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Purification and Characterization of Enoyl-acp Reductase From Euglena Gracilis

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Purification and characterization of enoyl-ACP reductase from *Euglena gracilis*

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PURIFICATION AND CHARACTERIZATION OF ENOYL-ACP REDUCTASE FROM EUGLENA GRACILIS

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by

Margie M. Tucker

May 1990

APPROVAL

This is to cerfity that the Graduate Committee of

MARGIE M. TUCKER

met on the

fifth day of April, 1990

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

PURIFICATION AND CHARACTERIZATION OF ENOYL-ACP REDUCTASE FROM EUGLENA GRACILIS

by

Margie M. Tucker

Enoyl-(acyl-carrier-protein) reductase was purified from the phytoflagellate Euglena gracilis. Its purification employed DEAE-Sephacel chromatography, Matrex Orange chromatography, and affinity chromatography using acyl carrier protein (ACP) covalently bound to Sepharose as the affinity ligand. Matrex Orange chromatography resolved two different enoyl-ACP reductases having different characteristics. Euglena gracilis appears to resemble higher plants in the possession of two isoforms of this enzyme.

Antibodies specific for the cofactor binding site of NADP(H)-requiring dehydrogenases were obtained. They were isolated from a polyclonal population of antibodies directed against yeast glucose-6-phosphate dehydrogenase by affinity chromatography using chicken liver malic enzyme as the affinity ligand. The affinity purified antibodies were covalently bound to Sepharose. Glucose-6-phosphate dehydrogenase and malic enzyme were both bound by the antibody column and were eluted by their cofactor, $NADP^+$, identifying the site of recognition of the enzymes by the antibodies as the cofactor binding site. The utility of this antibody affinity column was demonstrated by its ability to bind enoyl-ACP reductase, which was eluted by its cofactor, NADPH.

Preliminary studies of the E. gracilis fatty acid synthase (FAS) genes were undertaken using the plasmid pFAS4 (Witkowski et al., 1987), which contains a cDNA insert to part of the rat liver FAS mRNA and was a gift of Dr. Stuart Smith. The insert was cleaved with KpnI and PstI to generate probes specific for the ketoreductase, ACP, and thioesterase domains of the FAS. DNA from wild type E. gracilis and from a mutant, $W_{10}BSML$, which lacks chloroplast DNA, was subjected to field inversion gel electrophoresis and the DNA alkaline-blotted onto Nylon membranes. Hybridization of the three probes to the DNA was performed; all three probes hybridized to nuclear DNA, but none of the three hybridized to chloroplast DNA. The three probes also hybridized to a band which was neither nuclear nor chloroplast DNA. This DNA, which was larger than the chloroplast genome, may represent E. gracilis mitochondrial DNA sequences.

DEDICATION

This is dedicated with love and gratitude to Jim.

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Whatever I have done here has been made possible by the help and encouragement of a large number of people. The members of my committee, Phillip Musich, David Johnson, Ellen Rasch and Paul Monaco have been tremendously helpful. I thank Dave for running the VA plus much preferred advice, Phil for answering many questions, beginning with "What is a ribosome?". Ellen took photomicrographs of my Euglena and shared her fish and eels with my children. I especially thank Paul for his assistance in photography, and for urging me, once upon a time, to consider graduate school. I also thank Dr. Ken Herd for serving on my committee for a time, and for much pediatric advice.

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To know Lou
is to know the original belly laugh
celebratory, exuberant
a passionate intent
to experience each moment
to the hilt.
You showed me riches where I saw only duty.

To know Lou
is to see the possible
recognition of quality
by total integrity
creates a possibility
that wasn't.
You showed one a person I didn't know was there.
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Lastly and most importantly, for all you have done, thank you, Jim.

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ABBREVIATIONS

ACP	acyl carrier protein
BSA	bovine serum albumin
DTT	dithiothreitol
FAS	fatty acid synthase
EDTA	ethylenediamine tetraacetic acid, disodium salt
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
TES	N-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid
MGDG	monogalactosyldiacylglycerol
DGDG	digalactosyldiacylglycerol
SDS	sodium dodecyl sulfate
RNase	ribonuclease
DNase	deoxyribonuclease
PEG	polyethylene glycol
Tris	Tris hydroxymethylaminomethane

CHAPTER 1

Introduction

The living cell is both limited and defined by its plasma membrane, which forms a hydrophobic barrier between its internal environment and the external universe. All interactions between the cell and its environment are necessarily mediated by the membrane. The composition of the membrane plays a crucial role in such processes as nutrient uptake, responses to stimuli, and cell division and reproduction. Although membranes are composed of protein as well as lipid, it is the fatty acid composition of the lipids which primarily determines the overall physical characteristics, particularly fluidity, of the membrane (Stubbs and Smith, 1984). The synthesis of fatty acids and their incorporation into membrane lipids is an energetic, and not surprisingly, a tightly regulated process. At the same time, it is enormously complicated, involving competing pathways which are not fully understood. Many of the enzyme systems involved in fatty acid and lipid biosynthesis are membrane-bound, making their study difficult. The various enzymatic transformations and reactions required to synthesize the full battery of fatty acids and lipids found in a normal cell are also compartmentalized, necessitating the transfer of both from one part of the cell to another. This adds further complications to the elucidation of the

myriad reactions which are involved in fatty acid and lipid biosynthesis.

The significance of the diversity of fatty acid species and their distribution among lipids, the diversity of lipids, and the differential distribution of lipid species among different cellular membranes and between different faces of the same membrane is not at all clear. In the case of the highly specialized thylakoid membranes, differential distribution of the lipids peculiar to these membranes has been observed (Sundby and Larsson, 1985). In this instance, the galactolipids monogalactosyl-diacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were found to be asymmetrically distributed in the two leaflets of the thylakoid membranes of spinach, 60% in the outer and 40% in the inner half of the bilayer. Since these two lipids together account for 75% of the lipids of the thylakoid membrane, it was concluded that the remaining thylakoid lipids phosphatidylglycerol and sulphoquinovosyl-diacylglycerol, both of which have anionic headgroups, must also be asymmetrically distributed. They were predicted to be present preferentially in the inner leaflet of the bilayer; it was suggested that exposure of these anionic lipids in the inner half of the bilayer would allow them to act as counterions for the H^+ which flow into the thylakoid space during photosynthesis.

Certain integral membrane proteins exhibit a requirement for particular lipid species in order to function properly. For instance, many enzymes involved in the biosynthesis of lipids are integral membrane proteins. UDP-galactose:diacylglycerol galactosyltransferase is found in the innermost chloroplast envelope, where it catalyzes the transfer of galactose from UDP-galactose to diacylglycerol, forming the polar lipid MGDG. The partially purified enzyme from spinach was extensively delipidated and required the addition of lipids which had been extracted from chloroplast membranes for expression of activity (Coves et al., 1988). The acidic glycerolipids were most effective at restoring activity, especially phosphatidylglycerol. The effect of the lipid was due to specificity for the headgroup rather than for the fatty acid chains, as demonstrated by the results of experiments in which egg phosphatidylglycerol was used rather than the lipid from thylakoids. The fatty acid compositions of the lipid from these two sources is quite different; phosphatidylglycerol from egg is high in palmitate (16:0) and oleate (18:1), while the lipid from spinach is high in linolenic acid (18:3) and in the plant-specific fatty acid trans-3-hexadecenoic acid ($16:1_t$).

One important variable controlled by the fatty acid and lipid composition of a particular membrane is its fluidity. This is a rather imprecise term which describes both the lateral mobility of lipids within a membrane, and the range

of motion possible to individual fatty acids within the membrane. Since the lipid bilayer forms a two-dimensional solvent for the integral membrane proteins, its fluidity will have consequences on protein activity (Devaux and Seigneuret, 1985). For most creatures the control of membrane fluidity is critical, since fluctuations in environmental temperature and composition are frequently encountered. The ability of cells to respond to changing environmental conditions by adjusting the fatty acid and lipid compositions of their membranes is both relevant and significant.

In eukaryotic systems, fatty acids, either synthesized de novo or taken up from the environment, are transferred to the endoplasmic reticulum to be elongated, desaturated and/or incorporated into lipids. The phospholipids are then transported to other cellular membranes, where the combined action of resident phospholipases and acyltransferases results in the eventual production of the full complement of fatty acids and lipids found in eukaryotic cells. The acyltransferases have specificity for both the fatty acid and for the lipid species, so the distribution of fatty acids among the various lipids is not random (Chen et al., 1988). Furthermore, lipids tend to partition into different faces of a single membrane, and specific lipids are enriched in the membranes of certain organelles. How lipid species are routed to various cellular membranes is not clear,

although the Golgi apparatus has been implicated in the sorting of lipids destined for different domains of the plasma membrane (Simons and van Meer, 1988). Phospholipid binding proteins may also mediate the transportation of lipids from their site of synthesis in the endoplasmic reticulum to intracellular membranes such as those of mitochondria (Yaffe and Kennedy, 1983).

In plants fatty acids are synthesized entirely within the chloroplast stroma (Ohlrogge et al., 1979; Walker and Harwood, 1985). The primary product is palmityl-acyl carrier protein (ACP), which can be elongated to stearyl-ACP (Jaworski et al., 1974), desaturated to oleoyl-ACP (McKeon and Stumpf, 1982), incorporated into lipids by glycerol-3-phosphate acyltransferase or monoacylglycerol-3-phosphate acyltransferase (Frentzen et al., 1983), or may exit the chloroplast. Exiting the chloroplast requires the joint efforts of an acyl-ACP thioesterase (Joyard and Stumpf, 1980) to release the free fatty acid, and an acyl-CoA synthetase, which is located on the outer membrane of spinach chloroplasts (Joyard and Stumpf, 1981). Once a fatty acid enters the cytoplasmic compartment of a plant cell it travels to the endoplasmic reticulum, where it enters the so-called "eukaryotic pathway" of lipid bisynthesis (Kunst, 1988). In this compartment, fatty acids are incorporated into lipids and may undergo further elongations and desaturations. Due to the different

specificities of the elongating/desaturating enzymes and the acyltransferases of the chloroplast and endoplasmic reticulum, the types of fatty acids and lipids synthesized in these two compartments are different. Specifically, chloroplast-synthesized glycerolipids contain oleate esterified at the 1 position and palmitate at the 2 position, while glycerolipids synthesized in the "eukaryotic pathway" exclude palmitate, and oleate is esterified to the 2 rather than the 1 position of glycerol (Heinz and Roughan, 1983). The details of the interaction between these two compartments are just beginning to be elucidated. Kunst et al. (1988) have described a mutant of Arabidopsis which is deficient in the chloroplast glycerol-3-phosphate acyltransferase, which routes fatty acids into the "prokaryotic" pathway of lipid biosynthesis. In this mutant, lipid biosynthesis was largely redirected through the "eukaryotic" pathway. The fatty acid composition of the various lipids was altered to compensate for the loss of the "prokaryotic" pathway, but there was no apparent impairment in growth or development of the plants.

The structures of the unsaturated fatty acids produced by plant cells are different from those produced by non-plant cells, the former group being represented by α-linolenic acid (an 18-carbon fatty acid with double bonds in the 9, 12, and 15 positions) and the latter by γ-linolenic ($6,9,12\text{-}C_{18}$). The types of lipids which are synthesized are

also quite different depending on whether the lipid originated in a plant or nonplant cell. Photosynthetic membranes characteristically contain monogalactosyl diglycerides and digalactosyl diglycerides as well as sulfoquinovosyl diglycerides; galactolipids are found in the central nervous system, but otherwise these lipids are not found in significant quantities in nonphotosynthetic creatures.

Reactions of Fatty Acid Biosynthesis

In spite of the eventual differences in fatty acids produced by different cells, the early reactions which lead to the first fatty acid product proceeds in all cells by the cyclic series of reactions outlined below.

1. acetyl-CoA + ACP --> acetyl-ACP --> acetyl-enzyme + ACP
2. malonyl-CoA + ACP --> malonyl-ACP
3. acetyl-enzyme + malonyl-ACP --> acetoacetyl-ACP + CO_2
4. acetoacetyl-ACP + NADPH + H^+ --> ß-hydroxybutyryl-ACP $NADP^+$
5. ß-hydroxybutyryl-ACP --> crotonyl-ACP + H_2O
6. crotonyl-ACP + NADPH + H^+ --> butyryl-ACP

The product of the first round, butyryl-ACP, serves as the primer in the second round, replacing acetyl-ACP; this cyclic series of reactions proceeds until the product, usually the saturated 16-carbon fatty acid palmityl-ACP, is formed. The reactions are catalyzed by acetyl-CoA:ACP

transacylase, malonyl-CoA:ACP transacylase, ß-ketoacyl-ACP synthetase (also called condensing enzyme), ß-ketoacyl-ACP reductase, ß-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase in that order. These enzymes plus ACP are collectively referred to as the fatty acid synthase, or FAS.

Structurally, FASs can be divided into two groups, Type I and Type II (Bloch and Vance, 1977). Although artificial, this division does seem to reflect a fundamental difference in the ways in which different organisms synthesize and use fatty acids. Type I FASs can be further divided into two subgroups, IA and IB. Both IA and IB consist of multienzyme complexes of multifunctional enzymes. Their molecular weights range from 0.5×10^6 to several million daltons. They are characterized by the fact that they contain an ACP domain as an integral part of the multifunctional peptide. IA and IB FASs differ from one another in their sources, molecular architectures, substrate requirements, and product forms. Type II FASs characteristically are composed of separate monofunctional enzymes and a discrete ACP, and their substrate requirements and products are different from those of the Type I FASs.

Characteristics of Type IA Fatty Acid Synthases

The Type IA fatty acid synthases include the enzymes from all vertebrates and insects (Gavilanes et al., 1979), and the heterotrophic marine dinoflagellate Crypthecodinium

Cohnii (Sonnenborn and Kunau, 1982). The avian and mammalian FASs have been the object of much scrutiny. They are all homodimers with subunit molecular masses of 200,000 to 250,000 daltons (Bloch and Vance, 1977). Their structures have been worked out by a combination of proteolytic digestion and peptide mapping (Mattick et al., 1983a and 1983b, Wong et al., 1983, and Tsukamoto et al., 1983), active-site specific labelling (Poulose and Kolattukudy, 1980a and 1980b; Vernon and Hsu, 1984), cross-linking (Stoops and Wakil, 1981), and, most recently, cloning and sequencing of the mRNA for the monomer (Amy et al., 1989; Holzer et al., 1989, Yuan et al., 1988). The combined results of all of these studies have shown that these enzymes are splendidly complex. The active form is a head-to-tail dimer (Stoops et al., 1987), and the monomer is divided into three domains, I, II, and III. Domain I contains the ß-ketoacyl synthetase and the acetyl and malonyl transacylase activities, domain II contains the ß-ketoacyl reductase, the ß-hydroxyacyl dehydrase, the ACP component and the enoyl reductase, and domain III contains the thioesterase activity.

The thioesterase activity is responsible for the product form; once a chain length of 16 carbons has been achieved, the thioesterase hydrolyzes the fatty acid from the ACP component, releasing a free fatty acid. This FAS domain is easily cleaved by a number of different proteases,

leaving a core which retains the ability to synthesize long chain fatty acids. The thioesterase domain, once cleaved, has little propensity to reassociate with the core structure, and fluorescence resonance energy transfer has shown that in the intact dimer this domain is very flexible (Yuan and Hammes, 1986). A possible origin of the Type I multienzyme complexes through fusion of the genes for the component enzymes has been proposed (McCarthy et al., 1983; Chang and Hammes, 1989). The Type II FASs, which are presumed to resemble the ancestral FAS, release acyl-ACP as their product; since in the Type I FASs ACP is an integral part of the protein, the gene fusion theory requires that some other means of releasing the acyl chain from the protein must have been found. In the case of the Type IA synthases, a thioesterase was apparently recruited for that purpose. The properties of the thioesterase, in particular its covalent attachment to the fatty acid synthesizing core without an accompanying physical affinity, may lend credence to this view of the origin of the Type IA FASs.

Head-to-tail dimerization of the FAS was demonstrated by cross-linking studies which showed that the essential sulfhydryl of the condensing enzyme component of one monomer is located spatially near the phosphopantetheine site of the other monomer (Stoops and Wakil, 1981). That this juxtaposition of active sites from two different chains creates two centers for fatty acid synthesis was confirmed

by hybridization of fatty acid synthetase monomers which had been chemically modified at one or the other of these groups (Wang et al., 1984). The two centers were shown to be functionally independent by studies employing dimers whose thioesterase domains had been removed by proteolysis (Smith et al., 1985).

Extensive kinetic analyses of chicken liver FAS have been performed. One characteristic of the Type I FASs is the cofactor requirement; both the ß-ketoacyl reductase and the enoyl reductase require NADPH. Because of the size and structure of the FAS, some aspects of its physical and catalytic behaviour are quite novel. Many details of the catalytic mechanism have been elucidated by the work of Dr. Gordon Hammes. He demonstrated that the enzyme tends to dissociate to monomers at low ionic strength and/or low temperature (Kashem and Hammes, 1988). The monomer is inactive; reassociation is facilitated by salt, raising the temperature and NADPH. Several studies have indicated that conformational changes play an important role in catalysis (Yuan and Hammes, 1986; Tian et al., 1985) and in regulation (Stern and Smith, 1987).

The linear order of the enzymatic activities on the polypeptide chain has been deduced and confirmed by the results of cloning of the FAS mRNA from chicken liver and rat liver. The deduced amino acid sequences of the two proteins have been compared to one another and to the

deduced amino acid sequence of yeast FAS (Chang and Hammes, 1989). In comparing the chicken and rat active sites, matches of 70-80% were found, while comparison of the active sites of the yeast and chicken enzymes revealed matches of 14-30%. In another study, smaller regions of the rat and chicken FAS comprising the thioesterase domain and flanking regions were examined (Kasturi et al., 1988). The amino acid sequence of the linker regions between domains, regions which are proteinase-accessible, are not conserved between the two species. Other regions, most notably the thioesterase active site, are conserved up to 90%.

Characteristics of Type IB Fatty Acid Synthases

Type IB FASs are found in some advanced prokaryotes such as Mycobacterium smegmatis and in yeast. These FASs are very large, with molecular weights in the millions (Wakil and Stoops, 1983) and structurally are multimeric complexes of multifunctional enzymes. The yeast FAS has been examined in some detail. This large enzyme is composed of six copies each of two subunits, α and ß. Each multimeric complex has six centers for fatty acid biosynthesis. The α subunit has a molecular weight of 212,000 daltons and includes the ß-ketoacyl synthetase, the ß-ketoacyl reductase, and the ACP moiety. The ß subunit has a molecular weight of 203,000 daltons and includes all the remaining activities. A palmitoyl transacylase activity on

the ß subunit is responsible for the product form, acyl CoA thioesters. This activity is identical with the malonyl transacylase (Engester et al., 1979), illustrating a slightly different way of solving the problem of releasing the completed fatty acid. In the yeast FAS, the enoyl reductase is a flavin enzyme, unlike either the Type IA or Type II FASs. The active center of the condensing enzyme site contains juxtaposed thiols from neighboring α subunits. Each of the six fatty acid synthesizing centers is thus composed of two 1/2 α subunits and one ß subunit (Singh et al., 1985). The α and ß mRNAs have been cloned (Chirala et al., 1987; Mohamed et al., 1988) and their amino acid sequences deduced from the nucleotide sequence of the cloned cDNA.

Characteristics of Type II Fatty Acid Synthases

The Type II synthases are found in lower prokaryotes and in plants (Stumpf, 1987). These FASs are composed of a minimum of six discrete enzymes and ACP. The enzymes from E. coli were the first to be isolated and characterized (Vagelos et al., 1969), but the FAS enzymes from plants have recently been studied in more detail. All of the enzymes have been purified from spinach leaf, and these are the best characterized of the plant FAS enzymes. One interesting feature of the Type II FASs is the presence of multiple isoforms of several of the constituent enzymes, including

the noncatalytic component ACP. For instance, two isoforms of the malonyl-CoA transacylase have been observed in both soybean (Guerra and Ohlrogge, 1986) and leek (Lessire and Stumpf, 1982); they appear to be expressed in a tissue-specific fashion. Only one isoform of the acetyl-CoA:ACP transacetylase has been so far described. This enzyme was thought to catalyze the rate-limiting step in fatty acid biosynthesis in plants, although recent evidence from E. coli has raised some doubts as to the validity of that interpretation. Only one form of the ß-hydroxyacyl-ACP dehydrase has been described, but two isoforms of the enoyl-ACP reductase and the ß-ketoacyl-ACP reductases have been identified in various plants (Caughey and Kekwick, 1982; Shimakata and Stumpf, 1982a and 1982c; Cottingham et al., 1988).

There are two forms of condensing enzyme in plants, ß-ketoacyl-ACP synthetase I and II; the second of these is responsible for the elongation of palmitoyl-ACP to stearoyl-ACP (Jaworski et al., 1974). Both of these are inhibited by the antibiotic cerulenin, but to different degrees. In E. coli, there are also two condensing enzymes having different specificities for the acyl-ACP substrates; ß-ketoacyl-ACP synthase I is primarily responsible for elongation, while ß-ketoacyl-ACP synthase II is responsible for the temperature-dependent regulation of fatty acid composition (Rock and Cronan, 1982). Recently, a third ß-ketoacyl-ACP synthase

has been described in E. coli (Jackowski and Rock, 1987). This third condensing enzyme catalyzes the condensation of acetyl-ACP and malonyl-ACP to form acetoacetyl-ACP, and is thus acetoacetyl-ACP synthase rather than a generic ß-ketoacyl-ACP synthase. This enzyme differs from its counterparts in its specificity for the very short acyl-ACPs, in its lack of sensitivity to the antibiotic cerulenin, and its sensitivity to the antibiotic thiolactomycin (Jackowski et al., 1989). It was proposed that this enzyme is the rate-limiting enzyme in bacterial fatty acid biosynthesis, and that the acetyl-CoA:ACP transacetylase activity of E. coli FAS was catalyzed by this enzyme. This new condensing enzyme activity was not isolated but was identified based on: 1) the ability of E. coli to accumulate octyl-, hexanoyl- and butyryl-ACP when grown in the presence of cerulenin, an inhibitor of ß-ketoacyl-ACP synthetase I and II, and 2) the synthesis of butyryl-ACP by cell-free extracts of E. coli in the presence of cerulenin. They further demonstrated that this activity was destroyed by the antibiotic thiolactomycin. The acetyl-CoA:ACP transacetylase activity of E. coli has been purified (Lowe and Rhodes, 1988); it remains to be seen whether this enzyme has the predicted condensing enzyme activity.

Most interestingly, a cerulenin-insensitive short chain ß-ketoacyl-ACP synthase activity has been recently identified in Spinacia oleracea leaves (Jaworski et al.,

1989). It appears that in plants as in E. coli the rate of fatty acid biosynthesis may be regulated by the activity of this newly recognized enzyme, which catalyzes the first condensation reaction in fatty acid synthase. If so, acetyl-CoA:ACP transacetylase in plants may also be a different manifestation of the activity of this condensing enzyme rather than existing as a separate entity.

ACP of plant and bacterial origin is a small (molecular weight about 9,000) acidic protein which serves as the carrier of the growing acyl chain during fatty acid biosynthesis (Majerus et al., 1964). The acyl group is attached to ACP in a thiolester linkage to the sulfhydryl of ACP's 4'-phosphopantetheine prosthetic group. In addition to its role in fatty acid biosynthesis, acyl-ACPs are the preferred substrates of many of the enzymes involved in lipid biosynthesis. Because it plays an important role in both fatty acid and lipid biosynthesis, ACP has been the object of much interest. The ACPs of several species have been purified and the sequences of some of these determined. These include E. coli (Vanaman et al., 1968), barley (Hoj and Svendsen, 1984), and spinach (Kuo and Ohlrogge, 1984b). Partial sequences have been determined for the ACPs from Rhodobacter sphaeroides (Cooper et al., 1987) and Streptomyces erythraeus (Hale et al., 1987), and the sequence of ACP from Brassica campestris has been deduced from the nucleotide sequence of its cDNA (Rose et al.,

1987). Comparisons of the amino acid sequences of ACPs from E. coli and plants as well as the sequences of the ACP domains of the rat, chicken and yeast FASs have been made (Huang et al., 1989). Chicken and E. coli ACP were only 23% homologous, while rat and chicken were 65% homologous, and spinach and E. coli ACPs were 35% homologous. All ACPs are relatively conserved (Walker and Ernst-Fonberg, 1982), particularly in the central region of the proteins, but they tend to be more divergent in the N-terminal regions. The central, highly conserved region includes the serine where the 4'-phosphopantetheine prosthetic group is attached to the protein. The conservation of sequence at this site is most likely the explanation for the ability of E. coli ACP to substitute for plant ACPs in the reactions of fatty acid synthesis and the ability of E. coli acyl-ACP synthetase to acylate plant ACPs (Kuo and Ohlrogge, 1984a). At the same time, the sequences at the N-terminus of ACP are apparently important in imparting species-specificity to the interactions of the various ACPs and Type II FASs/lipid biosynthetic enzymes. Differences in this region are thought to explain the observation that E. coli ACP serves as a better substrate for fatty acid synthase from barley and spinach than do the species-specific ACPs (Hoj and Svendsen, 1984; Simoni et al., 1967). Also, while E. coli ACP is active with spinach FAS, incubation of spinach ACP with E. coli FAS resulted in an aberrant distribution of

fatty acid products, implying that the plant ACP does not interact with the bacterial enzymes in quite the same way that it does with its own FAS. Equally interesting results have been obtained in comparing the immunological relationship of the various ACPs. Anti-E. coli ACP antibodies cross-react with ACP of Euglena, a cross reactivity that was the basis for purification of the Euglena protein (Ernst-Fonberg et al., 1977), but there is some evidence to suggest that the interaction of E. coli ACP and the Euglena FAS is subtly different from the interaction of the FAS and its own ACP. The ACP of E. coli, in spite of its ability to substitute for the spinach protein as a substrate for the spinach FAS, is only weakly cross-reactive with antibodies directed against the ACP of spinach.

Our understanding of the unusual relationships between the spinach and E. coli ACPs and FASs has been extended by the realization that barley (Hoj and Svendsen, 1984) and spinach have two isoforms of ACP which are expressed in a tissue-specific manner (Ohlrogge and Kuo, 1985). The oleoyl-derivatives of the two spinach isoforms suffered different fates; oleoyl-ACP I was the preferred substrate for the stromal thioesterase, routing it to the cytoplasmic compartment, while oleoyl-ACP II was preferred for the acylation of glycerol-3-phosphate. Thus the relative amounts of the two isoforms are speculated to play a role in the coordination of the "eukaryotic" and "prokaryotic"

pathways of lipid biosynthesis in plants (Guerra et al., 1986). A synthetic gene for ACP I of spinach was cloned and expressed in a ß-alanine auxotroph of E. coli (Beremand et al., 1987), and the recombinant ACP I was purified and characterized (Guerra et al., 1988). It was observed that 50% of this recombinant protein was acylated; the acyl moiety was cis-vaccenic acid, a major unsaturated fatty acid product of the E. coli FAS. As it was in spinach chloroplasts, this isoform of ACP was a poor substrate for the bacterial acyltransferases, leading to the accumulation of acylated ACP I (Guerra and Browse, 1989). This supports the proposal that the two isoforms of ACP play a role in routing fatty acids to different subcellular compartments of plant cells.

In the higher plants, ACP is encoded in the nucleus as part of a multigene family (Safford et al., 1988). Translation of the mRNA for ACP takes place in the cytoplasm, as does attachment of the pantetheine prosthetic group (Elhussein et al., 1988). The precursor form of ACP is then routed to the chloroplast by means of its transit peptide, taken up, and proteolytically cleaved to its mature form. By analogy with other nuclear-encoded chloroplast proteins, uptake of precursors into chloroplasts is a process which most likely requires ATP (Schmidt and Mishkind, 1986).

Chloroplast Induction in Euglena Gracilis

Euglena gracilis is a unicellular organism which is distinguished by its ability to live as either a heterotroph or an autotroph. When grown in the dark and supplied with an appropriate carbon source, *Euglena* contain structures called proplastids, membrane-enclosed organelles lacking the characteristic internal membranous structures of chloroplasts. Transfer of such dark-grown cells into the light results in the induction of numerous enzyme systems, whose eventual product is a mature, functioning chloroplast (Schiff and Schwarzbach, 1982). This light induction of chloroplast development is controlled by two receptor systems. One of these, the blue light receptor, is cytoplasmic; its activation results in an increase in central metabolism which provides the energy and metabolites necessary for chloroplast biogenesis. This allows *Euglena* to develop chloroplasts independently of its ability to photosynthesize, as demonstrated by studies in which chloroplast development was induced in the presence of 3,(3,4-dichlorophenyl)1,1-dimethyl urea (Schiff *et al.*, 1967). The other light receptor is sensitive to red and blue light and is located within the chloroplast. This receptor is responsible for initiating chloroplast-specific responses such as translation of mRNA for plastid encoded proteins. The coordinated response of both systems results in the transcription of nuclear genes encoding chloroplast-

specific proteins and their translation and uptake into the organelle.

Following illumination, there is a lag of six hours during which no structural changes are visible in the proplastid, and chlorophyll begins to accumulate. After 12 hours chlorophyll content begins to rise rapidly, and structural changes in the proplastid become apparent (Klein et al., 1972). During this period the proplastid grows and thylakoids begin to form, probably by invagination off the innermost chloroplast envelope. The synthesis of the various components of the chloroplast photosynthetic apparatus is tightly regulated and highly coordinated. This has been demonstrated in a number of studies which examined the reciprocal relationships between chlorophyll, carotenoids, lipids and proteins.

Chlorophyll is the most prominent component of the thylakoid membranes and is absent from dark-grown cells. Its biosynthetic pathway includes enzymes which are encoded in both the nucleus and the chloroplast of the cell, so it requires the coordinated effort of both cellular compartments. Cytochrome c-552, an electron carrier in photosynthetic electron transport in Euglena, is a 10,000 dalton protein present in very low amounts in dark-grown cells. It carries a heme prosthetic group, so its formation requires synthesis of the protein moiety in addition to the complicated biogenesis of heme and the assembly of the

holoprotein. Like chlorophyll biosynthesis, the synthesis of cytochrome c-552 is sensitive to inhibitors of both chloroplast and cytoplasmic ribosomes. In addition to the soluble stromal proteins such as cytochrome c-552, chloroplast biogenesis requires the synthesis and insertion of integral membrane proteins. These include the light-harvesting proteins as well as the components of the chloroplast ATPase in the thylakoid membranes, and the lipid biosynthetic enzymes of the chloroplast envelope. Unlike chloroplasts of most other plants and algae, the chloroplasts of Euglena are enclosed by three membranes (Lefort-Tran et al., 1980). The innermost of these, by analogy with spinach, is most likely the site of lipid biosynthesis within the chloroplast. Other components of the thylakoid membrane include the sulfolipids, which like cytochrome c-552 are present in low amounts in dark-grown cells, and the carotenoids.

During chloroplast induction, the levels of cytochrome c-552 and chlorophyll are closely regulated such that the ratio of chlorophyll to cytochrome c-552 is fairly constant (Freyssinet et al., 1979). Chlorophyll levels are correlated with and may be controlled by the levels of carotenoids, as demonstrated by studies using inhibitors of carotenoid biosynthesis (Vaisberg and Schiff, 1976). Sulfolipid also increases during chloroplast induction in such a way that the ratio of sulfolipid to chlorophyll is

constant (Bingham and Schiff, 1979b). Both sulfolipid and thylakoid polypeptides are coordinately regulated by carotenoids (Bingham and Schiff, 1979a). Thus, each component's synthesis is linked to the synthesis of other components of the chloroplast, and if the synthesis of one is blocked, the synthesis of the others also ceases.

A number of mutants of Euglena have been isolated and characterized. These mutants are lacking in plastid DNA and impaired in chloroplast development to varying extents. One of the better-characterized mutants, W_3BUL, has been extensively used in this laboratory in studies of fatty acid and lipid biosynthesis. Its ability to respond to light has been examined by a Osafune and Schiff (1980). Although no plastid DNA was detected in this mutant by a variety of techniques, it did retain the capacity to respond to light in a limited fashion. The proplastid remnants were observed by transmission electron microscopy to enlarge and reorganize, a limited amount of carotenoids were formed, and light-induced increases in transcription of cytoplasmic rRNA and in respiration took place. The extent of response of these mutants was felt to reflect initiation of the normal response to the cytoplasmic blue-light receptor, while the failure to sustain an appropriate developmental program was felt to be due to the absence of plastid DNA. Since the two responses are coordinated, the cytoplasmic program can only

be abortive in the absence of proper signals from the chloroplast.

