Fatty Acid Synthetase from Chlamydomonas reinhardi

SITES OF TRANSCRIPTION AND TRANSLATION*

(Received for publication, November 17, 1971)

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SUMMARY

Cell-free extracts of the unicellular green alga, Chlamydomonas reinhardi, catalyze the incorporation of acetyl-CoA and malonyl-CoA into long chain fatty acids. The fatty acid synthetase is dependent on added acyl carrier protein for activity, regardless if the cells are grown in the light or in the dark. The major products formed from acetyl-CoA and malonyl-CoA by cells in the light period of synchronous growth are palmitate, stearate, and arachidate, whereas in the dark period of synchronous growth more short fatty acids are formed. Cycloheximide has no effect on the appearance of the fatty acid synthetase in synchronous cultures of C. reinhardi, thus suggesting that transcriptional steps necessary for formation of fatty acid synthetase occur in the nucleus. Cycloheximide has no effect on the activity of fatty acid synthetase in the synchronous cultures, whereas spectinomycin reduced the activity significantly. These findings strongly indicate that the chloroplast ribosomes are responsible for the de novo protein synthesis required for fatty acid synthetase in C. reinhardi.

Current research has shown that although the chloroplast of the unicellular green alga Chlamydomonas reinhardi contains DNA and possesses the necessary equipment for protein synthesis (see Reference 1 for review), the synthesis of components of this organelle as well as its development are under control of both the nuclear-cytoplasmic genetic system and the genetic system of the chloroplast (2-4).

One characteristic feature of the chloroplast is its system of inner membranes or thylakoids that are known to be rich in galactolipids and sulfolipids (5). By their nature, the formation of these chloroplast-associated lipids has to be closely related to the biosynthesis of fatty acids, and thus when considering the genetic control over the formation of chloroplast components, it is of interest to determine the degree to which this organelle participates in the synthesis of fatty acids. In this paper we present the results obtained from an investigation of the synthesis of fatty acids and the cellular sites of transcription and translation of enzymes involved in their formation in C. reinhardi.

Antibiotics that selectively inhibit transcription and translation in either the nuclear-cytoplasmic system or in the chloroplast are important tools in studies on chloroplast formation. Rifampicin has been shown to inhibit RNA synthesis in the chloroplast of C. reinhardi (6, 7), Chlorella pyrenoidosa (8), and Acetabularia (9). Thus, the inhibitor apparently prevents the genes of the chloroplast from being transcribed. Spectinomycin has been shown to bind specifically to 70 S chloroplast ribosomes in C. reinhardi (10), while cycloheximide is known to inhibit protein synthesis on 80 S cytoplasmic ribosomes (2, 11). Thus, differential use of spectinomycin and cycloheximide gives information as to the sites of translation of various proteins. A system for synchronous growth of C. reinhardi has been described earlier (4) in which antibiotics were used in an attempt to determine the sites of genetic transcription and translation of certain important chloroplast components. Synchronous cultures are useful for such studies, since all the cells are in the same physiological state at any given time, and chloroplast and other cell components are synthesized at known times in preparation for a subsequent cellular division. Furthermore, an antibiotic can be added and its effects observed during a time period in which there is little RNA synthesis and the cells are nondividing. Also, the time of exposure of the cells to the antibiotic is relatively short, thus reducing the chances of secondary effects unrelated to protein synthesis.

Soluble enzyme systems that catalyze the formation of long chain fatty acids from acetyl-CoA and malonyl-CoA are known from a variety of organisms and tissues. In higher plants (12-14) and bacteria (15-17), the fatty acid synthetases consist of nonaggregating enzymes which can be separated into free ACP and enzyme components, which upon combination catalyze the synthesis of fatty acids, utilizing as substrates the ACP thioesters of the different acyl intermediates. The enzyme systems isolated from yeast (18) and animal tissue (19-22), on the other hand, are single multienzyme complexes, which are not dissociable into free ACP and active enzymes. Recently, it was reported that, depending on growth conditions, the phytoflagellate Euglena gracilis contains two independent fatty acid synthetases (23). When this organism was grown in the light on minimal medium, both an ACP-dependent and an ACP-independent fatty acid synthetase were present; the two different

* This study was supported by Grants GB 1866 and GB 29203 from the National Science Foundation.
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The abbreviations used are: ACP, acyl carrier protein; DTT, dithiothreitol.
enzyme systems could be separated either by ammonium sulfate fractionation or by centrifugation in a sucrose density gradient. In etiolated cells of *E. gracilis*, however, only the ACP-independent fatty acid synthetase was present. By the differential use of cycloheximide and chloramphenicol, it was further shown in regreening cells of *E. gracilis* that de novo synthesis of the ACP-dependent fatty acid synthetase was completely dependent on the chloroplast ribosomes (24).

