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Probing Protein-protein Interactions Among Proteins of a Nonaggregated Fatty Acid Synthetase From Euglena Gracilis Variety Bacillaris

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PROBING PROTEIN-PROTEIN INTERACTIONS AMONG PROTEINS OF A NONAGGREGATED FATTY ACID SYNTHETASE FROM *EUGLENA GRACILIS* VARIETY *BACILLARIS*

A Dissertation

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the Faculty of the Department of Biochemistry

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In Partial Fulfillment

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Doctor of Philosophy in Biomedical Sciences

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by

Sande Graves Williams

May 1993

APPROVAL

This is to certify that the Graduate Committee of

SANDE GRAVES WILLIAMS

met on the

16th of February, 1993

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Science.

Chair, Graduate Committee

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean of the Graduate School

ABSTRACT

PROBING PROTEIN-PROTEIN INTERACTIONS AMONG PROTEINS

OF A NONAGGREGATED FATTY ACID SYNTHETASE FROM EUGLENA

GRACILIS VARIETY BACILLARIS

by

SANDRA GRAVES WILLIAMS

Enoyl-acyl carrier protein (ACP) reductase from chloroplast nonaggregated fatty acid synthetase (FAS) of Euglena gracilis variety bacillaris was purified to a single band on a denaturing polyacrylamide gel. The enzyme was partially characterized with respect to substrate specificity, reduced nucleotide requirement, and the effect of ACP and Ca^{++} on enzyme activity. Antibodies against the purified protein were raised in hens and isolated from eggs.

ACP was purified from Euglena in yields of about 1mg/100g (wet weight) of cells. Antibodies were raised against the purified protein. ACP antibodies inhibited the Euglena chloroplast FAS using Euglena or E. coli ACP as a substrate. Comparisons with other ACPs included the following items: biological activity, pI, behavior in size exclusion media, and amino acid sequence of the N-terminal portion of the molecule.

ACPs from E. coli and Euglena have been shown to interact with melittin, a cationic peptide from bee venom. E. coli ACP is a small (Mr, 8847), acidic, Ca^{++}-binding protein which possesses some characteristics resembling those of regulatory Ca^{++}-binding proteins including interaction with melittin. Melittin inhibited activity of the nonaggregated FAS from Euglena using either E. coli or Euglena ACP as a substrate. The peptide also inhibited activity of the aggregated FAS from Euglena. Antibodies against melittin were raised. Anti-melittin inhibited activity of both the nonaggregated and aggregated FAS enzyme systems from Euglena relative to nonimmune antibody. Investigation of inhibition of the nonaggregated FAS enzyme system demonstrated that acetyl-CoA-ACP transacylase, malonyl-CoA-ACP transacylase, and keto-acyl-ACP synthetase activities were inhibited to different degrees by anti-melittin antibodies, while keto-acyl-ACP reductase and enoyl-ACP reductase enzyme activities were not inhibited.

DEDICATION

This dissertation is dedicated to the women who taught me the meaning of Margaret Fuller's statement "The especial genius of woman, I believe to be electrical in movement, intuitive in function, spiritual in tendency". I love you Mom.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

ABBREVIATIONS

ACP	acyl carrier protein
BCIP	5-bromo-4-chloro-3-indolyl phosphate
E. coli	*Eschercherichia coli*
FAS	fatty acid synthetase
HPLC	high pressure liquid chromatography
IgY	yolk immunoglobulin
Mr	molecular weight
NBT	nitroblue tetrazolium
O.D.	optical density
TBS	tris buffer saline
TCA	trichloroacetic acid
TES	N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tris	trishydroxymethylaminomethane
A	alanine
C	cysteine
D	aspartate
E	glutamate
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
N	asparagine

M	methionine
P	proline
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

CHAPTER 1

Introduction

Fatty acids are essential for membrane structures, energy storage, and second messenger systems. Biosynthesis of fatty acids is accomplished by the system of enzymes designated fatty acid synthetase (FAS). FAS is composed of six enzyme activities and a substrate carrier, acyl carrier protein (ACP). The enzyme system catalyzes the stepwise addition of carbons, two by two, until a chain 14-16 carbons in length is obtained. The building block for fatty acid biosynthesis is malonyl CoA and the reductant is generally NADPH. The reactions are as follows:

1. malonyl-CoA + ACP ←------→ malonyl-ACP + CoA

   enzyme - malonyl-CoA-ACP transacylase

2. acetyl-CoA + ACP ←-----→ acetyl-ACP + CoA

   enzyme - acetyl-CoA-ACP transacylase

3. acetyl-ACP + enzyme ←---→ acetyl-enzyme + malonyl-ACP ←---→ ß-ketobutyryl-ACP + CO_2

   enzyme - ß-ketoacyl ACP synthetase

4. ß-ketobutyryl-ACP + NADPH + H^+ ←-----→ D-3 hydroxybutyryl-ACP + $NADP^+$

   enzyme - ß-ketoacyl-ACP reductase

5. D-3-hydroxybutyryl-ACP ←---→ trans $^{\Delta 2}$-enoyl-ACP + H_2O

   enzyme - 3-hydroxyacyl dehydratase

6. trans $^{\Delta 2}$-enoyl-ACP + NADPH + H^+ ⟷ butyryl-ACP + $NADP^+$

enzyme - enoyl-ACP reductase

Butyryl-ACP is transferred to the cysteine SH of the condensing enzyme (ß-ketoacyl ACP synthetase) prior to condensation with malonyl-ACP, and the cycle continues. The mature fatty acid, in animals and yeast, is released from ACP by a thiolesterase. In plants and some bacteria the acyl-ACP product is used in subsequent metabolic reactions.

Among organisms, FASs vary in size and structural organization. FAS in vertebrates is a multifunctional polypeptide chain found as a dimer of two identical subunits. Each subunit has a molecular weight (Mr) of 272,000 (Stoops et al., 1989), and possesses an ACP domain and all six enzyme activities (Stoops et al., 1987). Each polypeptide contains three domains. Domain 1 consists of the transacylase and the condensing enzyme; domain 2 contains the two reductases, the dehydratase, and the ACP; and domain 3 is the thiolesterase. Yeast FAS is also a multifunctional polypeptide but it is a heterodimer. The A subunit (Mr 212,000) contains ACP, ß ketoacyl-ACP synthetase, and ß-ketoacyl-ACP reductase, while the B subunit (Mr 203,000) contains the remaining four activities which unlike the vertebrate FAS system, include separate transacylase activities for malonate and acetate. The active form of the enzyme is an aggregate of 6 heterodimers having a Mr of 2.3×10^6 (Stoops et al., 1978). Yet another

organization of the FAS enzyme system can be seen in E. coli and other lower bacteria (Alberts et al., 1963; Goldman and Vagelos, 1963; Lennarz et al., 1962) and higher plants (Overath and Stumpf, 1964; Brooks and Stumpf, 1966; Simoni et al., 1967). These FASs, are nonaggregated systems whose proteins can be readily fractionated giving free ACP and discrete enzymes. Unlike the aggregated enzyme systems whose ACP is tightly associated, ACP is easily separated from the nonaggregated FAS enzyme systems. The nonaggregated FAS systems are referred to as being ACP dependent due to the requirement of an extraneous ACP source for activity in vitro.

Euglena gracilis is a phytoflagellate whose lipid content and lipid biosynthetic enzyme expression vary depending on the presence or absence of chloroplasts. This ancient eukaryote is unique because it possesses two de novo FASs (Delo et al., 1971). In etiolated cells, the FAS is a true multienzyme complex with a Mr of 6.0×10^6 (Worsham et al., 1986) whose expression is controlled by cytosolic ribosomes (Ernst-Fonberg et al., 1974). In the presence of light, the major FAS activity is a nonaggregated, ACP dependent enzyme system, whose structure resembles that of plants and primitive bacteria (Ernst-Fonberg, 1973; Worsham et al., 1988)). Expression of the nonaggregated enzyme is dependent on chloroplast ribosomal protein synthesis (Ernst-Fonberg et al, 1974).

The enzyme responsible for the last step of fatty acid biosynthesis is enoyl-ACP reductase. This enzyme catalyzes the reduction of an enoyl bond using NADH or NADPH as a reductant (Table 1). In nonaggregated FAS systems, several isoforms of this enzyme are often present. These isoforms usually differ in substrate specificity with regard to fatty acid chain length and the source of reductant. Enoyl-ACP reductase has been isolated from nonaggregated FAS systems of the following organisms: E. coli, Mr 90,000 (Weeks and Wakil, 1968); avocado, Mr 62,400 (Caughey and Kekwick, 1982); safflower, Mr 83,000 (Shimakata and Stumpf, 1982); spinach, Mr 115,000 (Shimakata and Stumpf, 1982); and rape seed Mr 68,400(Slabas et al., 1986). The spinach enzyme is a homotetramer with subunits of 32,500 daltons, and the rape seed enzyme is a heterodimer with subunits of 34,800 and 33,600 daltons. Upon sequencing of rape seed enoyl-ACP reductase it was found that the two subunits differ by the addition of a six amino acid extension on the larger subunit (Cottingham et al., 1988). Enoyl-ACP reductase from the chloroplast nonaggregated FAS of Euglena gracilis has been partially purified by Hendren and Bloch, (1980) and by Tucker, (1990). Very little characterization has been done on the enzyme.

Of the nonaggregated FASs, 3 isoforms of enoyl-ACP reductase have been found. E. coli has an NADH specific enzyme that prefers longer chain length acyl-CoA or

Table 1

Nonaggregated FAS Enoyl-ACP Reductases

Source of Enoyl-ACP Reductase	Mr	Isoforms Reported	Reductant	pH Preference	Substrate (Fatty-Acyl) Chain-Length Specificity	Inhibitor
E. coli	90,000	2	NADH	7.0-8.0	Long	1 mM hydroxymercuribenzoate
			NADPH	6.5	Short	5 mM iodoacetic acid
Safflower	83,000	2	NADH	6.5	Short	Not Determined
			NADPH	7.1	Long	
Rape Seed	68,400	2	NADH	Not Det.	Not Det.	0.1 mM p-chloro-mercuribenzoate
			NADPH			
Avocado	62,400	No	NADH	6.0-7.5	-	10 mM N-ethylmaleimide 10 mM iodoacetimide
Spinach	115,000	No	NADH	6.4	-	0.1 mM p-chloromercuribenzoate

acyl-ACP substrates and an NADPH specific enzyme preferring shorter chain length acyl-ACP substrates (Weeks and Wakil, 1968). The NADH enzyme functions over a broad range of pH, 7.0-8.0 with no defined optimum, while the NADPH enzyme has a pH optimum of 6.5. Safflower has an NADH specific isoform that prefers shorter chain length substrates and has a pH optimum of 6.5. The other safflower reductase isoform is an NADPH dependent enzyme, with a pH optimum of 7.1, that demonstrates specificity for longer chain length substrates (Shimakata and Stumpf, 1982). During the purification of enoyl-ACP reductase from rape seed Slabas et al. (1986) encountered two reductases, one that required NADH and one that required NADPH. No characterization was done on the NADPH requiring reductase.

Enoyl-ACP reductase from several nonaggregated FASs are sensitive to thiol alkylating agents. Both isoforms from E. coli were inhibited by 1 mM p-hydroxymercuribenzoate and 5 mM iodoacetic acid (Weeks and Wakil, 1968). The spinach (Shimakata and Stumpf, 1982) and rape seed (Slabas et al., 1986) proteins were both inhibited by 0.1 mM p-chloromercuribenzoate. Inhibition of the rape seed enzyme was relieved by incubation with crotonyl CoA or NADH prior to the addition of inhibitor. Avocado enoyl-ACP reductase was inhibited by 10 mM N-ethylmaleimide and 10 mM iodoacetimide (Caughey and Kekwick, 1982). Incubation of the enzyme with crotonyl ACP, but not NADH or NADPH, prior

to addition of the inhibitor resulted in relief of inhibition.

Regardless of the structural organization of the FAS enzyme, ACP is the substrate carrier used *in vivo*. ACP is a small, acidic protein that is responsible for carrying the growing substrate during fatty acid biosynthesis. ACP, then, by nature of its physiologic function, must interact with several different proteins. Little is known about the interaction of ACP and enzymes of the FAS system.

At least 10 proteins interact with ACP. ACP is present in the mitochondria where at least a part of it is associated with the NADH:ubiquinone reductase (complex I) (Sackmann *et al.*, 1991; Mikolajezyk and Brody, 1990). ACP is implicated in biosynthesis of *E. coli* membrane derived oligosaccharide (Therisod and Kennedy, 1987), lipid A (Brozek and Raetz, 1990), complex polyketides (Donadio *et al.*, 1991) and the toxin hemolysin (Issartel *et al.*, 1991). ACP is not limited to enzymes for which it functions as a substrate carrier, it also inhibits (Ki 5 μM) an NADH-dependent acetoacetyl-CoA reductase from *Euglena* that is not part of an FAS (Ernst-Fonberg, 1986). Thus, ACP in its diverse metabolic role binds many different proteins, but the molecular basis of ACP interactions with any of these proteins has not been determined.

The best characterized ACP is that from *E. coli*, which is composed of 77 amino acids (Mr 8847) and has a pI of 4.2

(Vanaman et al., 1968). The 4'-phosphopantetheine prosthetic group is attached by a phosphodiester linkage to serine 36 and thus lies midway through the amino acid sequence. E. coli ACP contains one methionine at position 44 and no tryptophan or cysteine residues.

E. coli ACP was the subject of chemical modification studies in order to gain insight into which part of the molecule is important for activity. ACP was treated with trypsin yielding peptide 19 → 61; with cyanogen bromide yielding peptide 1 → 44; and with carboxypeptidase A yielding peptide 1 → 74. Of the modified peptides described above, only the peptide missing the 3 C-terminal amino acids was fully active in fatty acid biosynthesis; the other two peptides were inactive (Majerus, 1967; Prescott et al., 1969). When ACP was acetylated and cleaved with trypsin, the peptide 7 → 77 was obtained. Removal of the N-terminal hexapeptide caused the protein to be totally inactive. Further study of the 7 → 77 peptide by optical rotatory dispersion revealed the loss of all organized structure. It was concluded that the N-terminal hexapeptide has a role in stabilization of the overall structure. If the single tyrosine is nitrated, ACP's thermal stability is reduced, but its participation in fatty acid biosynthesis is not impaired (Abita et al., 1971). The arginine at position 6 is crucial for activity, although the guanidinium group may be substituted with retention of physiologic function

(Hancock et al., 1973). Amino acids that are nonessential for activity are methionine 44, and lysines 8,9, and 18. Acetylation of the amino group of all 3 lysine residues and of the amino terminal serine of ACP does not affect its biologic function. It is apparent that ACP can tolerate little structural modification and still retain conformation and function.

Despite differences among ACPs, evidence supports conservation of primary structure. Similarities in primary sequence are particularly striking around the centrally located serine where the phophopantetheine group is attached (Matsumura and Stumpf, 1968; Huang et al., 1989) and the N-terminal region (Slabas et al., 1987). This conservation might explain the ability of E. coli ACP to substitute for several plant ACPs in fatty acid biosynthesis and the ability of E. coli acyl ACP synthetase to acylate plant ACPs (Kuo and Ohlrogge, 1984). Ernst-Fonberg et al. (1977) found that anti-E. coli ACP antibodies would cross-react with Euglena ACP, and this cross-reactivity was used as the basis for purification of the Euglena ACP. E. coli ACP, in spite of its ability to substitute as a substrate carrier for spinach ACP in the nonaggregated spinach FAS, only weakly cross-reacted with anti-spinach ACP antibodies (Tucker, 1990).

NMR studies by Kim and Prestegard (1989) using E. coli ACP suggests a dynamic structure portrayed as a model of two

distinct conformers in dynamic equilibrium. The secondary structure of the largely helical protein remains mostly intact upon transition from one conformer to the other. The dynamic states, with one exception, involve concerted movement of helices rather than their dissolution, and substantial motion of two regions of extensive loop structure and the C-terminal peptide.

ACP exhibits several characteristics similar to calmodulin and EF hand Ca^{++} binding proteins, although their sequences are not homologous (Argos, 1977). The binding of Ca^{++} to ACP alters biological activity (Schulz et al., 1969) and causes pH dependent conformational changes (Schulz, 1977). Other common features include small size, acidic pI, interaction with several different proteins, and high alpha helix content. In addition, ACP is found in the same place on a hydrophobic moment plot as EF hand calcium binding proteins (Ernst-Fonberg, 1987).

Melittin is a 26 amino acid, amphiphilic peptide found in the venom of the European honey bee, Apis mellifera. This peptide binds Ca^{++} binding proteins, such as calmodulin. The complexing of melittin and Ca^{++} binding proteins has been studied as a model for interaction of Ca^{++} binding proteins and their respective physiologic interactive proteins. Such models have been invoked for interaction between melittin and calmodulin (Maulet and Cox, 1983), melittin and myosin light chains (Malencik and

Anderson, 1988), melittin and S100b (Baudier et al., 1987), and melittin and troponin C (Steiner and Norris, 1987). In further support of the biological relevance of this system, Ketzel and Dedman (1987) demonstrated that anti-melittin antibodies recognize the calmodulin binding domains of calmodulin acceptor proteins. Laine et al. (1988) found that the presence of anti-melittin antibodies inhibited complement lysis, leading to the identification of regions of sequence homology between melittin and human complement protein, C9. In these cases, melittin appears to mimic a common secondary or tertiary structure involved in protein interaction.

This dissertation research focuses on the possibility that ACP complexes with melittin. Interaction between ACP and melittin was examined by fluorescence, chemical cross-linking and Euglena FAS activity studies. Antibodies were raised to melittin. The physiologic significance of possible complex formation between ACP and melittin was probed using anti-melittin antibodies. If melittin mimics the ACP binding site on one or more of the FAS enzymes, it is possible that anti-melittin antibodies may react with the affected enzyme. The effect of anti-melittin antibodies on aggregated and nonaggregated FAS activities from Euglena were examined. Individual enzyme activities from the Euglena nonaggregated FAS system were also examined including: acetyl-CoA-ACP transacylase, malonyl-CoA-ACP

transacylase, ß-ketoacyl-ACP synthetase, ß-ketoacyl-ACP reductase, and enoyl-ACP reductase were also examined.

