

Purification and characterization of the fatty acid synthase from *Bugula neritina*

Jinghai Wen, Russell G. Kerr*

Department of Chemistry and Biochemistry, Center for Molecular Biology and Biotechnology, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431-0991, USA

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Abstract

The fatty acid synthase from *Bugula neritina* has been purified 100-fold using ammonium sulfate precipitation, ion-exchange and size exclusion chromatography. The purified enzyme has a molecular weight of approximately 382 000 Da, as judged by gel filtration. Polyacrylamide gel electrophoresis under denaturing conditions in the presence of SDS revealed one major protein band of approximately 190 000 Da suggesting that the enzyme is a homodimer. The size of the enzyme, together with the observation that the FAS activity is independent of the concentration of acyl carrier protein, indicate that the FAS from *Bugula neritina* is a type I. A detailed analysis of the products of the purified FAS indicated that palmitic acid is the primary product and longer chain fatty acids are not produced. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

Enzyme systems which are responsible for the biosynthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA are known from a variety of organisms and tissues (Harwood, 1988; Wakil, 1989). The enzyme systems from yeast (Lynen, 1961) and animals (Bressler and Wakil, 1961; Butterworth et al., 1967; Burton, et al., 1968) are generally single multi-enzyme complexes which

are not dissociable into a free acyl carrier protein (ACP) and individual active enzymes. They are referred to as type I fatty acid synthases. The fatty acid synthase (FAS) from higher plants (Overath and Stumpf, 1964; Brooks and Stumpf, 1966; Harwood, 1996) and bacteria (Alberts et al., 1963; Goldman et al., 1963; Simoni et al., 1967; Wakil, 1989) are comprised of proteins that can be separated into free enzyme components and an ACP and are referred to as type II fatty acid synthases. Interestingly, Delo and co-workers reported that the phytoflagellate *Euglena gracilis* contains two distinct fatty acid synthases which are expressed in response to different growth conditions (Delo et al., 1971). When grown in

* Corresponding author. Tel.: +1-561-297-3356; fax: +1-561-297-2759.

E-mail address: rkerr@fau.edu (R.G. Kerr).

light on minimal medium, both type I and type II FASs are present while in etiolated cells of *E. gracilis*, only the type II FAS was found.

Bugula neritina, a marine bryozoan, is the source of a family of potent anti-tumor macrolide polyketides known as the bryostatins (Pettit et al., 1982). One of these, bryostatin 1 (Fig. 1), is currently in clinical development as an anti-leukemic and anti-melanoma agent (Stone, 1997). Due to the documented sequence homology between fatty acid synthases and polyketide synthases (PKS) (Smith, 1994), and the greater relative abundance of the former, we have elected to purify and characterize the fatty acid synthase of *B. neritina* as an initial entry into the enzymology of this bryozoan. PKSs from bacteria consist of either a modular type I (Katz, 1997; Cane et al., 1998), or an iterative type II system (Hutchinson, 1997). A type I PKS is somewhat analogous to a type I FAS in which the active sites present in each module determine which extender is utilized, as well as the level of reduction or dehydration for that particular cycle. The number of modules in the protein constituting the PKS determines the length of the polyketide chain. In type II PKS systems, separate enzymes are responsible for different activities and are used repeatedly in the biosynthesis of the polyketide. Type I systems typically generate macrolide polyketides while type II systems generally afford an aromatic product (O'Hagan, 1991). Fungal PKS systems are iterative type I PKSs and thus represent a third group (O'Hagan, 1991).

While there are a number of reports concern-

ing fatty acid synthases, to our knowledge, there are no reports discussing the FAS of any member of the phylum Bryozoa. In fact, this report represents the first enzymology study from any bryozoan. We have recently developed a viable cell-free extract that was used in a preliminary investigation of the biosynthetic origin of the bryostatins (Kerr et al., 1996) and have used this as a starting point in our FAS purification studies.

2. Experimental procedures

2.1. Materials

The following reagents: NADH, NADPH, malonyl-coenzyme A, bovine serum albumin (BSA), acyl carrier protein (ACP) from *Escherichia coli*, dithiothreitol (DTT), and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO), while [³H]acetyl-CoA and [³H]malonyl-CoA were purchased from Du Pont. The Sephadex G-200 was obtained from Sigma Chemical Co. and the DEAE-Cellulose was obtained from Bio-Rad Laboratories. Protein concentration was determined using the Bradford assay, and polyacrylamide gel electrophoresis in 7.5% acrylamide gel slabs under both denaturing and non-denaturing conditions and gels were stained using a silver stain (Bio-Rad).