### Methods

**Organism and Culture Conditions**—*C. reinhardtii*, a substrain of wild type strain 137c, mating type plus, was used. This strain is capable of synchronous, phototrophic growth in the minimal medium of Sueoka (25) as modified by Armstrong et al. (4). Synchronous growth was obtained by using a 12-hour light-12-hour dark cycle as described by Kates and Jones (26). Light and temperature conditions were as described by Surzycki (27). When rifampicin was used, the light intensity was increased from 5000 to 8000 lux, since this drug is light-absorbing. This strain of *C. reinhardtii* was used. This strain was designed for the use of cycloheximide and chloramphenicol, it was further shown that the medium of Sueoka (25) as modified by Armstrong et al. (4).

The wild type strain 137c, mating type plus, was used. This strain was grown on the same medium as the wild type strain 137c, mating type plus, and was harvested by centrifugation at 3000 x g for 15 min. The final concentration of cycloheximide in the cultures was 2 pg per ml.

**Preparation of Enzyme**—Samples of 400 ml were taken from the synchronous cultures and harvested by centrifugation at 17,000 x g for 10 min. The cells were washed once with and resuspended in 0.02 M potassium phosphate buffer (pH 7.0), 0.001 M in DTT to a final volume of 1.6 ml. Cell-free extracts were obtained by exposing the cells to sonic oscillation for two 1-min intervals with a Mullard 20-kc ultrasonic disintegrator operated at full power. The preparation was centrifuged for 10 min at 17,000 x g to get rid of cell debris. The supernatant was centrifuged at 37,000 x g for 30 min. The soluble fraction (supernatant) was the crude extract.

Extracts of hetero- and mixotrophically grown cells were prepared in the same fashion, the concentration of cells before sonication being about 0.3 g per ml.

Ammonium sulfate fractionation of the crude extract was accomplished by adding a saturated salt solution to the crude extract to give 20, 35, 50, and 70% saturation. The precipitates were collected by centrifugation, dissolved to the original volume in the DTT-containing phosphate buffer, and assayed at once for enzyme activity. All operations were carried out at 0–4°C.

**Fatty Acid Synthetase Assay**—Fatty acid synthetase activity was measured by the radioactive assay of Delo et al. (23). The reaction mixture routinely contained in a total volume of 0.5 ml: [2-14C]malonyl-CoA, 30 nmoles; [3H]acetyl-CoA, 15 nmoles; NADPH, 15 nmoles; NADH, 15 nmoles; FMN, 0.5 nmoles; DTT, 2.5 nmoles, potassium phosphate buffer (pH 7.2), 0.5 mmole; *E. coli* ACP, 10 amoles; and 0.1 ml of enzyme preparation. The assay mixtures were incubated for 15 min at 30°C, the reaction stopped by adding 0.1 ml of 50% KOH, and the suspension saponified at 100°C for 30 min. After acidification with 8 to 9 drops 6 N HCl, the fatty acids were extracted three times with 5 ml of petroleum ether (b.p. 37–46°C). The solvent was evaporated and the residue dissolved in 15 μl of 2,5-diphenyloxazole (PPO)-toluene and counted in a Packard Tri-Carb liquid scintillation spectrophotometer, model 574.

The incorporation of [2-14C]malonyl-CoA and [3H]acetyl-CoA into long chain fatty acids was determined by computer analyses of the counting data. In experiments with synchronously grown cells, the results were calculated on the basis of 100 ml of synchronous culture, containing 106 cells per ml, since the cell number remains constant in these cultures during the experimental period.

**Identification of Fatty Acids**—The petroleum ether extracts of the synchronous culture were separated by thin layer chromatography on silica gel plates. The plates were then developed with a solvent system of chloroform-methanol-water (65:25:4) and exposure to iodine vapor.

