl-CoA.

The fatty acid synthetase assay solution was buffered with 0.10M-Tes. At lower and higher Tes concentrations, activity was impaired. Fatty acid synthesis was inhibited by assay potassium phosphate concentrations greater than 0.01M. Optimum activity required the presence of 2–8 mM-dithiothreitol, but the enzyme maintained 60% of maximal activity when assayed in the absence of dithiothreitol. Beyond 8 mM, dithiothreitol was inhibitory. The pH-rate profile was bell-shaped and revealed a pH optimum of 6.9–7.0. EDTA had little effect on activity.

Substrate requirements

Preliminary experiments were done to determine the dependence of reaction rate on acetyl-CoA, malonyl-CoA and NADPH concentrations. When one substrate was varied at constant concentrations of the other two substrates, the FAS reaction followed Michaelis–Menten kinetics within the concentration ranges used. As in the FAS multienzyme complexes from other unicellular organisms (Goldberg & Bloch, 1972; Knoche & Koths, 1973; Kawaguchi & Okuda, 1977; White *et al.*, 1971) NADH was required in addition to NADPH for optimal activity. The dependencies of the FAS reaction on NADPH alone, NADH alone, and NADPH plus NADH in equimolar amounts were compared. Identical maximal activities (80 units) were achieved with NADPH alone or with NADPH plus NADH, but the presence of NADPH plus NADH in equimolar amounts lowered the K_m^{app} for reduced nicotinamide nucleotide by almost 9-fold, from 69 μ M-NADPH to 7.6 μ M-(NADPH + NADH). The highest rate reached in the presence of NADH alone was only 68% of maximal, and K_m^{app} was 97 μ M. NADH was varied in the presence and absence of 100 μ M-NADPH (Fig. 1). NADPH alone (100 μ M) elicited 1.4 times more activity (40 units) than did saturating amounts of NADH (28 units). In

Fig. 1. Dependence of the FAS reaction on NADH in the presence and absence of NADPH

The radiochemical assay was used. Fixed substrate concentrations were: acetyl-CoA, 30 μ M; [2- 14 C]-malonyl-CoA (0.5 μ Ci/ μ mol), 60 μ M. Reaction was started with 29 μ g of enzyme and proceeded for 2 min. $\circ$, NADH alone; Δ , NADH in the presence of 100 μ M-NADPH. Specific activity is expressed as nmol of NADPH oxidized/min per mg of protein.

the presence of 100 μ M-NADPH, activity was optimal when NADH was present at 50–100 μ M; activity was almost twice as high as in the absence of NADH. Further increase in NADH concentration resulted in inhibition with respect to optimum activity, but not compared with activity with 100 μ M-NADPH alone. These experiments suggest that the two reduction reactions have different nicotinamide nucleotide specificities. In spite of the fact that both NADPH and NADH were required for maximal activity in an effort to simplify analysis, only NADPH, the superior single reductant, was used in further kinetics experiments.

Initial-velocity studies

When malonyl-CoA was varied from 1.5 to 30 μ M, at acetyl-CoA concentrations of 1.5–15 μ M (NADPH 50 μ M), a family of lines intersecting on the ordinate of the s/v -versus- s plot was obtained. When NADPH was varied from 10 to 160 μ M, at acetyl-CoA concentrations of 4.5 to 30 μ M (malonyl-CoA 60 μ M), the s/v -versus- s pattern was a series of lines intersecting on the s/v axis. When malonyl-CoA was varied from 4.5 to 30 μ M, at NADPH concentrations of 10–160 μ M (acetyl-CoA 30 μ M), an intersecting pattern was also obtained (Fig. 2). All three primary plots were of the Ping Pong or substituted-enzyme type; an example is shown in Fig. 2. Other plots (Lineweaver–Burk, Direct Linear) were also diagnostic for a Ping Pong mechanism in each initial-velocity experiment.

Kinetic parameters based on the results of initial-velocity studies are listed in Table 2.