2.2. Preparation of cell-free extract

Bugula neritina was collected from Palos Verde, California, immediately flash frozen and stored at -80°C . A typical cell-free extract was prepared by grinding 100 g frozen material with a chilled mortar and pestle, and adding the resulting fine powder to 200 ml buffer (100 mM, pH 7.8, 1 mM EDTA, 1 mM DTT, 1 μg /ml leupeptin and 1 μg /ml pepstatin A). The homogenate was centrifuged at $30\,000 \times g$ for 30 min and the resulting supernatant stored in 10-ml aliquots at -80°C .

2.3. Fatty acid synthase (FAS) assay

The FAS activity assay was conducted by incubating the following components: [³H]acetyl-CoA (1 μCi), malonyl-CoA (58 μM), NADH (70 μM), NADPH (60 μM) and BSA (0.1 mg/ml) with an enzyme preparation in a final volume of 5

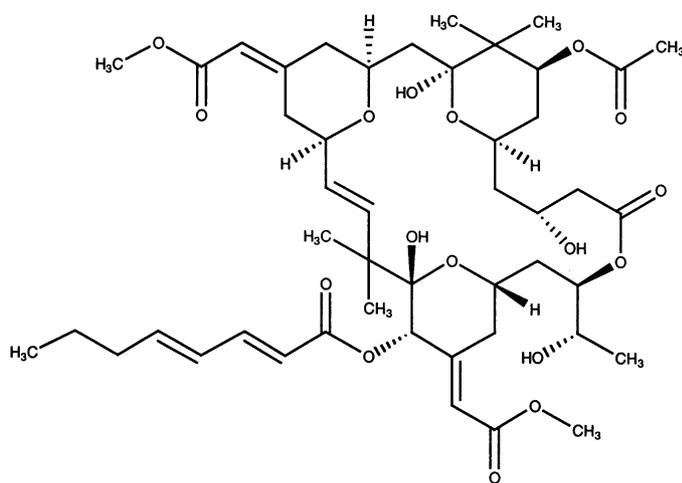


Fig. 1. Structure of bryostatin 1.

ml in phosphate buffer. The incubations were carried out at 30°C on an environmental shaker (Lab Line) at 200 rev./min for 15–20 h. Reactions were quenched by the addition of 200 μ l of 5 N NaOH and heating at 100°C for 30 min. Following acidification with 200 μ l conc. HCl, the fatty acids were extracted with hexanes, and the hexane layer divided into two equal aliquots and concentrated under N₂. The radioactivity of one aliquot was determined using a scintillation counter (LKB Wallace 1219 Rackbeta), and in cases which proved to be radioactive, the second aliquot was subjected to the following, more rigorous, assay: The hexane-extractable material was methylated using methanolic H₂SO₄ by dissolving in MeOH (1 ml), to which 0.3 ml conc. H₂SO₄ was added. The mixture was stirred at 60°C for 30 min, and after cooling, was extracted with hexanes. The fatty acid methyl esters were separated by HPLC using a RI detector (Waters Associates), and equipped with a reversed phase column (Altex Ultrasphere ODS, 1 \times 25 cm) and MeOH as eluent. Authentic samples of myristic, palmitic and stearic acid methyl esters were added as ‘cold carriers’. The radioactivity of the purified fatty acid methyl esters was determined using a scintillation counter.

2.4. Purification of the fatty acid synthase

Ammonium sulfate precipitation was achieved by adding (NH₄)₂SO₄ to the crude cell-free extract to generate a 23% solution. Following centrifugation at 2400 \times g (10 min), the supernatant was further precipitated by increasing (NH₄)₂SO₄ to 40%. Pellets from the two precipitates were collected and re-dissolved in phosphate buffer (100 mM, pH 7.8, 1 mM EDTA, 1 mM DTT), and used in the FAS assay and for further purification.