Enoyl-ACP reductase from the chloroplast nonaggregated FAS of Euglena gracilis variety bacillaris was purified to a single band on a denaturing polyacrylamide gel. The enzyme was partially characterized with respect to substrate specificity and the effect of ACP and Ca^{++} on enzyme activity. Antibodies were raised to the purified protein.

E. coli ACP has been a useful substitute for Euglena ACP in studies of the Euglena nonaggregated FAS enzyme system. Previously, minute quantities of Euglena ACP have been purified from a related strain of Euglena, Euglena gracilis variety Z (Dinello and Ernst-Fonberg, 1973). It would be useful to obtain reasonable quantities of the Euglena protein for studies of the nonaggregated FAS system.

ACP was purified from Euglena gracilis variety bacillaris. Euglena ACP was compared with other ACPs regarding biological activity, pI, size, behavior in size exclusion media, and amino acid sequence of the N-terminal portion of the protein. Antibodies were raised against the purified protein and characterized with respect to their effect on the activity of Euglena nonaggregated FAS.

CHAPTER 2

Materials and Methods

E. coli grown in minimal media were purchased from Grain Processing Corporation. 2-[^{14}C]malonyl-CoA was from New England Nuclear. Crotonyl-CoA, acetyl-CoA, NADPH, and NADH were from Sigma. Ultima Gold scintillation fluid was from Packard. Column materials were DE-52 from Whatman, DEAE Sephacel from Pharmacia, Bio-Gel HPT, Bio-Gel A-15 m, and Bio-Gel A-0.5 m from Bio-Rad, Matrix Orange agarose gel from Amicon, Avid AL from BioProbe International, and Spectra/Gel A6 (Spectrum) from Fisher Scientific. High pressure liquid chromatography (HPLC) columns included 16 x 95 mm Hema-Bio 1000 Q (Tessek) from Fisher Scientific, (packed according to instructions into a Pharmacia HR 16 glass column), 7.5 x 300 mm Bio-Sil TSK 250 size exclusion column from Bio-Rad, and 5 x 200 mm Mono P chromatofocusing column from Pharmacia. Dimethyl suberimidate and bicinchoninic acid protein assay reagents were from Pierce. Nitrocellulose membranes were from Schliecher and Schuell and Zeta Probe membranes were from Bio-Rad. Gradient polyacrylamide gels were purchased from Jules Inc. Bio-Rad was the source of the prestained electrophoresis Mr standards. Sigma was the source of horseradish peroxidase conjugated rabbit anti-chicken IgG and alkaline phosphatase conjugated rabbit anti-chicken IgG. Horseradish peroxidase

conjugated goat anti-rabbit IgG, alkaline phosphatase conjugated goat anti-rabbit IgG, 4-chloro-1-naphthol, nitroblue tetrazolium (NBT) in dimethylformamide, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in dimethylformamide were from Bio-Rad. Brilliant blue G-colloidal concentrate was from Sigma, silver stain, copper stain and destain were from Bio-Rad and "Stains-all" was from Eastman. Spectra-Por 6 2000 Mr cutoff and Spectra-Por 6 10,000-12,000 Mr cutoff were from Fisher Scientific. Centricon microconcentrators were purchased from Amicon. Melittin was from either Sigma or Serva, a highly purified grade that had no detectable phospholipase A_2 activity. Keyhole limpet hemocyanin slurry in 65% ammonium sulfate, purity greater than 90% by gel filtration, was purchased from Calbiochem.

Growth of Euglena

Euglena gracilis variety *bacillaris* were grown, harvested, and stored at -80°C as described by Boehler and Ernst-Fonberg (1976) for light grown cells. Dark grown cells were grown and processed according to Ernst-Fonberg (1973).

Assays and Analyses

FAS

FAS activity was assayed by the incorporation of radioactivity from [2-^{14}C]malonyl-CoA into long chain fatty

acids (Worsham et al., 1988). Reactions (250μl total volume) were assembled at 4°C in 13x100 mm culture tubes and included the final concentration as indicated: 30 μM acetyl-CoA, 60 μM [2-^{14}C]malonyl CoA (1 μCi/μM), 800 μM NADPH (freshly prepared), 2 mM dithiothreitol, 100 mM TES, pH 8.0, 20 μl enzyme (36 μg protein), water, and ACP. After 10 minutes at 35°C, the reactions were stopped by the addition of 50 μl of 45% KOH and heated for 30 minutes at 100°C. The solutions were neutralized with 100 μl of 12M HCL. Fatty acids were extracted with pentane, three times, using 2 ml each time. Pentane was removed by evaporation and the residue was dissolved into 5 ml of scintillation fluid. Radioactivity was measured. Units of activity are defined as nanomoles of ^{14}C incorporated into long chain fatty acids per minute. Dark grown FAS assays were identical to the above assay with the exception of the exclusion of ACP from the reaction mix.

Enoyl-ACP Reductase

Enoyl-ACP reductase was assayed using crotonyl CoA as a substrate. The reaction (800 μl final volume) assembled in a quartz semimicro cuvette including the following components: 0.1 M TES, pH 8.0, 3 mM dithiolthreitol, 0.25 mM NADH (from stock solution freshly prepared in 0.01 M potassium phosphate, pH 7.4), 0.4 mM crotonyl-CoA, enzyme, and water. The reaction was initiated by the addition of crotonyl-CoA. The absorbance decrement at 360 nm as NADH

was oxidized was measured in a Cary 3 spectrophotometer. The millimolar extinction coefficient of NADH under these conditions was 4.283. One unit of activity is defined as the oxidation of one nanomole of NADH per minute. For an 800 μl reaction volume a decrease of 0.005 O.D. units equals one unit of activity.

Protein Determinations

Protein concentration was determined with bicinchoninic acid at 60°C according to the procedure provided Pierce. Protein concentration of IgY solutions were calculated using the absorbance at 280 nm; a 1% solution has an absorbance of 13.5.

Gel Electrophoresis and Blotting

Analytical polyacrylamide gel electrophoresis was performed using a Hoeffer Mighty Small apparatus and the Laemmli discontinuous buffer system (Laemmli, 1970). Proteins were precipitated by the addition of cold TCA (to 10% v:v) followed by 30 minutes incubation on ice. The precipitates were collected by centrifugation in a microfuge and washed twice, with cold 7.2% TCA and twice with acetone. The pellets were resuspended in sample buffer 0.125 M Tris, pH 6.8, 5% sodium dodecylsulfate, 2 mM dithiolthreitol, 10% glycerol, and 0.01% bromophenol blue, and boiled for 3 minutes. For native gels, the Laemlli procedure was modified by the omission of sodium dodecylsulfate and

boiling. The gels were run at a constant current of 20 mAmps. The electrophoresed proteins were visualized using the Coomassie stain described by Rock et al., (1981). Gels stained with "Stains-all" were fixed overnight with 25% isopropanol and washed with 25% isopropanol (7 changes) to remove sodium dodecylsulfate. The gel was placed in stain for 48 hours in the dark. The stain was prepared freshly as follows: 0.0025% "Stains-all", 25% isopropanol, 7.5% formamide, and 30 mM Tris, pH 8.8 (Campbell et al, 1983).

Proteins were Western blotted from 1.5 mm gels to 0.1 or 0.2 μm pore size nitrocellulose or zeta probe membranes. Membranes were treated according to Bio-Rad's recommended procedure. Protein transfer was accomplished by using a Bio-Rad trans blotter for 12 hours at 30V and 40 mAmps or a Bio-Rad semi-dry blotter for 30 minutes at 15V. Blots were cross-linked with 0.025% glutaraldehyde in TBS for 30 minutes and blocked with 5% blotto for 60 minutes. Membranes were incubated overnight with primary antibody and for 1 hour with secondary antibody at the recommended dilution. Proteins recognized by specific antibodies were visualized using 4-chloro-1-naphthol or BCIP and NBT as substrates for horseradish peroxidase and alkaline phosphatase, respectively. Controls for endogenous enzyme were preformed for each sample.

Chemical Cross-linking

Chemical cross-linking with dimethyl suberimidate of ACP to melittin and ACP to enoyl-ACP reductase was done as described by Ernst-Fonberg et al. (1990). Electroeluted enoyl-ACP reductase was electrophoresed on a 12% denaturing polyacrylamide gel and Western blotted to 0.2 μ pore size nitrocellulose using a Bio-Rad trans blotter for 12 hours at 30V and 40 mAmps. The blot was cross-linked with 0.025% glutaraldehyde, blocked with 5% blotto, and washed with 2 changes of 10 mM potassium phosphate, pH 7.6, 0.15 M NaCl. Blot was incubated for 30 minutes, at room temperature, with 100 μg of E. coli ACP in 20 ml of 10 mM potassium phosphate, pH 7.6, 0.15 M NaCl. ACP was cross-linked to the blot by a 7.5 minute incubation with 2 ml of 0.1 M dimethyl suberimidate followed by another 7.5 minute incubation with an additional 2 ml of 0.1 M dimethyl suberimidate. The reaction was stopped with 360 μl of 7.5 M ammonium acetate. The cross-linked blot was equilibrated with 20 mM Tris, pH 7.5, 0.5 M NaCl, 0.05% Tween-20 and incubated with primary antibody as usual (refer to prior blotting procedure). Controls for cross-linked blots included incubation of the blot with dimethyl suberimidate without additional protein.

Electroelution of Proteins from Polyacrylamide Gels

Protein bands from 1.5 mm nondenaturing polyacrylamide gels were visualized by incubating the gel with Copper stain for 5 minutes. The gel was washed with water for 3 minutes

and the band of interest was excised. The gel pieces were destained and electroeluted into 7.5 M ammonium acetate using an IBI Model UEA unidirectional electroeluter. The system was run at 50V for 2 hours using 0.1% sodium dodecylsulfate, 25 mM Tris, 192 mM glycine electroelution buffer. The salt solution was dialyzed against 10 mM potassium phosphate, pH 6.5, and the protein was concentrated using centricon microconcentrators.

ACP Affinity Column Construction

An ACP affinity column was constructed as described by Harlow and Lane (1988), with a few modifications. Cyanogen bromide, 25 mg, was dissolved in 25 ml of acetonitrile and stirred gently for 30 minutes with 50 ml of Spectra/Gel A6 equilibrated in 50 ml of 1.5 M sodium carbonate, pH 11. The gel was collected in a Buchner funnel and washed with 1000 ml of water followed by 1000 ml of 0.1 M sodium phosphate, pH 7.8. The activated gel was gently stirred with 50 mg of ACP (dissolved in 0.1 M sodium phosphate, pH 7.8) overnight, at 4°C. The gel with the absorbed protein was warmed to room temperature and stirred with 2 ml of 0.1 M ethanolamine for 30 minutes. The gel was washed with 500 ml of 0.2 M sodium acetate, pH 4.5, 0.5 M NaCl followed by 500 ml of 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol.

Protein Isolations

Aggregated FAS

Aggregated FAS was isolated from Euglena as described by Walker et al. (1981). Briefly, dark grown cells were resuspended in .01 M TES, pH 7.0, 2 mM dithiolthreitol, 2.2 M glycerol and broken by ultrasonication. The solution was centrifuged at 100,000 x g for 60 minutes and the supernatant solution was collected. Pellets were washed with buffer and supernatant solutions were collected by centrifugation at 100,000 x g for 60 minutes. Combined supernatant solutions were diluted with 10 volumes of .001 M potassium phosphate, pH 7.0, 2 mM dithiolthreitol, 2.2 M glycerol and applied to a calcium phosphate gel equilibrated in the dilution buffer. The column with absorbed protein was washed with 0.05 M potassium phosphate, pH 7.0, 2 mM dithiothreitol, 2.2 M glycerol and protein was eluted with 0.3 M potassium phosphate, pH 7.0, 2 mM dithiolthreitol, 2.2 M glycerol. The eluate was made 45% ammonium sulfate and stirred for 30 minutes at 4°C. Precipitates were collected by centrifugation at 27,500 x g for 45 minutes. Pellets were resuspended in 0.01 M TES, pH 7.0, 2 mM dithiolthreitol, 2.2 M glycerol and dialyzed overnight against 3 changes of buffer. The dialyzed solution was applied to a Bio-Gel A-15 m and eluted with the same buffer. Protein eluting in the void volume was collected, put into 1 ml aliquot and stored at -80°C.

Nonaggregated Chloroplast FAS

Nonaggregated FAS was isolated as described by Ernst-Fonberg et al. (1990). Light-grown cells were resuspended in 0.01 M Tes, pH 7.4, 0.15 M NaCl, 0.2 M sucrose, and 2 mM dithiolthreitol and disrupted by ultrasonic irradiation. The solution was centrifuged for 60 minutes at 100,000 x g and the supernatant solution was applied to a calibrated Bio-Gel A-15 column. Fractions that required the addition of ACP for FAS activity were pooled and stored in 1 ml aliquot at -80°C.

E. coli ACP

E. coli ACP was purified according to Rock and Cronan (1981). Briefly, E. coli were resuspended in 0.01 M Tris-glycine, pH 8.0, 0.0025 M EDTA. Cells were stirred with lysozyme (30mg/kg cells) for 2 hours at room temperature followed by the addition of an equal volume of 5% Triton-X 100 and stirred for 1 hour. The cell suspension was homogenized in a Waring blender for 2 minutes followed by the addition of an equal volume of 2-propanol. The homogenization step was repeated and the mixture was stirred for 1 hour. Supernatant solutions were collected by centrifugation at 4000 x g for 20 minutes, titrated to pH 6.1 with acetic acid and stirred with DE-52 (100ml/kg cells) overnight at room temperature. The gel with absorbed protein was collected in a Buchner funnel, washed with 5 volumes of 10 mM PIPES, pH 6.1, 0.25 M LiCl, 0.1% Triton-X

100, followed by 5 volumes of the same buffer without Triton-X 100. The gel was poured into a column and eluted with 0.6 M LiCl in the same buffer. Fractions with ACP were combined, titrated to pH 3.4 with acetic acid and allowed to stir overnight at 4°C. Precipitates were collected by centrifugation at 34,500 x g for 30 minutes. Pellets were resuspended in a minimal volume of water, titrated to pH 7.0 with 1 M Tris, and stirred with 4 volumes of saturated ammonium sulfate for 2 hours at 4°C. Supernatant solutions were collected by centrifugation at 34,500 x g for 30 minutes. Once again the supernatant solution was titrated to pH 3.4 and stirred overnight at 4°C. Precipitates were collected by centrifugation at 34,500 x g for 30 minutes. Pellets were dissolved in 10 mM PIPES, pH 6.8, 2 mM dithiolthreitol and stored in 5 ml aliquot at -20°C.

Euglena ACP

Euglena gracilis variety bacillaris, 105 g, were thawed and mixed with 105 ml of 10mM potassium phosphate, pH 6.2, 2 mM dithiolthreitol, 0.15M NaCl. The cells were passed through a Yeda press, twice, at 500 psi N_2. The solution was made 2% (v:v) Triton X-100 and stirred overnight at 4°C. The soluble fraction was collected by centrifugation for 2 hours at 27,500 x g. The pellet was resuspended in 100 ml of 10 mM potassium phosphate, pH 6.2, 2% (v:v) Triton X-100, 0.15 M NaCl, 2 mM dithiolthreitol and stirred for 1 hour at 4°C; the supernatant solution was collected and combined

with the first supernatant solution. The combined supernatant solution was made 70% saturation with ammonium sulfate (pH of the solution was adjusted to 6.2 with 1.0 M Tris base) and stirred for 30 minutes at 4°C. The solution was centrifuged for 1 hour at 27,500 x g, and the pellet was discarded. The supernatant solution was concentrated, at 4°C, to half of its volume using a 100 ml amicon cell at 60 psi with a YM5 membrane. The concentrated solution was put into 2000 Mr cut-off dialysis tubing and dialyzed against 10 mM potassium phosphate, pH 6.2. The solution was dialyzed until the conductivity equalled that of the dialysis buffer. The dialyzed solution was then gently stirred overnight, at 4°C, with Whatman DE-52 (10 mg protein/ml DE-52) equilibrated with 10 mM potassium phosphate, pH 6.2. DE-52 with adsorbed protein was collected in a Buchner funnel, washed with 10 mM potassium phosphate buffer, pH 6.2 and transferred into a column where it was washed with 10 mM potassium phosphate, pH 6.2 until the absorbance at 280 nm was minimal. Protein was eluted with 0.35M LiCl in equilibration buffer. Fractions that would support ACP dependent FAS activity without additional ACP, were pooled and diluted with water until the conductivity equaled that of 10 mM potassium phosphate, pH 6.2. The dilute ACP solution was pumped at 5 ml/min onto a 20 ml Hema-Bio 1000 Q column equilibrated in 10 mM potassium phosphate, pH 6.2. The column was washed, at 3 ml/min, with

equilibration buffer until the absorbance at 280 nm was zero. Protein was eluted using a linear gradient of 0 to 0.6 M LiCl at a flow rate of 3 ml/min. Fractions containing ACP were pooled. The ACP pool was then subject to nondenaturing electrophoresis and the ACP band was cut out of the gel. ACP was electroeluted from the gel pieces by the before mentioned procedure. ACP was also extracted from the gel by crushing the gel pieces and washing them with 10 mM potassium phosphate, pH 6.2. ACP was stored in 10 mM potassium phosphate, pH 6.2, 0.35 M NaCl, 2 mM dithiolthreitol in 1 ml aliquot at -20°C.

