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In vitro synthesis of arachidonoyl amino acids by cytochrome *c*

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ABSTRACT

Arachidonoyl amino acids are a class of endogenous lipid messengers that are expressed in the mammalian central nervous system and peripherally. While several of their prominent pharmacologic effects have been documented, the mechanism by which arachidonoyl amino acids are biosynthesized has not been defined. We have previously observed that the mitochondrial protein, cytochrome *c*, is capable of catalyzing the formation of the prototypic arachidonoyl amino acid, arachidonoyl glycine, utilizing arachidonoyl CoA and glycine as substrates, in the presence of hydrogen peroxide. Here we report that cytochrome *c* is similarly able to catalyze the formation of N-arachidonoyl serine, N-arachidonoyl alanine, and N-arachidonoyl gamma aminobutyric acid from arachidonoyl CoA and the respective amino acids. The identities of the arachidonoyl amino acid products were verified by mass spectral fragmentation pattern analysis. The synthetic reactions exhibited Michaelis–Menten kinetics and continued favorably at physiologic temperature and pH. Spectral data indicate that both cytochrome *c* protein structure and a +3 heme iron oxidation state are required for the reaction mechanism to proceed optimally. Reactions designed to catalyze the formation of N-arachidonoyl dopamine were not efficient due to the rapid oxidation of dopamine substrate by hydrogen peroxide, consuming both reactants. Finally, under standard assay conditions, arachidonoyl CoA and ethanolamine were found to react spontaneously to form anandamide, independent of cytochrome *c* and hydrogen peroxide. Accordingly, it was not possible to demonstrate a potential role for cytochrome *c* in the biosynthetic mechanism for either arachidonoyl dopamine or anandamide. However, the ability of cytochrome *c* to effectively catalyze the formation of N-arachidonoyl serine, N-arachidonoyl alanine, and N-arachidonoyl gamma aminobutyric acid *in vitro* highlights its potential role for the generation of these lipid messengers *in vivo*.

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1. Introduction

The prototypic arachidonoyl amino acid, N-arachidonoyl glycine (NAGly), was first isolated from bovine brain and subsequently found to be expressed in a variety of rat tissues [1]. Other arachidonoyl amino acids including N-arachidonoyl gamma aminobutyric acid (NAGABA), N-arachidonoyl alanine (NAAla), and N-arachidonoyl serine (NASer) have similarly been identified endogenously [1,2]. Although several of these compounds elicit cannabinoid-like behavioral responses in rodents, none are known to interact with established cannabinoid or vanilloid receptors [1–5]. Accordingly, it has been proposed that their effects are mediated through actions as competitive inhibitors of the enzyme fatty acid amide hydrolase (FAAH), which is responsible for the catabolism of anandamide [3,6]. Additionally, physiological actions of arachidonoyl amino acids may also be mediated independently through novel cell surface receptors. In this regard, Kohno et al. have recently reported evidence for the existence of an orphan G protein-coupled receptor in lymphoid cells that interacts with NAGly [7].

Despite the widespread natural occurrence of NAGly and other long chain fatty acyl amino acids in brain and other tissues, the mechanism of their biosynthesis remains undefined. Recently, our laboratory discovered that cytochrome *c* is capable of catalyzing the formation of oleoyl- and N-arachidonoyl glycine from their respective coenzyme A derivatives and glycine in the presence of hydrogen peroxide [8,9]. These findings prompted us to test whether or not cytochrome *c* is able to utilize other amino acids as substrates for the formation of additional naturally occurring N-arachidonoyl amino acids. Here we report on the catalytic properties of cytochrome *c* in the formation of NAGABA, NAAla, and NASer. Under the same conditions, however, the synthesis of N-arachidonoyl dopamine (NADA) by cytochrome *c* could not be demonstrated due to the rapid oxidation of dopamine by H_2O_2 and consequential consumption of both molecules. Efforts to demonstrate synthesis of anandamide by cytochrome *c* revealed that arachidonoyl CoA and ethanolamine react spontaneously to generate anandamide. The present finding that cytochrome *c* cat-

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alyzes the formation of NAGABA, NAAla, and NASer *in vitro* points to the possibility that cytochrome *c* mediates their biosynthesis *in vivo*.

2. Materials and methods

2.1. Reagents and biochemicals

[1-^{14}C]Arachidonoyl CoA (53 mCi/mmol) was purchased from Moravek Biochemicals, Brea, CA. NAGly, NAGABA, NAAla, NASer, and NADA were purchased from Cayman Chemical, Ann Arbor, MI. Bovine cytochrome *c*, H_2O_2, glycine, arachidonoyl CoA and other laboratory reagents were purchased from Sigma–Aldrich, St. Louis, MO.

2.2. Assay for arachidonoyl amino acid synthesis and thin layer chromatography (TLC)

Reactions for synthesis of N-arachidonoyl amino acids were performed at 37 °C (10 min) in 100 μL 50 mM Tris, pH 7.4, containing the desired amounts of cytochrome *c*, H_2O_2, [^{14}C]arachidonoyl CoA, potassium ferricyanide, sodium ascorbate and selected amino acid, dopamine, or ethanolamine. Reactions were terminated by the addition of 5 μL of 1 N HCl and extracted with three volumes of ethyl acetate. The organic layer was collected, dried under nitrogen, dissolved in 30 μL of ethyl acetate and spotted to heat-desiccated (45 min at 95 °C) Silica Gel G Uniplates™ (Analtech, Newark, DE). TLC plates were developed (70 min) using chloroform/methanol/glacial acetic acid, 90:10:1. Data were visualized using a FLA-5100 phosphorimager (FUJIFILM, Stamford, CT), and quantitative image analysis was performed with Multi Gauge V3.0 software (FUJIFILM). Kinetic analyses were performed using the EnzFitter program (Elsevier Biosoft, Cambridge, UK).

