Solubilization and purification of aldehyde-generating fatty acyl-CoA reductase from green alga *Botryococcus braunii*

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Abstract Membrane-bound fatty acyl-CoA reductase from the green alga *Botryococcus braunii* has been solubilized from the microsomal preparation by 0.1% octyl β-glucoside and purified to near homogeneity by Blue A agarose and palmitoyl-CoA agarose affinity column chromatography. The molecular mass of the enzyme was estimated by SDS-PAGE to be 35 kDa. The enzyme generates fatty aldehyde by reduction of fatty acyl-CoA with NADH as the reductant. The N-terminal amino acid sequence of this protein that represents the first eucaryotic aldehyde-generating reductase to be purified shows high homology with the N-terminus of fatty acid reductase from bacteria.

Key words: Acyl-CoA reductase; Purification; N-terminal sequence; Hydrocarbon; Aldehyde; *Botryococcus braunii*

1. Introduction

Biological reduction of fatty acids can provide fatty aldehyde or alcohol [1–3]. Fatty acyl-CoA reductase that generates fatty alcohol was first demonstrated in cell free preparations from *Euglena gracilis* [4], and subsequently particulate preparations that catalyze fatty acyl-CoA reduction from many animal and plant tissues have been described [5–9]. Fatty aldehyde is used for the generation of hydrocarbons by a recently discovered biological reaction, decarbonylation [10–13]. Hydrocarbons are widely distributed in all types of organisms including bacteria, fungi, algae, higher plants and animals [14]. In plants and insects, hydrocarbons are excreted as a component of surface waxes, where they function as a barrier to water exchange. Hydrocarbons are also present in mammalian nerve tissues and it has been suggested that certain central nervous disorders are related to drastically decreased hydrocarbon synthesis [15,16]. Thus organisms in both the animal and plant kingdoms probably contain fatty aldehyde-generating fatty acyl-CoA reductase. Such an acyl-CoA reductase was observed in the acetone powder extracts of leaves of *Brassica oleracea* [5]. Fatty alcohol-generating reductase has been solubilized and purified [17]. Aldehyde-generating reductase has been purified from bacteria that use aldehyde to generate light [18]. However, no aldehyde-generating reductase has been purified from any eucaryotic organism. *Botryococcus braunii* produces up to 32% of its dry weight as hydrocarbon [19,20] and it has been shown that a purified enzyme from this organism converts fatty aldehyde to hydrocarbon and carbon monoxide [21]. However, the enzyme that generates the aldehyde has not been purified. In this report, we describe solubilization and purification of the fatty acyl-CoA reductase from microsomal preparations from *B. braunii*. This enzyme catalyzes the reduction of fatty acyl-CoA to aldehyde with NADH as the reductant; its N-terminal amino acid sequence shows considerable homology with the N-terminal sequence of the bacterial fatty acid reductase [22,23].

2. Materials and methods

Chemicals: Blue A agarose, palmitoyl-CoA agarose, NADH, NADPH, Dithioerythritol and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. (St.Louis, MO). [1-^{14}C]Palmitoyl-CoA was from Du Pont New England Nuclear. Silica gel TLC plates were purchased from Whatman Co. Proteinase inhibitors leupeptin and pepstatin were supplied by Boehringer Mannheim.

2.1. Solubilization of fatty acyl-CoA reductase

Botryococcus braunii race A [20] was grown in a modified CHU13 medium of 2-fold strength [13] in a 20 liter Nalgene carboy with stirring, thermostated at 25°C and continuously illuminated at 10 $\mu Em^{-2}S^{-1}$ by cool-white fluorescent tubes. Cultures were harvested after 10 to 14 days of growth by centrifugation. The cells were washed with 0.5 M KCl and resuspended in 0.1 M phosphate buffer, pH 6.8, containing 1 mM DTE, 1 mM $MgCl_2$, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 15 μg/ml phenylmethylsulfonyl fluoride, and 0.3 M sucrose. After brief homogenization in a glass homogenizer, the cell suspension was passed through a French Press three times under pressure of 18,000 psi. The material was centrifuged at 3,000 × *g* for 1 min at 4°C, and the supernatant was centrifuged at 105,000 × *g* for 90 min to pellet the microsomes. The microsomal pellet was resuspended in the solubilization buffer (the phosphate buffer indicated above containing 0.1% octyl β-glucoside but without sucrose), homogenized in a glass homogenizer, and incubated at room temperature for 1 h with gentle shaking. The material was centrifuged at 105,000 × *g* for 90 min, and the supernatant was passed through a 0.8 μm filter and stored on ice for further purification.