Enoyl-ACP Reductase

Euglena gracilis variety *bacillaris*, 80 g wet weight, were suspended in 80 ml of 10 mM Tris, pH 7.6, 2 mM dithiolthreitol and subject to 60 seconds bursts (4) of ultrasonic irradiation (at a power setting of 4 using a Branson cell disrupter). The solution was centrifuged at 38,720 x g for 2 hours. The supernatant solution was loaded onto a DEAE-Sephacel column (5 mg protein/ml DEAE) equilibrated in 10 mM Tris, pH 7.6, 2 mM dithiolthreitol followed by a 1.5 column volume wash with equilibration buffer. The column was eluted with 3 column volumes of 0.1 M NaCl in equilibration buffer, which were put aside, followed by 3 column volumes of 0.35 M NaCl in equilibration buffer. The 0.35 M wash was brought to 70% saturation with ammonium sulfate and stirred for 30 minutes at 4°C. The

precipitate was collected by centrifugation at 27,500 x g for 1.5 hours. The pellet was resuspended in 10 mM potassium phosphate, pH 7.0, 0.2 M sucrose, 2 mM dithiolthreitol and the protein solution was applied to a Bio-Gel A-0.5 m column equilibrated in 10 mM potassium phosphate, pH 7.0, 0.2 M sucrose, 2 mM dithiolthreitol. Column was washed with equilibration buffer and fractions with enoyl-ACP reductase activity that were not in the void volume of the column were pooled. The pool was diluted with water until the conductivity was equal to that of 10 mM potassium phosphate, pH 7.0, 0.2 M sucrose, 2 mM dithiothreitol. The enzyme solution was applied to a Bio-Gel HPT column (5 mg protein/ ml HPT) equilibrated with 10 mM potassium phosphate, pH 7.0, 0.2 M sucrose, 2 mM dithiolthreitol. The column was washed with equilibration buffer and protein was eluted with 0.2 M potassium phosphate, pH 7.0, 0.2 M sucrose, 2 mM dithiolthreitol. Fractions with a high specific activity were pooled and desalted into 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol using a coarse Sephadex G-25 column. The desalted pool was gently stirred, at 4°C for 30 minutes, with Matrix orange gel (5 mg protein/ml of gel) equilibrated in 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol. The column was poured and washed with 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol and eluted with a linear gradient of 0 to 0.5 M NaCl in equilibration buffer.

Fractions with high specific activity were pooled and desalted into 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol using a coarse Sephadex G-25 column. The desalted pool was loaded onto an ACP affinity column equilibrated in 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol and allowed to bind for 30 minutes. The column was washed with 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol until the absorbance at 280 nm was minimal. The protein was eluted with 0.5 M NaCl in equilibration buffer. Pools of enzyme from the Matrix orange column and the ACP affinity column with high specific activity were electrophoresed on a nondenaturing polyacrylamide gel and the enzyme band was excised from the gel. The excised gel pieces were electroeluted as in the previous mentioned procedure. Active enzyme was extracted from nondenaturing gels by crushing the gel band with a glass rod and washing with 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol. Enzyme was stored in 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiolthreitol at -20°C.

Chicken Antibodies

Antigen Preparation

Antigens were melittin from Sigma, and ACP and enoyl-ACP reductase purified from *Euglena gracilis* variety *bacillaris*. The antigens were coupled to keyhole limpet hemocyanin according to the procedure described by Gullick

(1988). Antigens were dissolved in 0.1 M sodium phosphate, pH 8.0, and mixed with and equal amount of keyhole limpet hemocyanin (w:w) in the same buffer (total reaction volume of 500 μl). Proteins were incubated at room temperature with 5 μl of 25% glutaraldehyde for 15 minutes followed by the addition of 2.5 μl more of 25% glutaraldehyde and another 15 minute incubation. The reaction was stopped by the addition of 100 μl of 1.0 M glycine, pH 6.0. The coupled antigen was put into aliquot and stored at -20°C.

Immunization

Hens were immunized by multiple intradermal injections, in the breast, with 200-500 μg of antigen (dissolved in 20 mM potassium phosphate, pH 7.2, 0.14 M NaCl) mixed with an equal volume of Freund's complete adjuvant. After two weeks a booster of 100 μg of antigen mixed with an equal volume of Freund's incomplete adjuvant was given. The boost was repeated in two weeks and then given every 4 weeks. Eggs were collected and antibodies were isolated from the yolks.

IgY isolation

Antibodies were isolated from egg yolks according to the protocol supplied with Avid AL column material from Bioprobe International, Inc. Yolks were separated from the whites and washed with water. Washed yolks were removed from the yolk sac and mixed with 10 yolk volumes of 10 mM Tris, pH 7.4, 0.15 M NaCl. The supernatant solution was

collected by centrifugation at 650 x g for 20 minutes. Stock solutions of 10% dextran sulfate in 10 mM Tris, pH 7.4, 0.15 M NaCl (w:v) and 1.0 M calcium chloride in 10 mM Tris, pH 7.4, 0.15 M NaCl were made. The supernatant solution was stirred, for 30 minutes, with 3 ml of the 10% dextran stock solution and 7.5 ml of the 1.0 M calcium chloride stock solution per ml of original yolk volume. The solution was centrifuged at 650 x g for 20 minutes, and the supernatant was collected. The pellets were washed with 50 ml of 10 mM Tris, pH 7.4, 0.15 M NaCl and centrifuged at 650 x g for 20 minutes, and the supernatant solution was collected. Combined supernatant solutions were filtered through Whatman #1 paper by gravity filtration and adjusted to pH 7.4. Protein concentration was determined by bicinchoninic acid protein assay. A portion of the filtered supernatant solution (5 mg protein/ml gel) was applied to an Avid AL column equilibrated in 10 mM Tris, pH 7.4, 0.15 M NaCl. The column was washed with equilibration buffer and the IgY was eluted with 0.1 M acetic acid. Fractions were neutralized with 1.0 M Tris base. Antibodies were stored as a slurry in 50% ammonium sulfate at -20°C.

CHAPTER 3

Results

Purification of Euglena Chloroplast Enoyl-ACP Reductase

The purification of enoyl-ACP reductase from 135 g Euglena gracilis variety bacillaris is summarized in Table 2. All purification steps were performed at 4°C. Light grown cells were disrupted by ultrasound and centrifuged to remove particulate matter.

DEAE Sephacel Chromatography of Enoyl-ACP Reductase

The crude supernatant solution was applied to a DEAE Sephacel ion exchange column. The column was eluted stepwise with 0.1 M NaCl followed by 0.35 M NaCl. The low salt wash, containing most of the ß-ketoacyl-ACP reductase activity, was frozen at -20°C for later use. The 0.35 M salt wash, with enoyl-ACP reductase activity, contained 2173 mg of protein and 11,158 units of activity.

Bio-Gel A-0.5 m Chromatography of Enoyl-ACP Reductase

Enoyl-ACP reductase activity from the DEAE Sephacel was brought to 70% saturation with ammonium sulfate and stirred for 30 minutes. The pellet was collected by centrifugation and dissolved in 10 mM potassium phosphate, pH 7.0, 0.2 M sucrose, 2 mM dithiothreitol. The protein solution was applied to a Bio-Gel A-0.5 m molecular sieve column to separate any FAS multienzyme complex and to place the

Table 2

Purification of Enoyl-ACP Reductase from 100g of Euglena

Purification Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg proteins)	% Yield	Purification Factor
Broken Cells	3,000	13,530	4.4	100	1
DEAE Sephacel	2,173	11,070	5.1	82	1.2
Bio-Gel A-0.5 M	514	5,200	10.1	38	2.3
Hydroxylapatite	102	4,080	40.1	30	9.1
Matrex Orange	0.8	1,230	153.7	10	35
ACP-Affinity	0.12	402	3,350	3	761

Unit of enzyme activity is defined as the amount of protein needed to oxidize one nmole of NADH per minute under standard conditions.

reductase into phosphate buffer. Fractions with high specific activity were combined. The pool contained 514.5 mg of protein and 5200 units of activity.

Hydroxylapatite Chromatography of Enoyl-ACP Reductase

Activity from the Bio-Gel A-0.5 m was applied to a Bio-Gel HTP column in 0.01 M potassium phosphate. Hydroxylapatite (HTP), is a form of calcium phosphate gel. Enoyl-ACP reductase activity eluted from the column at 0.2 M potassium phosphate, pH 7.0 (Figure 1). At this point there were two pools of activity capable of reducing the substrate, crotonyl CoA; pool A, that did not bind to the hydroxyapatite column in 0.01 M potassium phosphate and pool B that did bind to the column in 0.01 M potassium phosphate. Pool B was deemed to be part of the nonaggregated FAS enzyme system for reasons discussed later and was reserved for further purification. It contained 202.5 mg of protein and 4088 units of activity.

Matrex Orange Chromatography of Enoyl-ACP Reductase

The enzyme eluted from the hydroxylapatite column with 0.2 M potassium phosphate was desalted on a Sephadex G-25 column. The protein was applied to a Matrex Orange column and allowed to equilibrate with column matrix for 30 minutes prior to elution. The column was developed with a linear gradient from 0-0.5 M NaCl, and fractions with high specific activity were pooled (Figure 2). Active protein eluted off

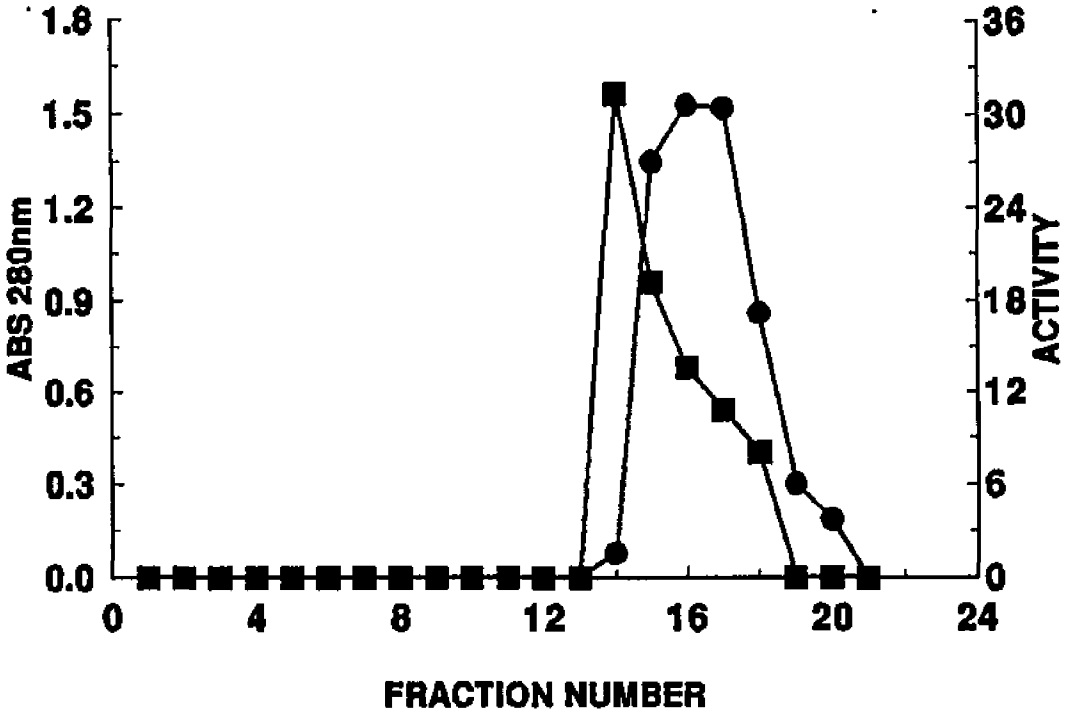

Figure 1. Hydroxylapatite Chromatography of Enoyl-ACP Reductase. Enzyme from a Bio-Gel A-0.5 M column was loaded onto a hydroxylapatite (2.5 x 21 cm) column equilibrated with 10 mM potassium phosphate pH 7.0, 0.2 M sucrose, 2 mM dithiothreitol. The column was eluted with 0.2 M potassium phosphate pH 7.0, 0.2 M sucrose, 2 mM dithiothreitol. The circles represent absorbance at 280 nm and the squares represent enzyme activity (units/100 μl). Fractions of 5 ml were collected and fractions 14-18 were combined.

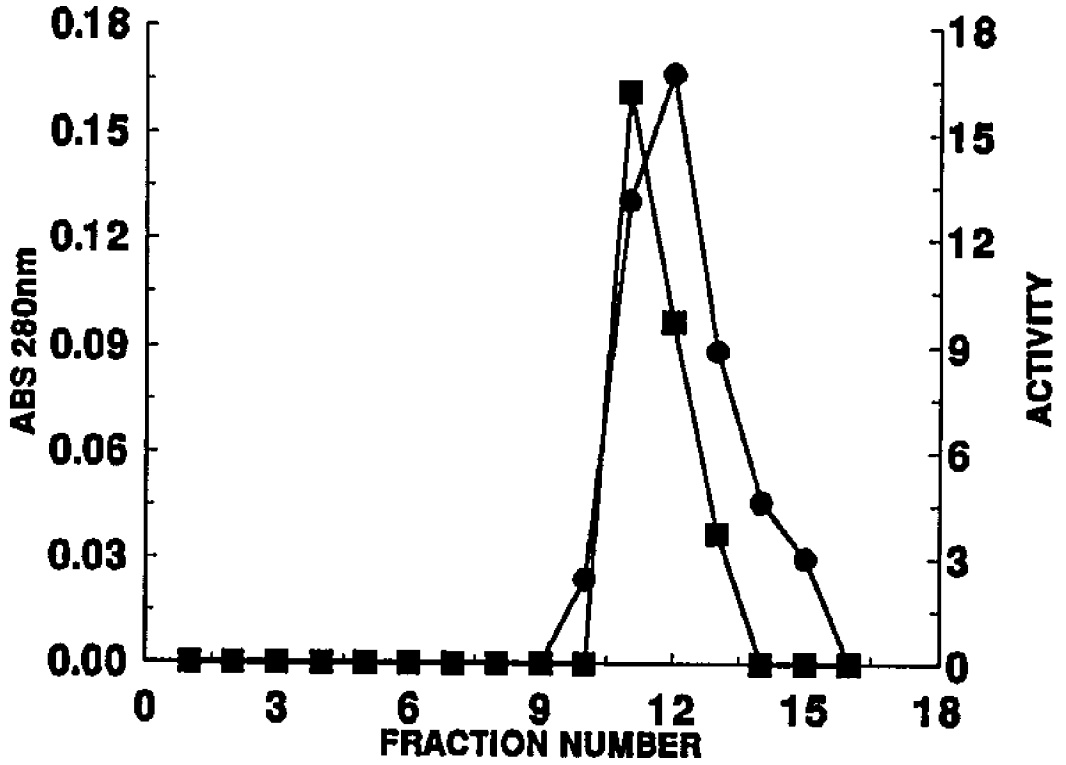

Figure 2. Matrex Orange Chromatography of Enoyl-ACP Reductase. Enzyme from a hydroxylapatite column was applied to a 2.5 x 8.0 cm matrex orange column equilibrated in 10 mM Tris pH 7.6, 0.2 M sucrose, 2 mM dithiothreitol. The column was developed with a linear gradient 0-0.5 M NaCl in equilibration buffer. Fractions of 5 ml were collected, and fraction 11-13 were combined. The circles represent absorbance at 280 nm and the squares represent activity (units/100 μl).

the column at 0.4 M NaCl. There was no evidence of enoyl-ACP reductase activity anywhere else in the salt gradient. The pool of fractions 11 through 13 contained 0.8 mg of protein and 1230 units of activity, a specific activity of 1537 units/mg.

ACP Affinity Chromatography of Enoyl-ACP Reductase

The enzyme from Matrex Orange was desalted on a Sephadex G-25 column and applied to an ACP affinity column in buffer with no salt. Protein was equilibrated with the column for 30 minutes prior to elution with 0.5 M NaCl (Figure 3). The combined eluant contained 402 units of activity and 0.12 mg of protein, a specific activity of 3350 units/mg. The enoyl-ACP reductase activity was desalted on a Sephadex G-25 column in 10 mM Tris, pH 7.6, 0.2 M sucrose, 2 mM dithiothreitol and stored at -20°C.

Electrophoresis of Enoyl-ACP Reductase

To obtain a pure protein, fractions from the Matrex orange column and the ACP column with high specific activity were electrophoresed on 7% nondenaturing polyacrylamide gels, and a band containing the enzyme was excised. Protein was extracted from pieces of gel either by washing crushed gel pieces with buffer or by electroelution of protein into 7.5 M ammonium acetate using an IBI unidirectional electroeluter. Although electroeluted protein did not retain activity, protein obtained by crushing gel pieces

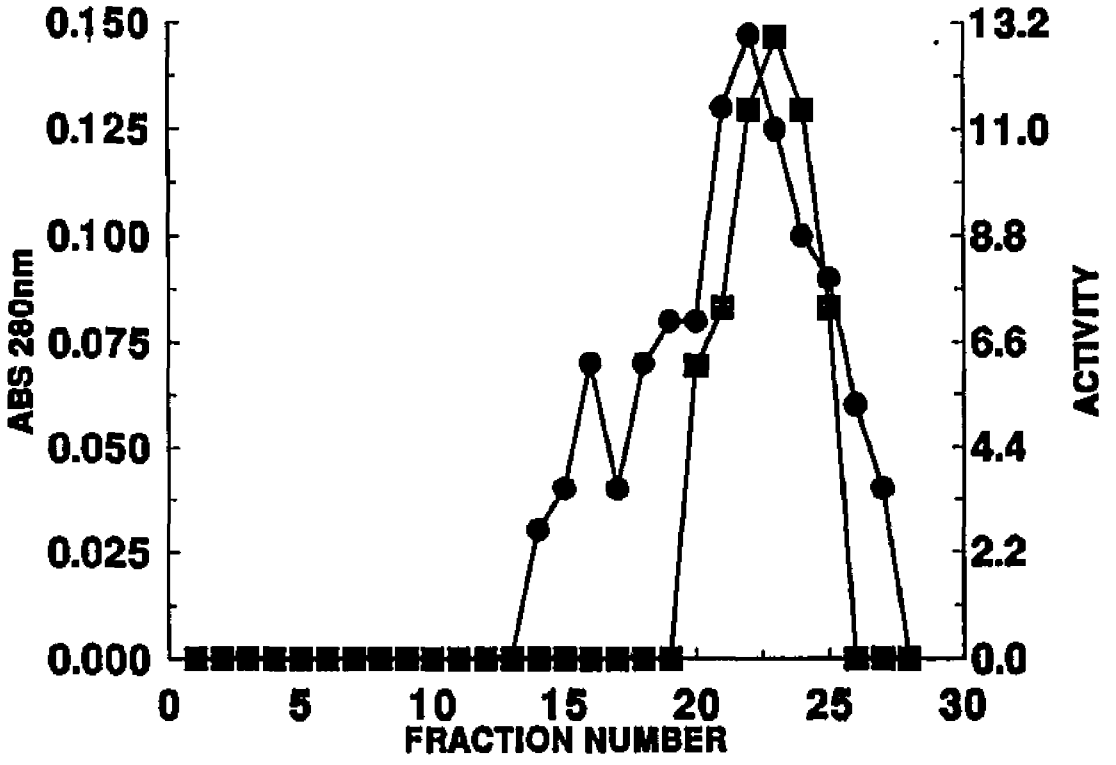

Figure 3. ACP Affinity Chromatography of Enoyl-ACP Reductase. Enzyme from a matrex orange column was applied to a 2.5 x 4.0 cm ACP affinity column equilibrated in 10 mM Tris pH 7.6, 0.2 M sucrose, and 2 mM dithiothreitol. Enzyme was allowed to equilibrate with the column for 30 minutes and was eluted with 0.5 M NaCl in equilibration buffer. Fractions of 1 ml were collected and fractions 20-25 were combined. The circles represent absorbance at 280 nm and the squares represent activity (units/100 μl).

retained some activity resulting in a final yield of 0.1 mg of pure protein with a specific activity of 943 units/mg protein.