The *Euglena* FAS multienzyme complex produces fatty acyl-CoA. Free CoA is required as a substrate for the termination reaction in which fatty acyl-CoA is formed. Since free CoA was not included in assay solutions, the availability of CoA as a substrate for the terminal transacylase reaction depended on the rates of the acetyl and malonyl transacylase reactions. As a result, the concen-

tration of CoA varied slightly, and kinetic parameters determined in the initial-velocity experiments contain an unquantified factor reflecting CoA concentration. This approach to the initial-velocity study was preferred to that of adding CoA and then trying to separate its 'small' role as a substrate from its 'large' role as a product of the reaction (1 mol of CoA substrate/7 mol of CoA product).

Fig. 2. Effect of varying the malonyl-CoA concentration at several fixed concentrations of NADPH: Hanes primary plot

Malonyl-CoA was varied at several fixed concentrations of NADPH: ○, 10 μM; ●, 20 μM; △, 40 μM; ▲, 80 μM; □, 160 μM. Acetyl-CoA was constant at 30 μM. Units on the ordinate are (μM-malonyl-CoA) (nmol of NADPH oxidized/min per mg of protein)⁻¹. Inset: secondary plot of the data: ([NADPH] × slope) versus [NADPH]. Units on the ordinate are (μM-NADPH) (nmol of NADPH oxidized/min per mg of protein)⁻¹.

Fig. 3. Effect of varying [malonyl-CoA] at several fixed concentrations of acetyl-CoA: Lineweaver-Burk plot The radiochemical assay was used. [2-¹⁴C]Malonyl-CoA was varied at several fixed concentrations of acetyl-CoA: ○, 4.5 μM (line 1); ●, 9.0 μM (line 2); △, 90 μM (line 3); □, 120 μM (line 4). NADPH was constant at 500 μM. The reaction was started with 30 μg of protein and proceeded for 2 min. Units of v are nmol of NADPH oxidized/min per mg of protein.

Table 2. Summary of initial-velocity studies

Experiment	Pattern	$K_{\text{acetyl-CoA}}$ (μM)	$K_{\text{malonyl-CoA}}$ (μM)	K_{NADPH} (μM)	V (nmol of NADPH oxidized/ min per mg of protein)
Acetyl-CoA and malonyl-CoA varied	Ping Pong	1.3	5.1	—	84
Acetyl-CoA and NADPH varied	Ping Pong	11	—	58	118
Malonyl-CoA and NADPH varied	Ping Pong	—	12	43	118

Substrate inhibition

When a wide range of substrate concentrations (malonyl-CoA varied from 6.0 to 600 μM , at acetyl-CoA concentrations of 4.5–120 μM) was used, the double-reciprocal plot showed the distinctive pattern of mutual competitive substrate inhibition by malonyl-CoA and acetyl-CoA (Fig. 3). There is a region of concentrations where there is no apparent substrate inhibition, illustrated by the parallel section of lines 1 and 2 (malonyl-CoA varied from 6 μM to about 24 μM , at acetyl-CoA concentrations of 4.5 μM and 9.0 μM). But as the concentration of malonyl-CoA is increased, inhibition occurs, as shown by the upsweep of all the lines as $(1/[\text{malonyl-CoA}])$ approaches 0. High acetyl-CoA concentrations are also inhibitory, as illustrated by lines 3 and 4. At high acetyl-CoA concentrations, the slopes of lines increased in a manner suggestive of simple competitive inhibition.

Product-inhibition studies

Product inhibition by NADP^+ and CoA was evaluated by examining substrate-dependence of the FAS-catalysed reaction at several concentrations of the product inhibitor. Inhibition by NADP^+ was linear competitive with respect to NADPH; plots of s/v versus s at several NADP^+ concentrations gave a series of parallel lines. $K_{\text{NADPH}}^{\text{app.}}$ increased with increasing NADP^+ concentration, whereas the value of $V^{\text{app.}}$ was virtually unchanged. NADP^+ was a linear uncompetitive inhibitor with respect to malonyl-CoA and acetyl-CoA; lines intersected on the ordinates of s/v -versus- s plots. Increasing the concentration of NADP^+ led to significant decreases in $V^{\text{app.}}$ and $K_m^{\text{app.}}$ for both malonyl-CoA and acetyl-CoA. Inhibition constants determined from secondary plots of NADP^+ -inhibition data are compiled in Table 3.