During the greening process, the lipids which are characteristic of photosynthetic tissues (sulfolipid, MGDG, DGDG) as well as the pigments, proteins and chlorophyll components of thylakoid membranes appear or increase drastically (Rosenberg and Pecker, 1964; Erwin, 1968). The remodelling of proplastid to chloroplast thus requires extensive synthesis of fatty acids and lipids. Precise details of the appearance of the chloroplast lipid biosynthetic apparatus are sparse. Glycerophosphate acyltransferase from Euglena chloroplasts has been isolated (Boehler and Ernst-Fonberg, 1976; Hershenson et al., 1983) and characterized. The enzyme was pelleted in the particulate fraction of cells, and was enriched in the chloroplast fractions of sucrose gradients. Solubilization of the acyltransferase required Triton-X 100. It is likely that the preparation included both a glycerol-3-phosphate acyltransferase and a monoacylglycerol-3-phosphate transferase activity, since diacylglycerol-3-phosphate was a major product. The former enzyme is a soluble stromal enzyme in most plants, while the latter is membrane-bound. The Euglena chloroplast enzyme preferred acyl-CoA substrates to acyl-ACP substrates, and palmitoyl-CoA was a better substrate than oleoyl-CoA. The product of the combined action of glycerol-3-phosphate acyltransferase and

monoacylglycerol-3-phosphate acyltransferase of peas and spinach is primarily phosphatidic acid bearing oleate at the sn-1 position and palmitate at the sn-2 position of glycerol. The diacyl product of the combined enzymatic activities from Euglena chloroplasts carried palmitate at the sn-1 position and oleate at the sn-2 position.

The existence of a "eukaryotic" pathway of lipid biosynthesis in Euglena was confirmed by the isolation of a microsomal glycerophosphate acyltransferase from the microsomal fraction of W_{10}BSML (Grobovsky et al., 1979; Hershenson and Ernst-Fonberg, 1983). This is a mutant of Euglena which is even more impaired than the W_3BUL mutant. Its properties resembled the properties of the chloroplast glycerol-3-phosphate acyltransferase in that acyl-ACP did not serve as a substrate, and palmitoyl-CoA was preferred over oleoyl-CoA. In fact, the microsomal and chloroplast acyltransferases resembled one another more than the Euglena chloroplast acyltransferase resembled other chloroplast acyltransferases, the most striking differences being the different positional specificities and the substrate specificity.

In addition to a glycerol-3-phosphate acyltransferase which is located within the chloroplast, Euglena contain a chloroplast-localized Type II FAS. This enzyme system is induced during greening, and its appearance in the cell is

accompanied by the concomitant diminution of a cytosolic FAS which is reminiscent of the Type I FASs.

The appearance of the chloroplast-associated system for fatty acid biosynthesis is accompanied by a diminution of cytosolic FAS activity. That percentage of total fatty acid synthesis which is attributable to the cytosolic FAS declines following transfer of dark grown cells into the light, from 100% of the total FAS activity to 47% of the total activity after 24 hours. In cells which have been grown for many generations in the light, the cytosolic fatty acid synthase, which is distinguished from the chloroplast system by its lack of a requirement for exogenously added ACP, still accounts for 24% of the total fatty acid synthetic capacity of the cells. As complicated as the lipid biosynthetic pathways are in other plant cells, which have a single FAS, it is staggering to contemplate the regulatory mechanisms which must exist in Euglena, with two separate FAS systems. These postulated regulatory interactions must include coordination of the two fatty acid synthases, both in terms of the relative levels of the two activities during the dark-light transition and prolonged growth in the light, and in terms of regulating the fates of the fatty acids synthesized by the two different FASs. The significance of the chloroplast-associated FAS and the role which it plays during the induction of chloroplast development is not known.

The two independent fatty acid synthesizing enzymes of Euglena gracilis strain Z were first demonstrated by Delo et al. (1971) in cells which had been grown either as autotrophs in the light or as heterotrophs in the dark. Dark-grown cells were observed to contain a high molecular weight fatty acid synthase which exhibited no requirement for added ACP for activity. This enzyme system was precipitated below 35% ammonium sulfate saturation and primarily synthesized palmitate, with some myristate and traces of stearate.

In additon to a FAS which was indistinguishable from the enzyme present in etiolated cells, light-grown Euglena contained a second FAS which differed from the first in several respects. This second FAS exhibited an absolute requirement for added ACP for activity. Its products were primarily stearate, with appreciable amounts of arachidate. It could be separated from the high molecular weight FAS by ammonium sulfate fractionation as it precipitated between 35-70% of saturation. The two FASs could also be separated by sucrose density gradient centrifugation; the ACP-dependent FAS sedimented near the top of the gradient while the high molecular weight FAS sedimented much lower in the gradient, with an estimated molecular weight greater than 650,000.

A more detailed examination of the two synthases (Ernst-Fonberg and Bloch, 1971) showed that the ACP-

dependent FAS was induced in dark-grown cells which were exposed to white light while being maintained in a resting medium. This induction therefore, like chloroplast induction, did not require growth and cell division. The appearance of the enzyme system was examined in dark-grown cultures which were exposed to white light in the presence of either chloramphenicol, an inhibitor of protein synthesis on 68S chloroplast ribosomes, or cycloheximide, which inhibits protein synthesis on 80S cytoplasmic ribosomes (Freyssinet and Schiff, 1974). After 24 hours in the light, the induced ACP-dependent FAS accounted for about 50% of the total FAS activity of control cells. In cells which were grown in the presence of cycloheximide, the ACP-dependent FAS still accounted for 57% of the total FAS activity. In these cells, chlorophyll levels were reduced to 15% of control. Chloramphenicol at 1 mg/ml totally suppressed the ACP-dependent FAS, while chlorophyll levels were still approximately one-third of control. The inhibition of chlorophyll biosynthesis by both chloramphenicol and cycloheximide is consistent with the role of both chloroplast and nuclear encoded proteins in its synthesis (Gomez-Silva et al., 1985).

In interpreting these results, the similarity of the high molecular weight FAS to the FASs of yeast and mammals was noted, as was the similarity of the ACP-dependent FAS to the FASs of E. coli and plants. There are thus two separate

fatty acid and phospholipid synthesizing systems in Euglena, one located in the cytosol and the other in the chloroplast. The different patterns of lipids present in light-grown or etiolated cells may reflect the presence or absence of the chloroplast "prokaryotic" pathway in these cells.

The two FASs of Euglena have been extensively characterized. It is clear that the high molecular weight enzyme is not structurally identical to the high molecular weight FASs of yeast or those of mammals and birds. Rather it is a multienzyme complex of at least nine separate proteins (Worsham et al., 1986). Unlike the Type IA FASs, which require only NADPH, optimal activity required both NADH and NADPH (Walker et al., 1981). Unlike the Type IB enzymes, it had no requirement for a flavin cofactor. Its products were primarily palmitate (69%) and stearate (21%) with traces of myristate (11%). It is a proteolipid, being composed of 40% lipid, primarily monoacylglycerol with some diacylglycerol and free fatty acids. The products were released as acyl-CoA thioesters, indicating the presence of a transacylase activity. Its molecular weight was determined by low angle light scatter to be approximately six million, and both light scatter and viscosity measurements revealed that at concentrations above 30 μg/ml, the enzyme dimerized. Like the vertebrate enzymes, dimerization occured in a side-to-side rather than end-to-end fashion; unlike them, dimerization of the Euglena enzyme

was accompanied by a reduction in activity. The existence of an ACP domain in the multienzyme complex was confirmed by the effect of antibodies directed against E. coli ACP, which inhibited the activity of the FAS.

One other FAS has been identified as a proteolipid; this is the FAS of an insect, Ceratitis capitata (Municio et al, 1977). This enzyme was a dimer in the active form, like the vertebrate FASs. It consisted of about 50% lipid in the form of triacylglycerol, diacylglycerol, phosphogycerides and free fatty acids. In this case the lipid apparently played an important structural role, as delipidation resulted in a loss of activity. Activity could be recovered by adding back phosphotidylethanolamine but not phosphatidylcholine (Gavilanes et al., 1979). The physical effect of phospholipid on the enzyme, as determined by circular dichroism studies, was an increase in the length of helical segments of the protein (Gavilanes et al., 1981). It was also proposed that the lipid may play a role in the release of palmityl-CoA, or may serve to anchor the FAS on cellular membranes.

Studies of the non-aggregated FAS of Euglena variety bacillaris confirmed the involvement of chloroplast ribosomes in the appearance of this enzyme system during greening (Ernst-Fonberg et al., 1974). Both spectinomycin and chloramphenicol when present in the medium during chloroplast induction resulted in decreased levels of ACP-

dependent FAS, to 50% and 30% of control levels, respectively. Cycloheximide did not affect the levels of the ACP-dependent FAS, but did result in decreased levels of the large multienzyme complex FAS. In one experiment, ACP-independent activity was undetectable, while in another it was reduced to 27% of control activity, when chloroplast development was induced in dark-grown cells in the presence of cycloheximide. These results indicate that this enzyme undergoes turnover during greening. The reduced levels of the ACP-dependent FAS in the presence of inhibitors of chloroplast ribosomes is suggestive of a role for chloroplast ribosomes in either the synthesis or the uptake of this FAS. However, the biogenesis of chloroplasts is so tightly regulated that the reduction in ACP-dependent FAS activity may be a secondary effect.

Characterization of the ACP-dependent FAS has been done (Ernst-Fonberg, 1973). Unlike the plant FASs, NADPH was the only reductant required. The kinetics of the reaction were complicated in that different graphs of initial velocity vs. ACP concentration and different values of V_{max} were obtained depending on the order of addition of enzyme, ACP and malonyl-CoA. The highest V_{max} was seen when enzyme and ACP were incubated together prior to the addition of malonyl-CoA. A graph of initial velocity vs. ACP concentration in this case was sigmoidal rather than hyperbolic. Initiation of the reaction with either enzyme or ACP resulted in graphs

which were hyperbolic, and yielded reduced values for V_{max}. These data were interpreted as evidence of complex formation which was organized by ACP. The most exciting evidence for complex formation was the result of experiments using antibodies directed against the high molecular weight multienzyme complex FAS. Ouchterloney double-diffusion showed that the two different FASs were immunologically cross-reactive but not identical. Incubation of the ACP-independent enzyme with these antibodies resulted in inhibition of its activity. Incubation of the ACP-dependent FAS with the antibodies resulted in a totally unexpected increase in activity. This increased activity was explained as stabilization by the antibodies of the FAS complex during catalysis. This would require that the junctional configurations or conformations of the enzymes comprising the ACP-dependent FAS while in the catalytically active complex resemble the junctions or conformations of the corresponding ACP-independent FAS enzymes.

One popular theory of the origin of both chloroplasts and mitochondria holds that both of these organelles derived from prokaryotes which were engulfed by a eukaryotic cell, where they established residency. Both organelles contain their own DNA, which usually encodes a handful of organellar proteins, rRNA, and tRNAs. They contain ribosomes which resemble prokaryotic ribosomes in structure and in sensitivity to inhibitors of protein synthesis, and their

rRNA and tRNA genes are arranged in an order reminiscent of the prokaryotes (Whitfield and Bottomly, 1983). The ribosomal proteins of Euglena chloroplast ribosomes resemble those of E. coli (Freyssinet and Schiff, 1974), and E. coli elongation factors are active on chloroplast ribosomes while cytoplasmic elongation factors are not (Spremulli et al., 1981).

It is proposed that, following the original engulfment, when the endosymbiotic relationship between the two cells was becoming established, the prokaryotic cell lost much of its coding capacity to the nuclear DNA of the host. This is supported by the existence of chloroplast and cytosolic isoforms of glyceraldehyde-3-phosphate dehydrogenase, both of which are nuclear encoded (Shih et al., 1986; Brinkmann et al., 1987). The chloroplast-specific enzyme has closer sequence homology to the enzyme from thermophilic bacteria than to the cytosolic form. It is synthesized on cytoplasmic ribosomes as a precursor, having an amino-terminal extension which is responsible for routing the protein to the chloroplast. Alternatively, in some cases the organellar gene might have been completely deleted from the genetic repertoire of the cell and the nuclear gene, by duplication and the addition of a transit peptide sequence, might have assumed the function of the deleted gene. This is apparently the case in glutamine synthetase of Pisum sativum, which is encoded by homologous nuclear genes

(Tingey et al., 1988). One of the genes is light-induced and codes for a chloroplast-specific protein, while the product of the other is cytoplasmic. In the case of some yeast mitochondial proteins, production of the organellar form of an enzyme results from differential transcription or translation of a common nuclear gene encoding both cytoplasmic and mitochondrial forms of the enzyme (Surgochov, 1987).

Little is known about the genetics of plant Type II FAS enzymes. The gene for the ACP component is nuclear in higher plants. The genes for the enzyme components have not been cloned, but are assumed to be nuclear. This is based on the observation that mutants of barley which lacked chloroplast ribosomes were able to synthesize the chloroplast-specific lipids (Dorne et al., 1982). The enzymes responsible for fatty acid and lipid biosynthesis must therefore be present, and thus cannot be encoded in the plastid. The only two organisms for which evidence to the contrary has been presented are Euglena and another unicellular alga, Chlamydomonas rheinhardi (Sirevag and Levine, 1972). In Chlamydomonas which were growing synchronously, the normal increase in fatty acid synthase activity seen during a light cycle was blocked by spectinomycin but not by cycloheximide, suggesting that fatty acid synthase in this organism is encoded in the organelle. The evidence implicating chloroplast ribosomes

as the site of synthesis of the Euglena chloroplast FAS has been presented.

The studies employing cycloheximide and chloramphenicol implicate protein synthesis on chloroplast ribosomes in the appearance of the chloroplast-associated FAS during greening; however, interpreting the results is not straightforward. First, since E. coli ACP is added during the assay, it is impossible to tell whether or not Euglena ACP appeared during greening, so this question is unresolved. Since ACP may play a regulatory role, knowing the location of its gene is likely to be critical in understanding the mode of synthesis and regulation of the catalytic components. Second, because greening in the presence of cycloheximide did not result in a decrease in ACP-dependent FAS activity, it is unlikely that some of the enzymes are encoded in the nucleus while others are encoded in the chloroplast (with the possible exception of ACP); however, the limited capacity of chloroplast DNA to encode proteins makes it seem equally unlikely that all seven of these proteins are chloroplast encoded. Only two other possibilities exist. The proteins could be encoded in the nucleus, the genes transcribed in the nucleus, and the mRNA translated in the chloroplast. There is no evidence of such a transfer of mRNA from the cytoplasm to the chloroplast, although transfer of RNA from the cytoplasm to mitochondria has been demonstrated. The 135-nucleotide RNA component of

mammalian mitochondrial endoribonuclease is nuclear-encoded, and following its synthesis is transported through the cytosol to the mitochondria, where it crosses the mitochondrial envelope and is incorporated into the ribonuclease (Chang and Clayton, 1987). Transfer of tobacco mosaic virus RNA into the chloroplast of tobacco has also been demonstrated, and its translation on chloroplast ribosomes has been suggested (Schoelz and Zaitlin, 1989). However, uptake of RNA by organelles is likely to be the exception rather than the rule.

The alternative explanation is that the large FAS, which was observed to experience turnover during greening, may be the source of the chloroplast-associated FAS. If a protein which is responsible for recognition, uptake and processing of the peptides of the cytoplasmic FAS is synthesized within the chloroplast, the appearance of the chloroplast FAS would be sensitive to chloramphenicol. The cytoplasmic FAS would serve as a precursor pool, eliminating an immediate sensitivity to cycloheximide on the part of the chloroplast FAS. Continuing depletion of the cytoplasmic FAS by transport and uptake into the chloroplast in the absence of cytoplasmic protein synthesis would cause a decrease in its levels, but the chloroplast FAS pool would be buffered and relatively insensitive. However, if this explanation were true, one would not expect to see a decrease in the activity of the cytoplasmic FAS in the

presence of chloramphenicol or spectinomycin. To reconcile the data with this hypothesis one would have to postulate multiple events in both compartments. Cytoplasmic events might include continuous turnover by dismantling of the Type I FAS followed by degradation during growth in the dark. During light induction, normal dismantling of the FAS would take place; however, a plastid encoded protein responsible for recognition and uptake of the FAS peptides would compete with degradation. When grown in the light, the distribution of FAS activity between cellular compartments would be determined by the competition between degradation and uptake. When cytoplasmic protein synthesis is blocked, the ACP-independent FAS pool would be decreased by normal dismantling, but for a short period of time the ACP-dependent FAS pool would be buffered. When chloroplast protein synthesis is blocked, the chloroplast would be unable to take up the FAS peptides, while normal dismantling of the cytoplasmic FAS would reduce its concentration to the appropriate level. This explanation would explain the cross-reactivity of the two FASs but it is bizarre in that it postulates the uptake not of precursor peptides but of mature proteins. We are left with the original hypothesis, that the Euglena Type II FAS is encoded in the chloroplast.

The purification of the enoyl-ACP reductase of Euglena chloroplast fatty acid synthase was undertaken as part of an ongoing investigation into the fatty acid and lipid

biosynthetic machinery of this organism. The immediate goal of the study was to purify and characterize this enzyme. The different cofactor requirement of the Euglena enoyl-ACP reductase compared to the analogous enzyme from other plants and E. coli make it an appealing subject for study. During the purification it became apparent that, like E. coli and the higher plants, the Euglena FAS may include more than one enoyl-ACP reductase. An affinity ligand specific for the dinucleotide-binding fold of dehydrogenases was constructed, based on the ability of antibodies directed against this site on one enzyme to recognize the same structure in unrelated enzymes (Katiyar and Porter, 1983). A method for disrupting Euglena which allowed the cells to be lysed without breaking the chloroplast membranes was developed, and resulted in an improvement in the published method for isolating chloroplasts from Euglena (Gomez-Silva et al., 1985). Since the location of the genes for the chloroplast FAS is ambiguous, preliminary studies were undertaken to begin to address that question. It is hoped that this work will serve as a prelude to more detailed studies of the relationship between the FAS of the cytosolic and chloroplast compartments of Euglena gracilis.

CHAPTER 2

Materials and Methods

Buffers and Solutions

$T_{10}E_1$ is 10 mM Tris-HCl, 1 mM EDTA, pH either 7.4 or 8.4, as indicated. $T_1E_{0.1}$ is 1 mM Tris-HCl, 0.1 mM EDTA, pH 7.4 or 8.4, as indicated. TBE buffer is 0.05 M Tris, 0.05 M boric acid, 1.25 mM EDTA, pH 8.3. TAE buffer is 0.05 M Tris, 0.04 M acetic acid, 1 mM EDTA, pH 8.4. SOG is 60% (w/v) sucrose, 0.1% (w/v) Orange G. SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0. CIA is chloroform/isoamyl alcohol (24:1 v/v). Phenol crystals were melted at 65°C and 8-hydroxyquinoline added to 0.1% (w/v), following which the solution was equilibrated with T_1E_{10}. Luria-Bertani (LB) medium contained 10 g bactotryptone, 5 g bacto-yeast extract and 5 g NaCl per liter, pH 7.4. LB medium was autoclaved to sterilize. Ampicillin (10 mg/ml) was filter-sterilized before adding to LB medium to a final concentration of 0.05 mg/ml for selection of *E. coli* transformed with plasmids containing an ampililin resistance gene. SET is 20% sucrose, 0.05 M Tris-HCl, pH 8.0, 0.05 M EDTA. LSS (low salt spermidine) is 20 mM Tris-HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 70 mM NaCl, 10 mM $MgClL_2$. 5M potassium acetate solution contained 60 ml of 5 M potassium acetate and 11.5 ml of glacial acetic acid in a

final volume of 100 ml. TBS is Tris buffered saline, 20 mM Tris, 500 mM NaCl, pH 7.5.

Growth of Cells

Euglena gracilis variety bacillaris were grown in Hutner's High Yield Heterotrophic Medium (Wolken, 1967) at 26°C under constant illumination and with constant rotary shaking (90 rpm). After five to six days cells were filtered through a double layer of cheesecloth and harvested at 4°C by centrifugation at 7974 x g in a Sorvall RC-5 centrifuge. Cells were stored at -85°C.

Protein Assay

Protein was estimated by the method of Bradford (Bradford, 1976), using Bio-Rad's Protein Assay dye reagent and following their instructions. A standard curve was prepared during each assay by measuring the binding of dye to BSA. A 1 mg/ml standard solution was prepared by dissolving 25 mg BSA in 20 ml of deionized water and adjusting the volume based on the absorbance at 278 nm. The absorbance at 278 nm of a 1 mg/ml solution of BSA is 0.667 in a 1 cm path length cell (Peters, 1975).

Counting Cells

Cells were counted in a hemacytometer. Viability was estimated based on the exclusion of the dye trypan blue by live cells. Cells (0.2 ml) were diluted to 0.5 ml with

growth medium, added to 0.5 ml of 0.4% trypan blue in saline, mixed, and counted within five minutes. Ten squares were counted each time, and the total cell count per ml was calculated as the average count per square multiplied by the dilution factor, five, and by the volume per square, 10^{-4} ml.

Chlorophyll Determination

Chlorphyll was determined by extracting 5-50 µl of whole cells or chloroplasts with 2 ml of 80% acetone as described in Gomez-Silva *et al*. (1985). Following five minutes in the dark at 4°C the solution was centrifuged for 3-5 minutes in a microfuge and the absorbance at 645, 663, and 652 nm determined. Concentration in mg/l of the acetone extract was calculated (Bruinsma, 1961) as

$$c = 20.2 \times A_{645} + 8.02 \times A_{663} = (1000 \times A_{652}) / 36$$

Gel Electrophoresis and Western Blotting

Polyacrylamide gel electrophoresis of proteins was performed using a Bio-Rad Model 360 Mini Vertical Slab-Cell and the discontinuous buffer system of Laemmli (1970). Protein was precipitated by the addition of cold TCA to 10% followed by incubation for 20 minutes at 4°C. The protein precipitate was collected by centrifugation for three minutes in a microfuge, washed once with cold 10% TCA and twice with cold acetone. The pellet was resuspended in sample buffer and boiled for five minutes before electrophoresis. Electrophoretic size markers were the low

molecular weight standards from Bio-Rad; Bio-Rad's prestained standards were used if the gel was to be Western blotted. Gels were electrophoresed at 20 milliamps.

Following electrophoresis proteins were visualized either by silver staining using the kit and procedure from Bio-Rad, or using the Coomassie Blue stain described by Steck et al. (1980).

Proteins were Western blotted from 0.75 mm gels onto 0.2 μm pore nitrocellulose in a Bio-Rad Mini Trans-Blotter at 30 volts and 40 milliamps for ten hours. Transfer was confirmed by silver staining of the gels. The nitrocellulose was treated following Bio-Rad's recommendations. Blocking was for five hours with 1% BSA in TBS; the membranes were incubated with primary antibody overnight, and for two hours with secondary antibody at the recommended dilution of 1:3000. The secondary antibody used usually was alkaline phosphatase-conjugated goat anti-rabbit IgG. The membranes were developed using either the fast red or the blue color development reagents from Bio-Rad.

Isolation of E. coli ACP

ACP was isolated from 500 g of E. coli as described by Rock and Cronan (1980).

Enzyme Assays

Fatty Acid Synthase

Fatty acid synthase activity was determined by measuring the incorporation of radioactivity from (2-^{14}C) malonyl-CoA into long chain fatty acids (Worsham, et al., 1988). The following were added to 13 x 100 mm culture tubes at 4°C to the final concentration indicated: acetyl-CoA, 30 μM; NADPH, 800 μM (freshly made); ACP, 9 μM; DTT, 2 mM; TES, pH 8.0, 100 mM; enzyme, and water. The reaction was initiated with (2-^{14}C) malonyl-CoA (1 Ci/μM), to a final concentration of 60 μM; the final volume of the reaction was 250 μl. Following a 20 minute incubation at 35°C, the reaction was stopped by the addition of 50 μl of 45% (w/v) KOH. Fatty acids were saponified by heating for 30 minutes at 100°C. The solution was acidified by the addition of 100 μl of 12 M HCl, and the fatty acids were extracted three times into 2 ml of pentane each time. The pentane was evaporated in a warm water bath and the residue dissolved in 5 ml of toluene containing 4 g 2,4-diphenyloxazole/liter, or in 5 ml of ECOLUME (ICN Radiochemicals). Radioactivity was measured in a Packard TriCarb liquid scintillation counter. Efficiency of counting was determined for each set of assays. Units of activity are defined as nanomoles of ^{14}C incorporated into long chain fatty acids per minute.

Enoyl-ACP Reductase

Enoyl-ACP reductase was assayed using the analogue crontonyl-CoA. The assay included 0.1 M TES pH 8.0, 3 mM DTT, 0.25 mM NADPH (freshly made in 0.01 M potassium phosphate pH 7.4), enzyme, and water. Following incubation for five minutes at 30°C the reaction was initiated by the addition of crotonyl-CoA to a final concentration of 0.2 mM in a final volume of 800 µl. Constant temperature was maintained by a Haake circulating water bath equipped with a tap water cooling coil. The decrease in absorbance at 360 nm as NADPH was oxidized was measured in a Gilford 240 spectrophotometer. The millimolar extinction coefficient of NADPH under these conditions was 4.283. One unit of activity is defined as the oxidation of one nanomole of NADPH per minute.

Malic Enzyme

The assay as described (Hsu and Lardy, 1967) contained 67 µM triethanolamine-HCl pH 7.4, 4 µM $MnCl_2$, 0.23 µM $NADP^+$ (freshly made in 0.01 M TES pH 7.4), enzyme (in 0.05 M Tris HCl, 10 mM EDTA, 0.2 M magnesium acetate pH 7.0), and water. Following incubation for five minutes at 30°C the reaction was initiated by the addition of potassium-L-malate pH 7.4 to a final concentration of 0.5 µM and a final volume of 1 ml. The increase in absorbance at 360 nm as $NADP^+$ was reduced was measured.

Glucose-6-Phosphate Dehydrogenase

The assay as described (Kanji et al., 1976) included 0.043 M glycine-NaOH pH 8.5, 1 mM $MgCl_2$, 5 x 10^{-5} M $NADP^+$, enzyme, and water. After incubation for five minutes at 30°C the reaction was initiated by the addition of glucose-6-phosphate to a final concentration of 1 mM and a final volume of 1 ml. The increase in the absorbance at 360 nm as $NADP^+$ was reduced was measured.

Isolation of Enoyl-ACP Reductase

Cells were thawed at room temperature and suspended in 0.01 M Tris-HCl, pH 7.6, 0.002 M DTT (1.5 - 2 ml per gram wet weight of cells). Cells were broken by sonication using a Bronson S-125 sonifier equipped with a microtip at 4-5 DC amps. Sonication was for a total of 90-120 seconds in 30 second bursts, cooling in a methanol/dry ice bath between bursts. Cell breakage was monitored microscopically. Broken cells were centrifuged at 38,720 x g for 90 minutes at 4°C and the pellet discarded. All subsequent manipulations were at 4°C.

DEAE Sephacel Chromatography of Crude Cell Extract

DEAE Sephacel was equilibrated in 0.01 M Tris-HCl, pH 7.6, 2 mM DTT. Generally, 2 ml of DEAE Sephacel was used per gram wet weight of cells. Crude cell supernatant solutions were applied directly to the column. Following a brief wash with 0.01 M Tris-HCl, pH 7.6, 2 mM DTT, the

column was developed with a 5 column-volume linear gradient of 0 - 0.2 M NaCl in 0.01 M Tris-HCl, pH 7.6, 2 mM DTT. DEAE Sephacel was used once and discarded. Active fractions were pooled and stored at -20°C.

Matrex Orange Chromatography

Pooled enzyme from DEAE Sephacel chromatography was concentrated by precipitation with ammonium sulfate at 70% of saturation. The protein collected by centrifugation was suspended in a minimal volume of 0.01 M Tris-HCl, pH 7.6, 0.002 M DTT and dialyzed against several changes of the same buffer. It was then chromatographed on a 2.5 x 17 cm Matrex Orange column which had been equilibrated in the same buffer. Protein was allowed to bind for 15 minutes; the column was then washed with buffer until the A_{280} dropped to zero, then developed with a linear gradient of 0 - 0.5 M NaCl in the same buffer. The column capacity was limited to no more than 6 mg of protein per ml of gel.

The Matrex Orange column was regenerated by washing with 6 M urea in 0.5 M NaOH, then rinsing with several column volumes of 0.01 M Tris-HCl, pH 7.6, 2 mM DTT. The column was used three or four times.

ACP-Affinity Chromatography

An ACP-affinity column which had been constructed by Dr. Lesa Worsham was kindly made available. The 12 ml column was composed of E. coli ACP cross-linked to Reactigel

6x from Pierce following their instructions. Enzyme was concentrated by precipitation with ammonium sulfate followed by chromatography on a G25 column equilibrated in 0.01 M Tris-HCl, pH 7.6, 2 mM DTT. Concentrated enzyme was applied to the ACP-agarose column equilibrated in the same buffer, and allowed to bind for 15 minutes. The column was washed with the same buffer until the A_{280} dropped to zero, then developed with a linear gradient of 0-0.5 M NaCl in 0.01 M Tris-HCl, pH 7.6, 2 mM DTT. The column was regenerated by washing with 1 M NaCL in 0.01 M Tris-HCl, pH 7.6, 2 mM DTT, and was stored in the same buffer containing 0.02% NaN_3.

Immunoaffinity Chromatography of Enoyl-ACP Reductase

An affinity column which was specific for the enzyme's nucleotide cofactor binding site was constructed. This was accomplished by raising antibodies which were directed against an NADP(H)-requiring enzyme, glucose-6-phosphate dehydrogenase and isolating IgG from antiserum. Those antibodies which cross-reacted with a second NADP(H)-requiring enzyme, malic enzyme, were isolated by affinity chromatography on malic enzyme-Sepharose, and were used in turn as an affinity ligand.

Preparation of Anti-Glucose-6-Phosphate Dehydrogenase Antibodies

Antibodies against yeast glucose-6-phosphate dehydrogenase were raised in male white New Zealand rabbits.

One mg of protein (Boehringer Mannheim) in 0.2 M TES pH 8.0 was emulsified in an equal volume (0.5 ml) of Freund's Complete Adjuvant and injected at multiple sites intradermally. The rabbits were boosted at one month with 1 mg of protein in Freund's Incomplete Adjuvant and bled biweekly thereafter. They were boosted at intervals when their antibody titer, as estimated by Ouchterloney double diffusion, began to drop. Ouchterloney plates were 1.5% agar in 0.01 M potassium phosphate buffer, pH 7.0, 0.15 M NaCl, 0.1% NaN_3. Serum was applied directly to the center well and varying concentrations of antigen to the surrounding wells.

Blood was allowed to clot overnight at 4°C and the serum collected by centrifugation at 11,950 x g for 10 minutes. The IgG fraction was isolated by chromatography of dialyzed serum on DEAE-Affigel Blue from Bio-Rad, following their recommended procedure. Before using the gel, unbound dye was removed by washing the gel in a sintered glass funnel with five bed volumes of 0.1 M acetic acid, pH 3.0, 1.4 M NaCl, 40% 2-propanol. The gel was then washed extensively with at least 10 bed volumes of column buffer, 0.02 M Tris-HCl, pH 8.0, 0.028 M NaCl, 0.02% NaN_3. A minimum of 4.7 ml of gel was used for each ml of serum. The volume of serum collected was measured, and it was dialyzed against three changes of column buffer before being chromatographed. Fractions equal in volume to the volume of

serum before dialysis were collected. IgG did not bind to the DEAE-Affigel Blue column, but was washed through as the column was rinsed with three bed volumes of column buffer; eight fractions were pooled, beginning with the first fraction containing protein. Pooled IgG was concentrated by precipitation with ammonium sulfate at 70% of saturation. The precipitated protein was collected by centrifugation and dissolved in 0.01 M potassium phosphate, pH 7.4, 0.15 M NaCl, 1 mM EDTA.