**Oxygen Evolution and Respiration**—To measure oxygen evolution and respiration, a Yellow Springs Instrument Co. oxygen monitor (model 53) and a Clark-type electrode were used. A cell sample of 65 ml was taken from the synchronous culture, centrifuged at 1,000 x g for 5 min, and resuspended in Tris-acetate-phosphate medium containing 0.0025 M NaHCO3. The measurements were done at 26°C in a thermostated Lucite vessel. For photosynthetic oxygen evolution the light intensity was 20,000 lux.
Cells were grown synchronously as described under "Methods" and harvested after 6 hours growth in the third light period. Crude extracts and (NH₄)₂SO₄ fractions were prepared and assayed for fatty acid synthetase activity as described. The reaction mixture for the radioactive assay contained in a total volume of 0.5 ml: [2-¹⁴C]malonyl-CoA, 30 nmoles; [¹⁹H]acetyl-CoA, 15 nmoles; NADPH, 15 nmoles; NADH, 15 nmoles; FMN, 0.5 nmole; DTT, 2.5 μmoles; potassium phosphate buffer (pH 7.2), 0.5 mmole; E. coli ACP, 10 μmoles; and 0.1 ml of enzyme preparation. Time of incubation: 15 min at 30°. The values given are nanomoles of [²⁻¹⁴C]malonyl-CoA plus [¹⁹H]acetyl-CoA incorporated into fatty acids by 100 ml of synchronous culture containing 10⁶ cells per ml. 

TABLE I
Dependence on ACP of fatty acid synthetase from C. reinhardtii, grown phototrophically in synchronous cultures

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Amount [²⁻¹⁴C]malonyl-CoA plus [¹⁹H]acetyl-CoA incorporated into fatty acids (nmols)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With ACP</td>
</tr>
<tr>
<td>Crude extract</td>
<td>1.100</td>
</tr>
<tr>
<td>Fraction 0 to 20% (NH₄)₂SO₄</td>
<td>0.008</td>
</tr>
<tr>
<td>Fraction 20 to 50% (NH₄)₂SO₄</td>
<td>0.003</td>
</tr>
<tr>
<td>Fraction 50 to 70% (NH₄)₂SO₄</td>
<td>0.004</td>
</tr>
<tr>
<td>Fraction 70 to 100% (NH₄)₂SO₄</td>
<td>0.825</td>
</tr>
</tbody>
</table>

CONTINUATION

The major products formed from malonyl-CoA and acetyl-CoA by the fatty acid synthetase from extracts of synchronously grown C. reinhardtii were identified by gas chromatography as palmitate (C₁₆), stearate (C₁₈), and arachidate (C₂₀). At the

RESULTS

Fatty Acid Synthetase from Synchronously Grown Cells of C. reinhardtii—Crude extracts from synchronously grown phototrophic cells of C. reinhardtii incorporated malonyl-CoA and acetyl-CoA into fatty acids in the presence of the cofactors described under "Methods." The incorporation was greatly stimulated upon addition of E. coli ACP (Table I). Fractionation of the crude extract showed that the enzyme activity was recovered in the protein precipitating between 50 and 70% saturated ammonium sulfate (Table I) as is the case for the ACP-dependent enzyme in E. gracilis (23). During the third light period of synchronous growth of C. reinhardtii, the activity of the fatty acid synthetase increases from the 1st to the 12th hour. The level of activity varied somewhat from one experiment to another, but in general the activity of the synthetase increased in the fashion shown in Fig. 1. The major products formed from malonyl-CoA and acetyl-CoA by the fatty acid synthetase from extracts of synchronously grown C. reinhardtii were identified by gas chromatography as palmitate (C₁₆), stearate (C₁₈), and arachidate (C₂₀). At the
TABLE III
Dependence on ACP of fatty acid synthetase from mixotrophically grown C. reinhardtii

The cells were grown mixotrophically for 40 hours as described under "Methods," harvested, washed, and resuspended in potassium phosphate buffer (pH 7.0), 0.001 M in DTT, to give a final concentration of 0.3 mg per ml, wet weight, before sonication. Crude extracts and ammonium sulfate fractions were assayed for fatty acid synthetase activity as described under "Methods" and Table I, except that unlabeled acetyl-CoA was used. The results are expressed as nanomoles [2-14C]malonyl-CoA incorporated into fatty acids by an amount of cell-free extract corresponding to 1 g of cells, wet weight.