The effects of adding CoA were complex. Primary plots of s/v versus s , when NADPH was varied at several CoA concentrations, showed a pattern of lines intersecting on the ordinate (Fig. 4), indicating uncompetitive inhibition. $K_{\text{NADPH}}^{\text{app.}}$ and

$V^{\text{app.}}$ were both significantly decreased as the CoA concentration was raised. However, the secondary plot of $1/V^{\text{app.}}$ versus $[\text{CoA}]$ was not linear below 100 μM -CoA (Fig. 4, inset). CoA was inhibitory at low concentrations. But only above 100 μM did CoA begin to behave as a typical simple product inhibitor (i.e. degree of inhibition linearly related to inhibitor concentration).

When malonyl-CoA was varied at several fixed concentrations of CoA, the overall pattern was one of competitive inhibition; the plot of s/v versus s was a series of parallel lines (Fig. 5). But little if any inhibition was observed below 45 μM -CoA; the lines for 0–45 μM -CoA were almost superimposable, so only the data for 30 μM -CoA are shown. As CoA concentration was increased from 45 to 180 μM , $K_{\text{malonyl-CoA}}^{\text{app.}}$ [equal to $(\text{intercept} \times V)]$ increased significantly, whereas $V^{\text{app.}}$ [equal to $(1/\text{slope})]$ hovered around 58 units.

When acetyl-CoA was varied at several fixed concentrations of CoA, the s/v -versus- s plot was again a series of parallel lines (Fig. 6). But CoA at low concentrations led to a decrease in $K_{\text{acetyl-CoA}}^{\text{app.}}$. At CoA concentrations up to 120 μM , although slopes $(1/V^{\text{app.}})$ of Hanes plots were fairly constant, ordinate intercepts $(K_{\text{acetyl-CoA}}^{\text{app.}}/V^{\text{app.}})$ were lower than the intercept observed in the absence of CoA. The lowest value of $K_{\text{acetyl-CoA}}^{\text{app.}}$ was seen in the presence of 30 μM -CoA (Fig. 6, line 1). From 30 to 300 μM , CoA behaved as a competitive inhibitor; $K_{\text{acetyl-CoA}}^{\text{app.}}$ increased significantly, while $V^{\text{app.}}$ hovered around 66 units. The inhibition by CoA with respect to acetyl-CoA did not become linear till about 180 μM -CoA (Fig. 6, inset).

Acyl-enzyme intermediates

The formation of stable acetyl-enzyme and malonyl-enzyme intermediates was demonstrated by the incorporation of ^{14}C -labelled acetyl or malonyl residues into acid-precipitable material. Under the conditions of the experiment, 2.24 nmol of ^{14}C -acetate or 1.68 nmol of ^{14}C -malonate was bound per 1.0 mg of protein. In another experiment, enzyme

Table 3. Summary of product-inhibition studies
Abbreviations: comp., competitive; uncomp., uncompetitive.

Varied substrate	Inhibitor	Pattern	$K_i(\text{comp.})$	$K'_i(\text{uncomp.})$
NADPH	NADP^+	Comp.	3.3 mM	
Malonyl-CoA	NADP^+	Uncomp.	—	6.6 mM
Acetyl-CoA	NADP^+	Uncomp.	—	1.7 mM
NADPH	CoA	Uncomp.	—	*
Malonyl-CoA	CoA	Comp.	*	—
Acetyl-CoA	CoA	Comp.	*	—

* Inhibition constants not determined; inhibition was nonlinear.

Fig. 4. Inhibition by CoA with respect to NADPH: Hanes primary plot

NADPH was varied at several concentrations of CoA: $\circ$, 0; $\bullet$, 50 μM ; $\triangle$, 100 μM ; $\blacktriangle$, 150 μM ; $\square$, 200 μM . Fixed substrate concentrations were: acetyl-CoA, 7.5 μM ; malonyl-CoA, 15.0 μM . Units on the ordinate are (μM -NADPH) (nmol of NADPH oxidized/min per mg of protein) $^{-1}$. Inset: secondary plot of the data: slopes versus [CoA]. Units on the ordinate are (nmol of NADPH oxidized/min per mg of protein) $^{-1}$.