Isolation of Anti-Dinucleotide Fold Antibodies

In order to select for those antibodies which recognized the cofactor binding site of glucose-6-phosphate dehydrogenase, the IgG was subjected to affinity chromatography using malic enzyme as the ligand. A malic enzyme affinity column was constructed from chicken liver malic enzyme (Sigma) and CNBr-activated Sepharose from Pharmacia, following their procedure. Malic enzyme (5.64 mg) was dialyzed against 0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl (bicarbonate buffer). CNBr-activated Sepharose (0.43 g) was allowed to swell for 20 minutes in 0.001 M HCl before being washed with 100 ml of the same. The gel was rinsed with 5 ml of bicarbonate buffer and transferred into a 15 x 125 mm culture tube. The malic enzyme was added, the tube was capped and shaken for 2 hours on a wrist action shaker at room temperature. The gel was then transferred into a

column, rinsed with 0.2 M glycine pH 8.0, and left overnight at 4°C to block unreacted amino groups. Then the gel was warmed to room temperature and rinsed alternately 4-5 times with bicarbonate buffer and with 0.1 M acetate, pH 4.0, 0.5 M NaCl. The gel was degassed, repoured and equilibrated with 0.01 M potassium phosphate, pH 7.4, 0.15 M NaCl, 1 mM EDTA. The column was used at room temperature, and stored in 0.02% NaN_3 in the same phosphate buffer.

Anti-glucose-6-phosphate dehydrogenase IgG (1.5 ml) in 0.01 M potassium phosphate, pH 7.4, 0.15 M NaCl, 0.001 M EDTA was applied to the malic enzyme-Sepharose affinity column and allowed to bind for 1.5 hours. The unbound IgG was run back through the column three to four times. The column was washed with phosphate buffer until the A_{280} dropped to zero. Bound IgG was eluted by 4.5 M $MgCl_2$ pH 6.4. The $MgCl_2$ was freshly made, its pH adjusted with 1 M Tris base, and filtered through glass wool before use. Eluant was pooled, dialyzed against phosphate buffer, and concentrated by Amicon ultrafiltration. This chromatography step was repeated until all of the IgG had been chromatographed. All of the IgG which bound to the column and was eluted by $MgCl_2$ is referred to as anti-dinucleotide fold antibody.

Construction and Use of the Affinity Column

Anti-dinucleotide fold IgG (1 mg) was coupled to CNBr-activated Sepharose (0.63 g) as previously described. The immunoaffinity column was used and stored at 4°C.

Glucose-6-Phosphate Dehydrogenase Immunoaffinity Chromatography

Yeast glucose-6-phosphate dehydrogenase was dialyzeed against 0.01 M Tris-HCl, pH 7.6, 0.15 M KCl and applied to the immunoaffinity column equilibrated in the same buffer. The column was washed with buffer until the A_{280} and glucose-6-phosphate dehydrogenase activity dropped to zero. Bound enzyme was eluted by 5.6×10^{-5} M $NADP^+$ in the same buffer. The gel was regenerated by washing with 0.2 M glycine pH 2.9, then rinsing with 0.01 M Tris-HCl, pH 7.6, 0.15 M KCl.

Malic Enzyme Immunoaffinity Chromatography

Chicken liver malic enzyme was dialyzed against 0.05 M Tris-HCl, pH 7.0, 0.01 M EDTA, 0.2 M magnesium acetate and applied to the immunoaffinity column equilibrated in the same buffer. The column was washed with buffer until the A_{280} and malic enzyme activity dropped to zero. Bound activity was eluted by 0.27 μM $NADP^+$ in the same buffer.

Enoyl-ACP Reductase Immunoaffinity Chromatography

Enoyl-ACP reductase purified by Matrex Orange chromatography was concentrated and desalted into 0.01 M

Tris-HCl, pH 7.6, 0.15 M KCl and applied to the immunoaffinity column equilibrated in the same buffer. The column was washed with buffer until the A_{280} and enoyl-ACP reductase activity dropped to zero. Bound enzyme was eluted by 0.23 mM NADPH in the same buffer.

Characterization of Enoyl-ACP Reductase

The effects of a variety of additives and also the effect of storage pH on the enzyme were evaluated. In the case of the former, enzyme was divided into aliquots of no more than 1 ml and kept frozen until immediately before use. Small volumes of the additive were included in the standard assay and compared to enzyme without additive. Comparisons were made only between identical batches of enzyme assayed on the same day. To examine the effect of pH on the enzyme during storage, small volumes of enzyme were dialyzed overnight at 4°C against two changes of buffer. The following buffers were used: imidazole, pK 7.05, for the pH range 6.5 to 7.5; TES, pK 7.5, for the pH range 7.0 to 8.0; and Tris-HCl, pK 8.3, for the pH range 7.5 to 9.0. The buffer concentrations were 0.01 M, and included 2 mM DTT. The ionic strength at each pH was calculated, and all buffers were made up to constant ionic strength (7.8 mM) by the addition of NaCl as necessary. The enzyme was then assayed under standard assay conditions.

Biogel A 0.5m Chromatography of Enoyl-ACP Reductase

A 2.5 x 41 cm Biogel A 0.5m columns was calibrated using the following proteins: bovine erythrocyte carbonic anhydrase, M_r 30,000; yeast alcohol dehydrogenase, M_r 141,000; bovine pancreatic RNase, M_r 13,700; and human transferrin, M_r 74,000, all obtained from Sigma, and blue dextran to mark the void volume. Pooled enzyme fractions destined for molecular sieve chromatography were concentrated by the addition of solid ammonium sulfate to 70% of saturation. The pH was maintained between 7.0 and 8.0 by the dropwise addition of 1 M Tris base. After stirring for 30 minutes at 4°C the precipitated protein was collected by centrifugation for 20 minutes at 11950 x g, resuspended in a minimal volume of 0.01 M TES, 0.2 M sucrose, 0.15 M NaCl, 0.002 M DTT, pH 7.4, and loaded directly onto a 2.5 x 41 cm Biogel A 0.5m column equilibrated in the same buffer. The A_{280} and enzyme activities of 2 ml column fractions were measured.

Chromatofocusing

A 10 ml chromatofocusing column was equilibrated in start buffer (0.025 M imidazole, pH 7.4). Enoyl-ACP reductase purified by Matrex Orange chromatography was dialyzed against several volumes of start buffer and applied to the column. The column was developed by a linear pH

gradient of pH 7.0 to pH 4.0, using Polybuffer 74 (Pharmacia).

Isolation of Intact Chloroplasts from Euglena Gracilis

Chloroplasts were isolated from E. gracilis using a modification of the procedure of Gomez-Silva et al. (1985). The modification was essentially a different way of breaking the cells, which allowed chloroplasts to be isolated from E. gracilis grown in the High Yield medium of Hutner. Previous protocols for isolating chloroplasts from Euglena called for the cells to be grown in a medium which was limiting in vitamin B_{12} (Ortiz et al., 1980).

One to four liters of cells were harvested at five or six days of growth while still in the logarithmic phase of growth, minimizing the accumulation of mucus (Cogburn and Schiff, 1984). The culture medium was filtered through two layers of cheesecloth, the volume measured, and the cells were counted. Cells were pelleted by centrifugation at 4,068 x g for five minutes, washed in incubation buffer (0.3 M sorbitol, 0.05 M potassium phosphate, pH 7.0) and their wet weight determined. The cells were then resuspended in incubation buffer (10 ml per gram wet weight of cells) and treated with the nonionic detergent Brij 35 (polyoxyethylene 23-lauryl ether, from Sigma), to a final concentration of 0.001%. Brij 35 was made up as a 5% stock in 1% 1-butanol. After 15 minutes on ice with occasional swirling, cells were

washed twice in incubation buffer centrifugation at 1,464 x g and resuspended in the same volume of incubation buffer. They were then digested briefly with crude pancreatic trypsin (Sigma) at a final concentration of 0.2%. Trypsin was freshly prepared by vortexing the proteinase in one ml of incubation buffer followed by centrifugation to remove insoluble material. Cells were examined microscopically at intervals and the digestion stopped as soon as cell breakage became evident, usually at about seven minutes. The trypsinized cells were washed twice in breakage buffer (0.25 M sorbitol, 4 mM Na_2EDTA, 0.02 M HEPES-KOH, pH 7.4) by centrifugation at 1,464 x g for 3 minutes and resuspension of the rather sticky cell pellet. Cells were broken by passage through a Yeda press at 400 psi N_2. The length of time spent in breakage buffer proved to be critical, and only stripped thylakoids were recovered from cells which had been left too long in that buffer.

Cell debris was removed by centrifugation at 119 x g for six minutes, and the supernatant solution clarified if necessary by repeating the centrifugation. Chloroplasts were then pelleted by centrifugation at 1,075 x g for five minutes, washed in 15 ml of gradient buffer (0.3 M sorbitol, 0.015 M NaCl, 2 x 10^{-4}% polyvinylsulfate, 1% ficoll, 5 mM 2-mercaptoethanol, 5 mM HEPES-NaOH, pH 6.8), and resuspended in two to five ml of gradient buffer. Using bent Pasteur pipets, the chloroplasts were layered onto linear gradients

of 10-80% Percoll in gradient buffer and centrifuged at 7,500 rpm for 20 minutes in an SW 27 rotor in a Beckman Model L5-65 ultracentrifuge. Intact chloroplasts were recovered as a dark green band about halfway down the gradient; stripped chloroplasts and thylakoid fragments barely entered the gradient. Chloroplasts were collected by puncturing the side of the polyallomer centrifuge tube with an 18 gauge needle just below the band, and gently drawing out the band with a syringe. Percoll was removed from the chloroplast pool by adding 20 ml of washing buffer (0.33 M sorbitol, 1 mM $MgCl_2$, 2 mM EDTA, 0.050 M Tricine-KOH, pH 7.8), and centrifuging at 1,075 x g for five minutes. The chloroplast pellet was resuspended in five ml of washing buffer, and any contaminating cell ghosts were pelleted by centrifuging at 119 x g for two minutes. Chloroplasts were brought down again, resuspended in one-to-two ml of storage buffer (0.33 M sorbitol, 0.05 M Tricine-KOH, pH 7.8), and stored at -85°C.

The effectiveness of two concentrations of Brij 35 (0.001% and 0.01%) at incubation times of either 15 or 30 minutes each was evaluated. The effect of incubation in water instead of detergent was also checked, as was the effect of incubation in 1-butanol, which was present in the 5% Brij 35 stock. This was done by centrifuging one liter of cells, resuspending in 6 mls of incubation buffer, and dividing the cells into six 1 ml aliquots. Two aliquots,

the water and 1-butanol controls, were incubated for 30 minutes at 4°C with added water or 1-butanol. Two aliquots were incubated for either 15 or 30 minutes with added Brij 35 at a final concentration of 0.01%. The last two aliquots were incubated for either 15 or 30 minutes with added Brij 35 at a final concentration of 0.001%. Following the detergent treatment, the six aliquots were handled identically, as described.

The isolation of chloroplasts from E. gracilis which had been grown in low vitamin B_{12} differed only in the treatment of the cells prior to breakage. No detergents were used, and trypsinization proceeded for up to one hour. All other manipulations were identical throughout.

Scanning Electron Microscopy of Euglena Gracilis

E. gracilis in the stationary phase of growth were centrifuged for five minutes at 2,987 x g in a Sorvall RC-5 centrifuge and washed twice with 0.01 M potassium phosphate buffer, pH 7.0, 0.15 M NaCl. Cells were fixed for two hours in 1 ml of 2% osmium tetroxide in phosphate buffer, washed five times with phosphate buffer, and placed on glass slides which were then glued to aluminum stubs. They were then dehydrated through a graded series of acetone baths at five minutes per bath, twice each in 50%, 80%, 95% and 100% acetone. Dehydrated cells were dried for one hour in a

critical point drying apparatus, gold coated, and viewed in a scanning electron microscope.

Isolation of Rat Genomic DNA

DNA was isolated from nuclei of rat spleen and kidney. The organs were rinsed and homogenized in 0.01 M Tris-HCl, pH 7.6, 0.25 M sucrose, 3 mM $CaCl_2$ to rupture cells and release nuclei. Cells were homogenized with a Teflon pestle in a Potter-Elvehjem homogenizing vessel. Nuclei were washed by centrifugation at 1,000 x g, gently resuspending the pellet in the same buffer, and recentrifuging. Nuclei were resuspended in 0.05 M Tris-HCl pH 8.4 and Protease K (20 mg/ml in $T_{10}E_{1}$ pH 8.4) was added to a final concentration of 1 mg/ml. In addition, 0.5 M EDTA was added to a final concentration of 7.5 mM, 5 M NaCl to a final concentration of 1 M, and 20% SDS to a final concentration of 0.5%. The viscous solution was incubated at 65°C for one hour, then transferred to 37°C for 16 hours. Residual proteins were extracted into an equal volume of 1:1 phenol:CIA (chloroform:isoamyl alcohol, 24:1 v/v) for 15 minutes. The phases were separated by centrifugation at 1,200 x g for 10 minutes at 22°C. The aqueous phase was removed with an upturned pipet and extracted with an equal volume of CIA to remove residual phenol. The aqueous phase was cooled, and an equal volume of cold 2-propanol was carefully layered on top. DNA was spooled-out at the

interface onto a glass rod. The DNA was rinsed with 70% ethanol, dried, rehydrated in $T_{10}E_1$ pH 8.4, and unspooled. Contaminating RNA was digested with pancreatic RNase (Sigma), at a final concentration of 0.01 mg/ml, for 15 minutes at 37°C. RNase was extracted into an equal volume of 1:1 phenol:CIA, and any residual phenol was extracted into CIA. The DNA was concentrated by extraction with 1-butanol (Maniatis et al., 1982).

Total genomic DNA was isolated from E. gracilis wild type and W_{10}BSML in the same way, except that nuclei were not isolated.

Isolation of Euglena Gracilis Nuclear DNA

Nuclei were isolated from one liter of light-grown Euglena by the method of Czupryn et al. (1987). Frozen cells were resuspended in 15 ml of 15 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.6 mg% polyvinylsulfate (PVS), 5 mM $Na_2S_2O_5$ (buffer A) containing 10% sucrose and 5% Triton-X 100 (w/v). The cells were refrozen in dry ice/methanol and thawed, followed by incubation for one hour at 4°C with gentle stirring. The cells were collected by centrifugation at 500 x g for 10 minutes and washed three times with buffer A containing 10% sucrose and 0.5% Triton-X 100. After the wash, the cell pellet was suspended in 10 ml of the same buffer. The cells were disrupted by sonication twice for 30 seconds each time, divided into two aliquots, and each

aliquot layered over 25 ml of buffer A containing 40% sucrose and 0.5% Triton-X 100. The nucleii were collected by centrifugation at 2,000 x g for 20 minutes and washed five times with 15 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 50 mM KCl, 0.6 mg% PVS, and 10% sucrose. The pellet was resuspended by gentle homogenization. The resulting pellets were combined and resuspended in 50 mM Tris-HCl pH 8.4 to a volume of 10 ml. DNA was isolated from the pellet by proteinase K digestion and phenol/CIA extraction as described above.

Isolation of Euglena Chloroplast DNA

Chloroplasts were isolated from one liter of Euglena grown in Hutner's medium by the method described above. Following isolation, the chloroplasts were washed as described, suspended in two ml of storage buffer, and divided into two aliquots. Following centrifugation to collect the chloroplasts, they were suspended in 900 µl of 50 mM Tricine pH 7.8, 0.33 M sorbitol, 3 mM EDTA, 10 mM $MgCl_2$, 0.1% BSA and 1 mM DTT. To one tube was added 100 µl of 10 mM Tris-HCl pH 7.4, 10 mM magnesium acetate (control) while to the other was added 100 µl of DNase I (Sigma) in the same buffer to a final concentration of 0.1 mg/ml. Following a one hour incubation on ice, 0.5 M EDTA pH 8.0 was added to a final concentration of 10 mM, and DNA

extraction from the treated and control chloroplasts was performed as described above.

Genomic DNA from other species were gifts of the following individuals: Sacchromyces cerevisiae, Robert Roudabush, Sigma Corporation, St. Louis, MO; Zea mays, Ralph Sinabaldi, Zoecon Corporation, Palo Alto, CA; Drosophila melanogaster, Chara fibrosa, Phaeoceros laevis, Conocephalum conicum amd Polytrichum pallidisetum, Lee Pike and Karen Renzaglia, Department of Biology, East Tennessee State University, Johnson City, TN.

The plasmid pFAS4 was a gift of Stuart Smith, Children's Hospital Oakland Research Institute, Oakland, CA. The plasmid pE7C738.1 (Koller et al., 1984) was a gift of R. B. Hallick, Department of Biochemistry, University of Arizona, Tucson, AZ.

Transformation of E. Coli

DH5α competent cells from Bethesda Research Laboratories were transformed following the recommended procedure with the plasmid pFAS4 (Witkowski et al., 1987). This plasmid contains the cDNA for part of the rat liver fatty acid synthase gene, including all of the ACP and part of the ketoreductase and thioesterase coding sequences, in the EcoRI site of pUC19. Frozen cells (-85°C) were thawed on ice and 50μl was removed into a chilled sterile 1.5 ml centrifuge tube. To this was added 1 μl of pFAS4 DNA, at a

concentration of 0.56 μg/μl; mixing was by moving the pipet through the solution as the DNA was added. The tube was tapped gently to mix and left on ice for 30 minutes. The cells were heat-shocked at 37°C for 20 seconds, then transferred back to ice. Luria-Bertani (LB) medium was added (0.95 ml) and the cells were incubated for one hour at 37°C, shaking at 150 rpm. They were then plated onto LB/ampicillin agar plates and incubated overnight at 37°C. Colonies were picked to inoculate 20 ml cultures of LB with ampicillin. Aliquots of cells from these cultures were frozen at -85°C after addition of glycerol to 10%; one of the frozen stocks was used as the inoculum whenever plasmid DNA had to be isolated.

Isolation of Plasmid DNA

E. coli transformed with pFAS4 recombinant plasmid were grown to the stationary phase of growth in 350 ml of LB and ampicillin overnight at 37°C with 150 rpm shaking. Cells were harvested by centrifugation at 2,600 x g for 15 minutes. The pellet was vortexed, transferred into a 15 ml Corex centrifuge tube, and washed with 1.8 ml of SET buffer, centrifuging for 10 minutes at 250 rpm in x g in a clinical centrifuge. The pellet was loosened by vortexing and 2 ml of SET buffer was added. Two ml of freshly made alkaline lysis solution (0.2 N NaOH, 1% SDS) was added, mixing by gentle inversion. The cells were incubated at 65°C for 30

minutes, mixing by inversion twice during the incubation. Three ml of 5 M potassium acetate solution was added, mixed by inversion, and incubated for 20 minutes on ice. This step precipitated cellular but not plasmid DNA, and cellular proteins and SDS. The floculant precipitate was removed by centrifugation at 12,000 x g for 15 minutes, and the supernatant solution was further clarified by recentrifugation. Plasmid DNA was precipitated by adding 2.4 ml of 27% PEG 8000-3.3 M NaCl. After a two hour incubation on ice, plasmid DNA was collected by centrifugation at 12,000 x g for 15 minutes, rinsed with 70% ethanol, and dried.

Plasmid DNA was further purified by chromatography on pZ523 columns (5'->3', Inc.). Following the manufacturer's directions, DNA was resuspended in 1.8 ml of 0.01 M Tris-HCl, 1 M NaCl, 1 mM EDTA, pH 8.0. The buffer was drained off of the column, and excess buffer removed by centrifugation for one minute at 2,500 rpm. Exactly 1.8 ml of DNA solution was applied to the column, which was then centrifuged for 24 minutes at 2,500 rpm. Plasmid DNA was precipitated from the effluent by the addition of 0.6 volumes of 2-propanol.

Restriction Enzyme Digestion of DNA

DNA was digested by restriction enzymes (2 units per μg of DNA) in LSS buffer for 16 hours at 37°C. Bovine serum

albumin (BSA) was added to 100 µg/ml, and 2-mercaptoethanol to 6 mM.

Preparative Fractionation of DNA

Intact pFAS4 recombinant plasmid DNA and the three insert fragments released from the plasmid by restriction with EcoRI, KpnI and PstI were isolated by electroelution following their separation by agarose gel electrophoresis. Electrophoresis and electroelution were done in TAE buffer. Plasmid DNA was electrophoresed through 0.8% agarose gels at 50 volts for 1.5 hours, and restricted plasmid was electrophoresed through 1.8% agarose gels at 50 volts for 2 hours. Gels were soaked briefly in buffer containing ethidium bromide, and gel slices containing the DNA of interest were excised under UV light and placed in the elution wells of an IBI electroeluter (International Biotechnologies, Inc.). The DNA was electroeluted at 50 volts for one hour onto a 150 µl bed of 7.5 M ammonium acetate and recovered by isopropanol precipitation.

Alcohol Precipitation of DNA

An equal volume of 5 M ammonium acetate was added to samples of DNA to be concentrated, followed by the addition of either 2.5 volumes of 95% ethanol or 0.6-1.0 volume of 2-propanol. The solution was chilled and the precipitated DNA collected by centrifugation at 12,000 x g in a Fisher Model

235B microcentrifuge at 4°C. The DNA pellet was rinsed with 70% ethanol and dried under vacuum.

Estimation of DNA Concentration

Standard and sample DNAs were subjected to agarose gel electrophoresis in the presence of ethidium bromide. The DNA bands were visualized by UV transillumination and photographed. The negative was scanned by a densitometer. Peak areas of the standard DNAs were used to generate a curve from which the sample DNA concentrations were calculated.

Agarose Gel Electrophoresis of DNA

Agarose was added to 100 ml of TBE to a final concentration of 0.7% to 1.8% depending on the size of the DNA to be electrophoresed. The solution was boiled to melt the agarose, cooled to 65°C, then poured into the gel mold and allowed to solidify at room temperature. Ethidium bromide, if used, was added to the buffer and agarose solution to a final concentration of 0.5 μg per ml. The DNA was mixed with 1/5 volume of SOG before loading and electrophoresed at 50-100 volts until the tracking dye reached the end of the gel. DNA was visualized by 305 nm UV-transillumination and photographed with either Polaroid type 55 positive/negative or type 665 high speed positive film.

Samples were prepared for field inversion gel electrophoresis by suspending cells at a concentration of 3 x 10^7 cells/ml in 1% Seaplaque agar and allowing to cool in molds. The non DNA components were removed by soaking the gel plugs at 50°C in 10% sarkosyl-0.5 M EDTA for 3 days, followed by equilibration in $T_{10}E_1$, pH 8.4 (Smith et al., 1988). The agar plugs were placed in the wells of 1% agarose gels and electrophoresed as in Caile et al. (1986).

Alkaline Transfer of DNA in Agarose Gels to Zeta Probe

DNA electrophoresed in an agarose gel was partially "depurinated" by treatment for 15 minutes at room temperature in 0.25 N HCl. The gel was then rinsed twice in deionized water and soaked for 15 minutes in 0.4 N NaOH, which causes double-stranded DNA to dissociate to single strands and cleaves the phosphodiester backbone of DNA at sites of depurination. DNA was transferred out of the gel onto Zeta Probe membranes (Bio Rad) by 0.4 M NaOH, using a VacuBlot apparatus (American Bionetics). Transfer was complete in 30 minutes. The membrane was baked for 30 minutes at 65°C.

Slot Blotting of DNA

DNA was made 0.2 N in NaOH and incubated at 65°C for 30 minutes to denature double stranded DNA and hydrolyze any contaminating RNA. It was then applied to Zeta Probe membranes in a Bio-Rad slot blotting apparatus. The

membrane was rinsed twice with 2XSSC for 15 minutes each, shaking at room temperature. Membranes were baked for 30 minutes at 65°C.

Preparation of Radioactive Probes

Radioactive probes were prepared by either nick translation of double stranded DNA (Rigby et al., 1977) or by random-primer labelling (Feinberg and Vogelstein, 1984). Plasmid DNA was nick translated using a kit and its accompanying instructions from Bethesda Research Laboratories. Ten microcuries of (α^{32}P)dCTP, with a specific radioactivity of 3000 Ci/mmole, were added to a final volume of 50 µl, containing one to two micrograms of DNA, and the reaction proceeded for one hour at 15°C. DNA was purified away from unincorporated nucleotides by alcohol precipitation. Random-primer labelling of DNA fragments employed a kit and instructions from Boehringer Mannheim. Double-stranded DNA was denatured by heating for 10 minutes in a boiling water bath. The (α^{32}P)dCTP had a specific radioactivity of 3000 Ci/mmole, and 40 µCi were added to a final volume of 20 µl, containing one to two micrograms of DNA. The reaction was at room temperature for three hours. DNA was alcohol precipitated after adding yeast tRNA to a final concentration of 0.06 µg/ml to act as a carrier.

The extent of incorporation of label was measured by adding 1 µl of probe to 100 µl of 1 mg/ml BSA. Twenty

microliters of this was spotted onto a Whatman GF/A filter and counted in a scintillation counter. This gave the total cpm in 20% of the 1 µl sample. The DNA was acid precipitated from the remaining 81 µl by adding 1 ml of 1 N HCl, 1% sodium phosphate, 1% sodium pyrophosphate and incubating 20 minutes on ice. Precipitated DNA was collected on a Whatman GF/A filter and counted. The ratio of acid precipitated to total counts gave an estimate of the extent of incorporation of radioactive nucleotides into DNA.

Filter Hybridization

Membranes to be hybridized were first treated with alkali to remove any DNA which was not tightly bound. The membranes were washed twice for 15 minutes each in 0.4 M NaOH at room temperature, rinsed three times with deionized water, and washed for 15 minutes in 3X SSC, 100 mM Tris-HCl pH 7.4 to neutralize. Membranes were then blocked by incubation for one hour at 65°C in 0.1X SSC, 0.5% SDS. Before hybridizing to the radioactive probe, the membranes were placed in a sealable plastic bag, hybridization buffer (0.25 M Na_2HPO_4, 0.25 M NaH_2PO_4, 1 mM EDTA, 7% SDS, 1% BSA) was added, and they were incubated for one hour at 65°C. The buffer was removed and fresh hybridization buffer containing the probe was added. Probes were first denatured by placing in a boiling water bath for 5 minutes. Hybridization was overnight at 65°C and was followed by a

low stringency wash of the membrane, twice for 15 minutes each at room temperature in 2X SSC 0.1% SDS, and a high stringency wash, twice for 30 minutes at 65°C in 0.1X SSC, 0.1% SDS. In some experiments the final wash was less stringent, twice for 30 minutes at 65°C in 3X SSC, 0.1% SDS; these are standard stringency conditions. The moist membranes were then placed in plastic bags, sealed, and subjected to autoradiography.

Stripping Filters

Filters which had been previously hybridized with a probe were stripped by denaturation of the double-stranded hybrid in alkali. Filters were first rinsed with deionized water, then incubated twice for 15 minutes each at 65°C in 0.4 N NaOH. The membranes were then rinsed with water, with 3X SSC, and neutralized by a 10 minute rinse in 3X SSC, 100 mM Tris-HCl, pH 7.4. In order to confirm that the filters were clean, they were exposed to X-ray film overnight.

Autoradiography of Filters

Membranes were exposed to Kodak XAR-5 films with two Dupont Cronex Lightning Plus intensifying screens in a Kodak X-ray exposure holder at -85°C for several hours to several weeks. Films were developed for up to 7 minutes in Kodak GBX developer, fixed in Kodak GBX Fixer for 10 minutes and then rinsed for 20 minutes in water before drying.

CHAPTER 3

Results

Purification of Enoyl-ACP Reductase from Euglena Gracilis

DEAE-Sephacel Chromatography of Enoyl-ACP Reductase

The supernatant solution obtained from centrifugation of disrupted Euglena gracilis cells contains both a large multienzyme complex fatty acid synthase and a nonaggregated chloroplast-associated fatty acid synthase composed of six separate enzymes and ACP (Delo et al., 1971). This latter system includes two NADPH-dependent oxidoreductases, β-keto acyl-ACP reductase and enoyl-ACP reductase. The first step in the purification of enoyl-ACP reductase separated these two reductases. The supernatant solution, obtained from 28 g (wet weight) of Euglena containing 798 mg of protein in 60 ml of 0.01 M Tris-HCl, pH 7.6, 2 mM DTT was applied to a 2.5 x 9.0 cm DEAE-Sephacel column. This solution contained 12,441 units of enoyl-ACP reductase activity and 6,613 units of acetoacetyl-ACP reductase activity. As shown in Figure 1, the acetoacetyl-ACP reductase activity (7,212 units) washed through the column, while the enoyl-ACP reductase activity bound to the column and was eluted by a linear gradient of 0-0.2 M sodium chloride in the same buffer. Activity began to elute at a sodium chloride concentration of 0.14 M and had not completely washed off at the end of the gradient, so the column was washed with a small volume

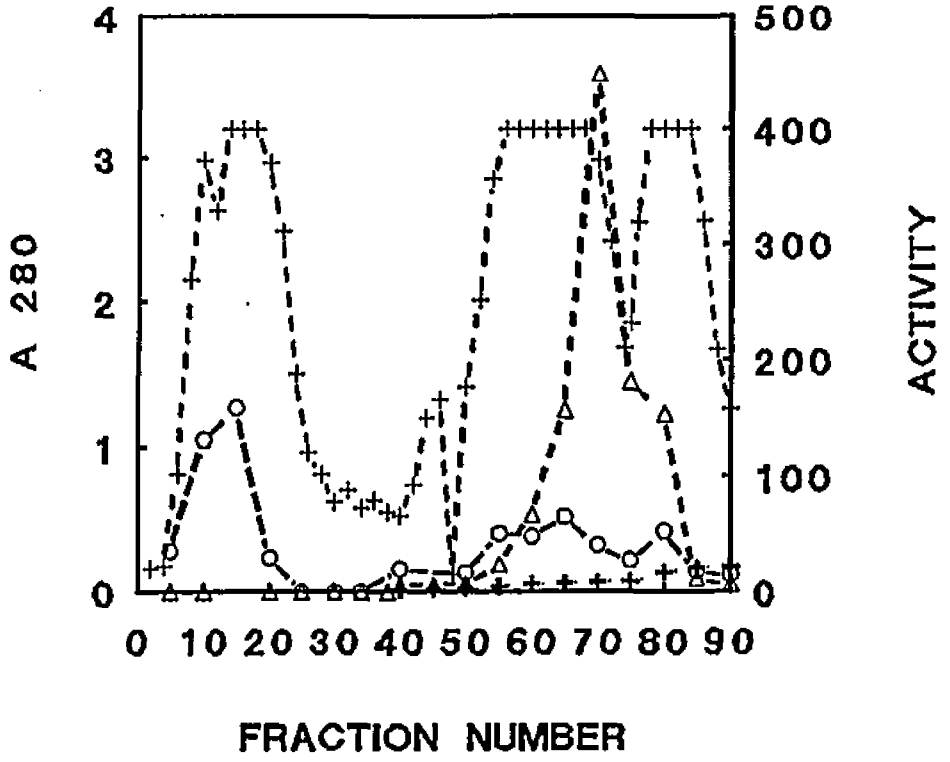

Figure 1. DEAE-Sephacel chromatography of enoyl-ACP reductase. The 38,720 x g supernatant solution of disrupted Euglena gracilis was applied to a 2.5 x 9.0 cm column of DEAE-Sephacel equilibrated in 0.01 M Tris-HCl, pH 7.6, 2 mM DTT. Enoyl-ACP reductase was eluted by a linear gradient of 0-0.2 M NaCl in the same buffer, which began at fraction 45. Fractions of 2 ml were collected. Enoyl-ACP reductase activity is in units/ml. (+--+) absorbance at 280 nm. (O--O) activity of acetoacetyl-ACP reductase. (Δ--Δ) activity of enoyl-ACP reductase.

of 0.5 M NaCl. The recovery of activity was generally greater than 100%; in this case, 31,472 units of activity, or 253% of the starting activity, were recovered.

Matrex Orange Chromatography of Enoyl-ACP Reductase

The pooled protein eluted from DEAE-Sephacel was concentrated by precipitation with ammonium sulfate at 70% of saturation. Precipitated protein was collected by centrifugation and desalted into 0.01 M Tris-HCl, pH 7.6, 2 mM DTT by chromatography on Sephadex G-25. A final volume of 60 ml containing 135 mg of protein and 8,070 units of activity were applied to a Matrex Orange column as shown in Figure 2. When the column was developed with a linear gradient of 0 - 5.0 M sodium chloride, two peaks of enzymatic activity eluted, at sodium chloride concentrations of 0.04 M (Pool A) and 0.41 M (Pool B). A total of 36% of the applied activity was recovered, 876 units in the fall-through peak, 1,185 units in Pool A and 807 units in Pool B. The specific activities of the three pools were 14.1 units/mg protein, 502.1 units/mg protein, and 1,120.8 units/mg protein respectively.