Assessment of Purification and Identification

Denaturing polyacrylamide gel analysis of enzyme from later steps in the purification showed the enhancement of a protein band with an approximate Mr of 39,800 (Figure 4A). The electrophoretic pattern of the active enzyme extracted from nondenaturing gels also was a single band at 39,800 (Figure 4B). Antibodies raised against the purified protein showed reactivity with a single band on Western blots of the enoyl-ACP reductase purified through a Matrex orange column (Figure 4C). A protein migrating at the same position cross-reacted with anti-Euglena FAS antibodies and anti-dinucleotide fold specific antibodies (Figure 5A and 5C).

Characterization of Enoyl-ACP Reductase

Euglena enoyl ACP reductase was characterized with respect to estimation of Mr, substrate specificities, and the effect of Ca^{++} on biological activity. Matrex orange pure enoyl-ACP reductase was used as the enzyme source and CoA was the substrate carrier unless otherwise specified. The Mr of the enzyme estimated by size exclusion HPLC of the native enzyme and denaturing polyacrylamide gel electrophoresis was 39,800. The enzyme demonstrated broad

Figure 4. Gels and Blots of the Enoyl-ACP Reductase Preparation. All gels were 12% polyacrylamide gels and were developed according to the references mentioned in Methods. Protein samples were precipitated with cold 10% trichloroacetic acid, washed twice with acetone, redissolved in 125 mM Tris-HCl pH 6.8, 10% glycerol, 5% sodium dodecyl sulfate, 2 mM dithiothreitol (fresh), and 0.01% bromophenol blue, and boiled for 5 minutes. (A) Coomassie blue-stained gel to which enzyme from the following sources was applied: lane 1, 30 μg hydroxylapatite; lane 2, 30 μg Matrex orange (not bound); lane 3, Matrex orange (bound to column and eluted with 0.4 M NaCl) lane 4, (B) 20 μg of pure enoyl-ACP reductase electroeluted from a nondenaturing polyacrylamide gel, (C) Western blot of a 12% polyacrylamide denaturing gel of 5 μg of enzyme (from a Matrex orange column) transferred to 0.2 μ nitrocellulose. Transfer conditions are discussed in Methods. Detection was with chicken anti-Euglena enoyl-ACP reductase antibodies and horseradish peroxidase-conjugated rabbit anti-chicken IgG. Mr standards were as follows: a) bovine serum albumin (75,000), b) ovalbumin (50,000), c) carbonic anhydrase (39,000), d) soybean trypsin inhibitor (27,000), and e) lysozyme (17,000).

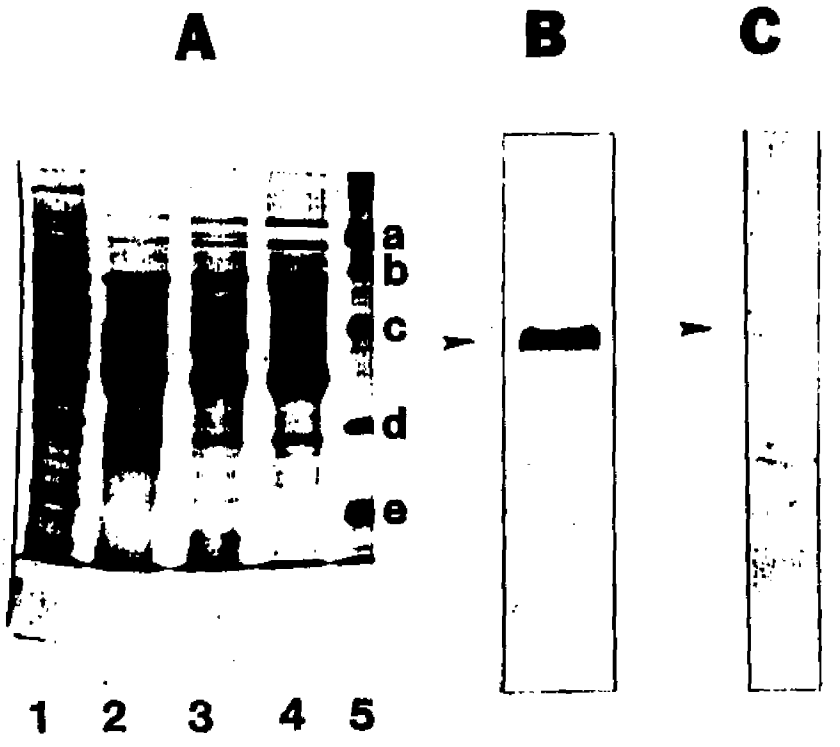
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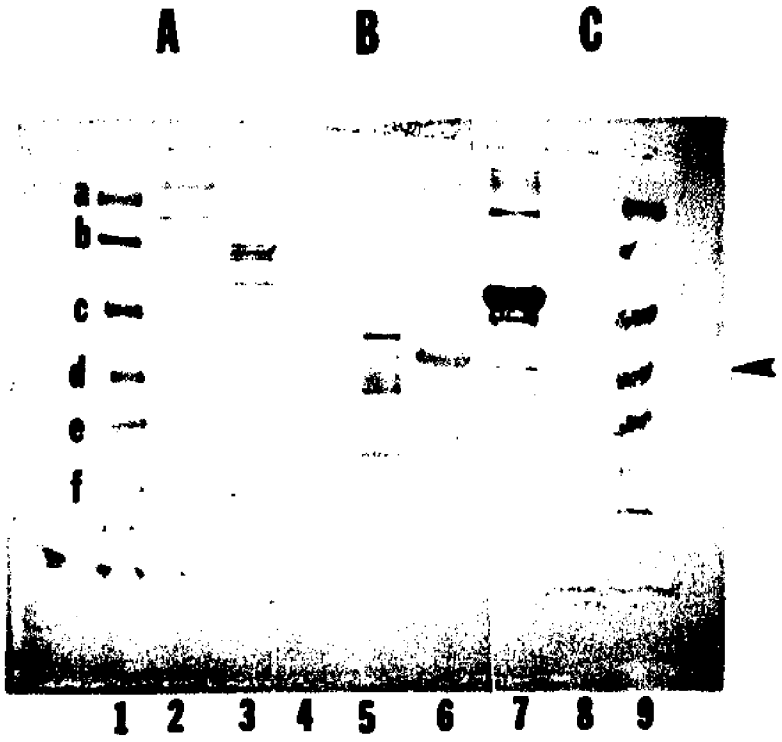
Figure 5. Western Blots of Enoyl-ACP Reductase. Euglena enoyl-ACP reductase (from a Matrex orange column), E. coli ACP, Euglena chloroplast FAS, and yeast glucose-6-phosphate dehydrogenase were electrophoresed on a 12% polyacrylamide gel, then transferred onto 0.2 μM nitrocellulose. Primary antibodies used were hen anti-Euglena FAS for lane 1-3 (A), affinity purified rabbit anti-E. coli ACP lane 4-6 (B), and affinity purified hen anti-dinucleotide fold antibodies in lane 7-9 (C). Horseradish peroxidase-conjugated rabbit anti-chicken IgG and horseradish peroxidase-conjugate goat anti-rabbit IgG were used to detect immune complexes: lane 1 Bio-Rad low Mr prestained standards; lane 2, Euglena chloroplast FAS (20 μg); lane 3, enoyl-ACP reductase (10 μg); lane 7, yeast glucose-6-phosphate dehydrogenase (5 μg); lane 8, enoyl-ACP reductase (10 μg); and lane 9, Bio-Rad low Mr prestained standards. Mr standards were as follows: a) bovine serum albumin (75,000); ovalbumin (50,000); c) carbonic anhydrase (39,000), d) soybean trypsin inhibitor (27,000), and e) lysozyme (17,500). (B) E. coli ACP (5 μg/ml) was generally cross-linked to blotted protein in lane 4-6. Lane 4 Euglena chloroplast FAS (20 μg); lane 5 E. coli ACP (5 μg); enoyl-ACP reductase (10 μg).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nucleotide specificity utilizing NADH, NADPH, or a combination of the two reductants (Figure 6). Of the nucleotide concentrations examined, the enzyme was most active with 0.25 mM NADH. Enzyme activity was stimulated by the presence of 0.01 μM concentrations of Ca^{++} in assays and inhibited by 1.0 mM concentrations of Ca^{++} in assays (Table 3). The addition of 10 μM Ca^{++} resulted in an increase in activity to 137% while 10 mM Ca^{++} inhibited activity 80%.

Interaction of enoyl-ACP reductase with ACP was probed by examining the effect of increasing amounts of E. coli ACP in enzyme assays and chemical cross-linking of ACP to enzyme that had been Western blotted. Increasing amounts of ACP in enoyl-ACP reductase assays inhibited biological activity (Figure 7). Enzyme activity was inhibited 71% by the addition of 4.5 μM ACP to assays using NADH as the reductant. When NADPH was the reductant source, 9.0 μM ACP was needed to achieve the same percent inhibition.

Enoyl-ACP reductase was Western blotted and E. coli ACP chemically cross-linked to the enzyme with dimethyl suberimidate. When anti-E. coli ACP antibodies were used as the primary antibody, the blot showed a reactive band with an estimated Mr of 39,800 (Figure 5B). Control blots without ACP cross-linked did not cross-react (data not shown).

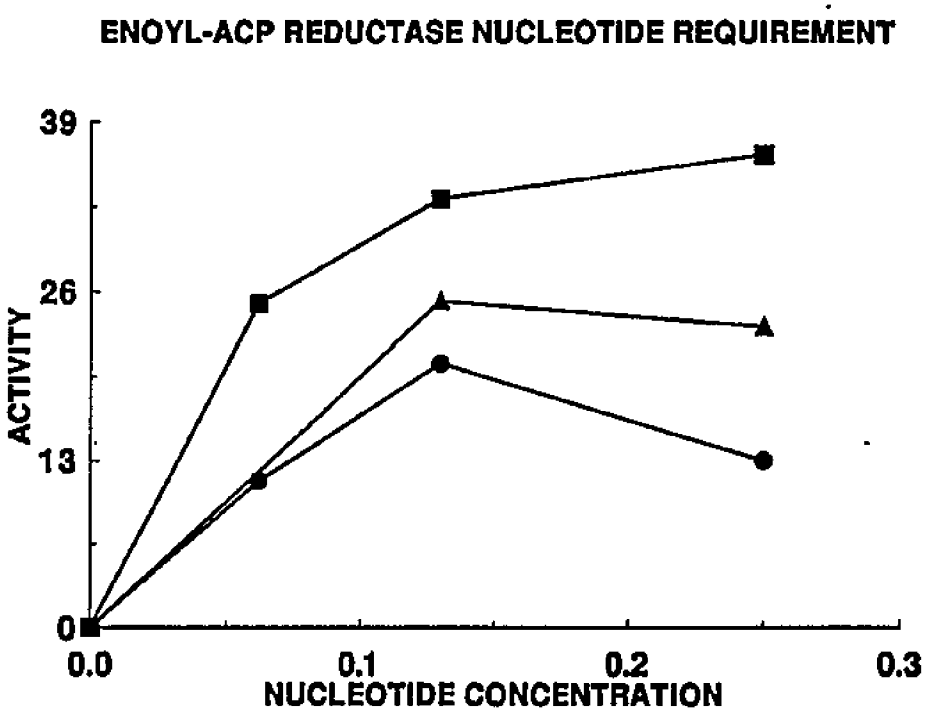


Figure 6. Nucleotide Specificity of Enoyl-ACP Reductase. The enoyl-ACP reductase assay was described in Methods. The squares represent NADH, the circles represent NADPH, and the triangles represent both NADH and NADPH. A unit of enzyme activity is defined as the amount of protein needed to oxidize one nmole of NADH or NADPH per minute under standard conditions.

Table 3

Effects of Ca^{++} on Activity of Enoyl-ACP Reductase from Euglena

$[Ca^{++}]$	Activity nmol/min	% Control Activity
5 μM	24.2	122
10 μM	26.0	131
10 μM	27.0	137
1 mM	12.1	61
5 mM	7.1	36
10 mM	1.9	9
10 mM	3.9	18
0 Ca^{++}	19.7	100
0 Ca^{++}	19.0	100

Assays were performed as described in Methods. A unit of enzyme activity is defined as the amount of protein needed to oxidize one nanomole of NADH per minute under standard conditions.

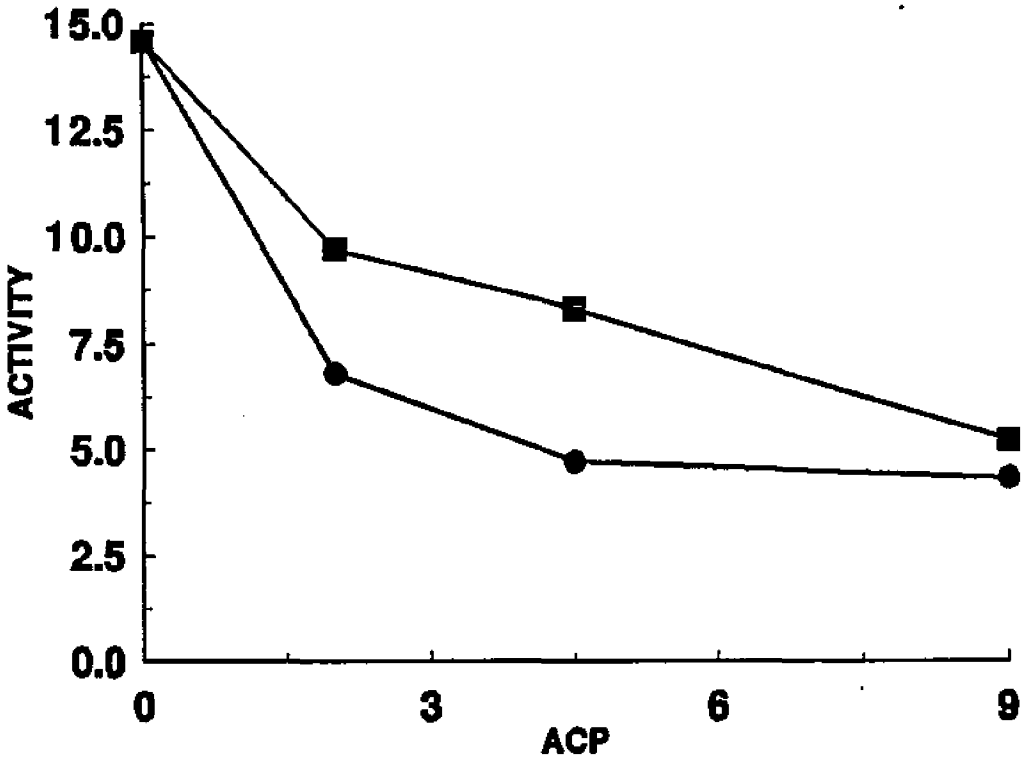


Figure 7. The Effect of E. coli ACP on Enoyl-ACP Reductase Activity. Enoyl-ACP reductase (from a Matrex orange column) was allowed to incubate with E. coli ACP for 5 minutes at room temperature. Assays were started by the addition of 0.4 mM crotonyl CoA. Assays were done as stated in the Methods. The circles represent assays with 0.25 mM NADH as the reductant; the squares represent assays with 0.25 mM NADPH as the reductant. ACP concentration in μmoles. A unit of enzyme activity is defined as the amount of protein needed to oxidize one nmole of NADH or NADPH per minute under the defined assay conditions.

The efficiency of enoyl-ACP reductase using crotonyl-ACP vs crotonyl-CoA as a substrate was compared. Although kinetic constants were not determined, crotonyl-ACP was the better substrate (data not shown). With all other conditions equal, the enzyme required only 1.6 nmoles of crotonyl-ACP to produce the same activity achieved with 37 nmoles of crotonyl-CoA.

Antibodies Raised Against Enoyl-ACP Reductase

Anti-enoyl-ACP reductase antibodies were detected 6 weeks after immunization of hens using dot blots of Euglena enoyl-ACP reductase. Increasing amounts of antibody in enoyl-ACP reductase assays (without dithiothreitol) showed inhibition of enzyme activity by anti-enoyl-ACP reductase antibodies relative to control antibodies (data not shown). The inclusion of 2000 μg of anti-enoyl-ACP reductase antibodies inhibited enzyme activity 80%.

Purification of Euglena ACP

DE-52 Chromatography of ACP

Purification of ACP from 105 g of Euglena gracilis variety bacillaris is summarized in Table 4. After disruption of cells, the solution was made 2% Triton X-100 and stirred overnight to solubilize any ACP that may have been bound to membrane proteins. The supernatant solution was collected by centrifugation, and the pellet was washed with 100 ml of 10 mM potassium phosphate, pH 6.2, 0.15 M

Table 4

Purification of ACP from 105 g of Euglena

Purification Step	Total Protein[a]	Total Units	Fold Enrichment	% Yield
Triton X-100 Supernatant Solution	5688	168	0	100
Concentrated dialyzed $(NH_4)_2SO_4$ supernatant solution	444	92	5	55
DE-52	13	71	182	42
Hema-Bio Q pool A	1	29	966	17
Electrophoresis of pool A	0.7	27	1290	16

[a]Protein is in mg.

One unit of ACP is defined as the amount required for the incorporation of 1 nmol of [2-^{14}C]malonyl-CoA into fatty acid per minute under the conditions described. Specific activity is units of ACP per milligram of protein.