Fig. 5. Inhibition by CoA with respect to malonyl-CoA: Hanes primary plot

Malonyl-CoA (MalCoA) was varied at several concentrations of CoA: (0); (7.5 μM); (15.0 μM); $\circ$, 30.0 μM ; (45.0 μM); $\bullet$, 60.0 μM ; $\triangle$, 90.0 μM ; $\blacktriangle$, 120 μM ; $\square$, 180 μM . Lines for 0–45 μM -CoA were practically identical; for clarity, only the line for 30 μM -CoA is shown. Fixed substrate concentrations were: acetyl-CoA, 15 μM ; NADPH, 200 μM . Units on the ordinate are (μM -malonyl-CoA) (nmol of NADPH oxidized/min per mg of protein) $^{-1}$. Inset: secondary plot of the data: intercepts versus [CoA]. Units on the ordinate are (μM -malonyl-CoA) (nmol of NADPH oxidized/min per mg of protein) $^{-1}$.

Fig. 6. Inhibition by CoA with respect to acetyl-CoA: Hanes primary plot

Acetyl-CoA (AcCoA) was varied at several concentrations of CoA: ●, 0 (line 0); ○, 30 μM (line 1); ▲, 90 μM (line 2); △, 120 μM (line 3); □, 180 μM (line 4); ■, 240 μM (line 5); ●, 300 μM (line 6). Fixed substrate concentrations were: malonyl-CoA, 30 μM ; NADPH, 150 μM . Units on the ordinate are (μM -acetyl-CoA) (nmol of NADPH oxidized/min per mg of protein) $^{-1}$. Inset: secondary plot of the data: intercepts versus [CoA]. Units on the ordinate are (μM -acetyl-CoA) (nmol of NADPH oxidized/min per mg of protein) $^{-1}$.

was incubated with [^{14}C]malonyl-CoA or with [^{14}C]malonyl-CoA plus [^3H]acetyl-CoA. When enzyme was incubated with [^{14}C]malonyl-CoA alone, 1.52 nmol of [^{14}C]malonate was bound per 1.0 mg of protein; when both [^{14}C]malonyl-CoA and [^3H]acetyl-CoA were present, 0.88 nmol of [^{14}C]malonate and 1.28 nmol of [^3H]acetate were bound per 1.0 mg of protein. When [^3H]acetyl-enzyme or [^{14}C]malonyl-enzyme was isolated and incubated with unlabelled substrates, radioactivity was incorporated into long-chain fatty acids.

Discussion

Functionally, the FAS multienzyme complex from *E. gracilis* variety *bacillaris* most resembles the mycobacterial and yeast fatty acid synthetases. These systems synthesize fatty acyl-CoA and require both NADPH and NADH for maximal activity. Fatty acid synthesis by the *Euglena*

multienzyme complex would optimally involve five substrates, acetyl-CoA, malonyl-CoA, NADPH, NADH and CoA. These are the free substrates. Reaction intermediates, presumably bound to the acyl-carrier prosthetic group of the FAS, also serve as substrates in the individual site reactions. Effects of varying the concentrations of the free substrates reflect events occurring not only at the substrate-binding sites but also at the reaction sites involving the bound intermediates. Data presented here, where, for simplicity, only three substrates, acetyl-CoA, malonyl-CoA and NADPH, were manipulated, are consistent with the view that some of the partial reactions of *Euglena* fatty acid synthesis proceed via Ping Pong mechanisms. The Hanes-plot initial-velocity patterns showing the relationships between acetyl-CoA and malonyl-CoA, acetyl-CoA and NADPH, and malonyl-CoA and NADPH, are all of the Ping Pong type, series of lines intersecting on the s/v axis. The rate equation (Walker, 1980) for a multisite mechanism with Ping Pong reactions at one or more sites predicts that the initial-velocity patterns will be of the Ping Pong type regardless of which substrate is varied at several fixed concentrations of a second substrate (Cleland, 1973).