2.2. Acyl-CoA reductase purification

The supernatant solubilized from the microsomal preparation was applied to the Blue A agarose column (25 ml, 2.5 cm diameter) with a flow rate of 20 ml/h. The column was washed with 400 ml phosphate buffer (0.1% octyl β-glucoside), and the bound acyl-CoA reductase was eluted with 50 ml phosphate buffer without sucrose but containing 1 M NaCl. The acyl-CoA reductase eluted from this column was dialyzed against phosphate buffer overnight and subsequently applied to a palmitoyl-CoA agarose affinity column (6 ml, 1.5 cm diameter). The column was washed with 200 ml phosphate buffer, and acyl-CoA reductase was eluted with 10 ml NADH (10 mM) at a flow rate of 10 ml/h.

2.3. Assay for fatty acyl-CoA reductase activity

Fatty acyl-CoA reductase activity was assayed anaerobically in 16 × 100 mm tubes sealed with serum stoppers essentially as previously described [17] with the following exception: 300 μM NADH was used instead of NADPH, and 20 μM [1-^{14}C]palmitoyl-CoA (10 Ci/mole) was used in the final reaction volume of 0.5 ml. Phenylhydrazine hydrochloride was added to a final concentration of 50 mM. After incubation at room temperature for 60 min, the reaction mixture was heated in a boiling water bath for 5 min, followed by treatment with 10 μl of pyruvic acid (99%). After 20 min at room temperature, the reaction mixture was extracted twice with chloroform (10 ml), and the solvent was removed by a stream of nitrogen. The recovered lipid was applied to a TLC plate (LK6D Silica gel 60 Å) and developed with hexane:ethyl

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ether:formic acid (40:10:1 v/v) as the solvent system. The silica gel from the region representing the product that co-migrated with non-radioactive authentic octadecenal was scraped from the TLC plate and assayed for radioactivity in a Beckman LS3801 scintillation counter.

3. Results and discussion

Acyl-CoA reductase in *B. braunii* was found exclusively in the microsomal preparations, with little enzyme activity in the soluble fraction from the cell-free preparations, consistent with previous observations [13]. When the microsomes were treated with 0.1% octyl β-glucoside, the solubilized material did not yield any labeled fatty aldehyde when incubated with [1-^{14}C]palmitoyl-CoA with NADH under anaerobic or aerobic conditions. The labeled fatty aldehyde that might have been generated could have been decarbonylated, oxidized to acid or converted to some other products. When phenylhydrazine was added in an attempt to trap the aldehyde and pyruvic acid treatment was used to release the aldehyde at the end of the enzymatic reaction, TLC of the products showed ^{14}C-labeled aldehyde; $NaBH_4$ reduction of this product yielded alcohol (data not shown). Therefore, for routine assays, the silica gel from thin layer plate corresponding to the aldehyde was scraped off and assayed for ^{14}C. With such an assay, it was found that 30 to 40% of the total microsomal acyl-CoA reductase was solubilized by the octyl β-glucoside. This appears to be the first case of the solubilization of an aldehyde-generating acyl-CoA reductase.

To purify the solubilized reductase, 100–120 g batches of green alga yielding 5–8 g of microsomes were used for each purification. Such a high yield of membrane was obtained only when French Press was used for cell breakage; ultrasonication and glass homogenizer were not effective. When the reductase preparation solubilized from the microsome was applied to Blue A agarose column, about 40% of reductase and more than 90% of proteins were found in the flow through fractions. The reductase retained on the column was eluted by 1 M NaCl after washing the column with phosphate buffer to baseline absorbance (Fig. 1). The enzyme eluted from the Blue A column was relatively stable for a few days in the presence of 1 M NaCl. When the enzyme preparation from this step was applied to a palmitoyl-CoA agarose affinity column, virtually all of the reductase was retained on this column. The reductase was eluted with 10 mM NADH and the reaction product was identified on radio-TLC (Fig. 2); further elution with NADPH did not yield any reductase activity.