NaCl, 2% (v:v) Triton X-100, 2 mM dithiothreitol. The wash supernatant solution was collected by centrifugation and combined with the first supernatant solution. The combined supernatant solutions contained 5688 mg of protein and 168 units of activity. A 70% saturation ammonium sulfate treatment of the combined supernatant solution resulted in precipitation of 92% (5244 mg) of the proteins, including FAS enzymes, while ACP remained in solution. The supernatant solution was collected by centrifugation, concentrated in an Amicon cell, and dialyzed to remove salt. The protein was applied to a DE-52, ion-exchange column. Protein bound to the DE-52 column was eluted with 0.35 M LiCl. Of the 444 mg of protein applied to the column, the 0.35 M LiCl eluant (containing ACP) had 13 mg of protein and 71 units of activity, resulting in a 182 fold increase in specific activity.

Hema-Bio Q Chromatography of ACP

The DE-52 protein pool was diluted with water until the conductivity was equal to that of 10 mM potassium phosphate, pH 6.2 and applied a Hema-Bio Q column HPLC ion exchange column. The dilute solution was pumped at a rate of 5 ml/min onto a 20 ml Hema-Bio Q column, and protein was eluted with a 3 ml/min linear gradient of 0-0.6 M LiCl. ACP eluted off the column in 0.35 M LiCl, at the front of the second protein peak, giving a sharp activity peak as seen in Figure 8. Fractions with high specific activity were

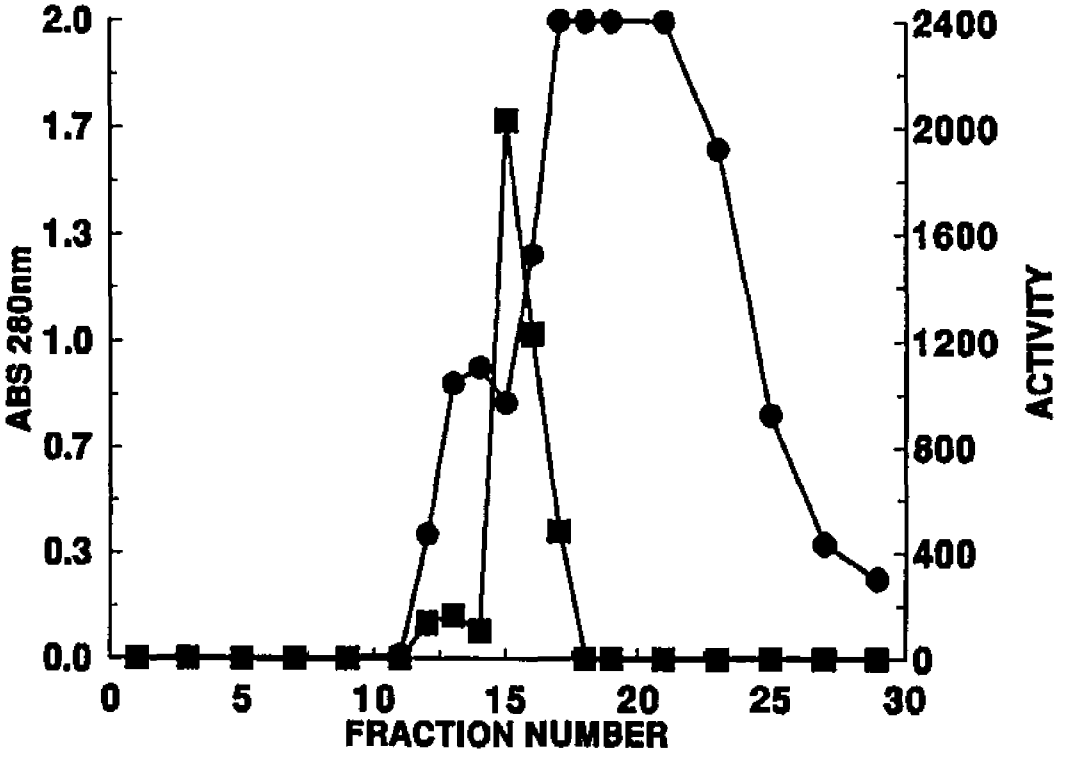


Figure 8. Hema-Bio 1000 Q Chromatography of Euglena ACP. Euglena ACP off of a DE-52 column was pumped at 5 ml/min onto a 16 x 63 mm Hema-Bio 1000 Q equilibrated with 10 mM potassium phosphate pH 6.2. The column was developed with a linear gradient of 0.5 M LiCl in equilibration buffer at a flow rate of 3 ml/min over 20 minutes. Fraction 15-17 were combined. The squares represent activity (DPM) and the circles represent absorbance at 280 nm.

combined. The pool contained 29 units of activity and 1 mg of protein, the majority of which was ACP.

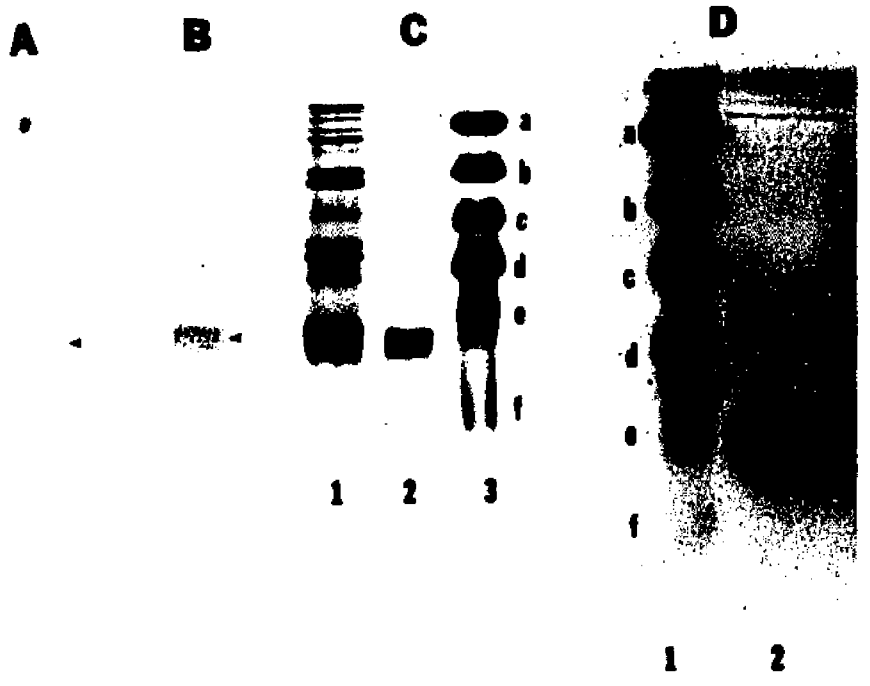
Electrophoresis of ACP

To obtain pure ACP, the Hema-Bio Q ACP pool was electrophoresed on 18% nondenaturing polyacrylamide gels and the ACP band excised from the gel. Protein was extracted from the gel by either washing crushed gel pieces with buffer or electroelution of protein from gel pieces into 7.5 M ammonium acetate using an IBI unidirectional electroeluter. Although electroeluted protein did not retain much activity, protein that was washed from the gel maintained activity resulting in a final yield of 0.7 mg of pure ACP with a specific activity of 38.6. ACP was stored in 10 mM potassium phosphate, pH 6.2, 0.35 M LiCl, 2 mM dithiothreitol at -20°C.

Assessment of Purification

Analysis of the protein and ACP activity in pooled fractions from the DE-52 and Hema-Bio Q columns on denaturing polyacrylamide gels demonstrated the enrichment of a low Mr band (Figure 9C). ACP purity following HPLC ion-exchange chromatography was estimated to be greater than 90%, while the electrophoresis of the final step yielded a single band identical with the low Mr protein enhanced in the previous steps (Figure 9D). Worsham et al, (1990) have shown the persistence of oligomers of ACP in spite of the

Figure 9. Gels and Blots of ACP Preparations. All gels were 18% polyacrylamide and developed according to the reference given in the Methods. Protein samples were precipitated with cold 10% trichloroacetic acid, washed twice with acetone, then suspended and boiled for 5 minutes in 0.125 M Tris-HCl (pH 6.8), 10% glycerol, 5% sodium dodecylsulfate, 0.002 M dithiothreitol, and 0.01% Bromophenol blue. A is a Western blot of an 18% polyacrylamide gel electrophoresis of *Euglena* ACP which was transferred onto 0.1 μm nitrocellulose for 30 minutes in a Bio-Rad Minitrans-Blotter. Detection was with chicken anti-*Euglena* ACP antibodies and horseradish peroxidase conjugated rabbit anti-chicken IgG. B is a Western blot prepared like that shown in A except that the primary antibody was rabbit anti-*E. coli* ACP. The secondary antibody was alkaline phosphatase conjugated goat anti-rabbit IgG. C is a Coomassie blue stained gel to which the following samples were applied: lane 1, 33 μg protein, DE-52 concentrating column; lane 2, 10 μg protein, Hema-Bio Q pool ; lane 3, standards. D is a Stains-all stained gel to which the following samples were applied: lane 1, standards; lane 2, 10 μg, purified *Euglena* ACP. Standard Mrs were as follows: a) 46,000; b) 30,000; c) 21,500; d) 14,300; e) 13,000; f) 3,400.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presence of sodium dodecyl sulfate and sulfhydryl reducing agents, thus explaining the small amount of dimer present in (lane 2 of Figure 9D) when a relatively large amount of purified Euglena ACP was applied to the gel. Antibodies raised against the purified protein showed reactivity with a single band on Western blots of the ACP purification (Figure 9A). The same band cross-reacted with anti-E. coli ACP antibodies (Figure 9B).

Characterization of Euglena ACP

Analysis on denaturing polyacrylamide gels provided a Mr of about 8000 for Euglena ACP. The pI of this protein was estimated by HPLC chromatofocusing to be 3.9. Staining of denaturing polyacrylamide gels with "Stains-all", a cationic carbocyanine dye, gave a deep blue band verifying the presence of anionic groups on Euglena ACP (Campbell et al., 1983). The UV spectrum of this protein indicated few aromatic amino acids with no evidence of the presence of tryptophan (data not shown). Purified Euglena ACP was sent to Dr. Mark Lively (Protein Core Analysis Lab, Cancer Center of Wake Forest University, Bowman Gray College of Medicine) for amino acid sequencing, yielding the first 17 amino acids (Table 5).

Fatty acid biosynthesis catalyzed by the chloroplast FAS from Euglena was compared using E. coli ACP and Euglena ACP (Figure 10). Although kinetic constants were not measured, it is clear that Euglena ACP was the more

Table 5

Amino Acid Sequence of N-Terminus of ACP from

Euglena gracilis variety bacillaris

1	2	3	4	5	6	7	8	9
S	?	A	G	A	S	P	E	L
10	11	12	13	14	15	16	17	18
F	E	K	V	R	S	I	V	I

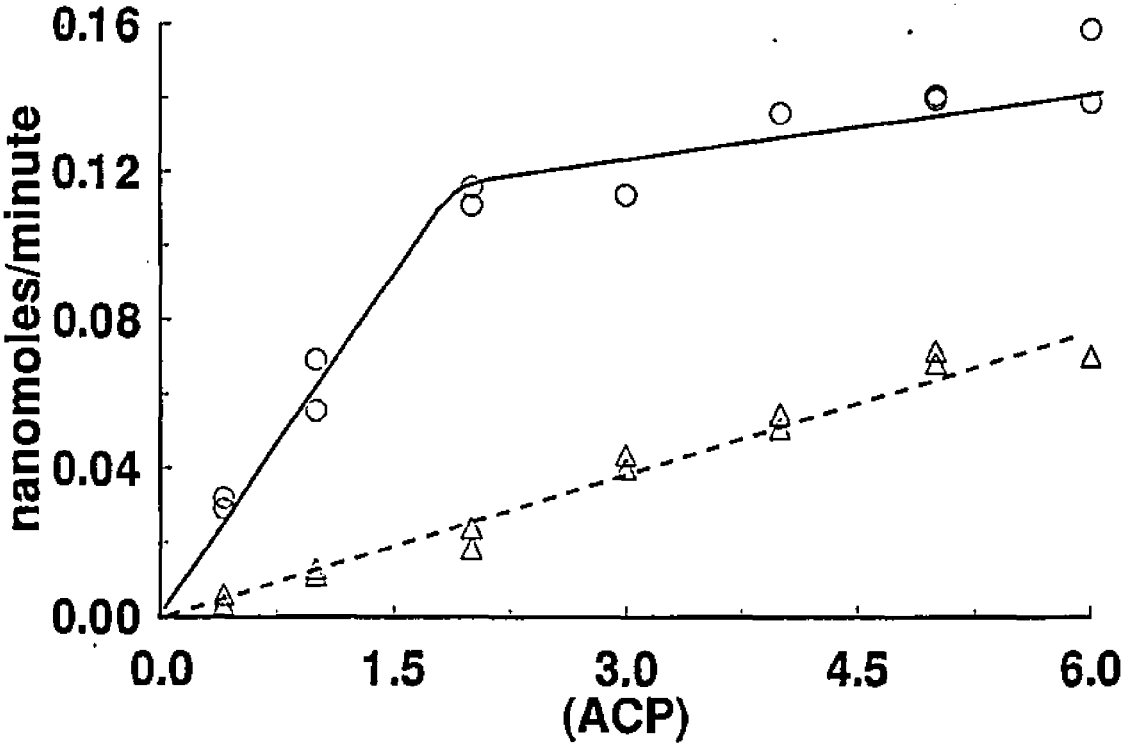

Figure 10. Rates of Fatty Acid Biosynthesis Catalyzed by the Euglena Nonaggregated FAS With Different ACPs. The assay technique is described in the Methods. Circles are rates where Euglena ACP was the substrate while triangles are rates where E. coli ACP was the substrate. ACP concentration in μmoles. A unit of activity is defined as the amount of ACP required for the incorporation of 1 nmol of [2-^{14}C]malonyl-CoA into fatty acid per minute under the defined assay conditions.

efficient of the two. The reaction rates in assays with the Euglena protein approach zero order at concentrations at which the E. coli protein did not evidence saturation.

Antibodies Raised Against Euglena ACP

Anti-Euglena ACP antibodies were detected 6 weeks after immunization of hens using dot blots of Euglena ACP . The anti-Euglena ACP antibodies were shown to cross-react with E. coli ACP in two ways. Anti-Euglena ACP antibodies, used as the primary antibody on Western blots, cross-reacted with E. coli ACP (data not shown). Anti-Euglena ACP antibodies inhibited Euglena chloroplast FAS when E. coli ACP was used as the substrate carrier (data not shown). The antibodies also inhibited the Euglena chloroplast FAS when Euglena ACP was used as the ACP source (data not shown).

ACP Interaction with Melittin

Interaction of melittin with EF hand Ca^{++} binding proteins has been a useful model for studying the interaction of Ca^{++} binding proteins and their respective physiologically interactive proteins. Parallels between ACP and EF hand Ca^{++} binding proteins prompted the investigation of the possibility of using the complexing of ACP with melittin as a model for studying the interaction of ACP and enzymes of the FAS enzyme system. Ernst-Fonberg et al. (1990) demonstrated that ACP and melittin interact. If the melittin-ACP model is applicable to the interaction of ACP

with the proteins of the FAS enzyme complex, it is possible that melittin will have an affect on activity of the nonaggregated, ACP dependent, FAS enzyme system.

Effects of Melittin on Biological Activity

Relevancy of the protein-protein interaction model of melittin binding ACP as a tool for investigating the binding of ACP with nonaggregated FAS proteins was investigated. The first step was to see if melittin bore any structural resemblance to any Euglena proteins. Membranous and soluble fractions of greened Euglena were Western blotted and probed with anti-Apis melifera antibodies, revealing 2 reactive bands in the soluble proteins and 4 reactive bands in the membrane proteins (Figure 11). The 4 membrane peptides had estimated Mr values of 60,300, 36,900, 27,800, and 17,400 while those of the soluble peptides had a Mr of 60,300 and 16,400.

If the ACP-melittin model is applicable to physiological ACP-protein interaction, it is possible that the presence of melittin may have an effect on FAS activity. The effects of melittin on the activity of the Euglena chloroplast FAS using E. coli ACP or Euglena ACP, as the ACP source, were explored (Figure 12). Lysozyme, another cationic protein, was used instead of melittin as a control. All assays were done in the presence of 0.15 M NaCl to minimize nonspecific ionic interaction. The presence of melittin inhibited fatty acid biosynthesis whereas

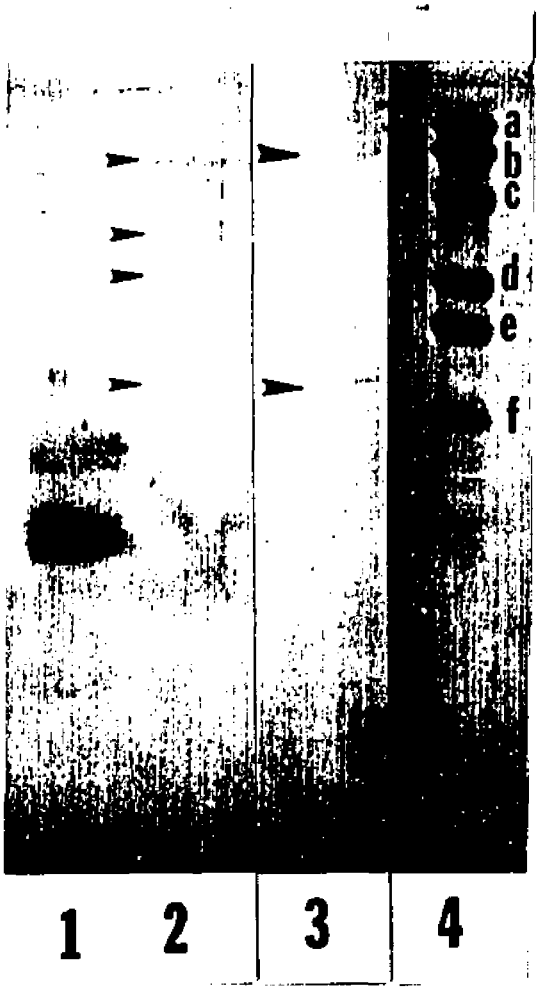

FIGURE II

Figure 11. Western Blot of Euglena Membrane and Soluble Proteins. Proteins (90 μg) were electrophoresed on a denaturing 10-20% gradient polyacrylamide gel and transferred to 0.2 μm nitrocellulose. Anti-melittin and horseradish peroxidase conjugated goat anti-rabbit antibodies were used to probe for anti-melittin antibody-reactive proteins. Lane 1, melittin; lane 2, membrane fraction; lane 3, soluble fraction; and lane 4, prestained standards. Standard Mrs were as follows: a) bovine serum albumin (75,000), b) ovalbumin (50,000), c) carbonic anhydrase (39,000), d) soybean trypsin inhibitor (27,000), and e) lysozyme (17,000).