DEAE-Cellulose chromatography was performed with a 1 \times 30 cm column and a gradient program consisting of buffer A (Tris-HCl 100 mM, pH 7.8 with 1 mM EDTA) and buffer B (A with 1 M KCl) and was used as follows: 0.40 min 100% A, 40–180 min gradient from 100% A to 100% B, 180–200 min 100% B, 200–210 min 100% B to 100% A, 210 min–225 min 100% A, at a flow rate of 1 ml/min. Fractions were concentrated by ultra filtration (Bio-Max, MWCO 30K) and analyzed on native polyacrylamide gel electrophoresis and subjected to the FAS assay. Active fractions were combined and applied to a Sephadex G-200 column (100 \times 1.5 cm) which was developed with phosphate buffer (0.1 M, pH 7.8, 1 mM EDTA). Fractions were concentrated and analyzed as described above. Active fractions were combined and further purified on a Bio-Gel A column (50 \times 1.5 cm) and fractions subjected to the FAS assay and electrophoretic analysis (Table 1). The Bio-Gel column was calibrated with protein standards; thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B-12 (1.35 kDa).

3. Results

As we have previously reported (Kerr et al., 1996), a crude enzyme extract was prepared by grinding flash frozen *B. neritina* in a chilled mortar and pestle and adding the powder to a phosphate buffer. Centrifugation of the resulting homogenate removed insoluble debris. The supernatant was stored as a viable cell-free extract at –80°C. It was determined that incubation times of 6 h were required for the assay to demonstrate significant activity. Increasing buffer strength bey-

Table 1
Purification of the fatty acid synthase from *B. neritina*

Protein preparation	Protein (mg)	Specific Activity ^a (mU/mg \times 10 ⁻³)	Purification factor (-fold)
Cell-free extract	2532	1.8	1
23% (NH ₄) ₂ SO ₄ ppt.	1125	3.7	2
DEAE-Cellulose (pk 1)	98	46	26
Sephadex G-200 (pk 1)	20	120	67
Bio-Gel A	3	190	106

^a1 mU of FAS activity is defined as the amount of enzyme which catalyzes the incorporation of 1 nmol of [³H]acetyl-CoA into hexane-extractable material in 1 h.

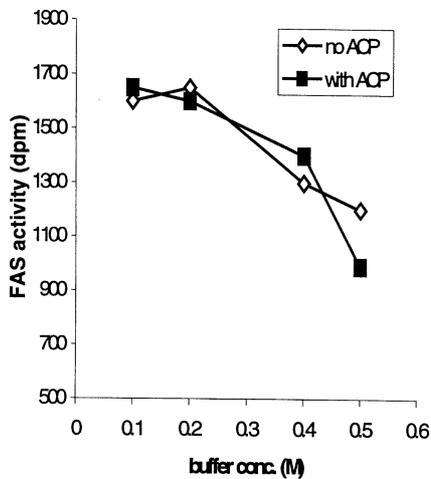


Fig. 2. Effect of changes in buffer concentration in the presence and absence of acyl carrier protein on fatty acid synthase activity.

and 0.2 M led to a decrease in FAS activity although no significant difference was noted from 0.1 to 0.2 M (Fig. 2).

The 23% ammonium sulfate precipitate contained the majority of the FAS activity and was further purified by DEAE-Cellulose ion exchange chromatography. Fractions with the highest synthase activities were pooled and concentrated by ultra-filtration. Subsequent elution on a Sephadex G-200 column gave two fractions with the FAS activity found to be associated with the first of two peaks. Native PAGE analysis of this G-200 fraction revealed the presence of two bands, and final purification was achieved with a Bio-Gel A column. The latter column was calibrated with molecular weight standards and the active protein found to have a molecular weight of 382 kDa. SDS-PAGE analysis of the protein indicated one band with a molecular weight of approximately 190 kDa.

The optimal pH (in phosphate buffer), for the purified protein in the assay described above was found to be 7.8 (Fig. 3). An examination of the cofactor and substrate requirements indicated that maximal FAS activity is found at 80 μ M NADH and 80 μ M NADPH. The data described in Fig. 4 indicate that changes in the concentration of NADPH has a larger effect on the FAS activity than do changes in the concentration of NADH. As described in Fig. 5, changes in the concentration of acetyl Co-A and of malonyl Co-A indicated that the FAS was more susceptible to

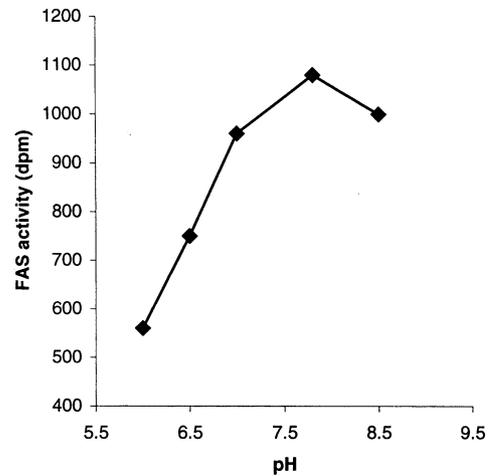


Fig. 3. pH optimum of fatty acid synthase activity.

fluctuations in the concentration of the former. The addition of ACP (10 μ M) to the ammonium sulfate precipitate did not result in an increase in activity.