ACP-Affinity Chromatography of Enoyl-ACP Reductase

The final step in the purification of the enoyl-ACP reductase was affinity chromatography on ACP-agarose. Enzyme which had been purified by Matrex Orange chromatography (Pool B) was concentrated by ammonium sulfate

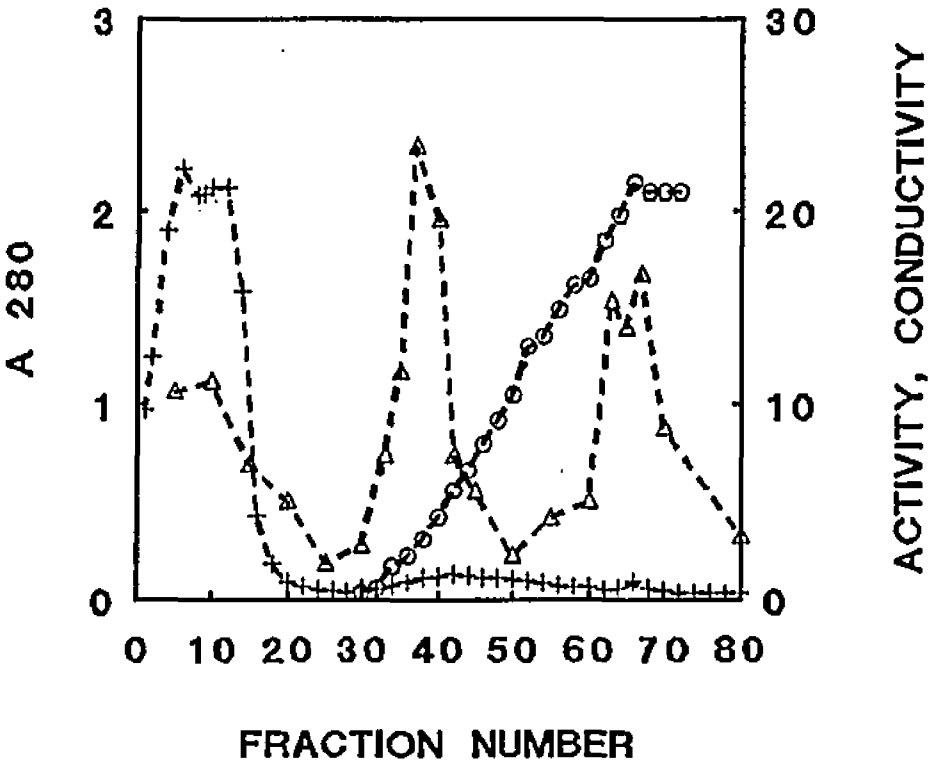

Figure 2. Matrex Orange chromatography of enoyl-ACP reductase. Enzyme purified by anion exchange chromatography was precipitated with ammonium sulfate and collected by centrifugation. It was chromatographed on Sephadex G-25 equilibrated in 0.01 M Tris-HCl, pH 7.6, 2 mM DTT, and applied to a 2.5 x 17 cm Matrex Orange column equilibrated in the same buffer. Enoyl-ACP reductase was eluted by a linear gradient of 0-0.5 M NaCl in the same buffer, beginnning at fraction 30. Fractions of 2 ml were collected. (+--+) absorbance at 280 nm. (Δ--Δ) enoyl-ACP reductase activity, units/ml. (O--O) conductivity, mmhos.

precipitation, collected by centrifugation, desalted into 0.01 M Tris-HCl, pH 7.6, 2 mM DTT by chromatography on Sephadex G-25, and applied to the ACP-agarose column (Figure 3) equilibrated in the same buffer. A total volume of 21 ml containing 1.3 mg of protein and 686.5 units of activity was applied. A large fall-through peak of activity was seen. The major peak of activity eluted off the column at a salt concentration of 0.04 M NaCl.

The purification of enoyl-ACP reductase employed three chromatographic steps as shown in Table 1. In the first step, DEAE-Sephacel chromatography, 690 mg of protein and 12,441 units of activity were applied to the column in a volume of 60 ml. The recovery of protein was 343 mg or 50% yield, and the recovery of activity was 31,472 units or 253% yield. The specific activity increased from 18.03 units/mg of protein to 91.76 units/mg of protein, achieving a purification of 5-fold. In the next step, Matrex Orange chromatography, 327.6 mg of protein and 34,190 units of activity were applied. Of this 4.24 mg or 1.3% of the protein and 4,484 units or 13% of the activity were recovered in pool B. The specific activity was 1,057 units/mg of protein, and a 59-fold purification was achieved. In the final step, ACP-agarose affinity chromatography, 1.38 mg of protein and 686 units of activity were applied to the column. Recovery of protein in the eluted peak was 0.04 mg or 2.9%, while 243 units of activity

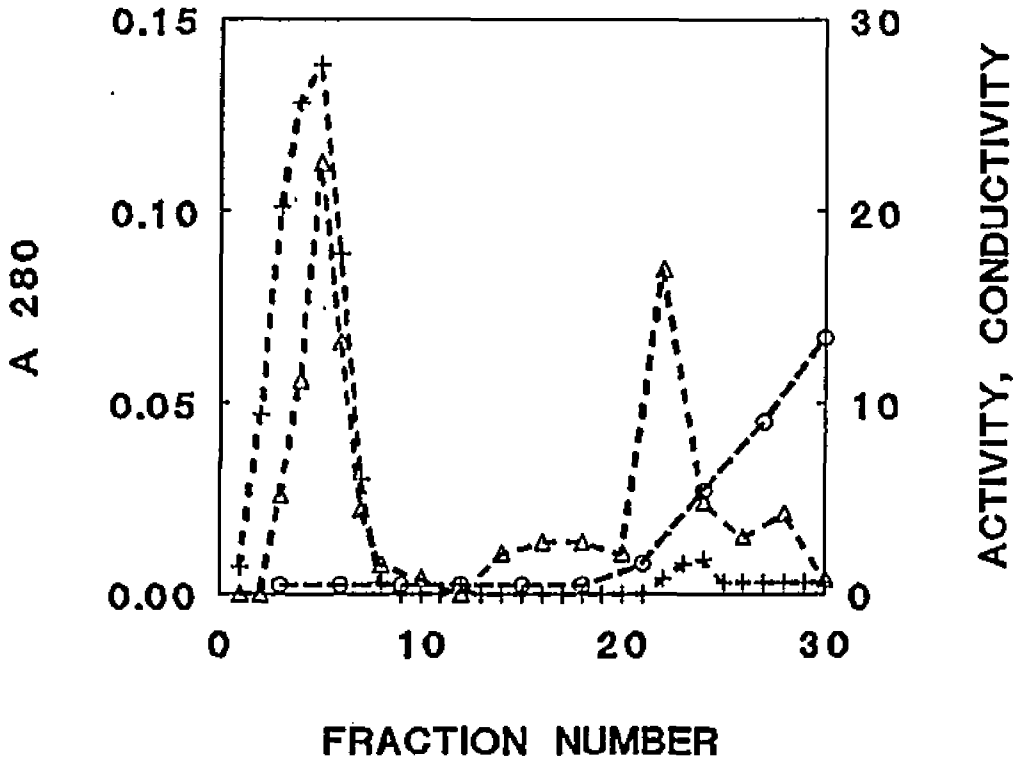

Figure 3. Affinity chromatography of enoyl-ACP reductase. Enzyme which was purified by Matrex Orange chromatography (pool B) was concentrated by ammonium sulfate precipitation. Following chromatography on Sephadex G-25 equilibrated in 0.01 M Tris-HCl, pH 7.6, 2 mM DTT, the enzyme was applied to a 12 ml affinity column composed of E. coli ACP covalently linked to agarose. Elution was with a linear gradient of 0-0.5 M NaCl in the same buffer, and 2 ml fractions were collected. (+--+), absorbance at 280 nm. (Δ--Δ) enoyl-ACP reductase activity, units/ml. (O--O) conductivity, mmhos.

Table 1. Purification of enoyl-ACP reductase

Step	Vol. (ml)	Total Protein (mg)	Total Units*	Specifc Activity**	Purification -fold	% Yield
Crude Supernatant	60	690	12,440	18	-	100
DEAE Sephacel	156	343	31,472	91	5	253
Matrex Orange	101	4.24	4,484	1,057	59	36
ACP Sepharose	18	0.04	243	6,090	338	2

*A unit of enzyme activity is defined as the amount of protein that will oxidize one nmole/min of NADPH under standard assay conditions.

**Specific activity is in units/mg of protein.

or 35.5% were recovered. The specific activity of the eluted peak was 6,090 units/mg, for a purification of 338-fold.

Characterization of Enoyl-ACP Reductase

The stability of enoyl-ACP reductase which had been purified by anion exchange chromatography was examined over the pH range 6.5 to 9.0. The residual activity was determined following dialysis for 24 hours at 4°C against the buffers indicated in Table 2. The same data are graphically presented in Figure 4. It was necessary to include the sulfhydryl reagent DTT in all buffers, as the enzyme rapidly lost activity in its absence. For instance, following 24 hours at 4°C in 0.01 M imidazole-HCl at pH 7.5, 114% of the original activity was recovered if the buffer included 2 mM DTT, while only 79% of activity was recovered if DTT was not included. The same effect was observed with 0.01 M TES at pH 8.0, where 99% of activity was retained when DTT was included in the buffer, and only 46% of activity when DTT was not present.

A comparison of the residual activity following dialysis for 24 hours at 4°C in three different buffers showed full retention of activity over the pH range 7.5 to 8.0. In 0.01 M Tris-HCl, 2 mM DTT at a pH of 7.5 the enzyme retained 98% of its original activity, as shown in Table 2. The amount of residual activity dropped to 53% as the pH of

Table 2. Activity of enoyl-ACP reductase following incubation for 24 hours at 4°C in various buffers.

Buffer	Specific Activity*	Mean ± S.E.M.	% of Original Activity**
0.01 M Tris-HCl, pH 9.0, 2 mM DTT	7.0 7.0	7.0 ± 0	53
0.01 M Tris-HCl, pH 8.5, 2 mM DTT	10.5 9.3 9.8 8.3	9.5 ± 0.9	72
0.01 M Tris-HCl, pH 8.0, 2 mM DTT	9.6 10.5 9.0 10.5	9.9 ± 0.7	75
0.01 M Tris-HCl, pH 7.5, 2 mM DTT	13.6 12.4 12.7	12.9 ± 0.6	98
0.01 M TES, pH 8.0, 2 mM DTT	12.9 13.2 12.3 13.9	13.1 ± 0.7	99
0.01 M TES, pH 7.0, 2 mM DTT	11.7 11.4 9.9 12.7	11.4 ± 1.2	86
0.01 M imidazole, pH 7.5, 2 mM DTT	16.2 15.6 14.6 13.9	15.1 ± 1.0	114
0.01 M imidazole, pH 7.0, 2 mM DTT	10.2 12.6 10.8 12.0	11.4 ± 1.1	86
0.01 M imidazole, pH 6.5, 2 mM DTT	6.5 6.4 6.7	6.7 ± 0.3	51

*Specific activity is units/mg of protein.

**The original activity was the activity of the enzyme measured under standard assay conditions, before the overnight incubation at 4°C.

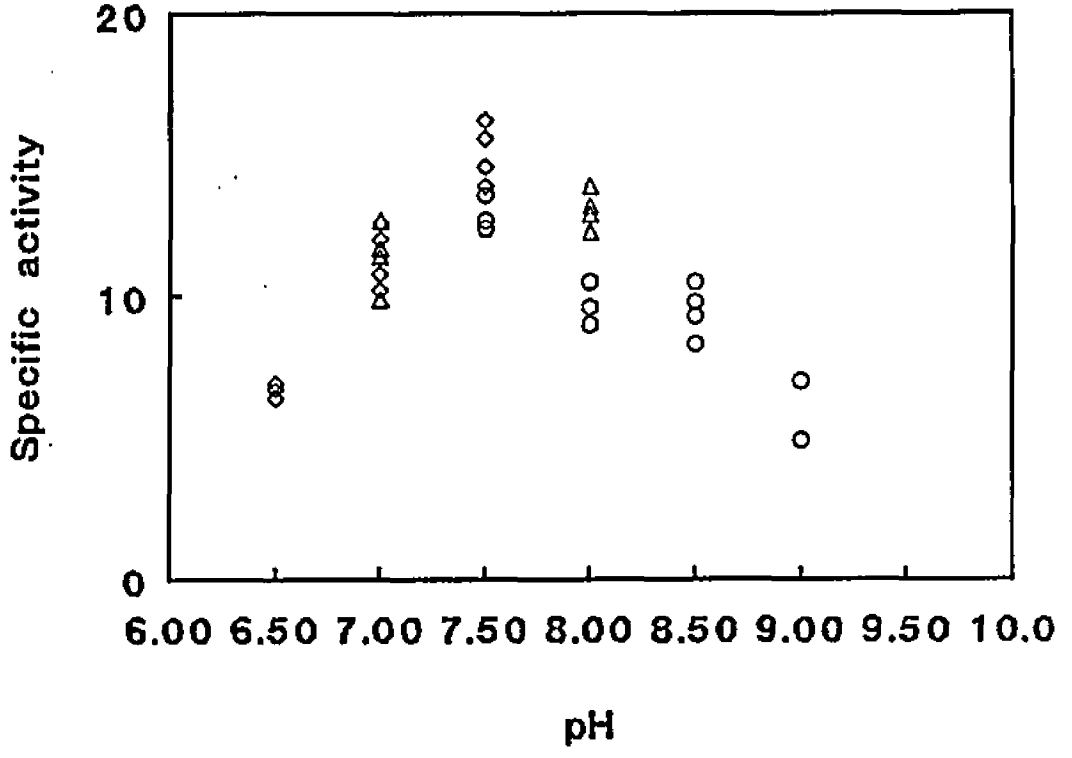

Figure 4. Stability at 4°C of enoyl-ACP reductase at different pH's. Enoyl-ACP reductase purified by anion exchange chromatography was dialyzed for 24 hours at 4°C against the buffers indicated. Specific activity is in units/mg of protein. The specific activity of the enzyme before dialysis was 13.2 units/mg of protein. (◇) 0.01 M imidazole-HCl, 2 mM DTT. (△) 0.01 M TES, 2 mM DTT. (○) 0.01 M Tris-HCl, 2 mM DTT.

that buffer was raised to pH 9.0. In 0.01 M imidazole-HCl, 2 mM DTT at pH 7.5, the activity recovered was 114% of the starting activity and dropped to 51% as the buffer's pH was dropped to pH 6.5. The third buffer, 0.01 M TES, 2 mM DTT, was checked at pH 8.0, where it supported 99% of the original activity, and at pH 7.0, where it supported 86% of activity.

The effect on enzyme which had been purified by anion exchange chromatography of including several reagents in the standard assay was also examined. As shown in Table 3, the addition of either $MgCl_2$ or $CaCl_2$ to a final concentration of 10 mM in the assay caused a marked enhancement of activity, to 145% and 138% of control, respectively. Potassium chloride at concentrations ranging from 10 mM up to 188 mM in the assay did not affect activity appreciably; at the higher concentration, activity was 93% of the control. The activity of the enzyme in the presence of EDTA at 2 mM was 89% of the control. There was a strong requirement for DTT in the assay, as enzyme assayed in its absence exhibited only 69% of control activity.

Enoyl-ACP reductase at this stage of its purification was quite stable in the buffer in which it was eluted from the DEAE-Sephacel column. As shown in Table 4, no activity was lost following 24 hours at 4°C in 0.01 M Tris-HCl pH 7.6 containing 2 mM DTT and 0.2 M NaCl. Even after 72 hours at 4° C, 55% of the original activity was recovered.

Table 3. Effect of various reagents on the activity of enoyl-ACP reductase. Enoyl-ACP reductase purified by anion exchange chromatography was assayed in the presence of the following reagents in the concentrations indicated.

Reagent added	Specific Activity*	Mean ± S.E.M.	% of Control**
none	19.4 19.4 17.0	18.6 ± 1.4	100
10 mM $MgCl_2$	29.4 24.9 26.6	27.0 ± 2.3	145
10 mM $CaCl_2$	27.7 25.3 24.2	25.7 ± 1.8	138
10 mM KCl	17.0 16.6 19.4	17.7 ± 1.5	95
188 mM KCl	18.0 17.6 16.3	17.3 ± 0.9	93
2 mM EDTA	17.3 16.6 15.9	16.6 ± 0.7	89
without DTT	11.4 13.5 13.5	12.8 ± 1.2	69

*Specific activity is units/mg of protein.

**Control activity was measured in the standard assay with no additive.

Table 4. The stability over time of enoyl-ACP reductase at 4°C. The enzyme, purified by anion exchange chromatography, was maintained at 4°C in 0.01 M Tris-HCl pH 7.6, 2 mM DTT, 0.2 M NaCl.

Time	Specific Activity*	Mean ± S.E.M.	% of Initial Activity
initial	28.0	26.0 ± 2.8	100
	24.0		
24 hours	27.4	27.7 ± 0.4	107
	28.0		
48 hours	17.3	17.7 ± 0.5	68
	18.0		
72 hours	15.3	14.3 ± 1.4	55
	13.3		

*Specific activity is units/mg of protein

Similarly, there was no loss of activity during storage for two months at -20°C, and, in fact, an increase to 116% of the original activity was observed (Table 5). The same effect was seen following storage for two months in 0.01 M TES pH 7.4, 0.15 M NaCl, 0.2 M sucrose, 2 mM DTT. In this case, 115% of control activity was recovered following two months at -20°C, and 126% of activity was recovered following two months at -85°C.

The M_r of enoyl-ACP reductase was determined by gel filtration using Biogel A 0.5m. The activity chromatographed as two peaks, at M_rs of 52,600 and 36,800, as shown in Figure 5. Attempts to achieve a better separation of the two peaks of activity by using molecular sieves with a narrower range or different column geometries were to no avail.

Bovine serum albumin (BSA) is known to enhance the activity of some FASs (Knoche et al., 1973), apparently by facilitating product removal. Its effect on the activity of enoyl-ACP reductase purified by gel filtration chromatography on Biogel A 0.5m was checked. It was inhibitory to this enzyme when included in the assay, reducing the activity to 74% of control when present at levels of 0.125 mg/ml as shown in Table 6.

Polyethylene glycol 4000 (PEG 4000) stabilized the pooled active peaks eluted off the Biogel A 0.5m column to a remarkable extent (Table 7, graphically depicted in

Table 5. The effect of storage at -20°C or -85°C on the activity of enoyl-ACP reductase. Enoyl-ACP reductase purified by anion exchange chromatography was stored for two months at -20°C in 0.01 M Tris-HCl, pH 7.6, 0.2 M NaCl, 2 mM DTT (part A) or for two months at either -20°C or -85° in 0.01 M TES, pH 7.4, 0.15 M NaCl, 0.2M sucrose, 2 mM DTT (part B).

		Specific Activity*	Mean ± S.E.M.	% of Initial Activity
A.	initial	10.0	10.4 ± 0.5	100
		10.7		
	after 2 months	11.3	12.1 ± 1.1	123
	at -20°C	12.8		
B.	initial	16.3	16.8 ± 0.6	100
		17.2		
	after 2 months	18.5	19.3 ± 1.1	115
	at -20°C	20.1		
	after 2 months	21.1	21.2 ± 0.1	126
	at -85°C	21.3		

*Specific activity is units/mg of protein.

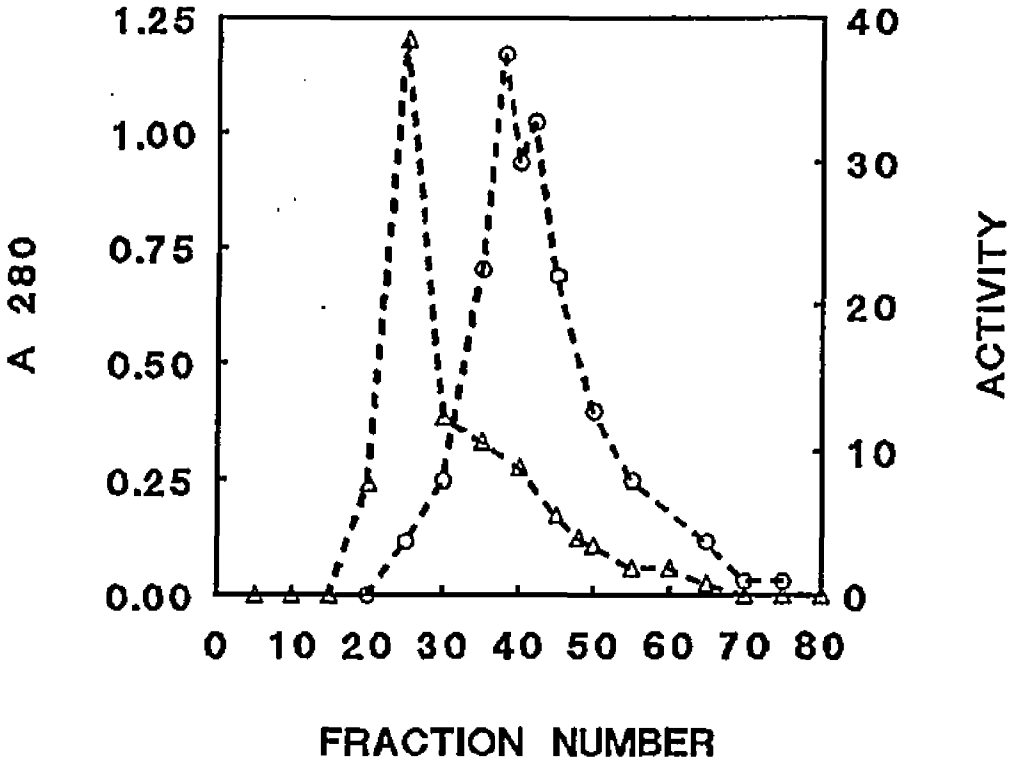

Figure 5. Biogel A 0.5m chromatography of enoyl-ACP reductase. Enoyl-ACP reductase purified by anion exchange chromatography was concentrated by precipitation with ammonium sulfate, collected by centrifugation, resuspended in a minimal volume of 0.01 M TES, pH 7.4, 0.15 M NaCl, 0.2 M sucrose, 2 mM DTT, and applied to a 2.5 x 41 cm Biogel A 0.5m column equilibrated and developed in the same buffer. Fractions of 2 ml were collected. The column was calibrated with blue dextran (void volume), yeast alcohol dehydrogenase (M_r 141,000), human transferrin (M_r 74,000), bovine erythrocyte carbonic anhydrase (M_r 30,000), and bovine pancreatic ribonuclease (M_r 13,700). (Δ--Δ) absorbance at 280 nm. (O--O) enoyl-ACP reductase activity, units/ml.

Table 6. The effect of BSA added to the assay on the activity of enoyl-ACP reductase purified by gel filtration.

Concentration of BSA (mg/ml)	Specific Activity*	Mean ± S.E.M.	% of Control
0	54.0	51.7 ± 3.3	100
	49.3		
0.038	42.0	43.9 ± 2.7	85
	45.8		
0.125	40.2	38.3 ± 2.7	74
	36.4		

*Specific activity is units/mg of protein.

Table 7. The effect of 30% (w/v) PEG 4000 on the stability of enoyl-ACP reductase. Enoyl-ACP reductase purified by DEAE sepharose chromatography and gel filtration was diluted with an equal volume of buffer (0.01 M TES, pH 7.4, 0.2 M sucrose, 0.15 M NaCl, 2 mM DTT) or buffer containing 60% (w/v) PEG 4000. The enzyme control (A) and enzyme containing 30% PEG 4000 (B) were then incubated for the times indicated at room temperature.

	Time (hours)	Specific Activity*	Mean ± S.E.M.	% of Initial Activity
A	2	24.3	23.9 ± 0.6	78
		23.4		
	8	21.5	20.1 ± 2.0	65
		18.7		
	24	19.6	20.1 ± 0.7	65
		20.6		
	48	14.0	13.1 ± 1.3	43
		12.1		
B	2	39.2	40.2 ± 1.3	131
		41.1		
	8	34.6	37.4 ± 4.0	121
		40.2		
	24	31.8	31.8 ± 0	103
		31.8		
	48	22.4	22.9 ± 0.7	74
		23.4		

*Activity is units/ml

**The initial activity of the diluted enzyme was 30.8 units/ml

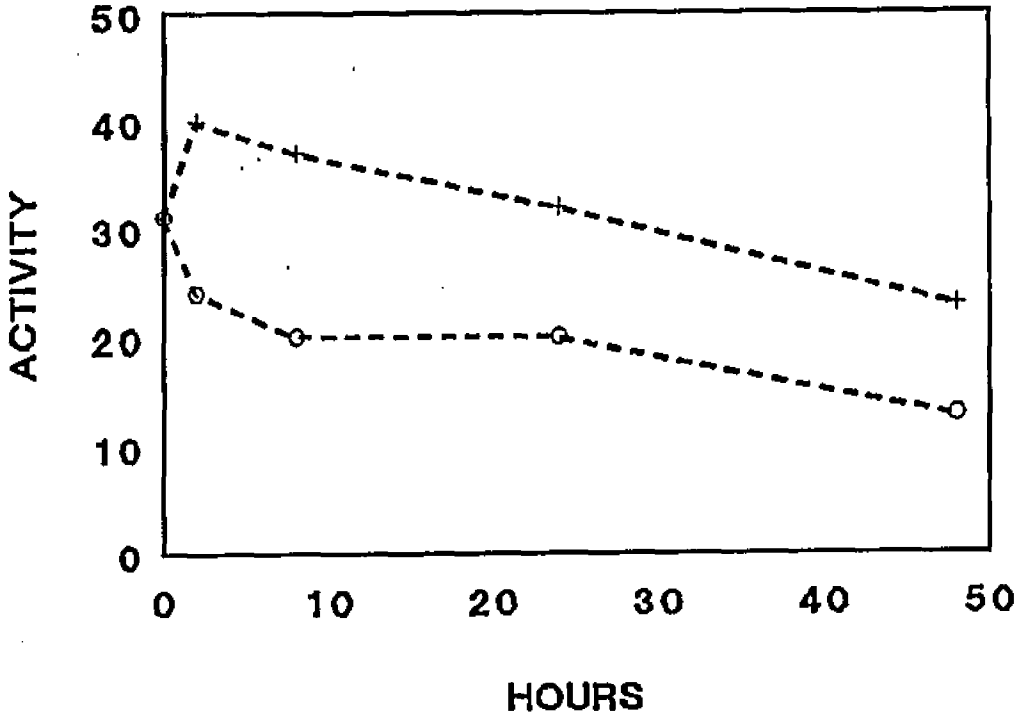

Figure 6. The effect of 30% PEG 4000 on the stability of enoyl-ACP reductase. The enzyme, purified by gel filtration, was diluted 1:1 with either 0.01 M TES, pH 7.4, 0.15 M NaCl, 0.2 M sucrose, 2 mM DTT (O--O) or with 60% (w/v) PEG 4000 in the same buffer (+--+) and incubated at room temperature. Aliquots were removed at the times indicated and the activity in units/ml determined.

Figure 6). A sample of the pooled enzyme was diluted 1:1 with either 0.01 M TES, pH 7.4, 0.15 M NaCl, 0.2 M sucrose, 2 mM DTT for the control, or with 60% (w/v) PEG 4000 in the same buffer, and incubated at room temperature for the times indicated. At early time points the activity of enzyme in 30% PEG 4000 was enhanced, up to 131% of the initial activity after two hours at room temperature. The activity was still 121% of control activity after eight hours, and full activity was retained by the enzyme following 24 hours at room temperature in 30% PEG 4000. Even after 48 hours, the activity had only decreased to 74% of the control activity. The effect of incubation at room temperature on the control was equally interesting. Following an initial rapid loss of activity over the first eight hours, the loss of activity stabilized and was quite slow over the next 24 hours. Even after 48 hours at room temperature, the enzyme still retained 43% of activity.

The two peaks of activity eluted by NaCl from the Matrex Orange column were compared by varying the assay pH and by adding several different reagents to the assay. Pool A eluted at 0.04 M NaCl while Pool B eluted at 0.41 M NaCl. Pool A and pool B showed optimal activities at the two extremes of assay pH tested. As shown in Table 8, pool A was most active at a pH of 7.0, and its activity decreased as the assay pH increased. At an assay pH of 8.0, the activity had decreased to 52% of the activity measured at a

Table 8. Effect of assay pH on the activities of enoyl-ACP reductases purified by Matrex Orange chromatography (pool A and pool B).

pH	Specific Activity*	Mean ± S.E.M.	% of Control
Pool A			
7.0	758.9	735.6 ± 33.0	100
	712.2		
7.2	665.5	683.0 ± 24.7	93
	700.5		
7.6	642.1	583.8 ± 82.5	79
	525.4		
8.0	397.0	379.5 ± 24.8	52
	361.9		
Pool B			
pH			
7.0	1068.0	1068.0 ± 0	83
	1068.0		
7.2	756.5		
	778.8	771.4 ± 12.9	60
	778.8		
7.6	979.0	1001.3 ± 31.5	78
	1023.5		
8.0	1268.3		
	1201.5	1290.5 ± 102.0	100
	1401.8		

*Specific activity is units/mg of protein.

pH of 7.0. Pool B was most active at an assay pH of 8.0, and its activity decreased as pH decreased; its activity at a pH of 7.2 was 60% of the activity measured at pH 8.0. As the pH was further decreased to pH 7.0 the activity increased, to 83% of the activity measured at pH 8.0. The enzyme being assayed in this particular experiment may have been contaminated by pool A. Matrex Orange proved to have a limited lifespan; following two to three uses, it continued to bind the reductase but failed to resolve the two peaks of activity. Incomplete resolution of the two peaks of activity would explain the observed increase in activity at pH 7.0.

Sodium chloride was quite inhibitory to pool A as shown in Table 9. Activity, which was measured at an assay pH of 7.0, decreased steadily as the sodium chloride concentration increased. At a concentration of 0.075 M in the assay, activity was 70% of the activity without added salt. Dithiothreitol was not necessary for the enzyme, as enzyme assayed without added DTT was only slightly inhibited, to 87% of control. The effect of divalent cations was the opposite of the effect seen with enzyme purified through DEAE-Sephacel chromatography. Calcium chloride was slightly inhibitory; activity in the presence of 10 mM $CaCl_2$ was 91% of the activity in its absence. Magnesium chloride at the same concentration reduced the activity to 83% of control. There was essentially no effect on activity when 10 μM ACP

Table 9. Factors affecting the activity of enoyl-ACP reductase purified by Matrex Orange chromatography (Pool A).

Reaction Mix 1	Specific Activity*	Mean ± S.E.M.	% of Control
Control	758.9 712.2	735.6 ± 33.0	100
+0.025 M NaCl	653.8 607.1	630.5 ± 33.0	86
+0.05 M NaCl	548.7 537.1	542.9 ± 8.2	74
+0.075 M NaCl	513.7 513.7	513.7 ± 0	70
-DTT	653.8 630.5	642.2 ± 16.5	87
+10 mM $CaCl_2$	653.8 677.2	665.5 ± 16.5	91
+10 mM $MgCl_2$	642.1 583.8	613.0 ± 41.2	83
+10 μM ACP	758.9 642.1	700.5 ± 82.6	95
Reaction Mix 2			
Control	502.0 455.3	478.7 ± 33.0	100
+10 μM ACP	455.3 397.0	426.2 ± 41.2	89

*Reaction mix 1 contained 0.1 M TES pH 7.0, 3 mM DTT, 0.025 mM NADPH, enzyme, water, and 0.2 mM crotonyl-CoA in a final volume of 800 μl.

**Specific activity is units/mg of protein.

***Reaction mix 2 contained 0.1 M TES pH 8.0, 3 mM DTT, 0.025 mM NADPH, enzyme, water, and 0.2 mM crotonyl-CoA in a final volume of 800 μl.

was included in the assay at pH 7.0. A slight decrease, to 89% of control, was observed when 10 μM ACP was included in the assay and activity was measured at pH 8.0.

By contrast, pool B was substantially inhibited by 10 μM ACP in the assay, as shown in Table 10. Pool B was assayed at pH 8.0. Its activity in the presence of 10 μM ACP was only 66% of the activity measured in its absence. The effects on pool B of 10 mM $CaCl_2$ and 10 mM $MgCl_2$ were also very different from their effects on pool A. The addition of calcium chloride to 10 mM nearly doubled the activity, to 191% of the activity measured in its absence. Magnesium chloride had no effect on activity, while 2 mM EGTA slightly increased activity, to 119% of control. The effect of 10 μM ACP when included with each of these was also checked. ACP was found to be inhibitory only when included in the control (no additive) or when added with calcium chloride. Inhibition in both of these cases was about 44% of the activity measured in the absence of ACP. The effect of added ACP in the assay was negligible when magnesium chloride was also present, and activity was not affected by including both EDTA and ACP. The exclusion of DTT from the assay led to a decrease of almost 20% in the activity of pool B.