The product inhibition patterns support the Ping Pong mechanism. The rate equation predicts the following. Each product will be competitive against the substrates combining at the same site, regardless of the mechanism at the site. The product of a catalytic site will be uncompetitive against substrates binding at other sites. Finally, if the reaction at the very next site is random sequential, a product will be non-competitive against the substrate binding at the next site (Cleland, 1973). The inhibition of *Euglena* FAS by NADP^+ was linear competitive versus NADPH and linear uncompetitive versus acetyl-CoA and malonyl-CoA. The linear nature of inhibition by NADP^+ is curious in view of the fact that there are two reduction reactions which apparently differ in nucleotide specificity. Perhaps NADP^+ only binds at one of the reductase sites. Alternatively, the K_i values for NADP^+ of the two reductase sites may be identical. The inhibition by CoA was competitive with respect to acetyl-CoA and malonyl-CoA, but uncompetitive with respect to NADPH. The inhibition, however, was not linear; the secondary plots for CoA inhibition approached parabolic shapes. Classically, parabolic secondary plots are interpreted as indicators of multiple-site binding by the inhibitor molecule. The anomalous effects of CoA probably reflect the existence of more than one site for inhibitory binding of CoA (i.e. the acetyl transacylase and malonyl transacylase active sites) compounded with the dual role of CoA as both a product and a substrate for the overall reaction.

The initial-velocity and product-inhibition patterns observed for *Euglena* FAS resemble those for

pigeon liver FAS (Katiyar *et al.*, 1975). The major difference in the steady-state kinetic patterns from the two sources is in the behaviour of CoA. Since pigeon liver enzyme produces free fatty acid, CoA does not serve as a substrate at any step, and it behaves as a simple product inhibitor.

The initial-velocity and product-inhibition patterns suggest the existence of Ping Pong sites in the fatty acid synthesis reaction, but they give no information identifying the appropriate sites. Likely Ping Pong reactions are the acetyl, malonyl and palmitoyl transacylase reactions and the β -oxo acyl-enzyme synthetase reaction. The assignment of Ping Pong mechanisms to the acetyl and malonyl transacylase reactions is based on the demonstration of acyl-enzyme intermediates, [^{14}C]acetyl-enzyme, [^{14}C]malonyl-enzyme and [^3H]acetyl-[^{14}C]malonyl-enzyme. The analogous acetyl and malonyl transacylation reactions catalysed by *E. coli* enzymes have been shown to proceed in substituted enzyme fashion via acetyl-enzyme and malonyl-enzyme intermediates (Joshi & Wakil, 1971; Williamson & Wakil, 1966).

The assignment of a Ping Pong mechanism to the β -oxoacyl synthetase reaction is based on the assumption that *Euglena* FAS, like the pigeon liver and yeast enzymes, has a 'peripheral' thiol site which covalently binds acetyl residues (Chesterton *et al.*, 1968; Joshi *et al.*, 1970; Lynen *et al.*, 1967; Phillips *et al.*, 1970a,b) and is essential for condensation to occur (Nixon *et al.*, 1970; Phillips *et al.*, 1970b). Condensation in *E. coli* proceeds by a two part reaction in which an acetyl (or acyl) group is transferred from acetyl- (or acyl-) ACP to a covalent binding site on the β -oxo acyl-ACP synthetase enzyme, followed by condensation with malonyl-ACP (D'Agnolo *et al.*, 1975; Greenspan *et al.*, 1969; Toomey & Wakil, 1966). The mutual competitive substrate inhibition by acetyl-CoA and malonyl-CoA is consistent with the idea that the β -oxo acyl synthetase reaction in *Euglena* proceeds by a Ping Pong mechanism. This mutual inhibition is possibly due to a combination of effects at several sites. Inhibition by dead-end binding of malonate at the acetyl transacylase site and of acetate at the malonyl transacylase site would be expected. The acetyl-CoA:malonyl-CoA ratio would also indirectly control the relative amounts of -4'-phosphopantetheine-S-acetyl and -4'-phosphopantetheine-S-malonyl, the enzyme-bound intermediates which are the substrates at the condensing enzyme site. At a Ping Pong reaction site, a single binding region alternately accommodates first one substrate and then the other. If one substrate is present in great excess, the likelihood of incorrect binding of that substrate is increased, and substrate inhibition occurs. Substrate inhibition is strong evidence for a Ping Pong mechanism.

There is little difference in mechanistic and kinetic detail, in so far as studied, among fatty acid synthetases of diverse organizational complexity. Mechanisms of partial reactions catalysed by the multienzyme complex of pigeon liver, the multienzyme complex of *Euglena*, and the individual enzymes of *E. coli* appear to be analogous. Each of the systems synthesizes a different form of product, and there are differences in substrate specificity and probably in the compositions of the enzymes' active sites. But, in general, the mechanisms seem to be remarkably similar.