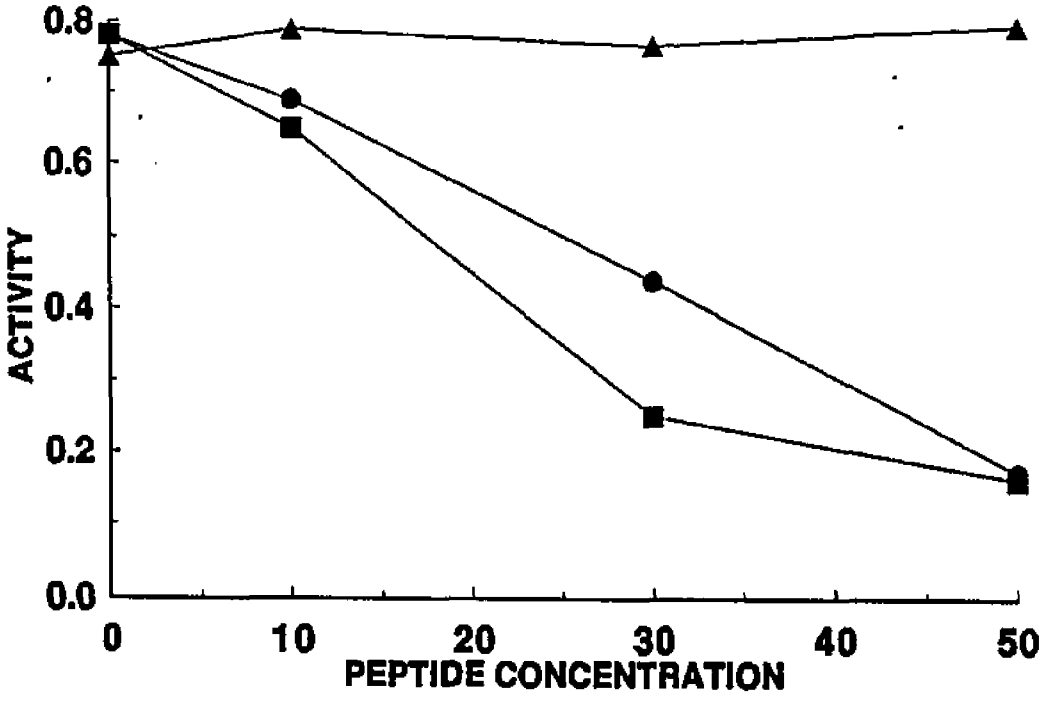

Figure 12. Inhibition of Euglena Nonaggregatted FAS by Melittin. Euglena nonaggregated FAS was assayed with E. coli ACP or Euglena ACP as a substrate according to assay conditions described in Methods with the following exceptions: 1) Either melittin or lysozyme was added to the assays in the concentrations shown. 2) All assays were adjusted to an ionic strength of 0.15 M with NaCl in order to prevent nonspecific interactions between anionic ACP and cationic peptides. 3) After the addition of all components to the assay except malonyl CoA, the assays were incubated 15 minutes at 4°C before the reaction was initiated. Enzyme activity is nmoles of [2-^{14}C] malonyl-CoA incorporated into long chain fatty acids. Peptide concentration is μM. Lysozyme containing assays are shown by triangles and melittin containing assays are shown by squares and circles (■ assays with Euglena ACP; ●, assays with E. coli ACP).

comparable amounts of lysozyme showed no inhibition. Identical results (no inhibition) were obtained when cytochrome C, another cationic peptide, was used as the control peptide (data not shown). FAS activity was inhibited in the presence of melittin regardless of whether Euglena or E. coli ACP was used as the substrate carrier. Melittin also inhibited activity of the Euglena aggregated FAS (Figure 13). Inclusion of 50 μM melittin inhibited aggregated FAS enzyme activity 75% as compared to the control peptide, protamine. The lack of inhibition by the other basic peptides suggested that the effect of melittin was specific and not nonspecific ionic binding between the anionic ACP and the cationic peptides.

The Effect of Anti-Melittin Antibodies on Biological Activity

If ACP-melittin binding occurs because melittin mimics a portion of the binding site for ACP on one or more of the FAS enzymes, anti-melittin antibodies might react with the "effected" enzyme. The effect of increasing concentrations of anti-melittin antibodies added to Euglena nonaggregated FAS assays using either Euglena ACP or E. coli ACP was examined (Figure 14). Assays were performed in the absence of dithiothreitol to avoid structural damage to the antibodies; for this reason, the control FAS activity was about half of that seen in the assays shown in Figure 13. Control antibodies were isolated from eggs collected before

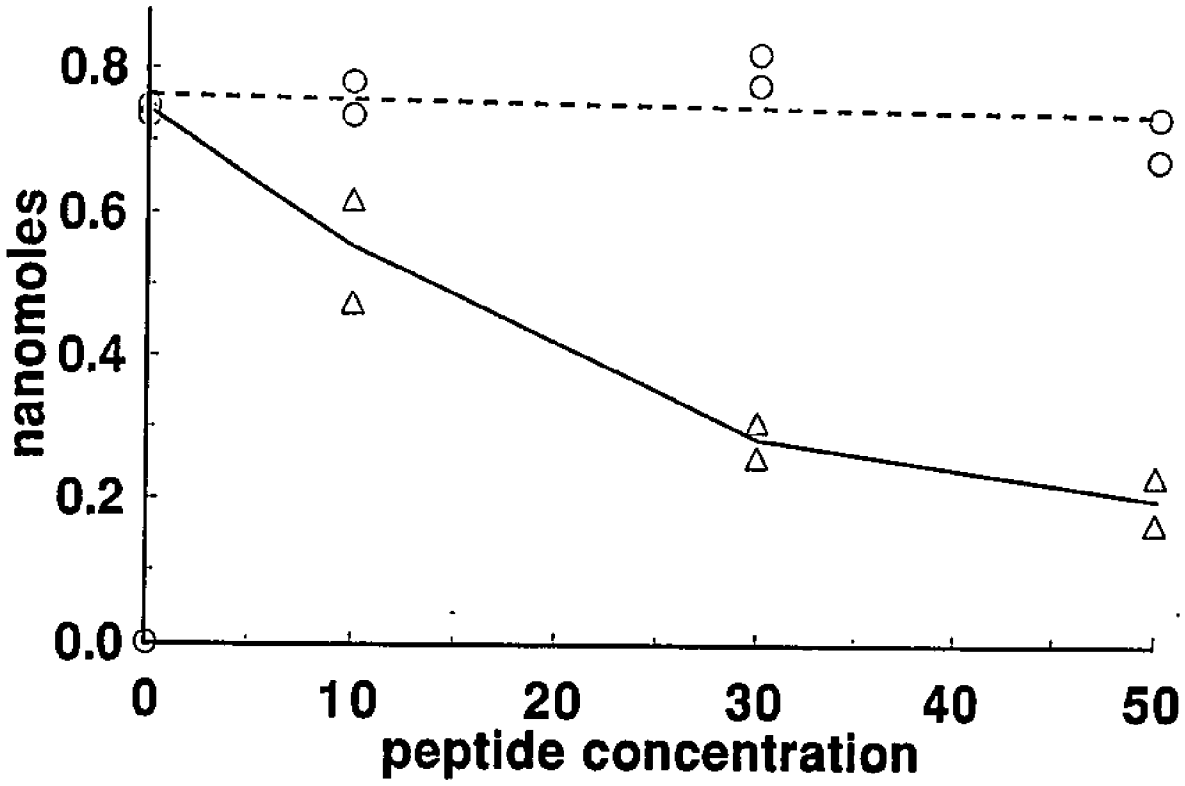

Figure 13. Effects of Melittin on Aggregated FAS Activity. Assays were performed as described in Methods. Melittin was allowed to incubate with Euglena FAS for 15 minutes on ice. The reaction was initiated by the addition of [2-^{14}C]malonyl-CoA. Each assay contained 30 μg of FAS. Peptides concentration is μmoles. Activity is defined as nmole [2-^{14}C] incorporated into long chain fatty acids. The circles show results of assays containing protamine; triangles show results of assays that contained melittin.

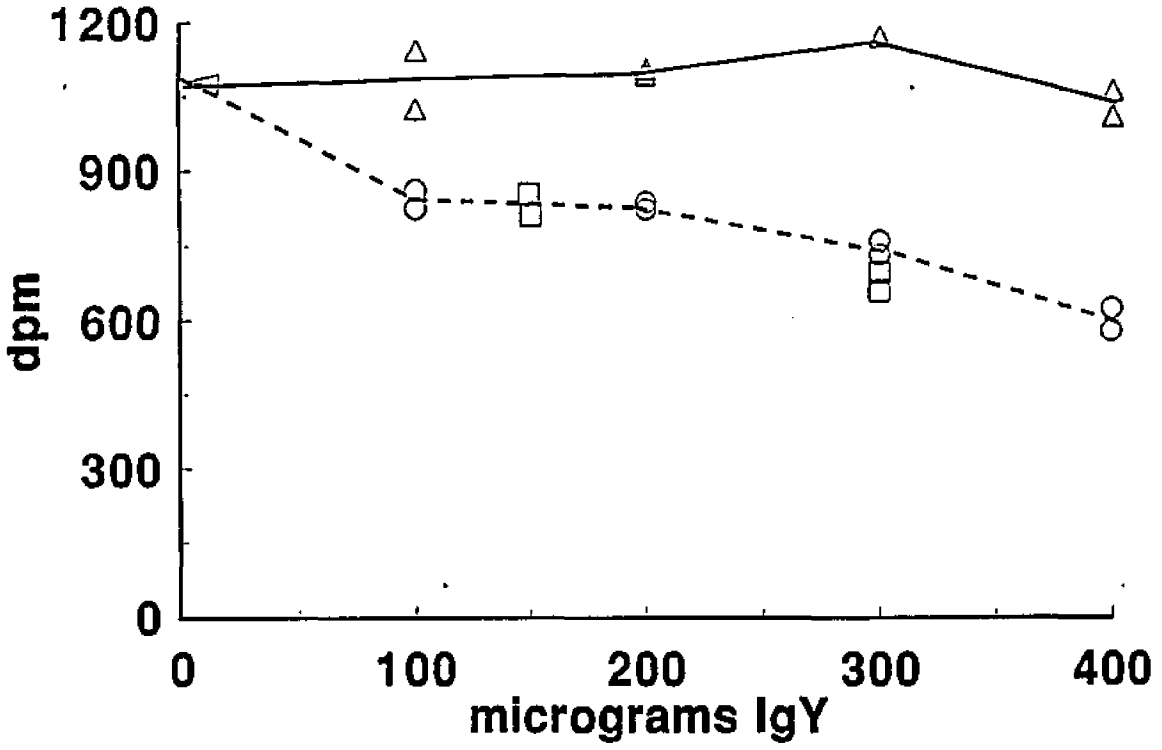

Figure 14. Inhibition of Euglena Nonaggregated FAS by Anti-Melittin Antibodies. Euglena nonaggregated FAS was assayed with either E. coli ACP or Euglena ACP as a substrate. Assays were performed as described in Methods with the following exceptions: (1) dithiothreitol was omitted from assays. (2) After the addition of all components to assays except malonyl-CoA, assays were incubated for 15 minutes at 4°C before initiating the reaction by the addition of malonyl-CoA. Enzyme activity is dpm of [2-^{14}C] malonyl-CoA incorporated into long chain fatty acids. Assays that contained none or normal immunoglobulin are designated by triangles. Assays the contained anti-melittin immunoglobulin are designated by circles and squares (□, assays with Euglena ACP; O, assays with E. coli ACP.

immunization. Anti-melittin antibodies inhibited nonaggregated FAS activity compared to assays with equal amounts of control antibody. FAS activity was inhibited regardless of the ACP source. The effect of increasing amounts of anti-melittin antibodies on Euglena aggregated FAS activity was also examined (Figure 15). Like the nonaggregated FAS, the aggregated FAS was inhibited by the presence of anti-melittin antibodies. Lack of inhibition by control antibodies suggests, that the effect of anti-melittin antibodies was a specific interaction with at least a portion of the FAS enzyme system.

In order to better define the interaction of anti-melittin antibodies with Euglena nonaggregated FAS proteins, the effect of these antibodies on individual enzyme activities was examined. Increasing amounts of anti-melittin antibodies were included in assays of acetyl-CoA-ACP transacylase, malonyl-CoA-ACP transacylase, ß-ketoacyl-ACP synthetase, and enoyl-ACP reductase. The two transacylase activities were inhibited by anti-melittin antibodies (Figures 16 and 17). Inclusion of 750 μg of hen antibody (62:1 antibody:protein) resulted in 23% inhibition of malonyl-CoA-ACP transacylase activity and 30% inhibition of acetyl-CoA-ACP transacylase activity. Addition of the same amount of anti-melittin antibodies to ß-ketoacyl-ACP synthetase assays resulted in 92% inhibition of enzyme activity (Figure 18). Enoyl-ACP reductase and

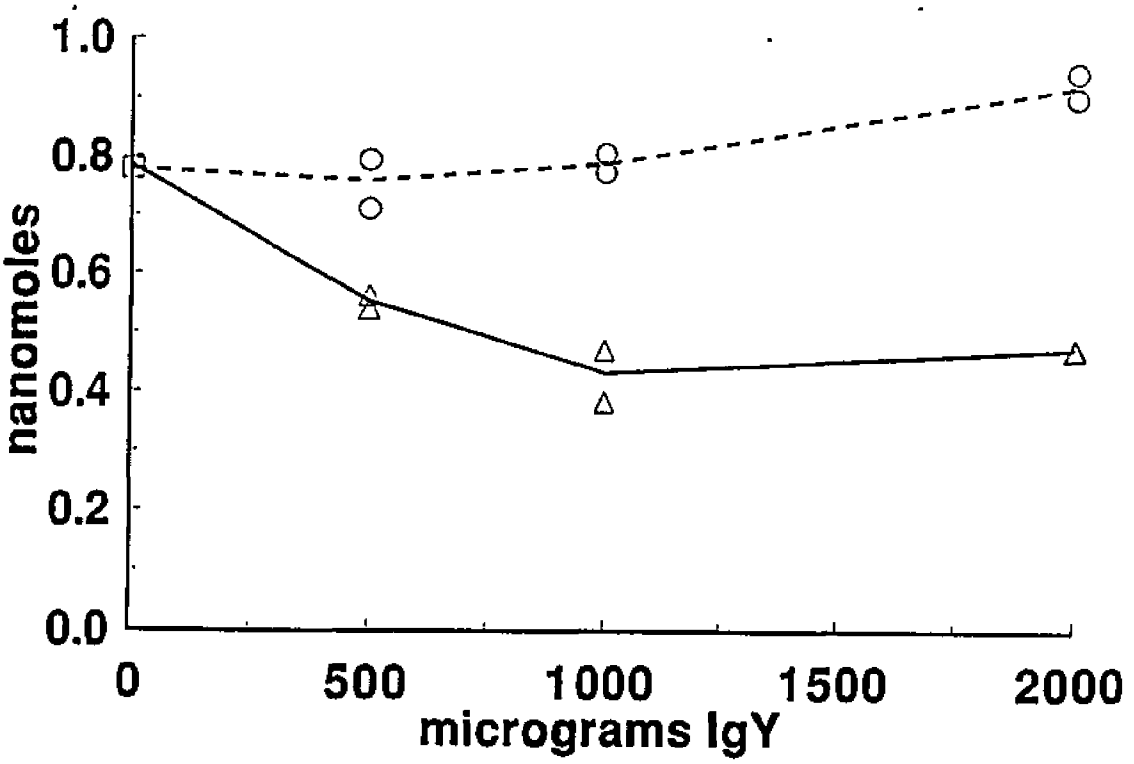

Figure 15. Effects of Anti-Melittin Antibodies on Euglena Aggregated FAS Activity. Aggregated FAS assays were done as described in Methods with the following exceptions: 1) Dithiothreitol was omitted from the assays. 2) After the addition of all components to assays except malonyl-CoA, assays were incubated for 15 minutes at 4°C before reaction initiation. Enzyme activity is incorporation of [2-^{14}C]malonyl-CoA into long chain fatty acids. Circles denote results of assays that contained normal IgY. Triangles denote results of assays that contained anti-melittin antibodies.