The approximate pI of enoyl-ACP reductase was determined. Chromatofocusing was performed on a 10 ml column equilibrated in 0.025 M imidazole-HCl, pH 7.4 and

Table 10. Factors affecting the activity of enoyl-ACP reductase purified by Matrex Orange chromatography (Pool B).

Reaction Mix*	Specific Activity**	Mean ± S.E.M.	% of Control
control	1545.5		100
+10 μM ACP	1012.4		66
+10 mM $CaCl_2$	2959.3		191
+10 mM $CaCl_2$, 10 μM ACP	1991.4		129
+10 mM $MgCl_2$	1590.9		103
+10 mM $MgCl_2$, 10 μM ACP	1479.6		96
+2 mM EGTA	1835.6		119
+2 mM EGTA, 10 μM ACP	1924.6		125
control	1268.3 1201.5 1401.8	1290.5 ± 102.0	100
-DTT	956.8 1134.8 1023.5	1038.4 ± 89.9	81

*The standard reaction mix contained 0.1 M TES pH 8.0, 3 mM DTT, 0.025 mM NADPH, enzyme, water, and 0.2 mM crotonyl-CoA in a final volume of 800 μl.

**Specific activity is units/mg of protein.

developed with a pH gradient of pH 7.0 to pH 4.0. As shown in Figure 7, 2,708 units of enoyl-ACP reductase, purified through Matrex Orange, was loaded onto the column. The reductase eluted in a major peak at a pH of approximately 4.8 with a leading shoulder. Only 314 units or 12% of the applied activity was recovered. Collecting fractions into concentrated Tris-HCl pH 7.6 in order to raise the pH as quickly as possible did not help overcome the deleterious effect of low pH on the enzyme.

Isolation of Dinucleotide-Fold Specific Antibodies

An affinity ligand for the dinucleotide binding site of dehydrogenases was constructed. Antibodies directed against yeast glucose-6-phosphate dehydrogenase were raised in white male New Zealand rabbits by standard methods. The immune serum was collected and the IgG fraction isolated by chromatography of serum on DEAE-Affigel Blue. The immunoglobulin was concentrated by precipitation by $(NH_4)_2SO_4$ at 70% of saturation in preparation for the next step, affinity chromatography on chicken liver malic enzyme-Sepharose. The IgG (2.89 mg) in 1.5 ml was applied to the column as shown in Figure 8. A total of 0.12 mg or 4% of the applied protein bound to the malic enzyme and was eluted by 4.5 M $MgCl_2$.

Gel electrophoresis of malic enzyme and glucose-6-phosphate dehydrogenase (Figure 9) showed a single major

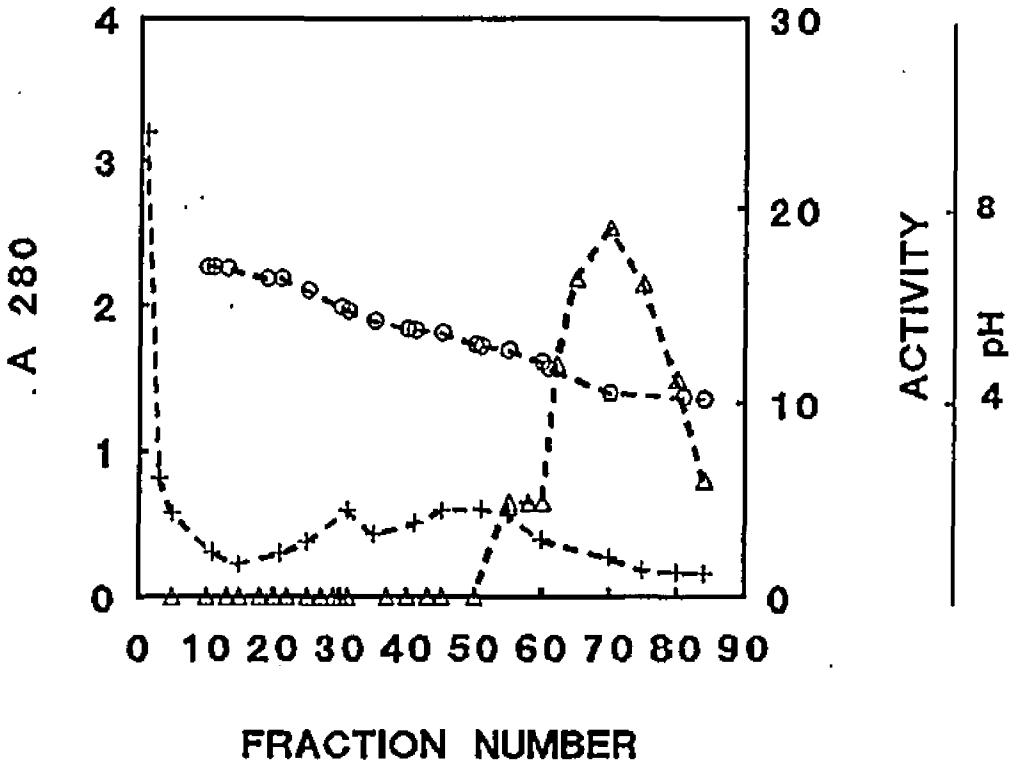

Figure 7. Chromatofocusing of enoyl-ACP reductase. Enzyme which had been purified by chromatography on Matrex Orange was concentrated by ammonium sulfate precipitation, dialyzed against 0.025 M imidazole-HCl, pH 7.4, and applied to a 10 ml chromatofocusing column equilibrated in the same buffer. The column was developed with a linear pH gradient of pH 7.0 to pH 4.0. (+--+) absorbance at 280 nm. (Δ--Δ) enoyl-ACP reductase activity, units/ml. (O--O) pH.

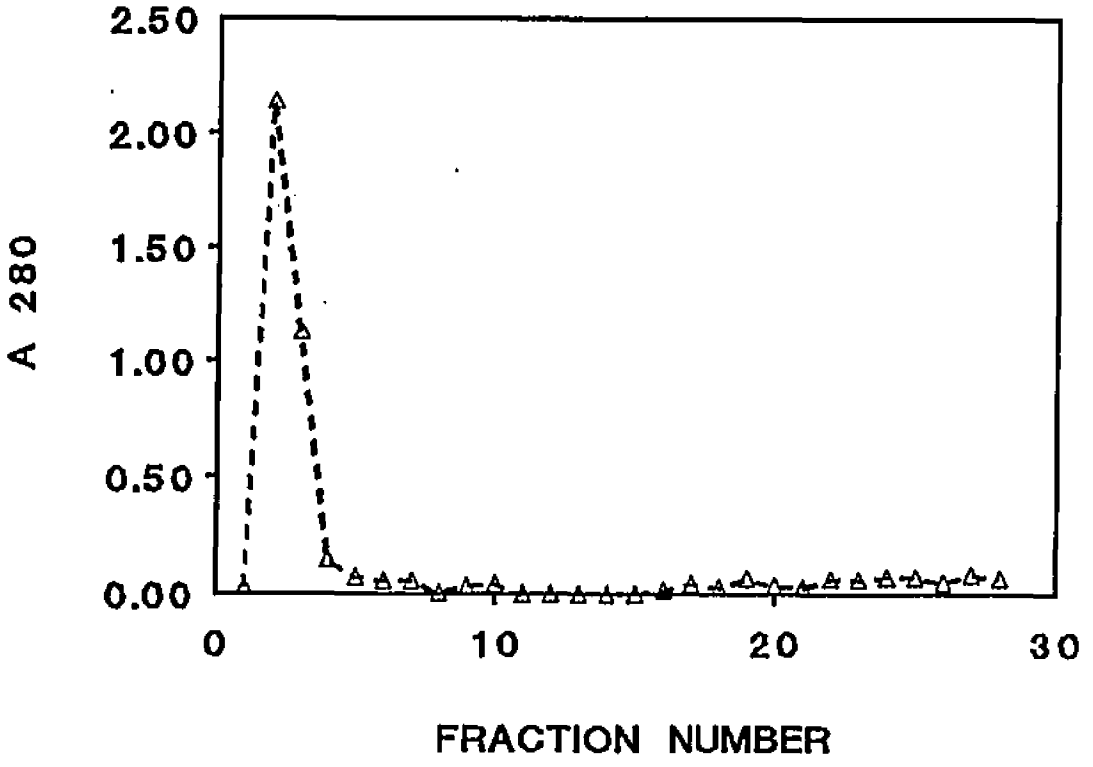

Figure 8. Affinity purification of dinucleotide-fold specific antibodies. The IgG fraction of antiserum raised against yeast glucose-6-phosphate dehydrogenase was applied to a 1.5 ml column composed of chicken liver malic enzyme covalently linked to CNBr-activated Sepharose. The IgG (2.89 mg) and column were in 0.01 M potassium phosphate, pH 7.4, 0.15 M NaCl, 1 mM EDTA. After allowing the IgG to bind for 1.5 hours the coumn was washed with the same buffer. Bound IgG (0.12 mg or 4%) was eluted 4.5 M MgCl titrated to pH 6.5 with 1 M Tris base, freshly made and filtered through glass wool before use. (+--+) absorbance at 280 nm.

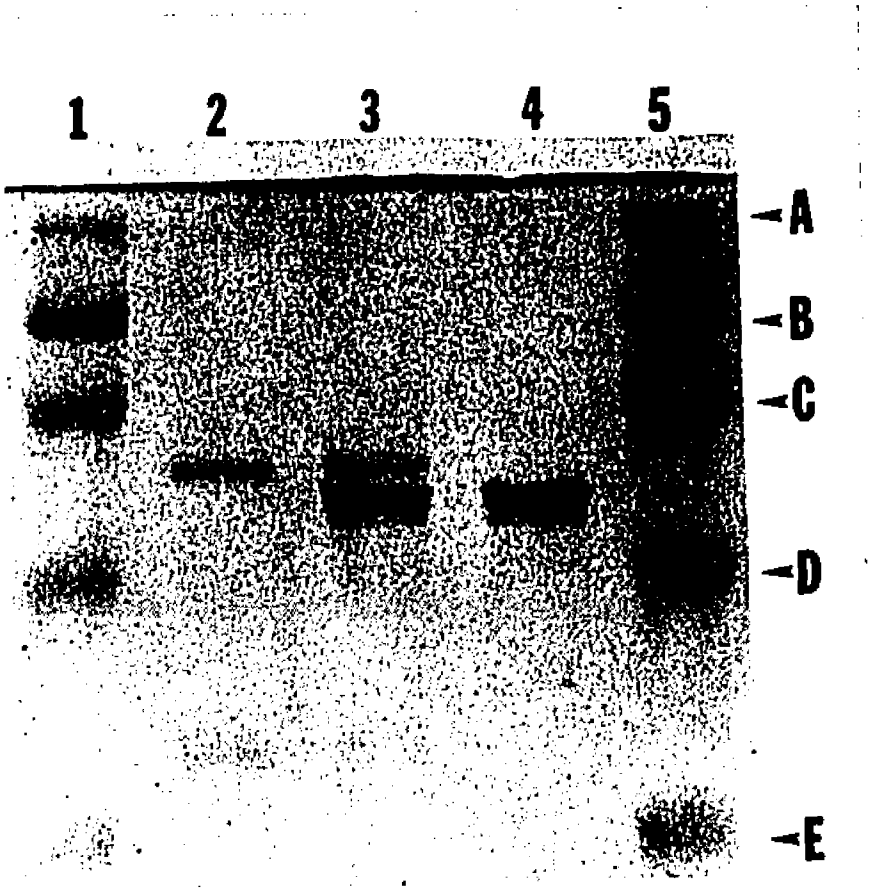

Figure 9. 12% polyacrylamide gel electrophoresis of glucose-6-phosphate dehydrogenase (Boehringer Mannheim) and chicken liver malic enzyme (Sigma). Glucose-6-phosphate dehydrogenase (500 ng) and malic enzyme (1 µg) and subjected to gel electrophoresis throuugh a 12% polyacrylamide gel using the discontinuous buffer system of Laemmli (1970). Lanes 1 and 5 are molecular weight markers, lane 2 is malic enzyme, lane 4 is glucose-6-phosphate dehydrogenase and lane 3 is a mixture of the two enzymes. Protein bands were visualized using the stain described by Steck et al. (1980).

band at apparent M_rs of 58,900 for glucose-6-phosphate dehydrogenase and 61,700 for malic enzyme. Literature values for the yeast and pigeon liver enzymes (monomers) are 64,000 and 70,000, respectively (Andrews, 1965; Hsu and Lardy, 1967). Glucose-6-phosphate dehydrogenase (100 ng) and malic enzyme (1 µg) were then subjected to gel electrophoresis and electrophoretically transferred onto nitrocellulose, as shown in Figure 10. The Western blot was incubated with anti-glucose-6-phosphate dehydrogenase antibodies, followed by alkaline phosphatase-conjugated goat anti rabbit IgG, and cross-reacting protein was visualized by the deposition of stain, using the red color reagents of BioRad. A similar Western blot was performed, using the same amounts of glucose-6-phosphate dehydrogenase and malic enzyme, but using the cross-reacting antibody affinity purified by chromatography on malic enzyme-Sepharose as the probe. The first Western blot demonstrates the recognition by anti-glucose-6-phosphate dehydrogenase antibodies of glucose-6-phosphate dehydrogenase (lane A) and malic enzyme (lane F). The second Western blot demonstrates that the cross-reactive antibody isolated by affinity chromatography is still capable of recognizing both glucose-6-phosphate dehydrogenase (lane C) and malic enzyme (lane H).

The cross-reacting antibody isolated by affinity chromatography was concentrated and pooled, and used to construct a second affinity column which would recognize and

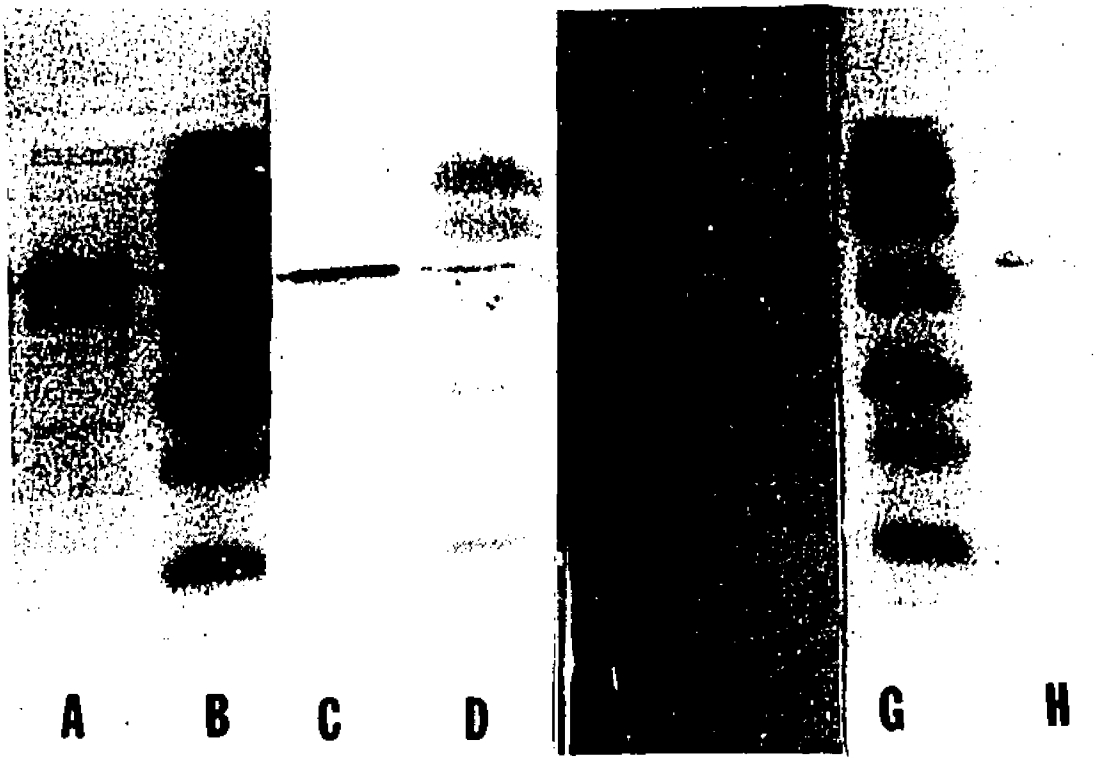

Figure 10. Western blot of glucose-6-phosphate dehydrogenase and malic enzyme. Yeast glucose-6-phosphate dehydrogenase (100 ng, lanes A and C) and chicken liver malic enzyme (1 μg, lanes F and H) were subjected to gel electrophoresis and transferred electrophoretically onto nitrocellulose paper. The Western blots were probed with either anti-glucose-6-phosphate dehydrogenase antibodies (lanes A and F) or with anti-dinucleotide fold antibodies (lanes C and H). Bound antibody was detected by incubating the membrane with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies, as visualized by the deposition of dye from the Fast Red alkaline phosphatase substrates obtained from BioRad. Lanes B, D, E and G are prestained molecular weight markers from BioRad.

bind the dinucleotide folds of unrelated enzymes. Glucose-6-phosphate dehydrogenase (0.1 mg of protein containing 9,863 units of activity) was applied to the column, which was then washed until activity dropped to zero as shown in Figure 11. Although the protein peak as measured by absorbance at 280 nm dropped to zero very quickly, the activity continued to trail off of the column for quite a long time. This was assumed to be due to a weak interaction of the enzyme with the antibody, which caused it to be retarded by the column without being tightly bound. When the activity reached zero, the column was washed with $NADP^+$ at a concentration of 5.6×10^{-5} M; this concentration was used so that the eluted protein could be assayed directly at the correct $NADP^+$ concentration without requiring additional cofactor. A peak of activity eluted when the cofactor was applied, 2.82 units or 0.03% of the total applied activity. No malic enzyme activity was measurable in the eluted protein peak.

The immunoaffinity column also recognized and bound malic enzyme as shown in Figure 12. Malic enzyme (2,839 units in 0.64 mg of protein) was applied to the column. In this instance, both the absorbance and the fall-through activity dropped to zero almost immmediately. A wash of the column with 0.27 μM $NADP^+$ resulted in the elution of a peak of activity, 12.3 units or 0.1% of the applied activity. No

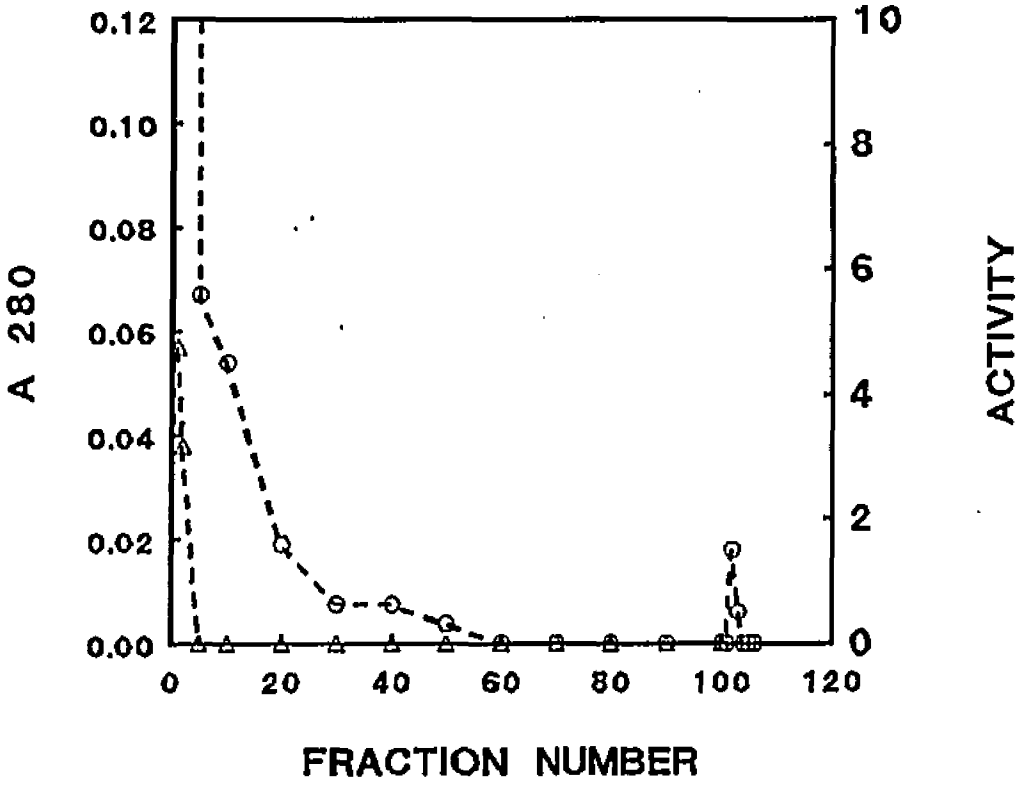

Figure 11. Immunoaffinity chromatography of yeast glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase, 9,863 units of activity, was applied to an affinity column composed of dinucleotide-fold specific antibodies covalently linked to CNBr-activated Sepharose. The column was equilibrated and run in 0.01 M Tris-HCl, pH 7.6, 0.15 M KCl. Bound enzyme (2.82 units or 0.03% of the applied acativity) was eluted beginning at fraction 100 by $5.6 * 10^{-5}$ $NADP^+$ in the same buffer. Fractions of 2 ml were collected. (Δ--Δ) absorbance at 280 nm. (O--O) activity, nm of $NADP^+$ reduced/min/ml.

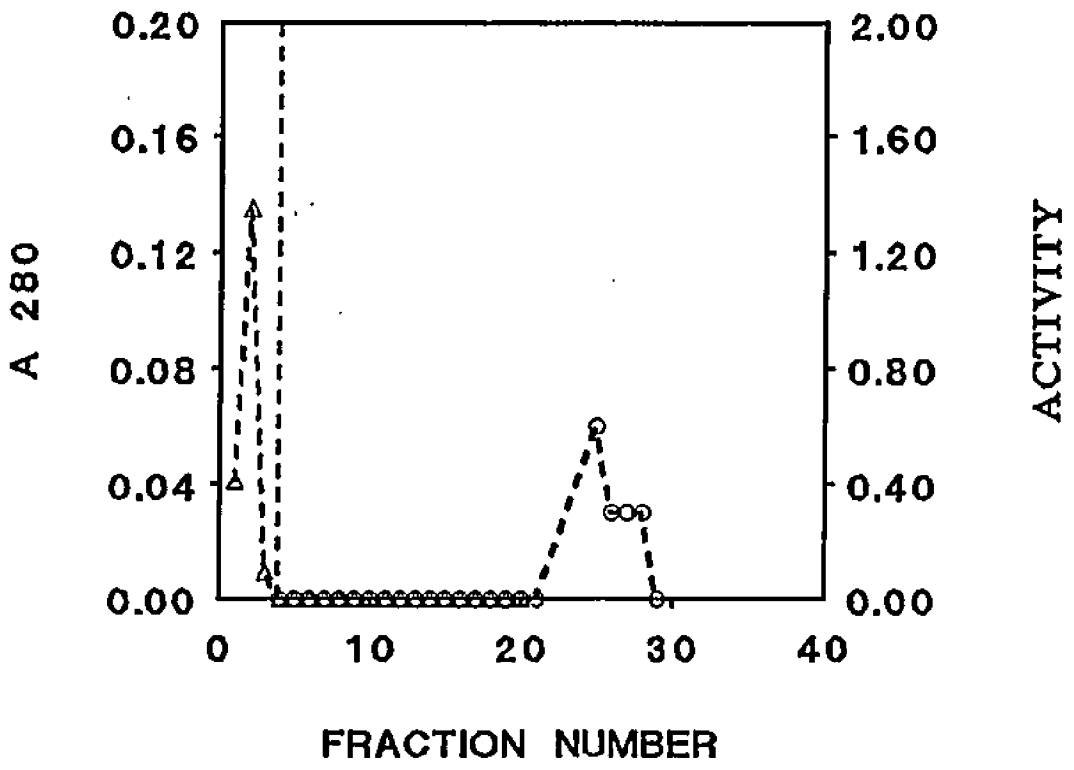

Figure 12. Immunoaffinity chromatography of chicken liver malic enzyme. Malic enzyme, 2,84 units of activity, was applied to the affinity column composed of dinucleotide-fold specific antibodies covalently linked to CNBr-activated Sepharose. The column was equilibrated and run in 0.05 M Tris-HCl, pH 7.0, 10 mM EDTA, 0.2 M magnesium acetate. Bound enzyme (12.3 units or 0.1% of the applied activity) was eluted by 0.27 μM $NADP^+$ in the same buffer, beginning at fraction 20. (Δ--Δ) absorbance at 280 nm. (O--O) malic enzyme activity, nm of $NADP^+$ reduced/min/ml.

glucose-6-phosphate dehydrogenase activity was measurable in the eluted protein peak.

Enoyl-ACP reductase (pool B from Matrex Orange chromatography, 41.3 units) was applied to the column as shown in Figure 13. Again, the absorbance at 280 nm and the fall-through activity peaks dropped to zero almost immediately. A brief wash of the column with 0.23 mM NADPH resulted in the elution of 2.4 units or 5.8% of the total applied activity.

Gel electrophoresis was performed on enoyl-ACP reductase which had been purified by Matrex Orange chromatography (pool B) and on the active peaks obtained by ACP-agarose affinity chromatography, and affinity chromatography on the anti-dinucleotide fold antibody-Sepharose column. The silver stained gel is shown in Figure 14. Clearly the enzyme is very impure following Matrex Orange chromatography, as shown by the multiple bands in lanes 1 (Matrex Orange pool A) and 2 (Matrex Orange pool B). In lanes 3, 4 and 5, the two prominent bands are artefacts, which appeared only in silver stained gels and were traced to an aging bottle of 2-mercaptoethanol. Beside the two artefactual bands, lane 3 contains a very prominent band at an apparent M_r of 41,300, and less intense bands at M_rs of 28,000 and 48,400. The only band visible in lane 4, the eluent from the immunoaffinity column, is a doublet at an M_r of 48,400. Lane 5 is from a different gel of enoyl-ACP

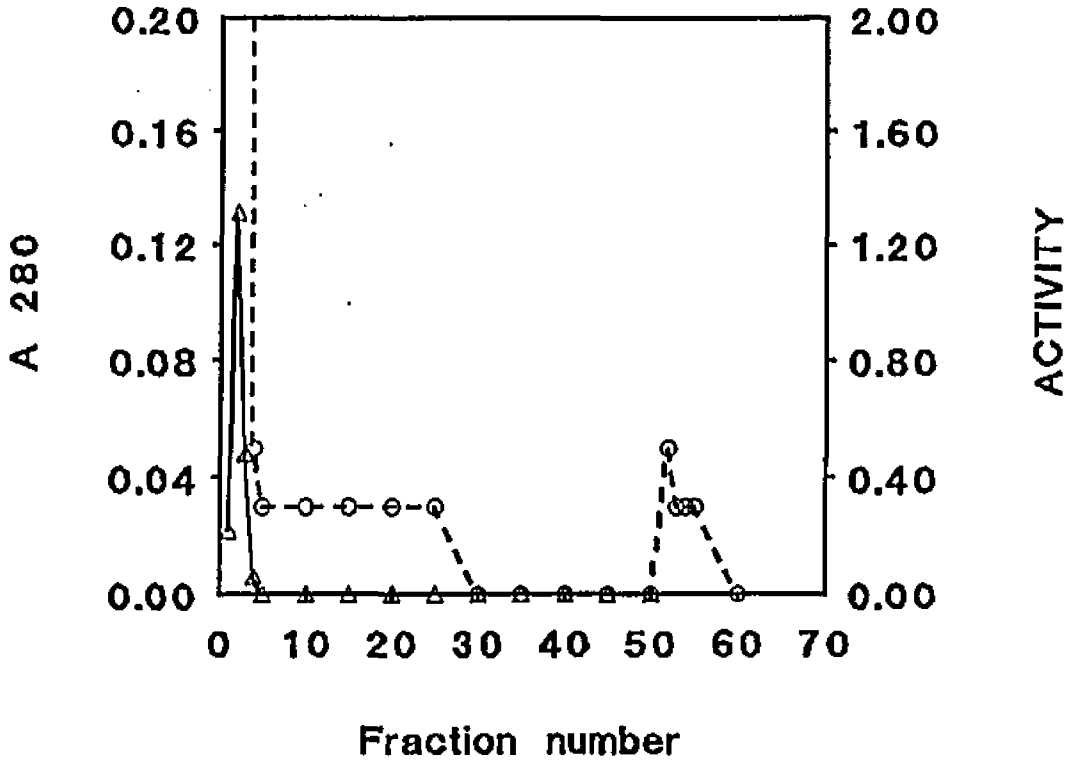

Figure 13. Immunoaffinity chromatography of Euglena gracilis enoyl-ACP reductase. Enzyme purified by chromatography on Matrex Orange (pool B, 41.3 units) was applied to the anti-dinucleotide fold affinity column equilibrated and run in 0.01 M Tris-HCl, pH 7.6, 0.15 M KCl. Bound protein (2.4 units or 5.8% of the applied activity) was eluted by 0.23 mM NADPH in the same buffer. (Δ--Δ) absorbance at 280 nm. (O--O) enoyl-ACP reductase activity, units/ml.

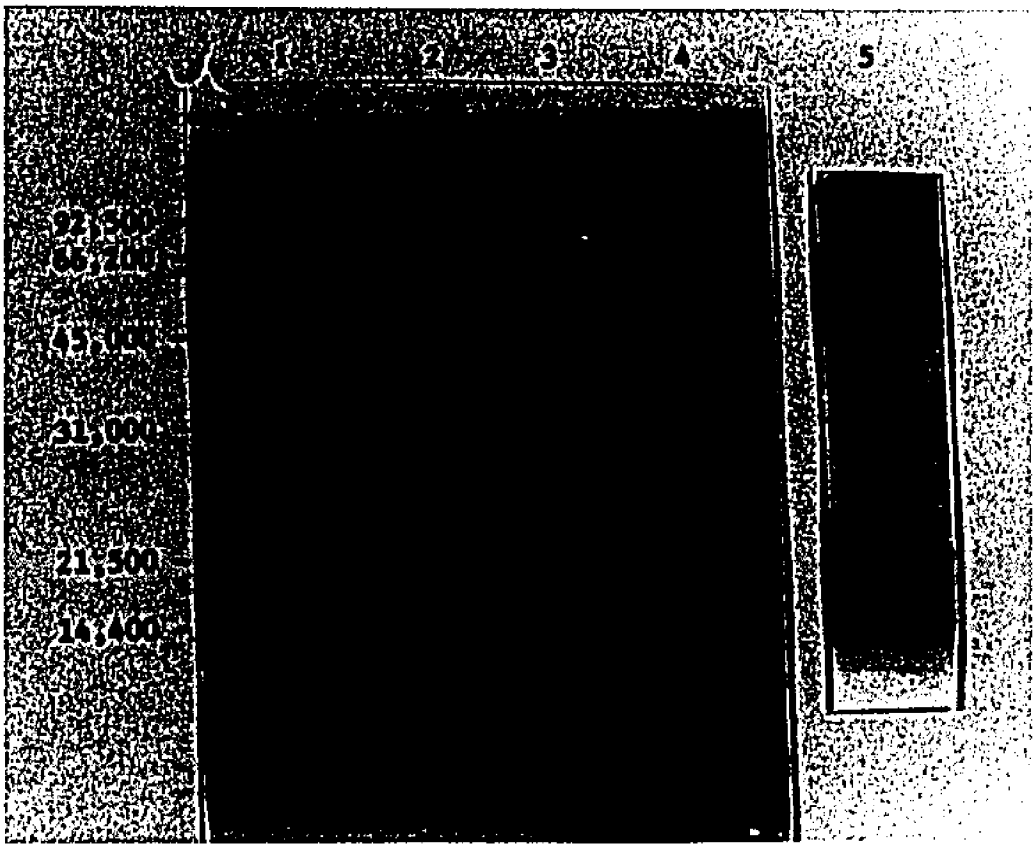

Figure 14. 12% SDS polyacrylamide gel electrophoresis of enoyl-ACP reductase. Lanes 1 and 2 are pool A and pool B, respectively, from Matrex Orange chromatography of enoyl-ACP reductase. Lane 3 is the active pool eluted from the ACP-agarose affinity chromatography. Lanes 4 and 5 are enoyl-ACP reductase purified by immunoaffinity chromatography on the dinucleotide-fold specific affinity column on two separate occasions. 12% SDS polyacrylamide gel electrophoresis was done following the procedure of Laemmli (1970). The gel was silver stained using the silver staining kit and instructions of BioRad.

reductase eluted from the immunoaffinity column. In this lane the major band at an M_r of 48,600 is very visible, as well as a minor band at an M_r of 86,900.