2.3. Heat and acid inactivation of cytochrome c enzymatic activity

Cytochrome *c* (5 mg in H_2O) was incubated at 100 °C for 3 h in the presence or absence of 6N HCl. Reactions were terminated by placing samples on ice. The acidified cytochrome *c* was adjusted to pH 7 with 6N NaOH and diluted to 5 mg/mL with H_2O. Treated and untreated samples were analyzed for enzymatic activity in the synthesis of NAGABA by incubating cytochrome *c* (500 ng) at 37 °C in the presence of 5 μM [^{14}C]arachidonoyl CoA, 150 mM GABA, and 1 mM H_2O_2 for 10 min. Reactions were terminated by the addition of 5 μL of 1 N HCl, extracted in three volumes of ethyl acetate and analyzed by thin layer chromatography as described above.

2.4. Spectrophotometric analysis of cytochrome c oxidation state

Cytochrome *c* (5 mg/mL) was incubated at 37 °C in the presence of 100 mM potassium ferricyanide or sodium ascorbate. Analysis of cytochrome *c* oxidation state was performed using a Evolution 300 UV–Vis spectrophotometer (Thermo Scientific, Waltham, MA). Samples were diluted 1:100 in 50 mM Tris pH 7.4 and scanned from 350 to 750 nm in a 1 cm path-length quartz cuvette.

2.5. Statistical analysis

Data were analyzed using analyses of variance (ANOVA) and Tukey testing. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using InStat software (GraphPad, La Jolla, CA).

2.6. Reverse phase HPLC

Separations were carried out at 45 °C on a 4.6 mm × 250 mm Luna 5 μ C_5 column (Phenomenex, Torrance, CA) using a Series 1100 HPLC (Agilent Technologies, Wilmington, DE) operated at a flow rate of 1 mL/min. The column was equilibrated with 50% solvent A (5% acetonitrile in water; 0.005% trifluoroacetic acid) and 50% solvent B (5% water in acetonitrile; 0.005% trifluoroacetic acid). Samples and standards were dissolved in 50 μL ethanol for injection. Following sample injection, initial conditions were maintained for 5 min followed by a linear gradient to 100% B over 10 min. The system was maintained at 100% B for 10 min and returned to initial conditions over 5 min. Elution profiles were monitored at 205 nm. Fractions were manually collected and dried by lyophilization.

2.7. Mass spectrometry

Mass fragmentation analysis was performed by electrospray ionization (ESI) mass spectrometry (MS) using a Q-Star®XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA). Arachidonoyl amino acid standards and lyophilized HPLC samples were first dissolved in 100% ethanol, then adjusted to 50% acetonitrile/water containing 0.1% formic acid and infused into the ionization source (5500 V) at a rate of 10 μL per minute. Fragmentation was performed at a collision energy which preserved a small amount of parent ion. Parent ion masses were confirmed for each sample by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS using a Voyager™ MALDI-TOF DE STR instrument (Applied Biosystems, Framingham, MA) operated in positive ion reflectron mode using gentisic acid matrix.

3. Results

3.1. Verification of arachidonoyl gamma aminobutyric acid synthesis by cytochrome c

Fig. 1 presents chromatographic and mass fragmentation analyses confirming the synthesis of NAGABA by cytochrome *c*. Panel A shows a TLC image demonstrating the co-migration of NAGABA product generated by the cytochrome *c* with NAGABA standard. The HPLC chromatogram represented in Panel B shows the purification and separation of NAGABA synthesized by cytochrome *c*. The retention time of the cytochrome *c*-generated NAGABA product corresponded precisely with that of NAGABA standard. Arachidonic acid (AA) was also present as a reaction byproduct. The NAGABA peak was collected, concentrated and subjected to ESI-MS/MS fragmentation analysis. The resulting spectrum is depicted in Panel C. Significant ions include NAGABA (*m*/*z* 390.58), the arachidonoyl aliphatic chain (*m*/*z* 287.47), GABA (*m*/*z* 104.15), and GABA minus H_2O (*m*/*z* 86.13). The fragmentation data for NAGABA synthesized by cytochrome *c* precisely matched the fragmentation spectra of NAGABA standard (not shown). None of these reaction products were generated in the absence of cytochrome *c*.

3.2. Optimum temperature and pH for the formation of arachidonoyl gamma aminobutyric acid by cytochrome c

Fig. 2 demonstrates that the formation of NAGABA is both temperature and pH dependent. Panel A shows the maximum conversion of arachidonoyl CoA to NAGABA under standard assay conditions from 4 to 80 °C. The optimum temperature for cytochrome *c* catalyzed synthesis of NAGABA was approximately 40 °C. Optimum pH (shown in Panel B) was determined by performing reactions in either 50 mM MES or Tris buffer, pH 4.5–9.5. The