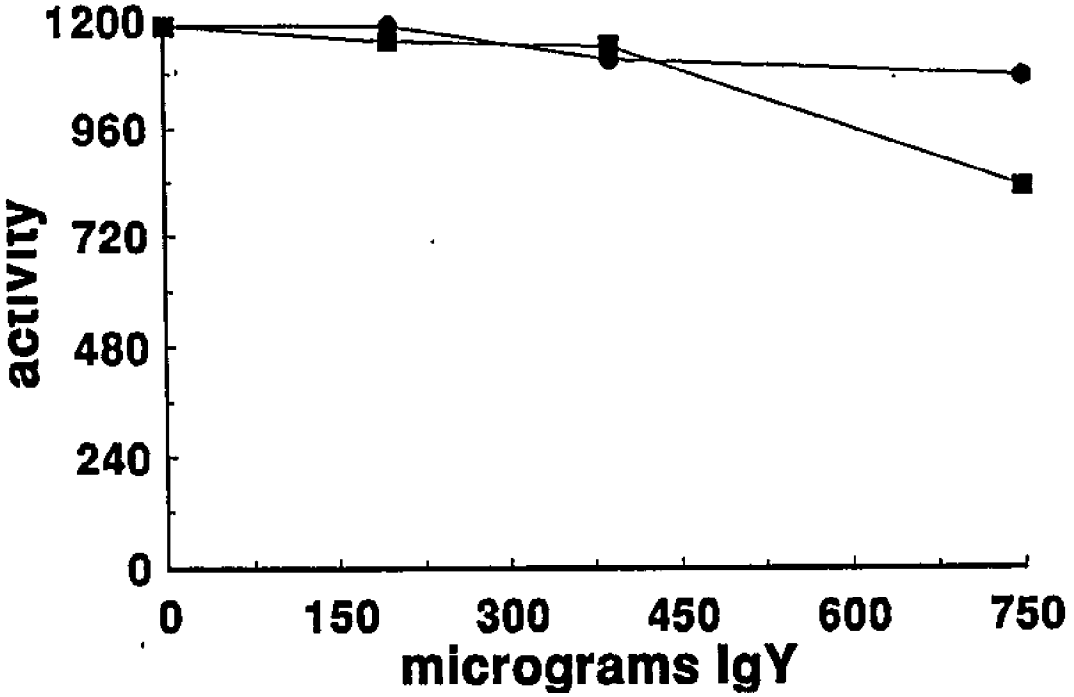

Figure 16. The Effect of Anti-Melittin Antibodies on Acetyl-CoA-ACP Transacylase Activity from Euglena Chloroplast FAS. Acetyl-CoA-ACP transacylase assays were done as follows: 1) Total assay volume was 100 µl. 2) All assays were done in 0.1 M TES pH 8.0 without dithiothréitol. 3) FAS (12 µg) and antibody were incubated together for 12 minutes, on ice. After the incubation period, 10 µl of 1 mmol [2-^{14}C]acetyl-CoA (with a specific activity of 1) was added to each assay. The reaction was initiated with 20 µg of ACP and was allowed to proceed for 5 minutes at 35°C. The reaction was stopped by TCA precipitation of proteins. Enzyme activity is dpm incorporated from [2-^{14}C]acetyl-CoA into acetyl-ACP. The squares represent results of assays that contained anti-melittin antibodies and the circles represent assays that contained normal antibodies.

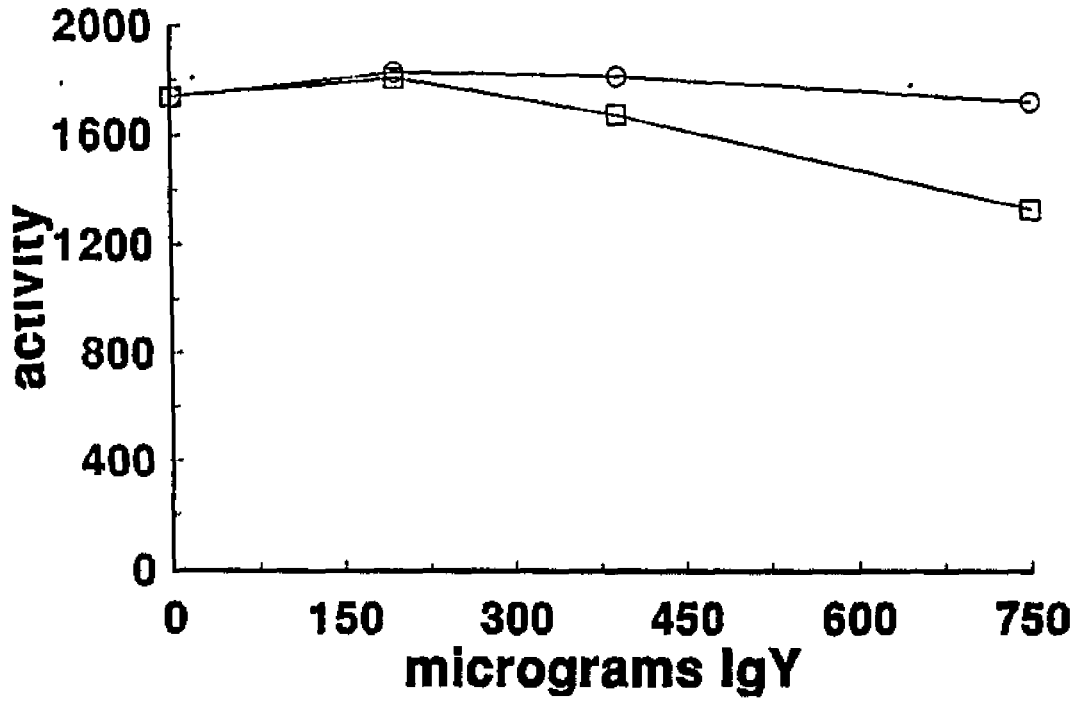

Figure 17. Effect of Anti-Melittin Antibodies on Malonyl CoA-ACP Transacylase Activity from Euglena Chloroplast FAS. Malonyl-CoA-ACP transacylase assays were done as follows. 1) The total assay volume was 100 µl. 2) All assays were done in 0.1 M TES pH 8.0 without dithiothreitol. 3) FAS (10 µg) was incubated with antibody on ice, for 12 minutes. The reaction was initiated by the addition of 3 µl of 3mmol [2-^{14}C]malonyl-CoA (with a specific activity of 1) and 20 µg ACP and was allowed to continue for 1 minute at 35°C. The reaction was stopped by TCA precipitation of the proteins. Enzyme activity is dpm incorporated from [2-^{14}C] malonyl-CoA into malonyl-ACP. The squares represent results of assays that contained anti-melittin antibodies and the circles represent assays that contained normal antibodies.

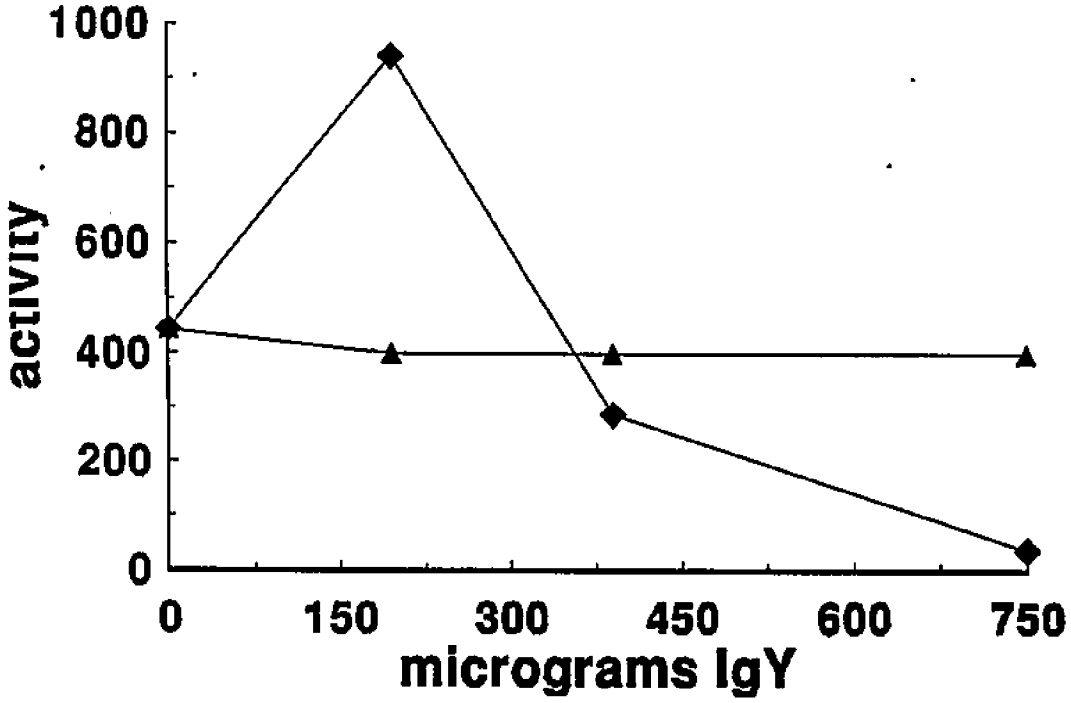

Figure 18. Effect of Anti-Melittin Antibodies on ß-Keto-Acyl-ACP Synthetase Activity from Euglena Chloroplast FAS. ß-keto-acyl synthetase assays were done as follows: 1) The total assay volume was 250 µl. 2) All assays were done in 0.1 M TES pH 8.0 without dithiothreitol. 3) FAS (30 µg) was incubated with antibody on ice, for 12 minutes. The reaction was started by the addition of 5 µl of '3 mmol [2-^{14}C]malonyl-CoA (with a specific activity of 1) and 20 µg of hexanoyl-ACP. The reaction was allowed to continue for 5 minutes at 35°C. The reaction was stopped by acidification, followed by saponification and pentane extraction as with the FAS assayed described in the Methods. Enzyme activity is incorporation of [2-^{14}C] malonyl-CoA into octonyl-ACP. The squares represent results of assays that contained anti-melittin antibodies and the circles represent assays that contained normal antibody.

ß-ketoacyl-ACP reductase activities were not inhibited by anti-melittin antibodies (data not shown). None of the enzymes showed inhibition by increasing amounts of control antibodies. Results are summarized in Table 6. It is important to note that the enzyme preparations used for this study were fairly impure, thus the native proportion of the enzymes were relatively undisturbed.

Table 6

Effect of anti-Melittin Antibodies on the Activity of ACP-Dependent Fatty Acid Synthetase From Euglena Gracilis

Enzyme	% Inhibition
Acetyl transacylase	30
Malonyl transacylase	32
Condensing enzyme	92
Acetoacetyl-ACP reductase*	0
Enoyl-ACP reductase**	0

*This enzyme was assayed with acetoacetyl CoA substrate

**This enzyme was assayed with crotonyl CoA substrate

CHAPTER 4

Discussion

Purification and Characterization of Enoyl-ACP Reductase

The enzyme responsible for catalyzing the last step of fatty acid biosynthesis is enoyl-ACP reductase. This enzyme catalyzes the reduction of an α,ß-unsaturated acyl-ACP to the saturated derivative using NADH or NADPH as the electron donor. An enoyl-ACP reductase was isolated from Euglena gracilis variety bacillaris grown in the light.

Euglena grown in the light possess a nonaggregated FAS enzyme system whose expression is dependent on chloroplast ribosomal protein synthesis and is located in the chloroplast (Ernst-Fonberg et al., 1974; Worsham et al, 1988). While the nonaggregated FAS is prominent in light grown cells, an aggregated, multienzyme complex, FAS enzyme system is also present in the cytosol (Worsham et al., 1986). In addition, two other enzyme systems for de novo fatty acid biosynthesis may occur in light grown Euglena, a microsomal FAS system found tightly associated with the membrane (Kahn and Kolattukudy, 1975) and a mitochondrial FAS system responsible for synthesizing long chain fatty acids for wax ester fermentation during anaerobiosis (Inui et al., 1982; Inui et al., 1984).

Enoyl-ACP reductase was purified from 135 g (wet weight) of Euglena, yielding 100 μg of enzyme. The enzyme

was purified by a combination of chromatographic steps including anion exchange, size exclusion, calcium phosphate, and ligand affinity. The enzyme was characterized with respect to estimation of Mr, substrate specificity, pyridine nucleotide requirement, and the effects of E. coli ACP and Ca^{++} on activity.

Of the nonaggregated FAS enoyl-ACP reductases isolated from different sources, the Mr of active enzyme varies. Size exclusion chromatography and denaturing polyacrylamide gel analysis of the Euglena enoyl-ACP reductase gave an estimated Mr of 39,800 and revealed that the native enzyme was a monomer. The enzyme size is closest to that of avocado, Mr 62,400 (Caughey and Kekwick, 1982) and rape seed, Mr 68,400 (Slabas et al., 1986). Other reductases of this type, are higher in Mr and include E. coli, Mr 90,000 (Weeks and Wakil, 1968), safflower, Mr 83,000 (Shimakata and Stumpf, 1982), and spinach Mr 115,000 (Shimakata and Stumpf, 1982). It is interesting to note that the spinach and rape seed reductases are multimers composed of subunits ranging from 32,500-34,800 daltons.

Another variation among enoyl-ACP reductases is the pyridine nucleotide requirement. The Euglena enoyl-ACP reductase was able to use NADH, NADPH, or a combination of the two nucleotides as an electron donor. The enzyme preferred NADH. The nonspecificity of the reductase towards the pyridine nucleotide suggests two possibilities. Either

there is one enzyme that is not specific for reduced pyridine nucleotides, or there are two distinct enzymes present in the preparation. There is probably only one enzyme present because when NADH and NADPH were used in combination as electron donors, enzyme activity was less than the sum of the enzyme activities observed with comparable amounts of the individual nucleotides. If there were two enzymes present one would expect the sum of the activity with NADH or NADPH to be equal or greater than the activity seen when comparable concentrations of NADH and NADPH were both present. However, since the sum of the activities was less, this suggests the nucleotides maybe competing for the same site.

In addition to nucleotide specificity, enoyl-ACP reductases vary in other substrate specificities. Although ACP is the substrate carrier used for fatty acid biosynthesis *in vivo*, enoyl-ACP reductases can often reduce CoA-bound substrates *in vitro*. This enoyl-ACP reductase was able to reduce crotonyl-CoA and crotonyl-ACP. Although kinetic constants were not measured, the enzyme reduced crotonyl-ACP about 20X faster than comparable concentrations of crotonyl-CoA. Substrate preference was further defined by the ability of *E. coli* ACP to inhibit enzyme activity when it was measured as a function of the rate of reduction of crotonyl-CoA (figure 7). Probably inhibition was due to ACP somehow blocking the binding site for the CoA-bound

substrate. It is interesting to note that although enzyme activity was inhibited regardless of the pyridine nucleotide used, when NADH was the electron donor, 9 μM ACP inhibited reductase activity 70%. When NADPH was used, 18 μM ACP was needed to achieve the same percent inhibition. It is likely that the binding of one substrate affects the binding of another. Perhaps NADPH causes some stearic hinderance due to charge or size differences and thus somehow interferes with the binding of ACP.

The responsiveness of some proteins to Ca^{++} is a well studied area. It is known Ca^{++} can induce conformational changes in Ca^{++} binding proteins, including ACP (Schulz, 1969), which can result in modification of biological activity. The presence of Ca^{++} moderately affected the activity of the Euglena reductase. The addition of 10 μM Ca^{++} to enoyl-ACP reductase assays using crotonyl-CoA, resulted in an increase in activity to 137%. While μM concentrations of Ca^{++} stimulated activity, mM concentrations of Ca^{++} were inhibitory. Tucker (1990) found that when comparable amounts of Mg^{++} were present in assays, the Mg^{++} had no effect on enzyme activity at comparable concentrations. We have demonstrated a similar effect of Ca^{++} on the activity of the Euglena nonaggregated FAS (data not published). When 500 μM Ca^{++} was present in nonaggregated FAS assays fatty acid biosynthesis was stimulated; however, when the concentration of Ca^{++} in

assays was increased, biosynthetic activity was inhibited. The addition of 10 mM Ca^{++} to nonaggregated FAS assays inhibited activity up to 30%. As seen with the reductase alone, the addition of comparable amounts of Mg^{++} to FAS assays had no effect on fatty acid biosynthesis. If the effect of Ca^{++} was due to nonspecific ionic effects, it is probable that Mg^{++} would have had the same effect, therefore the effect of Ca^{++} was more likely ion specific. While the mechanism of response of the Euglena nonaggregated FAS or the enoyl-ACP reductase to the Ca^{++} is not known, the ion specificity suggests protein conformation change rather than secondary Ca^{++} effects (Alken and Rasmussen, 1988). Interestingly, the inhibitory effect of Ca^{++} was greater with the reductase alone than that seen with the nonaggregated FAS enzyme system. One possible explanation for this difference is that transient interactions among the proteins of the nonaggregated FAS may in some way shield the enoyl-ACP reductase from interaction with Ca^{++}.

During purification of the enzyme, calcium phosphate gel resulted in the separation of two pools of enzyme activity able to reduce crotonyl-CoA. Pool A did not bind to the column in 10 mM phosphate buffer and pool B bound to the column in 10 mM phosphate buffer. Although isoforms of enoyl-ACP reductase have been described in other nonaggregated FAS enzyme systems (E. coli, Weeks and Wakil, 1968; safflower, Shimakata and Stumpf, 1982; rape seed,

Slabas et al., 1982), the existence of isoforms of enoyl-ACP reductase from the chloroplast FAS system in Euglena has not been documented. The two pools differed in some significant characteristics. Pool B was chosen for further purification because its characteristics suggested an involvement in fatty acid biosynthesis. Pool B enzyme activity was inhibited by the presence of E. coli ACP in assays with crotonyl-CoA, stimulated by μM concentrations of Ca^{++} and inhibited by mM concentrations of Ca^{++} in assays with crotonyl-CoA, and pool B enzyme was more active with crotonyl-ACP than with crotonyl-CoA. Pool A did not demonstrate these characteristics. It is not likely that the pool B Euglena reductase is one of the mitochondrial enoyl-CoA reductases because the mitochondrial enzymes are most active with CoA substrates, can only use NADH as an electron donor, and are not affected by the presence of Ca^{++} in assays (Inui et al., 1984). The pool B reductase is not the enoyl-ACP reductase from the microsomal FAS enzyme system because those enzymes are tightly associated with the membrane and would not be found in the soluble fraction of the cells which was the source of this enzyme (Khan and Kolattukudy, 1975). The aggregated FAS, found in the cytosol, can be ruled out because the high molecular weight complex is resistant to dissociation and is removed during size exclusion chromatography early in the purification scheme. Tucker (1990) detected pool B reductase activity in

chloroplast preps. This is important because the nonaggregated FAS enzyme system is found in chloroplasts. Therefore, it is most likely that the enoyl-ACP reductase I have purified and characterized is from the Euglena nonaggregated FAS enzyme system.

Diverse data suggests that Euglena is an ancient organism. Biochemical studies supporting the ancient origin are summarized by Buetow (1989). Phytogenetic trees inferred from small-subunit rRNA gene sequences place Euglena among the earliest of eukaryotes (Sogin et al., 1986). In this scheme, the Euglena diverge long before the radiation of the different eukaryotic living forms that became higher plants, fungi, ciliates, and animals. Based on the evidence of the low Mr of the active form of the enzyme, and the ability of the enzyme to use NADH and NADPH as an electron donor it is possible to speculate that this enzyme could be an early form of the enoyl-ACP reductase and that it has evolved into the reductase of higher plants with greater substrate specificity and strict nucleotide requirements.