Isolation of Chloroplasts from Euglena Gracilis

The light-induced nonaggregated fatty acid synthase of E. gracilis is a chloroplast-associated system, so the isolation of intact chloroplasts from the organism was undertaken. A reliable procedure for the isolation of intact chloroplasts from Euglena was first published in 1980 (Ortiz et al., 1980), and refined by Gomez-Silva et al. (1985). Euglena have, directly underneath the plasma membrane, a coat composed of interlocking strips of protein which form a helically striated coat as shown in a scanning electron micrograph in Figure 15. These strips are intimately associated with the underlying cytoskeleton, and the entire coat as a consequence is extremely tough and flexible. The combined cell membrane, proteinaceous coat, and underlying associated cytoskeletal elements are collectively referred to as the pellicle (Hofmann and Bouck, 1976). Any force which is extreme enough to break the pellicle is also extreme enough to break the more delicate chloroplast envelope, making isolation of intact chloroplasts difficult.

The solution to the problem of the pellicle was to grow cells in limiting amounts of vitamin B_{12}. Euglena have an

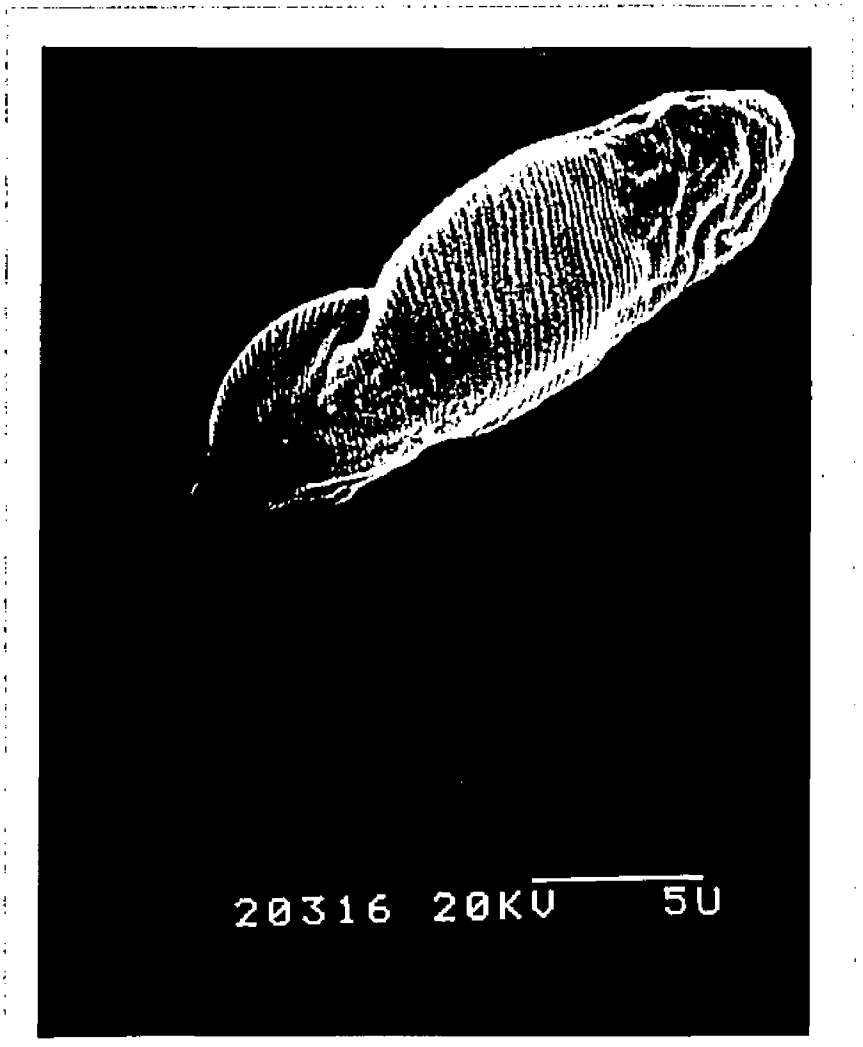

Figure 15. A scanning electron micrograph of a single cell of Euglena gracilis. Cells were fixed in 2% osmium tetroxide, dehydrated through a graded series of acetone baths, dried by the critical point drying method and gold-plated for viewing in a Zeiss model 940 scanning electron microscope. The cell is magnified 4,600x.

absolute requirement for this vitamin and, when it is in short supply, a variety of odd physiological effects ensue. The most apparent of these is the cessation of cell division, without cessation of protein, RNA or DNA synthesis (Carell et al., 1970). Cells grown in low vitamin B_{12} are much larger than cells grown in a normal medium, as shown in Figure 16. This photomicrograph shows a single cell of Euglena gracilis which was grown in the low vitamin B_{12} medium of Ortiz et al. (1980), in the same field with two Euglena gracilis cells which were grown in Hutner's High Yield medium. The cell which was grown in limiting vitamin B_{12} is much larger than the two cells grown in Hutner's medium and its cytoplasm is full of dark granular material. The cells are rounded due to contact with a glass surface.

Although these cells have been extensively used in studies of Euglena gracilis structure and function (for example, Gomez-Silva et al., 1985; Gomez-Silva and Schiff, 1985a and 1985b; Schurmann and Ortiz, 1982), we found that fatty acid synthesis in them is disturbed compared to fatty acid synthesis in cells grown in Hutner's medium, as demonstrated in Table 11. Cells were grown in either Hutner's medium or in low B_{12} medium for 5-6 days. Cells were harvested while in the logarithmic phase of growth, to aboid the mucus coat Euglena produce as they age (Cogburn, and Schiff, 1984) which might interfere with the chloroplast isolation. The concentration of cells grown in Hutner's

Figure 16. Photomicrograph of Euglena gracilis cells. Cultures were grown for five days in Hutner's High Yield medium, or for five days in the low vitamin B_{12} medium of Ortiz et al. (1980). An aliquot of cells from each culture were mixed, a drop applied to a slide, and examined under the light microscope. In this field, the two small cells on the right had been grown in Hutner's medium while the very large cell on the left had been grown in the low vitamin B_{12} medium. The photomicrographs were taken on Professional Pan X film, 2415 emulsion, and developed for four minutes in D19 developer to enhance contrast.

Table 11. A comparison of Euglena gracilis grown in Hutner's High Yield medium or in the medium of Ortiz et al. Cells were grown for six days in the medium indicated before harvesting. After determination of cell count and culture volume aliquots were removed and frozen at -85°C. Protein and chlorophyll determinations and FAS assays were performed on the following day.

	Medium	
	Hutner	Ortiz et al.
volume	3.6 l	6 l
cells/l	2.4×10^9/l	0.34×10^9/l
total cells	8.64×10^9	2.04×10^9 cells
protein concentration	0.6 mg/ml	0.37 mg/ml
total protein	2196 mg	2220 mg
protein/cell	0.25×10^{-6} mg/cell	1.09×10^{-6} mg/cell
normal range*	0.2 to 0.4 x 10^{-6} mg of protein/cell	
chlorophyll concentration	12 mg/l	15 mg/l
total chlorophyll	39.6 mg	90 mg
chlorophyll/cell	4.6×10^{-12} mg/cell	44.0×10^{-12} mg/cell
normal range*	0-35 x 10^{-12} mg of chlorophyll/cell	
FAS activity**		
Total	6373 units	1459.5 units
ACP-dependent	4843.5 units	569.2 units
% of total FAS activity	76	39
Total FAS activity/cell	7.38×10^{-7} units/cell	7.16×10^{-7} units/cell

*Cook, 1968

**Units of FAS activity are expressed in nm of ^{14}C from ^{14}C-malonyl-CoA incorporated into fatty acids/min/ml

medium was 2.4×10^9 ml, about 8 times that produced in low vitamin B_{12} medium, 0.34×10^9 ml. The protein concentration per cell for cells grown in Hutner's medium was 0.25×10^{-6} mg/cell, within the published range of $0.2 - 0.4 \times 10^{-6}$ mg/cell (Cook, 1968). Cells grown in limiting vitamin B_{12} contained 1.09×10^{-6} mg/cell, more than four times normal. Similarly, the concentration of chorophyll per cell for cells grown in Hutner's medium was 4.6×10^{-12} g/cell; for cells grown in low vitamin B_{12} it was 44×10^{-12} g/cell, nearly ten times as much, while the normal range is $0 - 35 \times 10^{-12}$ g/cell. Total fatty acid synthase activity was nearly the same in the two cultures, 7.38×10^{-7} nmoles of ^{14}C incorporated into fatty acids/min/cell in Hutner's medium and 7.16×10^{-7} nmoles of ^{14}C incorporated into fatty acids/min/cell in cells grown in limiting vitamin B_{12} medium. However, the fraction of the total activity which was due to the chloroplast-associated nonaggregated fatty acid synthase was quite different. In cells grown in Hutner's medium, 76% of the total fatty acid synthase activity was due to the chloroplast-associated fatty acid synthase, while in cells grown in limiting vitamin B_{12} only 38% of the total was due to the chloroplast-associated enzymes. Thus, in normal light grown cells most fatty acids were synthesized in the chloroplast. In contrast, in cells which had been grown in limiting vitamin B_{12}, the major fatty acid synthase activity was ACP-independent (61% of the

total), indicating that in these cells the chloroplast-associated fatty acid synthase was down-regulated in some fashion, with an increase in the activity of the cytoplasmic multienzyme complex fatty acid synthase. Alternatively, since the cells were frozen at -85°C for several days between harvesting and assay, it could be that the chloroplast-associated FAS of cells grown in low vitamin B_{12} was much less stable than the enzyme from cells grown in Hutner's medium. Because of the disturbance in fatty acid synthesis in the cells grown in low vitamin B_{12}, we felt it would be desirable to identify conditions which would allow us to isolate chloroplasts from cells grown in Hutner's medium.

Prolonged growth of Euglena in a low vitamin B_{12} medium causes the pellicle to become thin (Bre and Lefort-Tran, 1978). The standard chloroplast isolation procedure calls for these cells to be digested with proteinases, further weakening the pellicle and reducing the force necessary to break the cells. Our strategy in attempting to isolate chloroplasts from cells grown in Hutner's medium was first to treat the cells with a sublytic amount of a nonionic detergent for a short period of time. It was hoped that the detergent would weaken the pellicle by solubilizing some of the membrane lipid. The cells were then incubated with proteinases for very short periods of time, until cell breakage became evident under the light microscope. Beyond

that, the isolation procedure was identical to the standard published procedure for isolating chloroplasts from cells grown in limiting vitamin B_{12}.

Conditions for lysing the cells were initially determined by incubating aliquots of cells with Brij 35 at concentrations of 1%, 0.1%, 0.01%, and 0.001% for 15 minutes and examining them under the light microscope. Cell breakage was already evident in the first two aliquots at that time so the lower concentrations were checked in more detail. One liter of normally grown cells was divided into six aliquots and incubated with the concentrations of Brij 35 indicated in Table 12 for 15 or 30 minutes, as well as with a water control and a control of 0.0002% 1-butanol; this was to ensure that the butanol used in solubilizing the Brij 35 was not causing any cell breakage. Following the incubation the cells were handled identically as described. In the two controls, in which water or 0.0002% 1-butanol were added, there was no recovery of chloroplasts or of chloroplast-associated ACP-dependent FAS activity. Therefore the cell breakage is a consequence of the detergent incubation and not due to the proteinase treatment alone or to the 1-butanol which was used in solubilizing the Brij 35. The yield of chloroplast-associated fatty acid synthase activity from cells incubated in 0.01% Brij 35 increased when the incubation time was increased from 15 to 30 minutes, from 2.29 units to 2.87 units recovered, while

Table 12. Determination of optimal conditions for detergent treatment of Euglena gracilis. A one liter culture of Euglena gracilis grown for five days in Hutner's Medium was harvested by centrifugation and resuspended in 0.3 M sorbitol, 0.05 M potassium phosphate. The cells were divided into six aliquots which were incubated for the times indicated with either water, 0.002% butanol, 0.01% Brij 35/0.002% 1-butanol, or 0.001% Brij 35/0.0002% 1-butanol. Following incubation with controls or detergent the cells were washed twice and chloroplasts were isolated from each aliquot as described.

	Incubation Time (min)	Total Protein (µg)	Total Chlorophyll (µg)	FAS Activity (units)
Water	30	6	0.2	0.003
0.0002% 1-butanol	30	6	0.2	0.006
0.01% Brij 35	15	55	6.6	2.29
0.01% Brij 35	30	65	8.7	2.87
0.001% Brij 35	15	85	9.5	3.03
0.001% Brij 35	30	30	7.7	0.34

the yield decreased with increasing incubation time in 0.001% Brij 35. In this case the recovered activity went from 3.03 units following a 15 minute incubation to 0.34 units recovered following a 30 minute incubation. The best recovery of fatty acid synthase activity was obtained when cells were incubated for 15 minutes in 0.001% Brij which was chosen as the optimum condition for the detergent incubation. The incubation time in trypsin was determined by following cell breakage under the light microscope, and was usually about seven minutes.

Chloroplasts isolated in this manner were compared in Table 13 to chloroplasts isolated by the standard method. The chloroplasts were isolated from the cells described in Table 11. The recovery of ACP-dependent fatty acid synthase activity was higher for the cells grown in low vitamin B_{12} than for cells grown in Hutner's medium, 59% versus 11%, but more fatty acid synthase activity was recovered because of the increased number of cells in these cultures. Fatty acid synthase activity in chloroplasts isolated from either type of culture was ACP-dependent, indicating that there was no contamination of the chloroplasts by the cytoplasmic fatty acid synthase. The recovery of both protein and chlorphyll was 50% better for cells grown in Hutner's medium than for cells grown in low vitamin B_{12}. A total of 510 units of ACP-dependent FAS activity was recovered from 3.6 l of cells grown in the medium of Hutner, while 336.7 units of activity

Table 13. A comparison of chloroplasts isolated from Euglena gracilis grown in either Hutner's High Yield medium or in the medium of Ortiz et al. The chloroplasts were isolated from the cells described in Table 11.

Medium	Hutner's	Ortiz et al
total protein (mg)	1.66	1.16
total chlorophyll (mg)	0.36	0.26
total FAS activity (units)*	510	337
Recovery (%)**	11	59

*FAS activity was all ACP-dependent

**Recovery is based on the ACP-dependent FAS activity of the cells.

were recovered from 6 l of cells grown in the low vitamin B_{12} medium of Ortiz *et al.* (1980). The banding pattern of the chloroplasts on the Percoll gradient was identical, indicating that their densities were very similar, and the lower band of intact chloroplasts was cleanly separated from the upper two bands of broken chloroplasts and stripped thylakoid membranes.

The membrane and soluble fractions of chloroplasts can be separated by high speed centrifugation of disrupted chloroplasts (Walker and Harwood, 1985). This was done with chloroplasts isolated from cells grown in both the low B_{12} medium and in Hutner's medium. As shown in Table 14, 51 units of ACP-dependent fatty acid synthase activity derived from chloroplasts isolated from cells grown in Hutner's medium were centrifuged at 18,000 rpm for 2.5 hours in an SS34 rotor. Of this, a total of 22.57 units or 44% of the applied activity were recovered, 9.12 units or 40% in the supernatant solution and 13.45 units or 60% in the membrane fraction. The specific activities of the two fractions were 130.3 units/mg of protein and 103.5 units/mg of protein for the supernatant and membrane fractions, respectively. In contrast, of 33.7 units of ACP-dependent FAS activity derived from chloroplasts isolated from cells grown in the low vitamin B_{12} medium, only 3.08 units, or 9% of the applied activity, were recovered. Of this, 1.68 units or 54% were located in the supernatant fraction, while 1.41

Table 14. A comparison of the supernatant and membrane fractions of chloroplasts isolated from cells grown in either Hutner's High Yield medium or in the medium of Ortiz et al. The chloroplasts were described in Table 13. An aliquot (200 µl) of chloroplasts was centrifuged at 18,000 rpm for 2.5 hours in a Sorval SS34 rotor. The supernatant fraction was removed and the pellet resuspended in an equal volume of 0.33 M sorbitol, 0.05 M Tricine-KOH, pH 7.8.

Medium	Hutner's	Ortiz et al.
Initial FAS activity (units)	51.0	33.7
Supernatant fraction:		
total FAS activity (units)	9.12	1.68
% of recovered FAS activity	40	54
total protein (mg)	0.07	0.05
specific activity (units/mg)	130.3	33.6
Membrane fraction:		
total FAS activity (units)	13.45	1.41
% of recovered FAS activity	60	46
total protein (mg)	0.13	0.09
specific activity (units/mg)	103.5	15.7
total activity recovered (units)	22.57	3.08
% of initial activity	44	9

units or 46% of the total recovered activity were located in the membrane fraction. The specific activities were 33.6 units/mg of protein for the supernatant fraction and 15.7 units/mg of protein for the membrane fraction.

As shown in Table 15, chloroplasts isolated from Euglena gracilis contained an NADPH-dependent enoyl-ACP reductase which reduced crotonyl-CoA. E. coli ACP added to the assay to a final concentration of 9 μM inhibited the activity of the chloroplast enoyl-ACP reductase to 33% of control.

Molecular Biology of Euglena gracilis FAS

Dr. Stuart Smith isolated and described (Witkowski et al., 1987) a cDNA clone, pFAS4, which contained all of the coding sequence for the ACP and parts of the coding sequences for the thioesterase and the ketoreductase components of the multifunctional fatty acid synthase of rat liver. He kindly sent this clone for use in studies of the fatty acid synthase genes of Euglena.

Figure 17 shows an abbreviated diagram of pFAS4 and illustrates the strategy used in isolating fragments of the cloned DNA to use as probes for the ACP, ketoreductase and thioesterase genes. The plasmid was isolated and purified by electroelution from 1% agarose gels. Purified plasmid was then cleaved by the restriction enzymes EcoRI, KpnI, and PstI, releasing the vector and three fragments of the

Table 15. The effect of ACP on the activity of enoyl-ACP reductase. Activity of enoyl-ACP reductase was measured in chloroplasts isolated from cells grown for five days in Hutner's High Yield medium. The chloroplasts were lysed by freeze-thawing and assayed in the absence or presence of 9 μM *E. coli* ACP.

	Specific Activity (Units/mg Protein)	% of Control
Control	6.7	100
+ 9 μM ACP	2.2	33

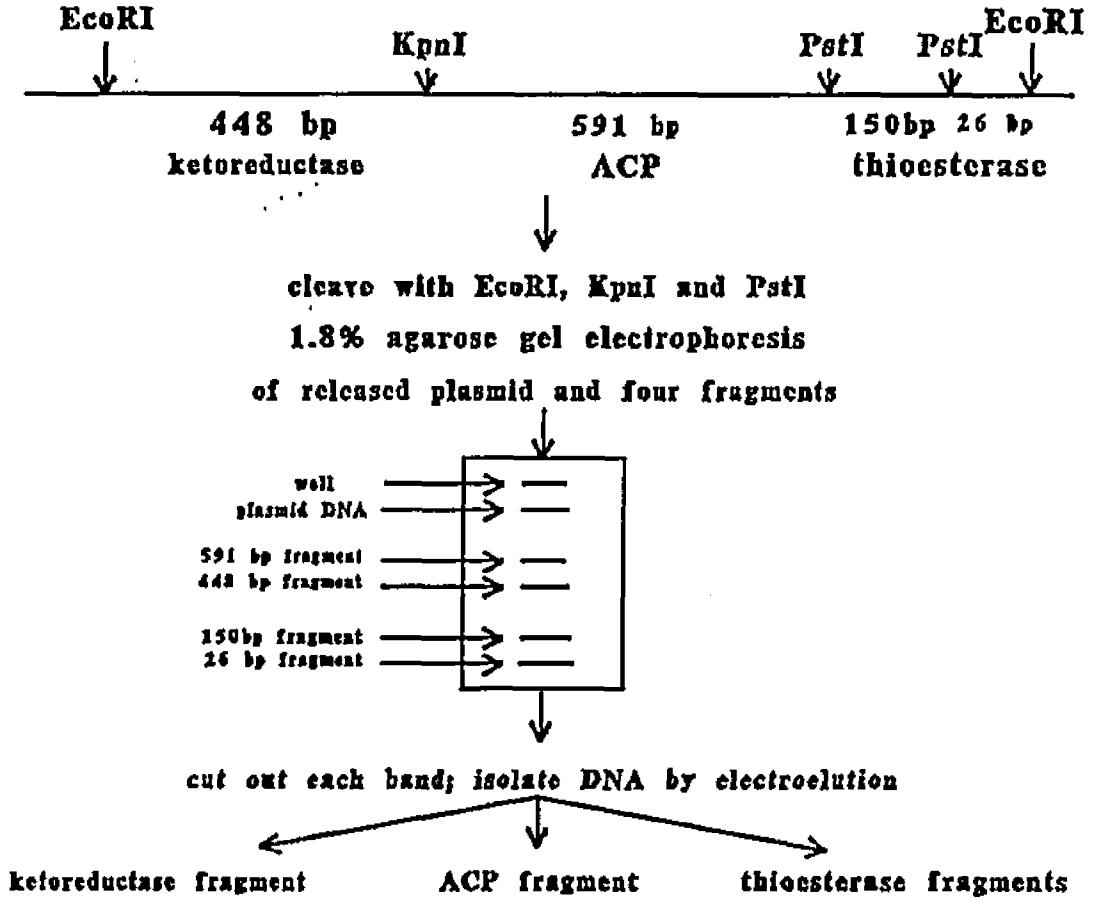

Figure 17. A digrammatic representation of the plasmid pFAS4. The cDNA is a 1215 bp insert into the EcoRI site of pUC19. The fragment defined by the EcoRI/KpnI restriction enzyme sites includes the active site of the ketoreductase domain. The 591 bp middle fragment defined by the KpnI/PstI restriction enzyme sites contains all of the ACP domain and part of the thioesterase domain. The 150 bp PstI/PstI fragment contains the thioesterase active site. Following cleavage with the restriction enzymes indicated, the DNA was electrophoresed through 1.8% agarose. The bands were excised and the DNA fragments were electroeluted from the agarose.

insert. The cleaved DNA was electrophoresed through 1.8% agarose gels, the bands excised, and the insert fragments purified by electroelution. Purity of the eluted fragments was assessed by gel electrophoresis, as illustrated in Figure 18. The fragment sizes are 591 bp, including all of the ACP coding sequences and partway into the thioesterase domain; 448 bp, including part of the reductase coding sequence; and 150 bp which includes the thioesterase active site.

Genomic DNA was obtained from several species, including spleen and kidney of rat, Euglena gracilis variety bacillaris wild type and W_{10}BSML, a white mutant of Euglena which is virtually devoid of chloroplast DNA. The concentrations of the DNAs were determined following gel electrophoresis, as described. A representative gel is shown in Figure 19.

All of the DNAs were applied to a nylon membrane at concentrations of 1 μg and 0.1 μg and hybridized to each of the three fragments of the pFAS4 insert. The results of the hybridizations are shown in Figure 20. All three fragments hybridized strongly to DNA from rat, human, mouse, Euglena gracilis wild type and W_{10}BSML, Chara fibrosa, Phaeocerus laevis, Polytrichum pallidesetum, and Conocephalum conicum. Hybridization to DNA from Zea mays, Saccharomyces cerevisiae, and Drosophila melanogaster was not seen under

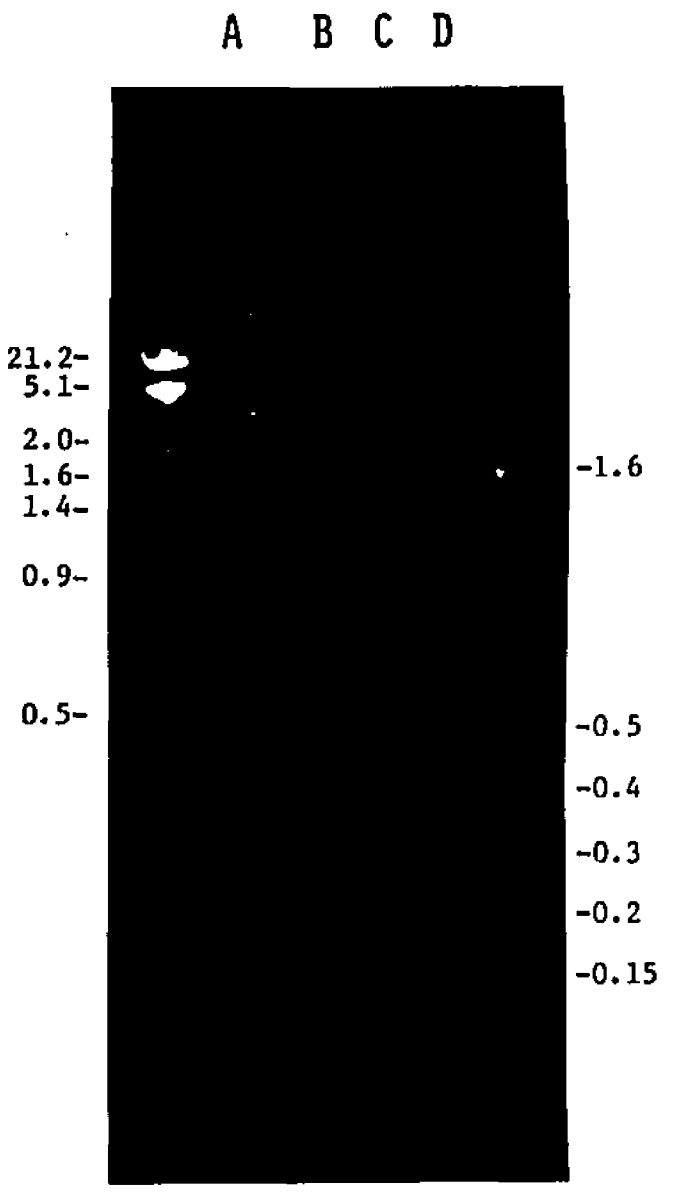

Figure 18. 1.8% agarose gel electrophoresis of pFAS4. pFAS4 which had been cleaved by the restriction enzymes EcoRI, KpnI and PstI (lane A) and the three purified fragments were subjected to electrophoresis through 1.8% agarose. Lane B contains the 591 bp ACP-specific fragment; lane C contains the 448 bp ketoreductase-specific fragment; and lane D contains the 150 bp thioesterase-specific fragment. The flanking lanes contain molecular weight markers / DNA cleaved by EcoRI and HindIII and on the left and pBr322 cleaved by Hinf on the right. Eithidium bromide-stained DNA was visualized by UV-transillumination.

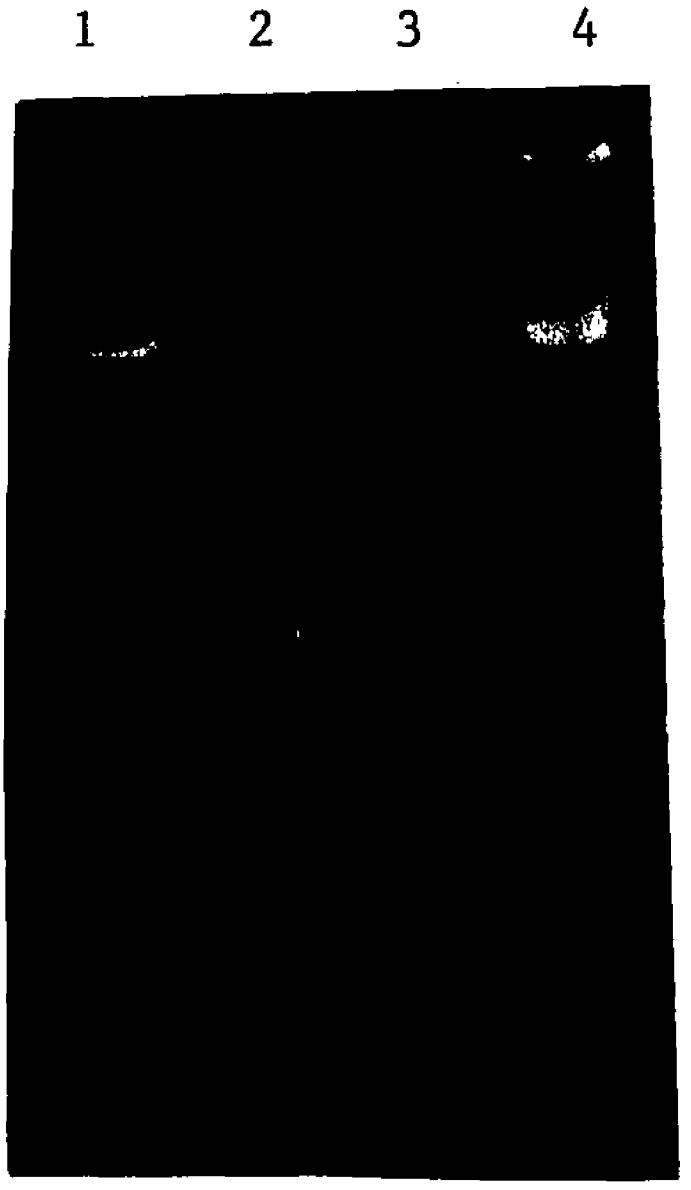

Figure 19. 1% agarose gel electrophoresis of genomic DNA from rat, wild type Euglena gracilis and W_{10}BSML. Lane 1 is λ DNA cleaved by EcoRI and HindIII, lane 2 is rat spleen and kidney DNA, lane 3 is DNA obtained from the E. gracilis mutant W_{10}BSML and lane 4 is DNA obtained from wild type E. gracilis. Ethidium bromide-stained DNA was visualized by UV-transillumination.

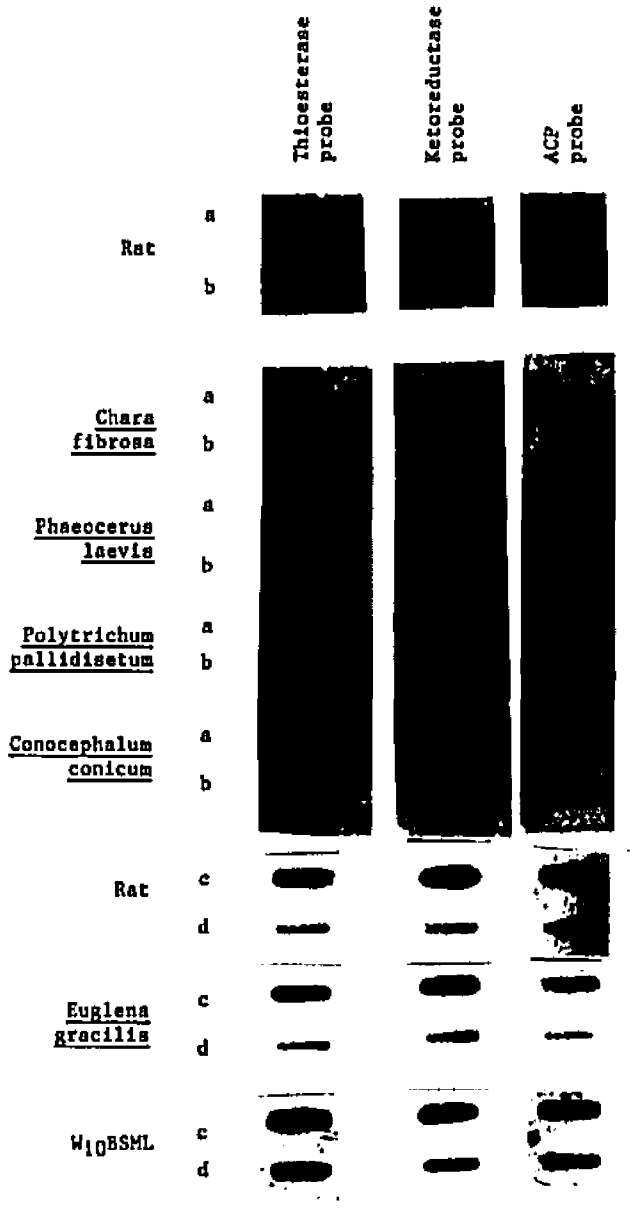

Figure 20. Autoradiogram of the ACP, ketoreductase and thioesterase probes hybridized at high stringency to membranes containing genomic DNA from several species. In part A, 1 μg (a) and 0.1 μg (b) of DNA from rat spleen and kidney, Chara fibrosa, Phaeocerus laeris, Polytrichum pallidesetum and Corocephalum conicum were slot-blotted onto nylon membranes (Zeta Probe, BioRad). In part B, 2 μg (c) and 0.2 μg (d) of DNA from rat spleen and kidney, wild type Euglena gracilis, and the E. gracilis mutant W_{10}BSML were slot-blotted onto Zeta probe membranes. Filters were hybridized using the high stringency conditions and were exposed to Kodak XAR-5 film at -85°C with two intensifying screens.

these conditions, but was apparent when the stringency of the hybridization was lowered as shown in Figure 21.