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Amount of [2-14C]malonyl-CoA incorporated into fatty acids</th>
<th>Amount of protein per g cells, wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With ACP</td>
<td>Without ACP</td>
</tr>
<tr>
<td>Crude extract</td>
<td>18.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Fraction 0 to 20% (NH₄)₂SO₄</td>
<td>2.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Fraction 20 to 35% (NH₄)₂SO₄</td>
<td>8.3</td>
<td>0.20</td>
</tr>
<tr>
<td>Fraction 35 to 50% (NH₄)₂SO₄</td>
<td>5.6</td>
<td>0.03</td>
</tr>
<tr>
<td>Fraction 50 to 70% (NH₄)₂SO₄</td>
<td>3.2</td>
<td>12.07</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of rifampicin on the increase in ACP-dependent fatty acid synthetase during the third light period of synchronous growth. Cells were harvested, broken, and assayed for the enzyme as described under "Methods" and Table I. ○, control; △, 250 μg per ml of rifampicin. Rifampicin was added 50 min before zero time.

Fig. 3. Effect of cycloheximide on the increase in ACP-dependent fatty acid synthetase during the third light period of synchronous growth. Cells were harvested, broken, and assayed for the enzyme as described under "Methods" and Table I. ○, control; △, 2 μg per ml of cycloheximide. Cycloheximide was added 3 hours after zero time.

Fig. 3A. Effect of cycloheximide on the increase in ACP-dependent fatty acid synthetase during the third light period of synchronous growth. Cells were harvested, broken, and assayed for the enzyme as described under "Methods" and Table I. ○, control; △, 2 μg per ml of cycloheximide. Cycloheximide was added 3 hours after zero time.

The relative low values for enzyme activity obtained for both the treated and the control are probably due to the light conditions that prevail in experiments with rifampicin (see "Methods").
FIG. 4. Effect of spectinomycin on the increase in ACP-dependent fatty acid synthetase during the third light period of synchronous growth. Cells were harvested, broken, and assayed for the enzyme as described under "Methods" and Table I. O, control; ●, 3 µg per ml of spectinomycin. Spectinomycin was added 50 min before zero time.

The synthesis of the ACP-dependent fatty acid synthetase in cell-free extracts of C. reinhardi was not affected by cycloheximide (Fig. 3), whereas chlorophyll synthesis in this experiment was inhibited by about 90%.

When spectinomycin was added to the synchronously growing cells, the synthesis of the ACP-dependent fatty acid synthetase increased in the first 6 hours after the onset of the light period, after this period the synthesis went down. This was the common pattern when cells were exposed to this drug. Results from a typical experiment are shown in Fig. 4. Although the rate of synthesis of the enzyme in treated cultures always begins to go down around the 6th hour, the early (0 to 3 hours) rapid rate in treated cultures is observed to vary from one experiment to another (compare Figs. 4 and 5). At the end of the light period the cells were viable and the level of chlorophyll was the same as for cells in the control.

To test the possibility that the decrease in appearance of the enzyme was due to secondary effects of the drug, i.e. inhibition of respiration, an experiment was performed in which the cells were exposed to higher concentrations of spectinomycin (4.5 µg per ml in contrast to 3 µg per ml usually employed). The rate of respiration and photosynthetic oxygen evolution by whole cells was measured at the onset of the third light period and after 11 hours in the light. The data from this experiment are given in Fig. 5. As is shown in the inset of the figure, photosynthetic oxygen evolution was completely inhibited by spectinomycin, whereas the increase in respiratory capacity of the cells was only slightly affected (4). The appearance of the fatty acid synthetase showed the same pattern as in the experiment shown in Fig. 4.

FIG. 5. Effect of an increased concentration of spectinomycin on the increase in ACP-dependent fatty acid synthetase. Cells were grown, harvested, broken, and assayed for the enzyme as described under "Methods" and Table I, except that unlabeled acetyl-CoA was used. The inset table shows the effect of spectinomycin on the increase in respiration and photosynthetic oxygen evolution. Samples were taken at zero time and after 11 hours and respiration and photosynthetic oxygen evolution measured as described under "Methods". The results are expressed as micromoles of O₂ consumed or evolved per hour by 50 ml of synchronous cultures. O, control; ●, 4.5 µg per ml of spectinomycin. Spectinomycin was added 50 min before zero time.