SDS-PAGE of the enzyme preparation eluted from palmitoyl-CoA agarose column showed a single Coomassie-staining band indicating that the enzyme has been purified to near homogeneity; the degree of purification achieved by the two steps can be seen from the SDS-PAGE (Fig. 3). The subunit molecular mass of the reductase was found to be 35 kDa. Each batch yielded only less than 10 μg of purified enzyme, even with the use of proteinase inhibitors during purification. The purified enzyme underwent degradation during storage at −20°C as indicated by the appearance of lower molecular weight protein bands in SDS-PAGE of the stored enzyme (data not shown).

The pyridine nucleotide specificity of the purified reductase was determined. Only NADH but not NADPH served as the reductant. When fatty acid, ATP and CoA were used as the substrates with the microsomal preparations from *B. braunii*, NADH but not NADPH supported the production of hydrocarbon, most probably by decarbonylation of the aldehyde generated by the reductase [13]. Since the aldehyde-generating acyl-CoA reductase purified from the microsomes also showed the same pyridine nucleotide specificity, it would seem likely that the present enzyme is the one that provides the aldehyde substrate for the decarbonylase. It is interesting to note that the only acyl-CoA reductase that yields aldehyde as the product from higher plants previously described also showed specificity for NADH [5]. The present enzyme is similar to the fatty acid reductase from bacteria, *Photobacterium phosphoreum* and *Vibrio fischeri*, in that they all use NADH as reductant to generate aldehyde [18,24]. Fatty acyl-CoA reductase from animal sources usually generate fatty alcohol with NADPH as the reductant [7,17,25].

The N-terminal amino acid sequence of the 35 kDa acyl-CoA reductase was determined yielding 26 clearly identified amino acid residues. The sequence was found to be similar to the fatty

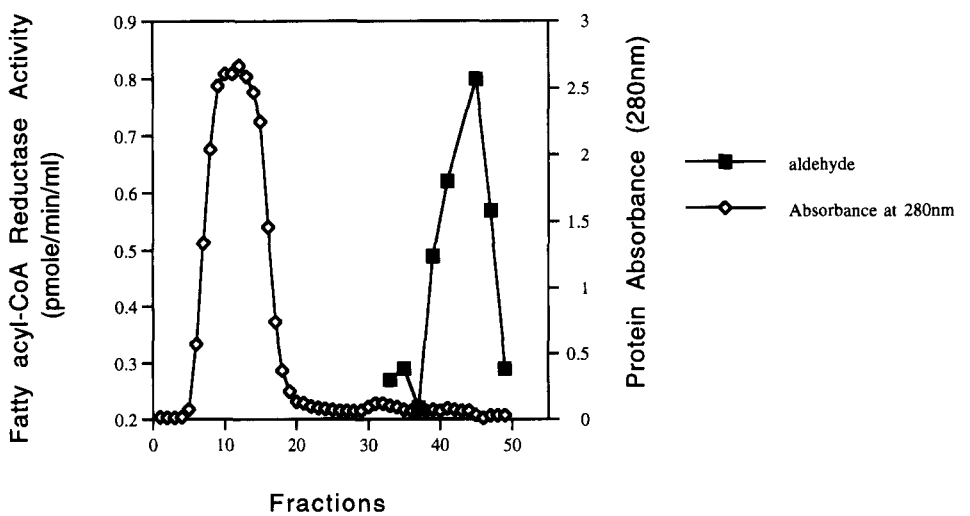

Fig. 1. Elution profile of acyl-CoA reductase from Blue A agarose column; 50 ml of phosphate buffer with 1 M NaCl was applied to the column with a flow rate of 20 ml/h; 2 ml fractions were collected in each fraction.

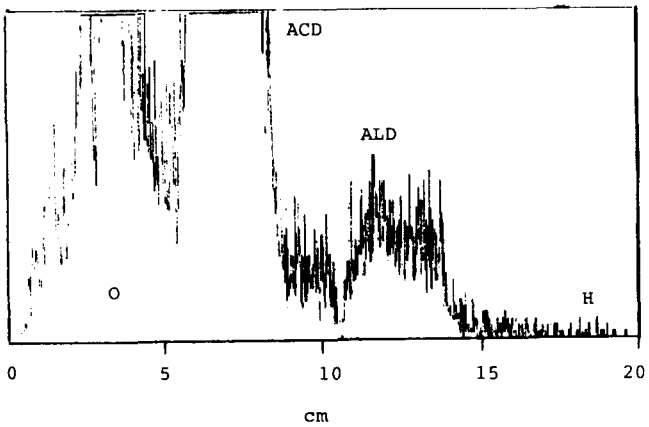

Fig. 2. Radio-thin layer chromatogram of lipids isolated from the reaction mixture containing NADH, [1-^{14}C]palmitoyl-CoA and the reductase eluted from palmitoyl-CoA agarose column. Chromatography was done on silica gel 60 Å with hexane : diethyl ether : formic acid (40 : 10 : 1 v/v/v) as the solvent system. O, original; ACD, fatty acid; ALD, aldehyde; H, hydrocarbon.