Field inversion gel electrophoresis is a technique which allows the separation of nuclear and organellar DNAs. Intact cellular genomes of Euglena, either wild type (light grown and dark grown) or W_{10}BSML, were subjected to field inversion gel electrophoresis; the fractionated DNAs were alkaline-blotted onto nylon membranes. Hybridization of these membranes was performed using either a cDNA probe for the chloroplast-encoded large subunit of ribulose-1,5-bisphosphate carboxylase from Euglena gracilis, or the three FAS probes. The results of hybridization with the chloroplast-specific probe pEZC738.1 are shown in Figure 22, A and C. Much of the chloroplast DNA remained in the well, trapped within the agarose plug. The chloroplast-specific probe hybridized to a band of ~150 Kb DNA very near the bottom of the gel (A and C). When the same gels were hybridized to the ketoreductase (B) and thioesterase (D) probes, both probes hybridized to material in the wells. In addition, hybridization of the probes to a band of DNA which lay in between the wells and the chloroplast DNA was apparent.

The lack of hybridization of the ACP probe to chloroplast DNA (Figure 22 E) was confirmed in a separate experiment. First, Euglena nuclei and chloroplasts were isolated; the chloroplasts were treated with DNase to digest

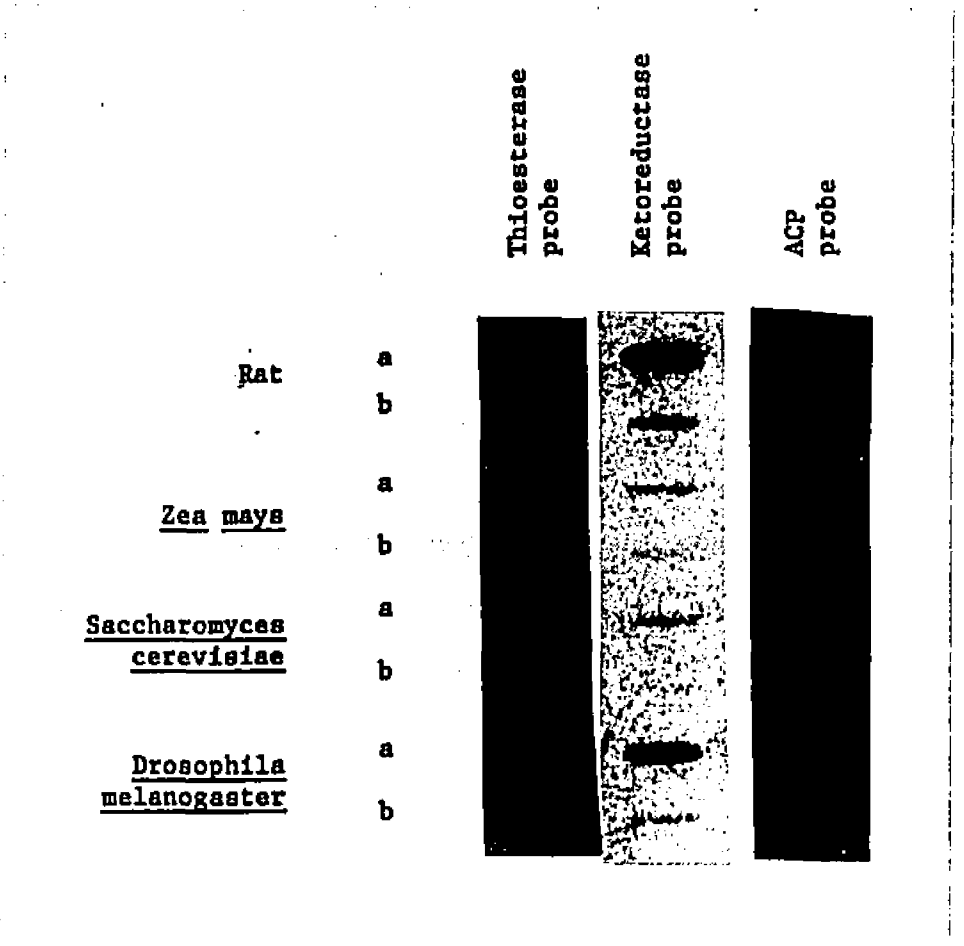

Figure 21. Autoradiogram of the ACP, ketoreductase and thioesterase probes hybridized at standard stringency to membranes containing genomic DNA from several species. One μg (a) and 0.1 μg (b) of DNA from rat spleen and kidney, Zea mays, Saccharomyces cerevisae and Drosophila melanogaster were slot-blotted onto Zeta Probe membranes. Filters were hybridized using the lower stringency conditions and were exposed to Kodak XAR-5 film at -85°C with two intensifying screens.

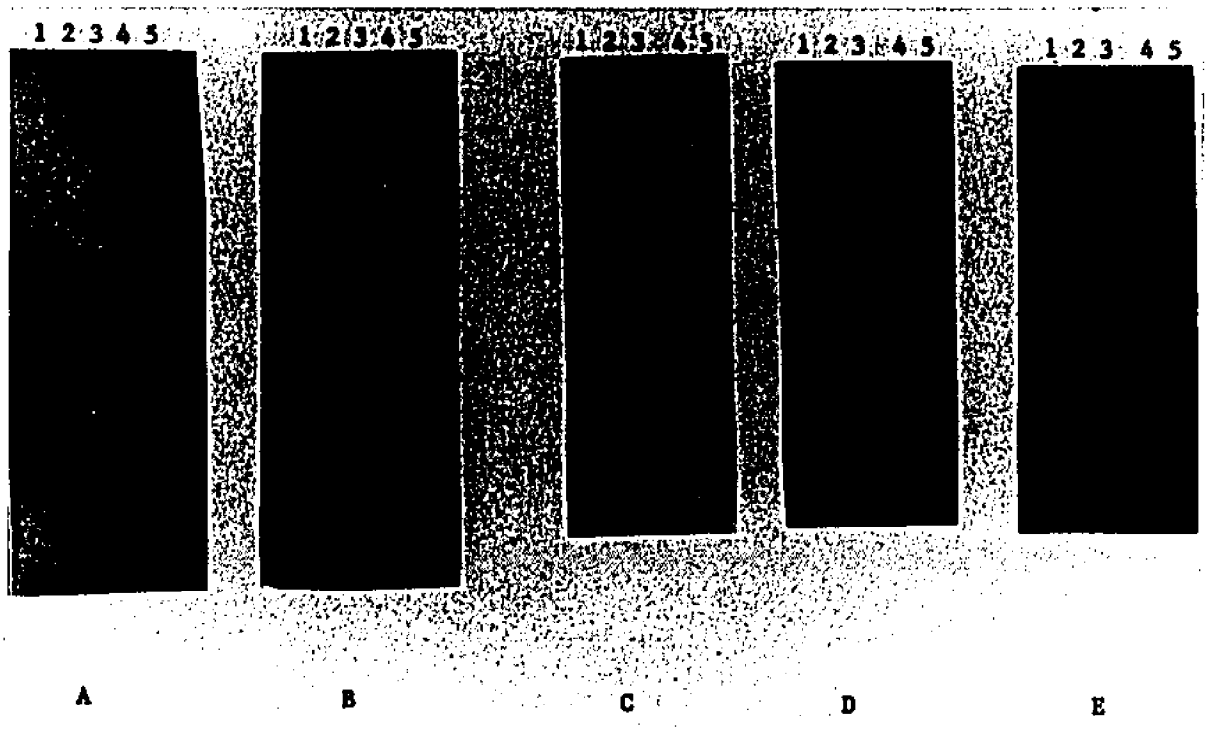

Figure 22. Hybridization of several probes to DNA from Euglena gracilis. Light grown and dark grown Euglena and W_{10}BSML were harvested and prepared for field inversion electrophoresis as described in Chapter 2. Following electrophoresis, the DNA was alkaline-blotted onto Zeta probe membranes and hybridized to the large subunit of ribulose-1,5-bisphosphate carboxylase (A and C). Following exposure and development of the film, the membranes were stripped and reprobed using thioesterase or ketoreductase probes. Figure 21E was hybridized to the ACP probe but not to ribulose-1,5-bisphosphate carboxylase. Figure 21A and 21C were hybridized using the high stringency conditions, and figures 21B, D and E were hybridized using the lower stringency conditions. Lane 1 is λ DNA, lane 2 is dark-grown wild type E. gracilis, lane 3 is light-grown E. gracilis, lane 4 is the mutant W_{10}BSML, and lane 5 is E. gracilis grown in low vitamin B_{12}.

any nuclear DNA which might be non-specifically bound, and DNA was isolated from the organelles. Gel electrophoresis of the nuclear DNA and of DNA from DNase-treated and untreated chloroplasts was performed (Figure 23A), and the gels were alkaline blotted. The blotted gel was then probed using the 591-bp ACP-specific probe. Hybridization of the probe to nuclear DNA was seen, as shown in Figure 23B, but no hybridization of the probe to chloroplast DNA was evident.

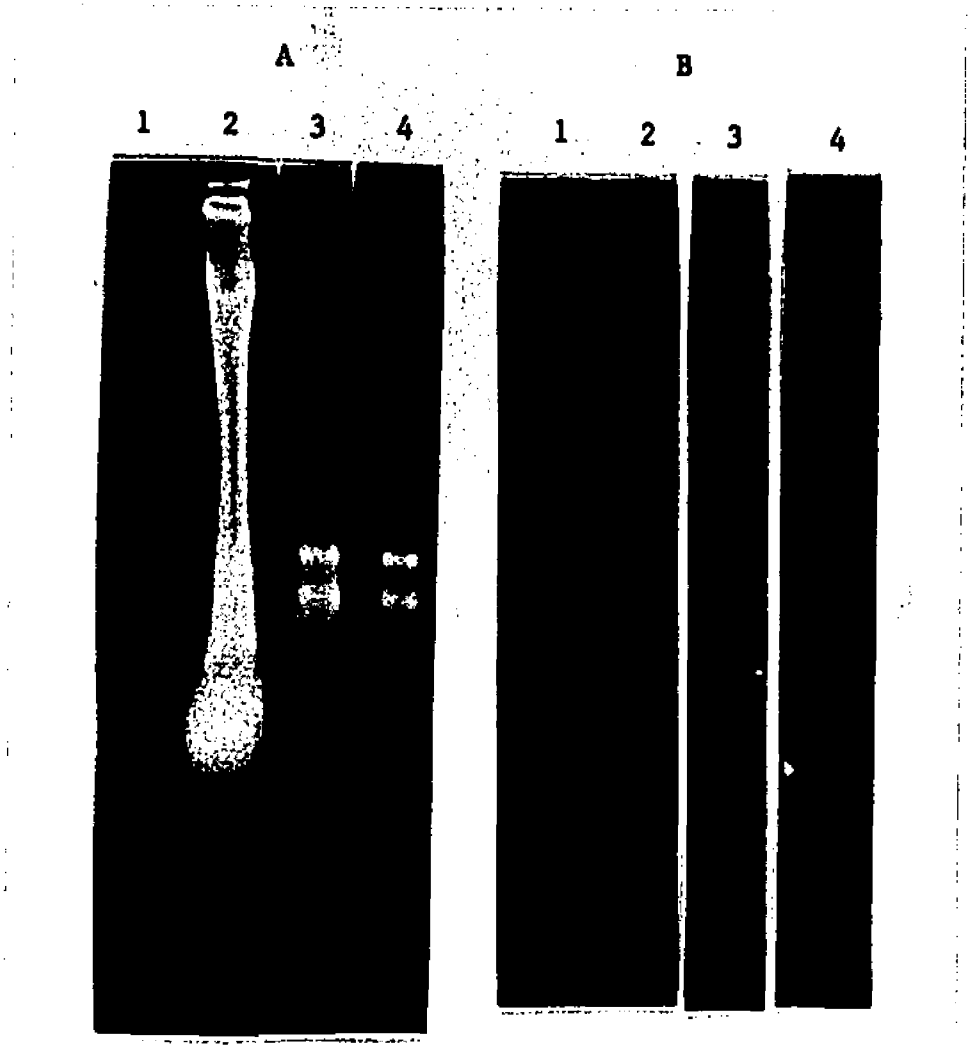

Figure 23. 1% agarose gel electrophoresis of Euglena gracilis nuclear and chloroplast DNA, and autoradiography of the alkaline-blotted DNA hybridized to an ACP probe. DNA isolated from E. gracilis nuclei (lane 2) and from chloroplasts which had (lane 3) or had not (lane 4) been treated with DNAse was electrophoresed through 1% agarose gels (Figure 22A). Lane 1 is λ DNA. The ethidium-bromide treated DNA was visualized by UV-transillumination. The DNA was subsequently alkaline-blotted onto Zeta Probe and hybridized to the ACP probe using the low stringency conditions (Figure 22B).

CHAPTER 4

Discussion

Purification and Characterization of Enoyl-ACP Reductase

Enoyl-ACP reductase has been purified 338-fold from light-grown Euglena gracilis (Table 1). It was purified by a combination of anion exchange chromatography (Figure 1), dye ligand chromatography using Matrex Orange (Figure 2), and affinity chromatography on an ACP-agarose affinity column (Figure 3). The enzyme had a pH optimum of at least pH 8.0 (Table 8), and it required DTT both in handling and in the assay. Its activity was nearly doubled when assayed in the presence of 10 mM $CaCl_2$, but was not affected by 10 mM $MgCl_2$ or 2 mM EDTA (Table 10). It was inhibited by E. coli ACP when present in the assay at 10 μM, but the inhibition was eliminated when either 10 mM $MgCl_2$ or 2 mM EDTA were also present. Upon gel filtration of the partially purified enzyme two peaks of activity were seen, with apparent M_rs of 52,600 and 36,800 (Figure 5). Denaturing gel electrophoresis of the active peak obtained from affinity chromatography of the enzyme showed a major band at an apparent M_r of 41,300, with minor bands at M_rs of 28,000 and 48,400 (Figure 14). It is likely that the band at an M_r of 48,400 is the reductase for the following reason. Following dye ligand chromatography, in parallel experiments, the active peak was subjected to affinity

chromatography on either an ACP-agarose column (Figure 3), or on a columnn designed to recognize the dinucleotide fold of NADP(H)-requiring enzymes (Figure 13). Enzyme purified by the latter chromatographic step was obtained in minute quantities; silver stained 12% SDS-polyacrylamide gels of the enzyme (Figure 14) revealed a doublet, with the lower band of the doublet having a molecular weight of about 48,400. This band coincides with the band at 48,400 seen in the active peak purified by ACP-agarose chromatography. The protein was so labile following affinity chromatography that no further purification or characterization could be achieved.

In their study of the nonaggregated FAS of Euglena, Hendren and Bloch (1980) described a single peak of NADPH-dependent enoyl-ACP reductase activity which eluted from a Biogel A 1.5m column at an apparent M_r of 56,500. The relative amounts of the two peaks which I observed varied somewhat from one preparation to another and were never well resolved; perhaps the lower molecular weight peak was overlooked by those investigators. One conclusion which can be drawn from the combined results of gel filtration chromatography and denaturing gel electrophoresis is that the active form of the enzyme is monomeric, though which of the two species (M_rs 52,600 and 36,800) identified by gel filtration corresponds to the protein which was purified by affinity chromatography is not certain. These molecular

weights are below the range of molecular weights reported for plant enoyl-ACP reductases. The smallest of these is the enzyme from avocado (Caughey and Kekwick, 1982) with a molecular weight determined by gel filtration of 62,400. The enzyme from safflower (Shimakata and Stumpf, 1982a) has an M_r of 83,000, very similar to the E. coli reductases, which both chromatograph with a M_r of 90,000 (Weeks and Wakil, 1968). The other two plant enoyl-ACP reductases which have been described, from spinach (Shimakata and Stumpf, 1982d) and from rape seed (Slabas et al., 1986) are tetramers, with native molecular weights of 115,000 and 140,000 respectively. The spinach enzyme is reported to be composed of four identical subunits with M_rs of 32,500. Oddly enough, in an earlier report the molecular weight of the spinach enoyl-ACP reductase was reported as 72,000 (Shimakata and Stumpf, 1982b). No explanation for the discrepancy was offerred. The rape seed enzyme is composed of two copies each of two subunits, having M_rs of 34,800 and 33,600. It was further found (Cottingham et al., 1988) that, except for a six amino acid N-terminal extension on the larger, the two proteins were identical. The difference in the two subunits may arise from differences in processing of a single gene product during uptake into the chloroplast, or alternate splicing sites in the mRNA for the protein. In addition to the heterogeneity in its subunits, enoyl-ACP reductase, like ACP from several plants (Safford et al.,

1988; Hoj and Svendsen, 1984), was also found to be a member of a multigene family. The presence of more than one enoyl-ACP reductase having rather different substrate specificities or cofactor requirements is well documented in plants and in E. coli; the same is true of other plant and E. coli FAS enzymes as well.

During the purification of Euglena enoyl-ACP reductase, the recovery of activity following anion exchange chromatography was greater than 100%. This is not a particularly uncommon phenomenon and was observed during the purification of component enzymes of the FAS of barley chloroplasts (Hoj and Mikkelsen, 1982). In this case, purification of the FAS away from inhibitors or substrate scavenging systems was invoked to explain the increase in total activity recovered following ammonium sulfate precipitation of chloroplast stromal proteins. In the case of Euglena enoyl-ACP reductase, one candidate for an inhibitor would be ACP. E. coli ACP, which in vivo presumably acts as a substrate-carrier for the chloroplast-associated FAS, was inhibitory to this enzyme (Table 10 and Table 15). Possibly the endogenous Euglena ACP was also inhibitory; separation of the enzyme from endogenous ACP would thus result in increased recovery of activity.

A similar effect was seen during storage of enoyl-ACP reductase purified through anion exchange chromatography. Following storage for two months at -20°C in 0.01 M Tris-

HCl, pH 7.6, 2 mM DTT, the activity of the enzyme increased to 123% of its initial activity as shown in Table 5. Similarly, following storage for two months at -75°C in 0.01 M TES, pH 7.4, 0.2 M sucrose, 0.15 M NaCl, 2 mM DTT, the activity of the enzyme increased to 126% of its initial value. The increased activity recovered following freezing for two months could be due to a contaminating cold-labile substrate scavenging system. Alternatively, it could be that the reductase was associated with other proteins, and the association partially blocked entry of the artificial substrate to the active site of the enzyme. Such an association was proposed (Hendren and Bloch, 1980) to explain the behaviour exhibited by the component enzymes of the Euglena FAS. In crude extracts, enoyl-ACP reductase, β-ketoacyl-ACP synthetase and β-hydroxyacyl-ACP dehydrase co-chromatographed during gel filtration chromatography, with an apparent M_r of 280,000. Enoyl-ACP reductase and β-ketoacyl-ACP synthetase continued to copurify through ammonium sulfate precipitation and DEAE-cellulose chromatography, and were only resolved by hydroxylapatite chromatography. The resolved enzymes were then subjected to gel filtration chromatography a second time, and chromatographed with apparent M_rs of 118,000 (β-ketoacyl-ACP synthetase) and 56,500 (enoyl-ACP reductase). Since their individual M_rs determined during the second gel filtration were much less than the M_r determined earlier, it is

apparent that the two proteins were associated in a complex which was only resolved by hydroxylapatite chromatography.

The formation of multienzyme complexes may be common among the enzymes which play a role in fatty acid biosynthesis. Associations of this sort have been invoked (Caughey and Kekwick, 1982) to explain the inability to detect an NADPH-specific enoyl-ACP reductase in avocado plastids. Such an enzyme must exist, since following gel filtration of disrupted plastids a peak of FAS activity which was NADPH-dependent was detected. They proposed that the NADPH-dependent enoyl-ACP reductase may be associated with other plastid enzymes in a complex which was impenetrable to crotonyl-ACP. Since in some cases the enzymes can be separated and purified, the associations may be transient, persisting only during the course of catalysis.

A specific association between the enzymes acetyl-CoA carboxylase, phosphoenolpyruvate carboxylase and malate dehydrogenase has been described in Euglena (Wolpert and Ernst-Fonberg, 1975a and 1975b). This complex was proposed to capture carbon dioxide for the acetyl CoA carboxylase as well as providing NADPH for the subsequent reductive steps in fatty acid biosynthesis. The K_m of phosphoenolpyruvate carboxylase for HCO_3^- was affected by complex formation; the Km of the isolated protein was 7.3-5.4 mM while the Km for HCO_3^- when the carboxylase was in the complex was reduced to

0.7-1.3 mM. The lowered Km and higher catalytic efficiency of phosphoenolpyruvate carboxylase relative to acetyl-CoA carboxylase could trap CO_2 while the association of the malate dehydrogenase and malic enzyme would help to create a microenvironment of high CO_2 concentration in the vicinity of the acetyl-CoA carboxylase active site.

An association of the Euglena ACP-dependent FAS has also been suggested. Both kinetic characteristics of the FAS and its increased activity upon incubation with antibodies directed against the ACP-independent FAS are most easily explained by the formation of a complex of the enzymes during catalysis. Protein-protein interactions which are cold-labile are also characteristic of the mammalian and avian multifunctional FAS complexes (Kitamoto et al., 1985).

If the NADPH-dependent enoyl-ACP reductase of Euglena is associated with other proteins in a complex, its dissociation during storage would result in increased accessibility of substrate to the reductase, and an apparent increase in activity. A logical candidate for its partner in the complex would be the β-ketoacyl-ACP synthetase, which has been shown to form a complex with enoyl-ACP reductase, although it is not the only possibility.

During the early stages of its purification the reductase was very stable, provided that it was handled and stored in buffers which contained DTT. It retained complete

activity following dialysis at 4°C for 24 hours at pH 7.5 in either 0.01 M imidazole, 2 mM DTT or 0.01 M Tris-HCl, 2 mM DTT, and at pH 8.0 in 0.01 M TES, 2 mM DTT (Table 2). Over the range examined, the loss of activity was not catastrophic at any pH; only about 50% of activity was lost at the two extremes, pH 6.5 and pH 9.0. It was very stable during longer periods of time at 4°C in 0.01 M Tris-HCl, pH 7.6, 0.2 M NaCl, 2 mM DTT (Table 4). No activity was lost during 24 hours at the 4°C, with a gradual decline to 55% after 72 hours.

The enoyl-ACP reductase purified by gel filtration chromatography was unusually stable when incubated at room temperature (Table 7, Figure 4). The loss of activity was biphasic; following an initial rapid loss of 35% of activity over the first eight hours the loss of activity stabilized, and remained constant over the subsequent 16 hours. Even after 48 hours at room temperature, the reductase still retained 43% of its initial activity. This biphasic loss of activity may reflect the complexity of the denaturation process. A more likely explanation is that the two enoyl-ACP reductase proteins comprising the pool have different rates of denaturation.

The stability of the reductase in 30% PEG 4,000 was remarkable (Table 7). Addition of PEG initially increased the activity of the enzyme, to 131% of its initial value after 2 hours at room temperature. Activity was

subsequently lost but in a linear fashion, rather than in the biphasic fashion seen with the control, and was always higher than the activity of the control at the same time points. The effect of the polymer thus is two-fold; both activating and stabilizing the enzyme. These effects or the combination of both somehow eliminated the complex pattern of denaturation which was observed in the control. PEG is a synthetic, chemically inert polymer which is commonly used in precipitation of proteins and DNA. Its ability to precipitate macromolecules is thought to be due to excluded volume effects. In analyzing the precipitation of proteins by PEG (Atha and Ingham, 1981), it was found that PEG had little tendency to interact physically with proteins. The effect of PEG was likened to a solvent sponge, which raises the effective concentration of all proteins present; precipitation occurs when the protein concentration exceeds its solubility. They also showed (Miekka and Ingham, 1978, 1980) that self-association or hetero-association between proteins in a mixture resulted in their precipitation at a concentration of PEG which was lower than the concentration required to precipitate either protein by itself. In these experiments, PEG was added at a level below that necessary to precipitate, where its only effect would be a tendency to concentrate the enzyme by reducing the available free water. Since conformational changes are intramolecular they should be unaffected by concentration, which indicates that the

effect of PEG on enoyl-ACP reductase must be to promote intermolecular associations. Such associations could be dimer or higher order associations of the reductase, or hetero-associations between the reductase and other proteins which have co-chromatographed. Possibly, the association is between the two enoyl-ACP reductases; this would explain both the activation, if the complex is more active than the monomeric forms, and also the elimination of the complex pattern of denaturation seen with uncomplexed enzymes.

This explanation of the activity and stability of the reductase in 30% PEG 4000 initially seems to be in direct conflict with the earlier suggestion that during storage of the enzyme, dissociation of a complex of the enoyl-ACP reductase and other unidentified proteins led to an increase in the amount of activity recovered. However, the reductase was very impure in that case and its behavior while in early stages of its purification was frequently not comparable to its behaviour in later stages. In fact, adding PEG 4000 to the enzyme prior to gel filtration resulted in the total loss of activity.

The reductase required DTT in both storage and column buffers, as well as in the assay. When incubated overnight at 4°C in the absence of DTT, the enzyme lost as much as 54% of activity, depending on the buffer. It may be that DTT is required in storage buffers for maintenance of proper conformation. When enzyme which had been handled and stored

in buffers containing DTT was assayed in the absence of additional DTT, activity was only 69% of the activity measured in the presence of DTT (Table 3). This requirement for DTT in both storage buffers and in the assay is true of the nonaggregated, chloroplast-associated FAS as well. The major requirement for a reduced sulfhydryl in catalysis is probably due to the condensing enzyme which has been shown in the mammalian FAS to have an essential cysteine at the active site (Varagiannis and Kumar, 1983). During catalysis, the acyl primer is transferred from the pantetheine moiety of ACP onto the cysteine sulfhydryl, and the incoming malonyl group is loaded onto the pantetheine. Condensation of the primer onto C2 of the malonyl group with the loss of CO_2 follows. Probably, the condensing enzyme of the Euglena chloroplast FAS also has an essential cysteine, which must be reduced for activity. However, there was a separate noncatalytic requirement for DTT, which may be due to an undefined structural requirement.

Of the enoyl-ACP reductases which have been purified and described in the literature, several were noted to be specifically inhibited by thiol alkylating reagents. The NADH-specific enoyl-ACP reductase of E. coli (Weeks and Wakil, 1968) was inhibited 95% by 10^{-3} M p-hydroxymercuribenzoate, while the same reagent at 10^{-4} M inhibited the NADPH-specific enzyme by 36-40%, and iodoacetic acid at 5×10^{-3} M inhibited both enzymes by over

90%. Both N-ethylmaleimide and iodoacetamide abolished the activity of the enoyl-ACP reductase of avocado (Caughey and Kekwick, 1982), and incubation of the enzyme with crotonyl-ACP but not NADH or NADPH prior to treatment with the alkylating reagent prevented the inhibition. The enzyme isolated from spinach (Shimakata and Stumpf, 1982a) was inhibited 87% by 0.1 mM p-chloromercuribenzoate but was not affected by N-ethylmaleimide. The rape seed enoyl-ACP reductase (Slabas et al., 1986) was completely inhibited by 0.1 mM p-chloromercuribenzoate, and the inhibition could be prevented by incubation of the enzyme with crotonyl-CoA but not NADH. N-Ethylmaleimide was also inhibitory, reducing the activity by 97% at a concentration of 10 mM, but this inhibition was not prevented by incubation with either substrate. Iodoacetamide did not inhibit the enzyme. It has been suggested that there may be more than one thiol group which interact with different alkylating reagents, which would explain the protection afforded by crotonyl-CoA towards inactivation by p-chloromercurbenzoate but not by the more hydrophilic reagent N-ethylmaleimide. The significance of these results in terms of catalysis and/or structure of the enoyl-ACP reductases have not been addressed, partly because none of their sequences are known. One can conclude though that these enzymes all contain at least one cysteine which must be in the reduced state either for catalytic activity or for correct conformation of the

enzyme. Although there has been no speculation about the role of this essential cysteine, it appears to be a conserved feature of enoyl-ACP reductases from bacteria, algae, and higher plants.

It is also interesting that the activity of several chloroplast enzymes is regulated by the light-dependent oxidation and reduction of vicinal thiol groups. Enzymes which are regulated in this fashion include fructose-1,6-diphosphate phosphatase, sedoheptulose-1,7-diphosphate phosphatase, ribulose-5-phosphate kinase, NADP-dependent malic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase (Anderson and Avron, 1976). The light-dependent reduction of oxidized dithiols can be mimicked in vitro by DTT, and it has been shown for isolated spinach chloroplasts (Sauer and Heise, 1983) that fatty acid synthesis in the dark is enhanced by DTT. Since fatty acid synthesis is light dependent and photosynthesis produces reducing equivalents, it is tempting to speculate that the inhibition of fatty acid biosynthesis in the dark may be partially due to the oxidation of dithiols. This might also explain the noncatalytic requirement for DTT of the ACP-dependent Euglena FAS, which is a chloroplast-associated enzyme system. Other regulatory effectors of chloroplast fatty acid synthesis, which include pH, Mg^{2+}, and the ratio of ATP

to ADP, most likely act on the acetyl-CoA carboxylase rather than having any direct effect on the FAS.

The effect of including divalent cations in the assay of enoyl-ACP reductase was interesting (Table 3). The addition of either 10 mM $MgCl_2$ or 10 mM $CaCl_2$ increased by 40% the activity of enzyme which had been purified through anion exchange chromatography. This was an effect of the divalent cation and not of the anion, since including KCl at concentrations of up to 188 mM had no effect on activity. Since no effort was made to chelate divalent cations in column buffers by including EDTA or EGTA, the enzyme may have been purified with bound cation. In this event the addition of a chelating agent to the assay should have resulted in inhibition. However, activity of the enzyme in the presence of 2 mM EDTA was 89% of control. This modest inhibition indicated that the enzyme was activated by divalent cations but did not require them, and probably was not being purified with bound cation. Following the next step in the purification of enoyl-ACP reductase, the effects of $MgCl_2$ and $CaCl_2$ became more complicated.

During further purification of the enzyme by dye ligand chromatography on Matrex Orange (Figure 3), two separate peaks of NADPH-dependent crotonyl-CoA reductase activity were resolved. These may represent the two peaks seen earlier during gel filtration, although this was never confirmed. The two pools exhibited different responses to

several parameters, particularly their pH optima, as well as the effect of including divalent cations, EDTA, or E. coli ACP in the assay (Tables 9 and 10). Specifically, $CaCl_2$ enhanced the activity of pool B but had little effect on pool A; Mg^{2+} inhibited pool A but had no effect on pool B; ACP inhibited the activity of pool B but not pool A; and pool A was most active at pH 7.0 while pool B was most active at pH 8.0 (Table 8). The presence of two peaks of activity having different properties is quite interesting. The two may both be members of the chloroplast FAS, or one or the other may be a totally unrelated enzyme. There are several known enzymes in Euglena which are capable of reducing crotonyl-CoA. These include component enzymes of the cytosolic high molecular weight FAS, the microsomal FAS, and the mitochondrial FAS. The cytosolic FAS has a molecular weight of several million, so it cannot account for either activity. The microsomal FAS (Khan and Kolattukudy, 1975) is tightly membrane associated, and would not be present in the soluble fraction of the cells, so it also can be ruled out. The mitochondrial FAS is a malonyl-CoA independent FAS which synthesizes long chain fatty acids for wax ester fermentation during anaerobiosis in Euglena (Inui et al., 1982; Inui et al., 1984). The mitochondrial FAS has been determined to require for NADH and FAD, but $CaCl_2$ does not affect its activity (Inui et al., 1986).

None of these known enzymes can account for the presence of two NADPH-dependent crotonyl-CoA reducing activities.