Purification and Characterization of Euglena ACP

ACP is a small, acidic protein responsible for carrying the growing substrate during fatty acid biosynthesis. The polypeptide contains a 4'-phosphopantetheine prosthetic group attached by a phosphodiester bond to a centrally located serine, and, in turn fatty acids are carried by a

thiolester linkage to the prosthetic group. The best characterized ACP is that from E. coli. E. coli ACP has been a useful substitute in studies of the Euglena gracilis variety bacillaris nonaggregated FAS enzyme system. Previously, minute quantities of ACP were isolated from a related strain of Euglena gracilis, strain Z (DiNello and Ernst-Fonberg, 1973). In the interest of obtaining larger amounts of the homologous ACP, a new purification procedure was developed for the purification of ACP from Euglena gracilis variety bacillaris with a more than 10 fold increase in yield.

Using the new procedure, about 1 mg of ACP was obtained from 100 g (wet weight) of light grown Euglena. The protein was greater than 90% pure after HPLC ion-exchange chromatography. Denaturing polyacrylamide gel analysis estimated the Mr of the protein to be about 8000. The pI of this protein was estimated to be 3.9 by HPLC chromatofocusing. Contrary to E. coli ACP, isoelectric precipitation of Euglena ACP resulted in activity loss.

The presence of anionic groups on Euglena ACP was verified by the fact that it stained deep blue on polyacrylamide gels with the cationic carbocyanine dye "Stains-all". Dye interaction with sialic acid residues, phosphoryl groups, or carboxylate groups, found in Ca^{++} binding sites will cause the protein to stain blue, while almost all other proteins stain pink or red (Campbell et

al., 1983). It is documented that E. coli ACP binds Ca^{++} (Schulz, 1977; Frederick and Prestegard, 1988), and the blue staining characteristic of Euglena ACP suggests it may also possess an anionic site capable of binding Ca^{++}.

Although fatty acid biosynthesis by the Euglena nonaggregated FAS goes readily with E. coli ACP, Euglena ACP was obviously preferred by the system. Perhaps this is not a surprising result considering that the homologous Euglena ACP is the native substrate carrier. However when E. coli ACP was assessed for its ability to support fatty acid biosynthesis with E. coli, spinach, and two different cyanobacterial nonaggregated FAS systems, the bacterial ACP was used equally as well as the native ACP of the respective FAS systems (Froehlich et al., 1990).

Despite amino acid sequence differences among ACPs, evidence supports conservation of primary structure. Conservation of amino acid sequence is found around the serine bound to the 4'-phosphopantetheine prosthetic group and in the N-terminal region. Table 7 was constructed by aligning serine residues which contained the prosthetic groups of numerous ACP sequences. The first 18 amino acids of Euglena ACP were aligned with matching amino acids of the conserved N-terminal region of the other ACPs. The Euglena segment showed the greatest identity with cyanobacterial ACP (57%), followed by two ACPs from Brassica (53%). Homology with other bacterial ACPs was less than that with higher

Table 7

Comparison of N-Terminal Amino Sequences of Different ACPs[a]

Source	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	% Identity
Euglena	S	?	A	G	A	S	P	E	L	F	E	K	V	R	S	I	V	I	
Synechocystis[b]					M	D	Q	E	I	F	E	K	V	K	K	I	V	V	50
Anabaena variabilis[b]					S	Q	S	E	T	F	E	K	V	K	K	I	V	I	57
Brassica campestris[c]				A	A	K	P	E	T	V	E	K	V	S	K	I	V	K	53
Brassica rapus[d]				A	A	K	P	E	T	V	E	K	V	S	K	I	V	K	53
Spinach I[e]					A	K	K	E	T	I	D	K	V	S	D	I	V	K	43
Spinach II[e]				A	A	K	P	E	M	V	T	K	V	S	D	I	V	K	47
Barley I[f]				Q	A	K	K	E	T	V	D	K	V	(C)	M	I	V	K	40
Barley II[f]					A	K	K	E	T	V	E	K	V	?	D	I	V	K	47
Arabidopsis Thaliana[g]				A	A	K	Q	E	T	I	E	K	V	S	A	I	V	K	47
Cryptomonas ϕ (plastid)[h]					N	E	Q	E	I	F	E	K	V	Q	T	I	I	S	43
Neurospora crassa[i]						K	D	E	V	F	S	R	I	A	E	V	L	S	36
Rhzobium melitoti[j]						M	S	D	I	A	E	R	V	K	K	I	V	V	31
Rhodobacter sphaeroides[k]							S	D	I	A	D	R	V	K	K	I	V	V	25
E. coli E15[l]							S	T	I	E	E	R	V	K	K	I	I	G	25
Streptomyces erythraeus[m]				M	D	R	K	E	I	F	E	R	I	E	Q	V	L	A	20
Cylindrotheca sp. Strain N1[n]						M	S	D	T	L	K	R	L	Q	K	I	V	S	16

Table 7 (Cont'd)

Comparison of N-Terminal Amino Sequences of Different ACPs[a]

[a]The alignment of the sequences is explained in the text

[b]Froehlich et al., 1990

[c]Rose et al., 1987

[d]Slabas et al., 1987

[e]Ohlrogge and Kuo, 1984

[f]Hoj and Svendson, 1984

[g]Post-Beittenmiller et al., 1989

[h]Wang and Liu, 1991

[i]Brody and Mikolajezyk, 1988

[j]Plah et al., 1990

[k]Cooper et al., 1987

[l]Vanaman et al., 1968

[m]Hale et al., 1987

[n]Huang and Tabita, 1991

plant ACPs. The N-terminal hexapeptide of E. coli ACP is essential for biological activity (Majerus, 1968). Also, according to the three dimensional structure predicted by Holak et al. (1988) from NMR, the portion of the structure represented in Table 5 includes one of the three of the major helices in which bound acyl groups are proposed to reside. Thus comparison of sequence in this region is not trivial.

ACP Interaction with Melittin

ACP occupies a pivotal position in fatty acid biosynthesis. Regardless of the molecular organization of the FAS enzyme system, ACP is the substrate carrier used in vivo. ACP, then by the nature of its physiologic function, must interact with several different proteins. Little is known about the interaction of ACP and proteins of the FAS enzyme system.

Melittin, a small basic peptide from bee venom, binds to several Ca^{++} binding proteins, including calmodulin. The binding of melittin to Ca^{++} binding proteins has been studied as a model of interaction between the Ca^{++} binding proteins and their respective physiologic interactive proteins. Parallels between calmodulin and ACP (mentioned in the introduction) suggest that perhaps the binding between ACP and melittin would be a useful model for studying the interaction of ACP with proteins of the nonaggregated FAS enzyme system from Euglena.

Interaction between E. coli ACP and melittin has been clearly demonstrated by Ernst-Fonberg et al. (1990). Evidence for E. coli ACP complexing with melittin included changes in the fluorescence (blue shift), anisotropy, and quenching constants of the tryptophan in melittin with the addition of ACP to melittin solutions. Chemical cross-linking with dimethyl suberimidate demonstrated that the interaction between E. coli ACP and melittin was specific. Another firm demonstration of the binding of ACP and melittin was the inhibition of nonaggregated FAS activity with the addition of melittin to assays. Activity of the chloroplast nonaggregated FAS is dependent upon the addition of extraneous ACP. It is possible that inhibition may stem from melittin binding ACP and thus removing an essential substrate. The degree of inhibition would then depend on the affinity of ACP for melittin or for the FAS enzymes, which in turn depends on binding constants and concentrations.

The melittin-binding model has been shown to be a valid model of protein interaction in some systems, but is it a relevant model for ACP interaction with Euglena nonaggregated FAS proteins? Western blots of membranous and soluble fractions of Euglena homogenates showed cross-reactivity of several peptides with anti-Apis mellifera venom antibodies. This suggested that a portion of some Euglena proteins bore structural resemblance to melittin.

Two of the peptides in the soluble fraction were identical in size to two peptides visualized with anti-FAS antibodies (Worsham, unpublished data). Although similarity in size does not equal identity, it does mean it is possible that the peptides are identical. Cross-reactivity of anti-melittin antibodies with calmodulin target proteins has been demonstrated in vertebrates (Kaetzel and Dedman, 1987). Perhaps it is not surprising that Euglena contains cross-reacting proteins since calmodulin, an evolutionarily highly conserved protein, has been isolated from Euglena (Kuzuicki et al., 1979).

E. coli ACP is a useful substitute for Euglena ACP in assays of the nonaggregated FAS enzyme system. Although the ACPs are not identical, they share regions of conserved sequence as well as common structural features, such as the 4' phosphopantetheine prosthetic group. Specific complexing of Euglena ACP and melittin has not been demonstrated. However, when Euglena ACP was used in FAS assays that included melittin, the enzyme inhibition seen was of a similar magnitude as seen with E. coli ACP. If inhibition of FAS activity was due to the complexing of melittin and ACP, this data then supports the hypothesis that Euglena ACP also interacts with melittin.

The E. coli ACP-melittin interaction has some characteristics resembling those described for the interaction of melittin with EF hand Ca^{++} binding proteins

and their respective target proteins. Another similarity between the two models, was the inhibition of nonaggregated FAS activity by anti-melittin antibodies. Anti-melittin antibodies identified a common binding domain among calmodulin acceptor proteins (Kaetzel and Dedman, 1987) and inhibited compliment lysis (Laine et al., 1988). Inhibition of compliment lysis by anti-melittin antibodies led to identification of regions of sequence homology between melittin and human complement factor C9. It is possible that inhibition of FAS activity by anti-melittin antibodies was due to recognition of a the binding site for ACP on one or more of its target proteins, the enzymes of the nonaggregated FAS complex.

Closer examination of the effect of melittin antibodies on the activities of some of the component nonaggregated FAS enzymes revealed that at least three of the individual enzyme activities were affected. At the highest concentration of antibody used, activities of the acetyl-CoA-ACP transacylase, malonyl-CoA-ACP transacylase, and ß-ketoacyl-ACP synthetase were specifically inhibited by anti-melittin antibodies 30%, 23%, and 92% respectively. Interestingly, the two reductase activities were not affected by the anti-melittin antibodies, nor did melittin protect against inhibition by ACP observed for these enzymes when they were assayed with CoA-bound substrates. If melittin mimics the structure of the ACP binding site, the

differential effect of anti-melittin antibodies on the component enzymes of the FAS suggests that differences might exist among the enzymes of the nonaggregated FAS with regard to the structure of the binding site for ACP.

The relationship between the two de novo FAS enzyme systems in Euglena is not clear. They are immunologically cross-reactive but not identical. They are also influenced differently by inhibitors of protein biosynthesis (Ernst-Fonberg et al., 1974). Their structures are vastly different with the multienzyme complex tightly associated at a Mr of 6,000,000 while the nonaggregated system of enzymes and ACP can be isolated as individual proteins with Mrs well below 100,000 (Worsham et al., 1986; Worsham et al., 1988; Williams et al., 1991). Despite these differences both FAS systems contain ACP (Walker et al., 1981). It is possible that the binding strategy of ACP and FAS enzymes may be conserved in both enzyme systems. This hypothesis is supported by the fact that the addition of melittin and anti-melittin antibodies to aggregated FAS assays resulted in inhibitions similar to those seen with the nonaggregated FAS system upon comparable treatment. It is probable that like E. coli ACP, the ACP moiety of the Euglena multienzyme complex binds melittin and that one or more of the component enzymes contains a site recognized by the anti-melittin antibodies.

It is a recognized motif in biological systems that structurally similar protein domains may be used in diverse proteins for related as well as unrelated functions. Melittin appears to mimic a binding strategy that is a recurrent motif in a number of systems that depend upon protein-protein recognition. Although the nonaggregated FAS enzymes appear to have differences in ACP binding sites, this model is potentially a valuable tool for clarifying the binding sites for ACP on as many as three of the nonaggregated FAS enzymes.

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APPENDIX

Data Table 1

Hydroxylapatite Chromatography of Enoyl-ACP Reductase

Frac. #	A_{280}	Act. (units/100 µl)
1	0.00	0.00
2	0.00	0.00
3	0.00	0.00
4	0.00	0.00
5	0.00	0.00
6	0.00	0.00
7	0.00	0.00
8	0.00	0.00
9	0.00	0.00
10	0.00	0.00
11	0.00	0.00
12	0.00	0.00
13	0.00	0.00
14	0.08	31.30
15	1.35	19.20
16	1.43	13.60
17	1.52	10.80
18	0.86	8.00
19	0.30	0.00
20	0.19	0.00
21	0.00	0.00

Data Table 2

Matrex Orange Chromatography of Enoyl-ACP Reductase

Frac. #	A_{280}	Act. (units/100 µl)
1	0.00	0.00
2	0.00	0.00
3	0.00	0.00
4	0.00	0.00
5	0.00	0.00
6	0.00	0.00
7	0.00	0.00
8	0.00	0.00
9	0.00	0.00
10	0.02	0.00
11	0.13	16.20
12	0.17	9.70
13	0.09	3.70
14	0.05	0.00
15	0.03	0.00
16	0.00	0.00

Data Table 3

ACP Affinity Chromatography of Enoyl-ACP Reductase

Frac. #	A_{280}	Act. (units/100 µl)
1	0.00	0.00
2	0.00	0.00
3	0.00	0.00
4	0.00	0.00
5	0.00	0.00
6	0.00	0.00
7	0.00	0.00
8	0.00	0.00
9	0.00	0.00
10	0.00	0.00
11	0.00	0.00
12	0.00	0.00
13	0.00	0.00
14	0.03	0.00
15	0.04	0.00
16	0.07	0.00
17	0.04	0.00
18	0.07	0.00
19	0.08	0.00
20	0.08	6.10
21	0.13	7.30
22	0.15	11.40
23	0.13	12.90
24	0.10	11.40
25	0.09	7.30
26	0.06	0.00
27	0.04	0.00
28	0.00	0.00

Data Table 4

Enoyl-ACP Reductase Nucleotide Requirement

Nucleotide Concentration	Activity (units/100 µl)		Nucleotide
0.062 mM	10.8	12.1	NADPH
	24.2	26.1	NADH
0.125 mM	18.4	22.7	NADPH
	32.8	33.4	NADH
	24.8	25.9	NADH + NADPH
0.250 mM	12.7	13.2	NADPH
	35.6	37.6	NADH
	19.4	27.2	NADH + NADPH

Data Table 5

Effect of *E. coli* ACP on Enoyl-ACP Reductase

Concentration ACP	Act. with NADH (units/ 100 µl)	% Inhibition	Act. with NADPH (units/ 100 µl)	% Inhibition
0.0	14.3	0	-	
	15.0		-	
2.0 µM	6.5	47	10.1	34
	7.1		9.3	
4.5 µM	4.3	67.5	8.3	46
	5.3		8.3	
9.0 µM	4.8	71	5.1	64
	3.9		5.4	

Data Table 6

HBQ Chromatography of Euglena-ACP

Frac. #	A_{280}	DPM
1	0.00	0
3	0.00	0
5	0.00	0
7	0.00	0
9	0.00	0
11	0.01	0
12	0.30	135
13	0.00	162
14	0.92	106
15	0.81	2029
16	1.27	1228
17	2.00	481
18	2.00	0
19	2.00	0
21	2.00	0
23	1.60	0
25	0.77	0
27	0.36	0
29	0.25	0

Data Table 7

Rates of fatty Acid Biosynthesis

Catalyzed by the Euglena Nonaggregated FAS

With Different ACP's

ACP Concentration (μMol)	Act. with *E. coli* ACP (nmol/min)	Act. with Euglena ACP (nml/min)
0.4	0.0060	0.010
	0.0034	0.014
1.0	0.0116	0.055
	0.0134	0.069
2.0	0.0193	0.111
	0.0251	0.116
3.0	0.0421	0.114
	0.0460	
4.0	0.0577	0.136
	0.0532	
5.0	0.0758	0.140
	0.0723	0.141
6.0	0.0742	0.139
	0.0740	0.159

Data Table 8

Inhibitin of Euglena Nonaggregated FAS by Melittin

Peptide Concentration (μM)	Act. with E. coli ACP (nmol/min)	Act. with Euglena ACP (nmol/min)	Control Peptide Act with Euglena ACP (nmol/min)
10	0.69	0.65	0.80
	0.67	0.66	0.75
30	0.56	0.25	0.79
	0.57	.022	0/80
50	0.22	0.16	0.75
	0.19	0.11	0.78

Data Table 9

Effect of Melittin on Aggregated FAS

Peptide Concentration	Activity with Melittin Act. (nmol/min)	Activity with Prolamine Act. (nmol/min)
10	0.64	0.76
	0.50	0.79
30	0.35	0.80
	0.28	0.82
50	0.27	0.78
	0.20	0.75

Data Table 10

Inhibition of *Euglena* Nonaggregated FAS by Anti-Melittin Antibodies

µg IgY	DPM with Anti Melittin IgY		DPM with Control IgY
	With *E. coli* ACP	With Euglena ACP	
100	880		1000
	830		1100
150		871	
		825	
200	865		1075
	858		1085
300	725	670	1150
	701	635	
400	630		1075
	590		1025

Data Table 11

Effect of Anti-Melittin Antibodies on

Euglena Aggregated FAS activity

µg IgY	Act. (nmol/min) Control IgY	Act. (nmol/min) Anti-Melittin IgY
500	0.82	0.565
	0.72	0.535
1000	0.80	0.450
	0.81	0.390
2000	0.92	0.480
	0.95	

Data Table 12

The Effect of Anti-Melittin Antibodies on Acetyl-CoA-ACP Transacylase Activity from *Euglena* Chloroplast FAS

µg IgY	Ratio µg/IgY/µg Protein	DPM with Control IgY	DPM with Anti-Melittin IgY
195	16	1197	1127
		1185	1187
390	32	1108	1025
		1126	1069
750	62	1085	833
		1080	838

Data Table 13

Effect of Anti-Melittin Antibodies on

Malonyl-CoA-ACP Transacylase Activity From

Euglena Chloroplast FAS

μg IgY	Ratio μg IgY/μg Protein	DPM with Anti-Melittin IgY	DPM with Control IgY
195	16	1767	1858
		1858	1808
390	32	1827	1619
		1814	1740
750	62	1747	1309
		1721	1372

Data Table 14

Effect of Anti-Melittin Antibodies on

ß-Keto-Acyl-ACP Synthase Activity From

Euglena Chloroplast FAS

µg IgY	Ratio µg IgY/µg Protein	DPM with Anti-Melittin IgY	DPM with Control IgY
195	16	943	400
		970	371
350	32	287	400
		290	400
750	62	36	399
		32	398

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