acid reductase from the bacterium *Photobacterium leiognathi* [17] (Fig. 4). The two classes of reductases thus far known can be classified as the alcohol generating one and the aldehyde generating one. They have different pyridine nucleotide specificity and different functions. Alcohols are esterified to form wax esters, and aldehydes are converted to hydrocarbons, both of which are common components of surface waxes [14].

The mechanism for alkane biosynthesis remained elusive until the 1980's [10]. With the discovery of decarbonylation, it was postulated that alkanes are generated from aldehyde by losing one carbon as carbon monoxide. Decarbonylase activity has been demonstrated from higher plants, mammals and insects [10,12,26] and the enzyme has been purified from the green alga *B. braunii* [21]. In all the above experiments, aldehyde serves as the substrate. In a recent report, it was confirmed that aldehyde is the immediate precursor of hydrocarbon in housefly microsomes [27]. However in this case, optimal conversion of the aldehyde to hydrocarbons was accompanied by formation of CO_2, required O_2 and NADPH, and suggested involvement of a Cytochrome P450. With *B. braunii* microsome, NADPH and O_2 inhibit hydrocarbon formation from aldehyde and over 90% CO does not inhibit hydrocarbon formation from aldehyde (Vioque, J. and Kolattukudy, P.E., unpublished results). In *B. braunii*, CO_2 is the product when microsome is used, but when the decarbonylase is purified, CO is the product. CO is oxidized to CO_2 by *B. braunii*. It is possible that in the housefly system, CO oxidation is tightly coupled to decarbonylation, and this oxidation may require molecular O_2 and NADPH. Alternatively, in the housefly a cytochrome P450 type enzyme might insert an oxygen between the carbonyl carbon and the methylene followed by transfer of the aldehydic H to the alkyl group with cleavage of the carbon oxygen bond to release CO_2. The acyl-CoA reductase may be functionally coupled to the enzyme that uses the product of the reductase. Thus the reductase that produces alcohol can be coupled to wax ester forming enzymes as previously observed with *Euglena gracilis* [2] whereas the aldehyde generating reductase may be functionally coupled to decarbonylase. Even though different organisms have characteristic chain length distribution for the hydrocarbons, the decarbonylase, and the acyl-CoA reductase that provides the aldehyde substrate for the decarbonylase, do not seem to show real chain length specificity. The chain elongation system that provides the acyl moiety for the reduction is probably what determines the chain length distribution of hydrocarbons.

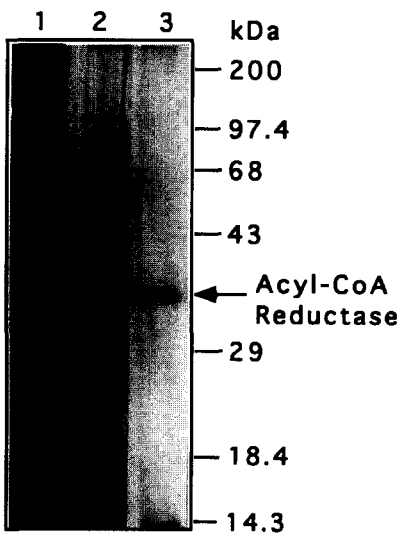

Fig. 3. SDS-PAGE of acyl-CoA reductase preparation solubilized from *B. braunii* microsomes (lane 1), and purified by Blue A agarose (lane 2) and palmitoyl-CoA agarose (lane 3) affinity chromatography.

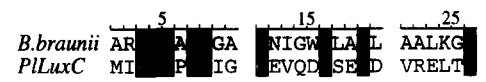

Fig. 4. N-terminal amino acid sequence comparison between acyl-CoA reductase from *B. braunii* and fatty acid reductase from *Photobacterium leiognathi*.

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