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References

- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279-285
- Buckner, J. S. & Kolattukudy, P. E. (1976) *Biochemistry* **15**, 1948-1957
- Chesterton, C. J., Butterworth, P. H. W. & Porter, J. W. (1968) *Arch. Biochem. Biophys.* **126**, 864-872
- Cleland, W. W. (1973) *J. Biol. Chem.* **248**, 8353-8355
- Cornish-Bowden, A. & Eisenthal, R. (1974) *Biochem. J.* **139**, 721-730
- Cornish-Bowden, A. & Eisenthal, R. (1978) *Biochim. Biophys. Acta* **523**, 268-272
- D'Agnolo, G., Rosenfeld, I. S. & Vagelos, P. R. (1975) *J. Biol. Chem.* **250**, 5283-5288
- Delo, J., Ernst-Fonberg, M. L. & Bloch, K. (1971) *Arch. Biochem. Biophys.* **143**, 384-391
- Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715-721
- Ernst-Fonberg, M. L. (1973) *Biochemistry* **12**, 2449-2455
- Ernst-Fonberg, M. L., Dubinskas, F. & Jonak, Z. L. (1974) *Arch. Biochem. Biophys.* **165**, 646-655
- Ernst-Fonberg, M. L., Schongalla, A. W. & Walker, T. A. (1977) *Arch. Biochem. Biophys.* **178**, 166-173
- Goldberg, I. & Bloch, K. (1972) *J. Biol. Chem.* **247**, 7349-7357
- Greenspan, M. D., Alberts, A. W. & Vagelos, P. R. (1969) *J. Biol. Chem.* **244**, 6477-6485
- Joshi, V. C. & Wakil, S. J. (1971) *Arch. Biochem. Biophys.* **143**, 493-505

- Joshi, V. C., Plate, C. A. & Wakil, S. J. (1970) *J. Biol. Chem.* **245**, 2857–2867
- Katiyar, S. S. & Porter, J. W. (1975) *Arch. Biochem. Biophys.* **170**, 220–227
- Katiyar, S. S., Cleland, W. W. & Porter, J. W. (1975) *J. Biol. Chem.* **250**, 2709–2717
- Kawaguchi, A. & Okuda, S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3180–3183
- Kinsella, J. E., Bruns, D. & Infante, J. P. (1975) *Lipids* **10**, 227–237
- Knoche, H. W. & Kothe, K. E. (1973) *J. Biol. Chem.* **248**, 3517–3519
- Lynen, F., Oesterhelt, D., Schweizer, E. & Willecke, K. (1967) in *Cellular Compartmentalization and Control of Fatty Acid Metabolism* (Gran, F. C., ed.), pp. 1–24, Academic Press, New York
- Moore, S. & Stein, W. H. (1963) *Methods Enzymol.* **6**, 819–831
- Nixon, J. E., Phillips, G. T., Abramovitz, A. S. & Porter, J. W. (1970) *Arch. Biochem. Biophys.* **138**, 372–379
- Phillips, G. T., Nixon, J. E., Abramovitz, A. S. & Porter, J. W. (1970a) *Arch. Biochem. Biophys.* **138**, 357–371
- Phillips, G. T., Nixon, J. E., Dorsey, J. A., Butterworth, P. H. W., Chesterton, C. J. & Porter, J. W. (1970b) *Arch. Biochem. Biophys.* **138**, 380–391
- Smith, S. & Abraham, S. (1970) *J. Biol. Chem.* **245**, 3209–3217
- Toomey, R. E. & Wakil, S. J. (1966) *J. Biol. Chem.* **241**, 1159–1165
- Walker, T. A. (1980) Ph.D. Dissertation, Yale University
- Walsh, K. A. & Brown, J. R. (1962) *Biochim. Biophys. Acta* **58**, 596–598
- White, H. B., III, Mitsuhashi, O. & Bloch, K. (1971) *J. Biol. Chem.* **246**, 4751–4754
- Williamson, I. P. & Wakil, S. J. (1966) *J. Biol. Chem.* **241**, 2326–2332