The chloroplast of C. reinhardi has a certain degree of genetic autonomy in that it contains DNA and a complete protein-synthesizing machinery (1). However, so far only a few components of the chloroplast have been found that are coded for by the chloroplast DNA (4). In the experiments where the synchronously grown cells of C. reinhardi were exposed to rifampicin, an inhibitor of algal chloroplast RNA synthesis (6-9), the activity of the fatty acid synthetase increased in the same fashion as in the control cultures, thus suggesting that the genetic information necessary for synthesis of this enzyme system is located in the nucleus. It should be kept in mind, however, that a lack of an effect of rifampicin must be interpreted cautiously since the lifetime of chloroplast messenger RNA is not known. Although the chloroplast of C. reinhardi may not be involved in transcriptional steps that lead to the formation of the fatty acid synthetase, the synthesis of the enzyme depends on translational steps that occur on chloroplast ribosomes. Spectinomycin, which binds specifically to the 70 S chloroplast ribosomes in C. reinhardi (10) inhibits the formation of the fatty acid synthetase drastically, under conditions where chlorophyll synthesis, which depends on translational steps on cytoplasmic ribosomes (4), was not affected at all. Furthermore, when the synchronous cultures were treated with cycloheximide, the inhibitor of protein synthesis on the 80 S cytoplasmic ribosomes, at a concentration (2 µg per ml) which inhibited chlorophyll
synthesis by 90%, they contained the same level of fatty acid synthetase as did the control cultures. Thus, the results obtained by the differential use of spectinomycin and cycloheximide strongly indicate that de novo protein synthesis necessary for synthesis of fatty acids take place on the 70 S ribosomes of the chloroplast in _C. reinhardtii_.

It appears from the results reported here that _C. reinhardtii_ contains a fatty acid synthetase similar to the one reported to occur in green plants (12-14) and bacteria (17). The fatty acid synthetase from _C. reinhardtii_ is completely dependent on the presence of ATP, regardless whether the organism is grown in the light or in the dark, and the major products formed from acetyl-CoA and malonyl-CoA in the light are palmitic, stearic, and arachidic acids. In contrast, it has been reported that _E. gracilis_ has the ability to form two distinct and separable fatty acid synthetases depending on the growth conditions. The two fatty acid synthetases from _E. gracilis_ differ from each other in molecular properties and give rise to fatty acids with different chain lengths (23). Thus _C. reinhardtii_ differs from _E. gracilis_ with respect to the synthesis of fatty acids. This difference between _C. reinhardtii_ and _E. gracilis_ in their enzymes for fatty acid synthesis probably mirrors the difference in mode of life of these two types of organisms. In _C. reinhardtii_, both chlorophyll synthesis and chloroplast formation are constitutive processes which occur in the dark as well as the light (32), whereas in _E. gracilis_ these processes are absent in the dark, and are only induced when the cells are exposed to the light (33).

Although only one stage of fatty acid synthetase was found in cell-free extracts of _C. reinhardtii_, it appears that the types and amounts of fatty acids formed are somewhat dependent on the stage of the cell cycle. This is indicated by the results from experiments with synchronous cultures, where the fatty acids formed in the dark (zero time) had shorter chain lengths than those formed later in the third light period (Table II and Fig. 1). When the cells in synchronous culture enter into a light period, many of their chloroplast components start to increase in amount in preparation for the cell division that will occur in the subsequent dark period. Thus the fact that relatively more of the long chain fatty acids are produced later in the light period probably reflects the need of the cell for precursors for oleic and α-linolenic acids, the major unsaturated fatty acids in the chloroplast membranes of _C. reinhardtii_ (23).

Acknowledgments—We wish to thank Professor Konrad Bloch for his generosity in providing the radioactive fatty acid derivatives and the ACP necessary for the experiments described here, for his advice and suggestions, and for the use of facilities in his laboratory. We also thank Dr. Barbara Talamo and Dr. Harold Wright for their assistance during the course of the experiments.

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