In three other non-associated FAS's, those of E. coli (Weeks and Wakil, 1968), rape seed (Slabas et al., 1986) and safflower (Shimakata and Stumpf, 1982b), the existence of at least two separate enoyl-ACP reductases has been documented. In addition, indirect evidence points towards the existence of two enoyl-ACP reductases in leek (Lessire and Stumpf, 1982) and avocado (Caughey and Kekwick 1982). In both E. coli and safflower, one of the two reductases of the pair exhibited an absolute specificity for NADH and the other exhibited an absolute requirement for NADPH. Both of the two enzymes from E. coli were able to use crotonyl-ACP as a substrate, but one of the two was much more active towards the longer chain derivatives of ACP than towards crotonyl-ACP. The NADH-requiring enzyme, which preferred the longer chain lengths, was able to reduce crotonyl-CoA, while the NADPH-requiring enzyme was inactive towards the CoA derivative. In the case of safflower, enoyl-ACP reductase I, which was specific for NADH, preferred shorter chain length acyl-ACP substrates and was able to reduce crotonyl-CoA. Its counterpart enoyl-ACP reductase II used NADPH, and would reduce neither crotonyl-ACP nor crotonyl-CoA. Furthermore, the pH optima of the members of the two pairs were substantially different. The NADPH-dependent, short chain-length specific reductase of E. coli had a pH optimum

of pH 6.5 and was inactive above pH 7.5, while its partner had a broad pH optimum, with maximum activity between pH 7.0 and pH 8.0. Enoyl-ACP reductase I of safflower, which used NADH and preferred short chain length substrates, had a pH optimum of 6.5, and its partner had a pH optimum of 7.1. The properties of the two enzymes of these two pairs suggest that one of the pair is responsible for the reduction of crotonyl-ACP and hexenoyl-ACP, the first two enoyl-ACPs which are formed during the cyclic process of fatty acid synthesis. The second enzyme would then be responsible for the reduction of the longer chain length enoyl-ACPs which are formed later in fatty acid synthesis.

An NADPH-dependent enoyl-ACP reductase was detected early in the purification of the NADH-dependent reductase of rape seed (Slabas et al., 1986). The activity was not detectable following HPLC DEAE chromatography, and no characterization of the enzyme was done.

The evidence for an NADPH-dependent enoyl-ACP reductase in avocado is more indirect. No NADPH-dependent enoyl-ACP reductase activity was detected in isolated, disrupted plastids. However, when plastids' stromal contents were subjected to ammonium sulfate precipitation and gel filtration chromatography, a peak of NADPH-dependent NADH-independent FAS activity was detected. This necessarily implies the existence of an NADPH-dependent enoyl-ACP

reductase, however no attempt was made to purify this enzyme.

In leek, fatty acid synthesis was studied in two tissue types, parenchyma and epidermis (Lessire and Stumpf, 1982). The only enoyl-ACP reductase activity present in parenchyma was NADH-dependent, while in epidermis enoyl-ACP reductase activity was detected when either NADH or NADPH was provided. It is clear that there are two separate enzymes present in leek. It is not clear whether one form is expressed in parenchyma and another, having very broad cofactor specificity, is expressed in epidermis, or whether a single NADH-dependent enzyme is expressed in parenchyma and epidermis, while an NADPH-dependent enzyme is expressed only in epidermis.

The two Euglena enoyl-ACP reductase activities which were resolved by dye ligand chromatography were most active at different pHs, pH 7.0 for Pool A, and pH 8.0 for Pool B. They were both NADPH-dependent, and their other substrate specificities were not examined. Whether they represent two forms of enoyl-ACP reductase analogous to the multiple forms seen in higher plants is not known.

In their study of the enoyl-ACP reductase of Euglena, Hendren and Bloch (1980) found that the enzyme was about equally active with either NADH or NADPH, and activity was slightly enhanced in the presence of both. In addition, when using either of the two cofactors, either crotonyl-CoA

or crotonyl-ACP would serve as substrate. The activity was less when crotonyl-CoA was the substrate, but there was little difference in the activity measured using crotonyl-CoA and either of the two coenzymes. This is contrary to the results seen with the enoyl-ACP reductase pairs of the FASs just described, which were absolutely specific for the cofactor. It seems likely that the activity measured by Hendren and Bloch (1980) was the combined activity of at least two separate enzymes.

Adding ACP had no effect on the activity of pool A (Table 9), but there was an inhibitory effect on the activity of pool B (Table 10). When 10 mM $CaCl_2$ was included in the assay, activity of the pool B enzyme was increased, to nearly double its activity in the absence of $CaCl_2$. When, however, the enzyme was assayed in the presence of $MgCl_2$, there was no increase in the enzyme activity. Therefore, the effect was specific for calcium and not a nonspecific effect of divalent cations. There was an enhancement of activity when EDTA was included in the assay, to 119% of the activity measured in its absence; since EDTA was not routinely included in column buffers, the absence of trace amounts of calcium in the enzyme preparation cannot be assumed. If, however, the enzyme were being purified with bound calcium, adding EDTA should have resulted in a decrease in activity as the EDTA chelates the cation. Also, adding $CaCl_2$ to the assay should not have

resulted in the dramatic increase which was observed. Therefore, we conclude that the reductase was not being purified with bound calcium. Possibly the effect of calcium was due to interaction of crotonyl-CoA with the cation, a neutralization of negative charge on the substrate. An effect similar to this was seen when the E. coli FAS enzymes were examined (Schulz et al., 1969). In this instance, the effect of divalent cations was nonspecific in that either calcium or magnesium caused an increase in activity, and both cations exerted their effect through an interaction with the substrate. Since this cation effect was nonspecific, it seems unlikely that in the case of the Euglena enzyme the same interaction of substrate with cation would be specific for the cation.

The mechanisms by which calcium enhances or inhibits the activity of the proteins with which it interacts have been the subject of much study. It is known for some Ca^{2+}-responsive proteins and also for ACP that the cation induces conformational changes. In the case of ACP, the effect of divalent cations was first described as an enhancement of activity of the E. coli FAS in the presence of Ca^{2+} and Mg^{2+} (Schulz et al., 1969). This was due to two effects; one was the nonspecific interaction of divalent cations with both ACP and CoA substrates, and the second was a specific effect of divalent cations on the structure of ACP which made it a better substrate for the enzymes. They showed that the Km

of β-ketoreductase was lower for the ACP-Ca^{2+} form than for the ACP form; similar effects were seen with other enzymes of the FAS, resulting in an increase in FAS activity in the presence of Ca^{2+}. It was later discovered that Ca^{2+} causes conformational changes in ACP, including an increase in the α-helical content of the protein (Schulz, 1977). These changes alter the hydrodynamic properties of the protein, and the structural changes must mediate the altered interaction of ACP-Ca^{2+} with these enzymes.

Similarly, calsequestrin (Slupsky et al., 1987) and calmodulin (van Eldik et al., 1980) bind Ca^{2+} with subsequent conformational changes; the altered conformers have different properties than the native conformers. A similar conformational change following binding of Ca^{2+} may underly the enhanced activity of enoyl-ACP reductase in the presence of Ca2+.

When the enoyl-ACP reductase was assayed in the presence of E. coli ACP, the activity was reduced by 44% relative to its value in the absence of ACP (Table 10). A reduction in activity of identical magnitude was seen when the enzyme was assayed in the simultaneous presence of ACP and Ca^{2+}; this inhibition was superimposed on the doubling of activity caused by the Ca^{2+}. When the enzyme was assayed in the presence of ACP and either EDTA or Mg^{2+}, there was no decrease in its activity. There are thus two separate effects at work here; one is the enhanced activity which was

seen only in the presence of Ca^{2+}, and is assumed to be due to an effect of Ca^{2+} on the structure of the enzyme. The other effect is an effect of ACP on the enzyme, and this inhibitory effect is not seen if either Mg^{2+} or EDTA are included in the assay. When Ca^{2+} was included the inhibition was seen; and when neither Ca^{2+}, Mg^{2+}, nor EDTA were included, that is in the absence of any added reagents, the inhibitory effect of ACP on the enzyme was also seen. Since this inhibition is not seen in the presence of EDTA, it must require divalent cation. The requirement is specific for Ca^{2+}, since Mg^{2+} cannot substitute. It may be caused by an effect of Ca^{2+} on either participant, that is either on the reductase or on the ACP. Whichever protein is affected, it must bind Ca^{2+} during its purification, since the inhibition was seen in the absence of added Ca^{2+}. The cation may be tightly bound endogenous Ca^{2+} or trace amounts of Ca^{2+} present in the lab water from which buffers were made. The results just presented argue that the enoyl-ACP reductase is not purified with bound Ca^{2+}; therefore, the inhibitory effect of ACP on the enoyl-ACP reductase must be mediated by or require Ca^{2+} bound to the ACP. Mg^{2+} can compete with Ca^{2+} for divalent cation binding sites on ACP but its effects on the structure of ACP may be slightly different; when Mg^{2+} is added to the assay with ACP, it probably displaces the bound Ca^{2+} from the protein, thereby removing the inhibitory effect. Similarly, when EDTA is

added to the assay with ACP, it chelates the bound Ca^{2+} off the ACP with the same effect.

The enzyme which eluted from Matrex Orange at a high salt concentration had properties which were consonant with the properties of the FAS, so this enzyme was chosen for further characterization. This decision was based on the high pH optimum, the inhibitory effect of added ACP, and the demonstration in intact chloroplasts of an enoyl-ACP reductase which used NADPH, accepted crotonyl-CoA as a substrate, and was inhibited by E. coli ACP in the assay (Table 15). The inhibition by ACP was used as the basis for the final step in the purification of the reductase, ACP affinity chromatography.

During fatty acid synthesis, the intermediates are carried as thiolesters of ACP, and the fatty acyl-ACP products of fatty acid synthesis are the substrates for the subsequent reactions of lipid biosynthesis. The acylated form of ACP thus interacts with a variety of different enzymes. The nonacylated form of ACP is also capable of recognizing and binding enzymes for which acyl-ACP is a substrate, as shown by the ability of ACP to act as an affinity ligand for the integral membrane enzymes 2-acylglycerolphosphoethanolamine acyltransferase/acyl-acyl carrier protein synthetase (Cooper et al., 1989) and glycerol-phosphate acyltransferase (Bayan and Therisod, 1989b) of E. coli, as well as a soluble NADH-dependent

acetoacetyl-CoA reductase of Euglena (Ernst-Fonberg, 1986). Presumably, this binding is a consequence of the specific interactions between ACP and the various enzymes for which it is a substrate. The binding of ACP to the membrane proteins of E. coli is thought to be the physical basis for the localization of E. coli ACP near the inner membrane. This was first described based on autoradiograms of a β-alanine auxotroph of E. coli grown in radioactive pantothenate or β-alanine (Van den Bosch et al., 1970). Immunoelectron microscopy failed to confirm these results and instead identified ACP as a soluble protein distributed throughout the cytoplasm (Jackowski et al, 1985). Another immunoelecton microscopy study found evidence for ACP localization near the chloroplast membranes in plants (Slabas and Smith, 1988). In still another study, evidence for the binding of radioactively-labelled ACP to purified E. coli membranes was presented (Bayan and Therisod, 1989a). This binding was explained based on the ability of ACP to bind to specific integral membrane lipid biosynthetic enzymes. Since acylation of ACP is known to alter its physical properties, it is tempting to speculate that the observed distribution of ACP between the soluble and particulate fractions may reflect the degree of acylation of the protein.

Following this affinity chromatography step, the recovery of activity was so low and the activity was so

labile that no further characterization or purification of the enzyme could be achieved.

Isolation of Chloroplasts from Euglena Gracilis

The localization of the ACP-dependent FAS of Euglena to the chloroplast stroma has been demonstrated in chloroplasts isolated from cells which were grown in low vitamin B_{12} (Worsham et al., 1988). Those results have been extended by this study, in which chloroplasts were isolated from cells grown in a medium which was not limiting in that vitamin. The effects on Euglena of growth in low or limiting vitamin B_{12} are not entirely understood. It is thought that the cells are blocked at the end of the S phase of the cell cycle (Bertaux et al., 1978). DNA levels in cells which have been grown in low vitamin B_{12} rise to 1.8 times the amount of DNA present normally per cell, and stay at that level (Bertaux and Valencia, 1973). The synthesis of RNA and protein meanwhile continues, with the end result that the cells' volumes increase to 10-30 times the volume of a normal cell (Cavel, 1969; Carrell et al., 1970). Chloroplast and mitochondria also continue to grow and divide in their normal rhythm, so the ratio of chloroplast to cytoplasmic protein remains constant throughout the blockade (Bre and Lefort-Tran, 1974). One important exception to the general rule of continuing protein synthesis is the loss of normal intussusceptive growth of

the proteinaceous component of the pellicle. During normal cell growth, new ridges are formed in the valleys between existing ridges, leading to an increase in volume as the cell coat uniformly expands. During cell growth in avitaminosis B_{12}, however, no new ridges are laid down. The increased volume of the cells is accomplished by stretching the existing structures rather than by their increase (Bre and Lefort-Tran, 1978). Other peculiar events observed in these cells include nucleolar an apparent fragmentation (Bertaux and Valencia, 1973) and alteration in chromosome structure (Bre et al., 1983). Unlike most animal and plant nuclei, chromosome condensation is displayed in Euglena throughout the cell cycle. In vitamin B_{12}-starved cells, chromatin is mainly dispersed through the nucleus rather than condensed. This may be due to alterations in histone H1, which has been reported in vitamin B_{12} starved cells, or to the decrease in polyamine synthesis which follows vitamin B_{12} starvation. In spite of these alterations in normal metabolism and growth, cells grown in low vitamin B_{12} have been used as the source of chloroplasts from Euglena by necessity. It would be desirable to be able to isolate chloroplasts from cells grown in a medium which more closely approximates normal, especially since all of the effects of growth in low B_{12} are not known. This is especially important for us because of the disruption in fatty acid synthesis which we observed in comparing cells which were

grown in limiting vitamin B_{12} to cells which were grown in the High Yield medium of Hutner (Table 11). In control cells, 24% of total FAS activity was due to the cytoplasmic FAS and 76% was due to the chloroplast FAS. In cells grown in low vitamin B_{12}, 61% of the total FAS activity was due to the cytoplasmic FAS, and 39% was due to the chloroplast FAS. Obviously, there are regulatory processes controlling the relative activities of these two enzyme systems which we do not understand. These control mechanisms are superimposed on the control mechanisms which regulate the levels of the two FASs during the chloroplast induction process and normal growth in light. For our purposes, finding a way to isolate chloroplasts from cells in which fatty acid biosynthesis was not disturbed by a vitamin deficiency was imperative.

Our approach to breaking cells grown in a normal medium was to first weaken the membrane, the outermost component of the pellicle, by adding detergent in very low amounts; then to weaken the exposed proteinaceous coat by digestion with trypsin. Cells could then be broken without destroying the chloroplast membranes in the process. In studies of the structure of the nuclear membrane and nuclear pore complexes, the detergents Triton X-100 and octylpoly (oxyethylene) have been used to strip the lipid layer off of nucleii, leaving the underlying protein coat intact (Jiang and Schindler, 1987). We attempted to weaken the pellicle and expose part of the underlying protein coat of Euglena in

an analogous fashion by treatment of intact Euglena with low concentrations of the nonionic detergent Brij 35. The data in Table 12 indicates that we were able to accomplish this, since treatment of the cells with proteinase but not with Brij 35 resulted in recovery of no visible chloroplast band, protein, chlorophyll, or FAS activity. Incubation of cells for 15 minutes in 0.001% Brij 35 was chosen as the best set of conditions, and was used in subsequent chloroplast isolations.

Chloroplasts isolated in this manner from the cells described in Table 11 are described in Tables 13 and 14. Although the yield of FAS activity per cell was reduced for chloroplasts isolated by this method compared to chloroplasts isolated by the method of Ortiz et al., the total FAS activity recovered was much more. This is due to the increased number of cells in cultures which are grown in Hutner's medium (2.4×10^9 cells/ml) compared to those which are grown in the low vitamin B_{12} medium of Ortiz et al (0.34×10^8 cells/ml). The chloroplasts were identical in density to intact chloroplasts isolated from the latter cells, banding in the same location on a Percoll density gradient. An identical banding pattern of thylakoid fragments on the top of the gradient, stripped chloroplasts underneath the thylakoid fragments, and intact chloroplasts in the gradient was seen. In both cases the FAS activity was ACP-dependent, indicating a lack of contamination by cytosol. Following

centrifugation of disrupted chloroplasts, further differences between chloroplasts from cells grown in Hutner's medium and cells grown in the medium of Ortiz et al. became apparent. When intact lettuce chloroplasts were gently disrupted and subjected to centrifugation, part of the FAS activity remained associated with the membrane fraction (Walker and Harwood, 1985). This association was destroyed if the chloroplasts were disrupted by harsher methods such as sonication. When chloroplasts isolated from cells grown in Hutner's medium were disrupted and centrifuged, 44% of the applied activity was recovered (Table 14). Of the recovered activity, 40% was recovered in the soluble supernatant fraction while 60% was recovered in the insoluble membrane fraction. By contrast, when disrupted chloroplasts isolated from cells grown in the medium of Ortiz et al. were centrifuged, only 9% of the applied activity was recovered; 54% of that was in the soluble supernatant fraction and 46% was in the insoluble membrane fraction. Because the yield was so low, it was impossible to come to any conclusions about the location of the FAS in these chloroplasts, but it does seem that the FAS was more sensitive to conditions and possibly less stable than was the FAS of chloroplasts isolated from cells grown in Hutner's medium. By comparison, when chloroplasts isolated from lettuce by freeze-thawing were centrifuged, 69% of the total FAS activity was recovered in the membrane

fraction. The observation of FAS activity loosely associated with the membranous fraction of chloroplasts is interesting in view of the findings of Slabas and Smith (1988), who showed that ACP in plants is primarily associated with thylakoid membranes. ACP thus forms two populations, one in contact with the internal membranes and presumably with the lipid biosynthetic enzymes which are localized to those membranes, while the other population remains soluble in the chloroplast stroma. A role for ACP in organizing the proposed catalytic complex of the chloroplast FAS of Euglena has been proposed; possibly the interaction of the FAS with the chloroplast membranes is mediated by ACP as well.

Isolation of Antibodies Specific for the Cofactor Binding Site of Dehydrogenases

One of the more useful and exciting concepts which has resulted from the study of protein structure and function has been the realization that for many proteins specific functions are performed by specific domains. The consequences of this domain structure of proteins in terms of evolution were expounded by Rossman et al. (1974), who postulated the existence of a primordial mononucleotide binding protein during precellular evolution. By a process of duplication the mononucleotide binding protein would gain the ability to bind dinucleotides. Gene shuffling would

combine this functional domain with functional domains of other proteins, resulting in enzymes with expanded functions and abilities. Evidence in support of this hypothesis has resulted from a comparison of the structures of genes of proteins containing the dinucleotide-binding domain (Michelson et al. 1985).

The basis of these speculations is the observed similarity of structure of the dinucleotide binding domains of several dehydrogenases for which the crystal structure had been determined, which included lactate dehydrogenase, malate dehydrogenase, liver alcohol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase. The structure consists of two mononucleotide-binding folds, each defined by a parallel sheet formed by three extended β-sheets, the first two connected by an α-helix and the second two by either an α-helix or an undefined loop. In the complete structure of two mononucleotide folds, the beta sheets are numbered βA-βF, and the helices are αB, αC, αE and β1F. The AMP moiety of NAD(H) is bound by the βA, αB, βB, αC and βC elements of the fold which form an enclosing hydrophobic pocket; the β-sheet structural elements tend to be more conserved in sequence than do the helices. In particular, when hydrogen-bonding patterns of analogous β-pleated sheets are compared, the conservation of the hydrophobic character of analogous amino acids was striking. The detailed interactions of the AMP and its binding fold are very

similar from one dehydrogenase to another. The sequences of the fold's elements and the interactions of the nicotinamide-binding fold with the nicotinamide portion of NAD(H) are not as highly conserved as the AMP-binding fold. These structural elements create a pocket for the nicotinamide which is hydrophobic on one side and hydrophilic on the other, from which substrate approaches. Either face of the nicotinamide may be exposed, depending on the dehydrogenase, and this is the basis for the stereospecificity of dehydrogenases. In examining the conservation of this structure, Rossmann et al. (1975) note that in conserving functional amino acids it is necessary not only that their physical and chemical characteristics be conserved, but also their spatial arrangement. This requirement that the β-sheets and the α-helices of the dinucleotide fold be maintained puts constraints on the amino acid alterations which can be tolerated.

Wierenga et al. (1985) examined the interactions of cofactor and fold in a number of dehydrogenases. They were particularly interested in the favorable electrostatic interaction of the α-helix (αB) with the pyrophosphate of NAD(H) (Hol et al., 1978). They observed an almost identical superposition of this helix and the AMP in several of the complexes which they examined, and suggested that the dipole moment of the helix plays a role in stabilizing the interaction of the protein with the pyrophosphate moiety of

NAD(H). They further observed that, in these β-α-β structural units of similar structure and dissimilar sequence, there is a characteristic "fingerprint". Part of the "fingerprint" is defined by the location of the pyrophosphate near the N-terminus of the α-helix; part is defined by the sequence GXGXXG, in which the first glycine is the first residue at the N-terminus of the α-helix. The other elements of the "fingerprint" are less well-defined, and include six small hydrophobic amino acid residues which form the core of the structure, a negatively charged residue at the C-terminus of the second β-sheet, and a hydrophilic residue at the N-terminus of the first β-sheet.

Although the sequences of the structural elements comprising the dinucleotide fold can vary, they still must recognize and bind the same cofactor. Thus the fold itself must retain a similar shape and size and electostatic character from one dehydrogenase to the next. Furthermore, the interaction with cofactor must discriminate between NAD(H) and NADP(H), and must be precise enough to bind the proper face of the nicotinamide, maintaining the stereospecificity of the dehydrogenase. At the same time binding cannot be so precise and exact that the cofactor cannot dissociate readily.

Katiyar and Porter (1983) reported that a polyclonal antiserum raised against an enzyme which uses NADPH as a cofactor contained a population of antibodies which

recognized a site in or near the dinucleotide binding site. These antibodies were capable of recognizing the dinucleotide binding site in unrelated enzymes. When the mixed population of antibodies was bound to a column matrix, the cross-reactivity allowed partial purification of unrelated dinucleotide-requiring enzymes (Stapleton and Porter, 1985). Antibodies against one NADH-requiring enzyme were found to inhibit the activity of other unrelated dehydrogenases, and the activity could be protected by prior incubation of the enzymes with NADH (Srivastava and Katiyar, 1988).

We isolated a subpopulation of antibodies which recognized the dinucleotide fold of dehydrogenases. To do this, we raised polyclonal antibodies against yeast glucose-6-phosphate dehydrogenase. These antibodies were then chromatographed on an affinity column of chicken liver malic enzyme covalently bound to Sepharose (Figure 8). A small portion, about 4% of the applied IgG, bound to the column and was eluted by $MgCl_2$. The presence of cross-reacting antibodies could be explained in a number of ways. The most obvious is that the glucose-6-phosphate dehydrogenase used to raise the antibodies might have been contaminated by malic enzyme, and the yeast and chicken enzymes may have been similar enough to cause cross-reaction. Alternatively, the malic enzyme may have been contaminated by glucose-6-phosphate dehydrogenase. Although gel electrophoresis of

the two enzymes did not show any major contaminants (Figure 9), and neither enzyme displayed any activity when assayed for the other, very low levels of inactive enzyme could have been present. To demonstrate that the cross-reactivity was not due to contaminants, the two proteins were separated by SDS-polyacrylamdie gel electrophoresis, Western-blotted onto nitrocellulose, and probed with either the polyclonal IgG or with the dinucleotide-fold specific IgG (Figure 10). If the glucose-6-phosphate dehydrogenase which was used to raise the antibodies was contaminated by malic enzyme, then the blot of glucose-6-phosphate dehydrogenase using the polyclonal antibodies as a probe should show two bands. Furthermore, the cross-reacting antibodies should be specific for malic enzyme and should not recognize glucose-6-phosphate dehydrogenase. Conversely, if the malic enzyme contained contaminating glucose-6-phosphate dehydrogenase, the cross-reacting antibodies should be specific for that enzyme and unable to recognize malic enzyme. The results of the Western blot do not support either of these possibilities. One band was visible when the glucose-6-phosphate dehydrogenase was probed with the polyclonal antibodies. The same band was visible when the cross-reacting antibody was used as a probe; therefore the presence of cross-reacting antibody is not attributable to contaminating malic enzyme in the glucose-6-phosphate dehydrogenase. Although the two proteins have very similar

molecular weights, the bands were resolved. In the malic enzyme lane, the band which was recognized by the polyclonal antibodies coincided with the major band of protein seen by gel electrophoresis of malic enzyme, which also corresponded with the size of the peak of activity seen when malic enzyme was subjected to gel filtration chromatography.

The antibodies which were isolated by chromatography on malic enzyme-Sepharose were used to construct a second affinity column. Chromatography of glucose-6-phosphate dehydrogenase on this column confirmed the results of the Western blot, demonstrating that the cross-reactivity is not due to contaminating malic enzyme (Figure 11). In addition, the antibodies were able to recognize and bind malic enzyme (Figure 12). They thus were not directed against contaminating glucose-6-phosphate dehydrogenase in the malic enzyme, but instead recognized a common antigenic site on both proteins. The most likely candidate for such a site is the dinucleotide fold, a structure which both of these enzymes are known to possess. The elution of bound malic enzyme and bound glucose-6-phosphate dehydrogenase from the column by their cofactor, $NADP^+$ strengthens this conclusion. The column was also able to bind a completely unrelated enzyme, enoyl-ACP reductase, which was eluted by its cofactor, NADPH (Figure 13).

The structural features of a protein which contribute to antigenicity are not well defined. It has been

demonstrated that in some cases where a particular epitope is known, small peptides having the sequence of amino acids of that region of the protein are capable of eliciting an immune response. This seems to be at odds with other studies which indicate that antibodies recognize native conformation of proteins (Amit et al., 1985). A number of studies of peptides which mimic the amphiphilic helix have shown, however, that some peptides can possess elements of secondary structure (Moe and Kaiser, 1985). Others have relied on computer graphics and molecular modeling to identify regions of a protein which are recognized by antibodies and try to determine the underlying rules which determine antigenicity. These studies have shown that, in general, regions of a protein which have a convex shape, high mobility and negative electrostatic potential, tend to be more antigenic (Geysen et al., 1987) They correlated the antigenicity of a particular region of a protein with the lack of bound water molecules, and remarked that the displacement of bound water might be important in the ability of antibodies to bind to protein. In another study, Amit et al. (1985) examined the binding of a monoclonal antibody to egg white lysozyme. In this instance the interaction of antibody and protein covered a large area on both proteins, and the region of lysozyme which was involved in the interaction was not significantly above average in mobility. In either case, a groove-like structure such as

that of the dinucleotide binding site would not be expected to be very antigenic. It is clear, however, that the interaction of the enzymes studied by myself and by Katiyar and Porter with these antibodies is disrupted by coenzyme. If this interaction is not within the binding site itself, it must be in close physical proximity.

The use of cross-reacting antibodies such as these should help to define the dinucleotide fold of enzymes for which no structural or even sequence information is available, as well as contributing towards our understanding of the physical characteristics of proteins which results in their antigenicity. Since determination of the structure of a protein is facilitated by knowledge of the structure of related proteins, it is also likely that structural information about related domains may be of use in determining the structures of otherwise unrelated oxidoreductases.

Molecular Biology of the Euglena Gracilis FAS

The preliminary data on the molecular biology of the Euglena FAS was surprising. Although some members of the FAS are quite conserved, it is puzzling that all three of the probes hybridized to DNA from the several different species examined. This is especially true since several investigators have noted the lack of hybridization of probes specific for one isoform of plant ACP to other isoforms of

the same species (for example, Safford et al., 1988). Non-specific binding of the probe to the membrane is an unlikely explanation, since the probe only bound to slots where DNA was applied. Non-specific interaction of the probe and the DNA is possible but seems unlikely for the following reason. Rat DNA, which was present as a positive control, was applied in amounts very similar to the amounts of DNA applied from each of the other species. Since the probes are specific for rat sequences, the specific hybridization of the probe should far outweigh any contribution of non-specific binding to those slots. Therefore, if the binding of the probes to the non-rat DNAs were due to non-specific interactions, the strength of the signal from those DNAs should have been much reduced compared to the rat controls. Since they are of about the same magnitude in most cases, this cannot be the case. Also, the probes did not bind to chloroplast DNA or lambda DNA.

Under the high stringency conditions of hybridization used, and taking into account the relatively high GC content (57-59% GC) of the three probes, mismatches of only about 10% could be tolerated (McConaughy et al., 1969). The rat (Amy et al., 1989) and yeast (Chirala et al., 1987; Mohamed et al., 1988) FAS have been cloned and their nucleotide sequences determined. The amino acid sequence of the active sites of these enzymes differ by more than 10%. Also, the nucleotide sequences for the ACPs of spinach (Scherer and

Knauf, 1987) and Brassica campestris (Rose et al., 1987) differ from the rat FAS ACP domain by more than 10%.

Whether the hybridization reflects true recognition of FAS genes or fortuitous homology to other sequences, the results of hybridization of the three probes to wild-type and mutant Euglena DNAs argued that the sequences being recognized were not located in the chloroplasts. The mutant is very deficient in or lacking chloroplast DNA, so if part of the contribution to the signal seen with wild-type DNA was due to sequences in chloroplast DNA, the signal should have been reduced in mutant DNA. Since the magnitude of the signals was about equal, the binding could not have been to chloroplast DNA. The results of hybridization of blots of field inversion gels raises another interesting possibility. In these gels, hybridization was seen in a region which is not chloroplast or nuclear and was seen in the mutant as well as wild type samples. Since Euglena contains a mitochondrial fatty acid synthase, it could be that these enzymes are mitochondrially encoded. ACP has been identified in the mitochondria of a number of species (Chuman and Brody, 1989) where its function is unknown. Alternatively, the hybridization might have been due to sequences present within the mitochondrial genome which are homologous to nuclear sequences. Sequence homologies have been demonstrated between the nuclear and chloroplast genome of spinach (Timmis and Scott, 1983) and between

mitochondrial and chloroplast DNA of several plants (Stern and Lonsdale, 1982; Stern and Palmer, 1984). The ability of DNA sequences to swap among the three genomes of plants may explain the presence of these homologous sequences. Lastly, although hybridization of these probes to DNA from diverse species is unusual, other proteins are known which are so highly conserved that probes specific for the protein in higher animals hybridizes to genes for the protein in plants. α-Tubulin is one example, and another is the so-called proliferating-cell nuclear antigen, which is a DNA-polymerase auxiliary protein (Suzuka et al., 1989). In any event, the hybridization of these three probes to DNA from Euglena provided a starting point for further studies of the molecular biology of the two FASs of this unique creature.

The structure and syntheses of lipids and fatty acids has been a poor cousin in the more glamorous world of nucleic acids and proteins. Nevertheless, because of their role as the structural component of membranes, they are of fundamental importance to all living things. It is perhaps not surprising that all organisms synthesize fatty acids by the same steps. What is surprising is that apparently part of what it means to be a eukaryote and not a plant is to have a very large multifunctional FAS, while part of being a plant is to have a non-associated FAS located within the chloroplasts. At some time in its passage through time, the cell which was destined to be the forerunner of all

vertebrate cells acquired a FAS which was a multifunctional product of seven fused genes. One could speculate about the advantages to a cell of having all the enzymes of fatty acid biosynthesis covalently linked, both in terms of the kinetic advantage to the cell and the lack of accumulation of potentially toxic intermediates, but in fact bacteria and plants seem to function quite nicely with their nonaggregated systems. Why do all eukaryotic cells studied to date have multifunctional multienzyme complex fatty acid synthases? One wonders whether the posession of such a FAS confers what one hopes is an advantage to eukaryotes, or whether it in fact limits nonplant eukaryotes by making their alternatives more restricted. In that sense one could speculate that the flexibility of lifestyles exhibited by prokaryotic cells, which is partly due to their ability to alter their fatty acid profile, has been traded in on a dazzling architectural complexity. While questions of when and how and why are seductive, they are also not amenable to easy answers. It is precisely for this reason that the unicellular alga Euglena gracilis is of such interest. These cells are something of a misfit, neither fish nor fowl nor good red meat. Like a unicellular protozoan, they can live and grow happily on an exogenously supplied carbon source such as glucose. Transfer of these cells into the light results in a drastic physiological realignment of the cell as chloroplast development is induced and they become

photosynthetic creatures. Our interest in Euglena resides in the gray area of the interface between the plant-like algal cell and the animal-like heterotroph. Their dichotomous life style is exemplified in the synthesis and the structure of the fatty acids and the lipids of these cells. Grown heterotrophically in the dark, Euglena contain the complement of lipids typical of a non-plant eukaryotic cell. The same cells grown in the light elaborate the enzymatic machinery necessary to synthesize the lipids characteristic of a plant cell. It is the simultaneous presence, in the same cell, of the enzymatic machinery characteristic of both plants and non-plant eukaryotic cells which has fascinated this laboratory and formed the thrust of much of its research.

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