To determine which fatty acids were produced by the purified FAS, a HPLC separation was performed on the fatty acid methyl esters following an incubation with 1.0 μ Ci [3 H]acetyl Co-A. The methyl esters of myristic, palmitic and stearic acids were added to the quenched incubation mixture as cold carriers. Subsequent purification of these standards revealed that the majority of the radioactivity was associated with

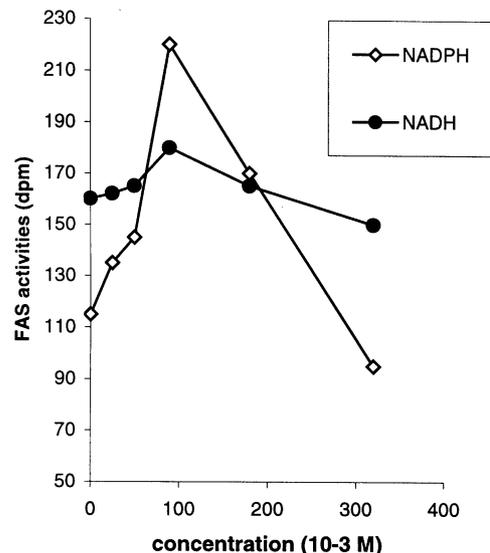


Fig. 4. Effect of changes in the concentration of NADPH and NADH on fatty acid synthase activity.

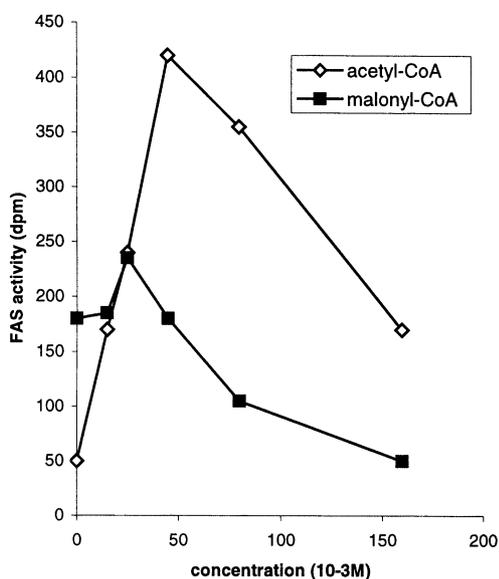


Fig. 5. Effect of changes in acetyl-CoA and malonyl-CoA concentrations on fatty acid synthase activity.

methyl palmitate (1150 dpm), with methyl myristate being less radioactive (870 dpm). No radioactivity was found in the methyl stearate fraction or fatty acids of higher molecular weight.

4. Discussion

There are a number of observations which suggest that the FAS from *Bugula neritina* belongs to the type I category. Firstly, it has previously been demonstrated that treatment of ACP with ammonium sulfate (23% or 40%) does not cause precipitation of this small protein (Sireag and Levine, 1972). ACP is required for type II FAS systems and we found that the FAS from *B. neritina* was insensitive to the concentration/presence of ACP. Secondly, changes in the concentration of acetyl Co-A and of malonyl Co-A indicated that the FAS was more susceptible to fluctuations in the concentration of the former. This is consistent with observations from other type I FAS systems (Goldman et al., 1963, Sonnenborn and Kunau, 1982). Furthermore the molecular weight of the purified protein is estimated to be 382 kDa from size exclusion chromatography. SDS-PAGE analysis of this protein under denaturing conditions showed one band of molecular weight of approximately 190 kDa, and thus the FAS of *B. neritina* appears to be a

homodimer of approximately 190 kDa subunits. This is to be expected for a type I FAS and is similar to the type I FAS purified from the dinoflagellate *Cryptocodinium cohnii* which was found to be a homodimer of 360 kDa (Sonnenborn and Kunau, 1982).

The data described in Fig. 4 indicate that the concentration of NADPH has a larger effect on the FAS activity of *B. neritina* than that of NADH which is consistent with observations from other FAS systems (Goldman et al., 1963). Thus, the fatty acid synthase from *Bugula neritina* is a type I FAS which produces palmitic acid as its primary product and has a requirement for NADPH.

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