Studies into Phosphopantothenoyl Cysteine Synthetase

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BACKGROUND INFORMATION

The Biosynthesis of CoA from Pantothenic Acid

A crucial process for the survival of organisms is the biosynthesis of coenzyme A (CoA) from pantothenic acid (vitamin B5). CoA functions to help the fatty acid metabolism and tricarboxylic acid. Some bacteria, plants, and yeast use *de novo* CoA biosynthesis from the pantothenic acid. Humans consume vitamin B5 from other organisms in order to complete the CoA biosynthesis. *E. coli* is generally the tested bacteria for CoA biosynthesis because there is more understanding about this particular bacteria.

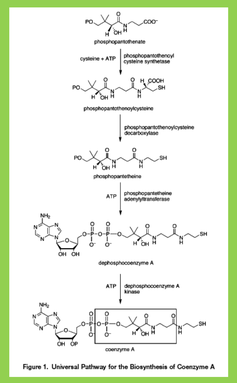


Figure 1. Universal Pathway for the Biosynthesis of Coenzyme A

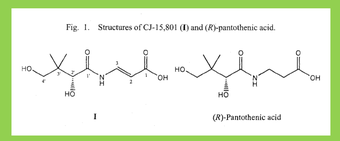
The Five Step Process

The biosynthesis of CoA takes place in five steps. Pantothenate is phosphorylated by phosphopantethenate kinase (PanK), the first step. This then reacts with cysteine and ATP by phosphopantothenoyl cysteine synthetase (PPCS) to produce phosphopantothenoyl cysteine. The third step goes from phosphopantothenoylcysteine to phosphopantetheine. This then reacts with ATP by phoshopantetheine adenylyltransferase (PPAT) to form dephospho-CoA. The last stage, dephosphocoenzyme A kinase (DPCK), uses ATP to form coenzyme A.

In our project, we are focusing on the second step in this pathway, PPCS: PPate + ATP + cysteine → PPC + AMP + iPP. ATP activates the acyladenylate intermediate in the PPCS reaction which then cysteine is added to form AMP.

Antibiotic Activity

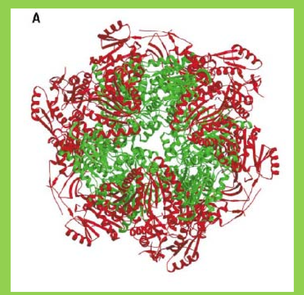
CJ – 15,801, a new antibiotic, is isolated from the fungus, Seimatosporium, which is found in the soil, possesses cytotoxic activity. Looking at the diagram on the right, the pantothenic acid and the CJ – 15,801 have similar structures, but the antibiotic has a double bond at C2 while the pantothenic acid has a single bond. Our hypothesis is that CJ – 15,801 inhibits phosphopantothenoylcysteine synthetase, the second step in CoA biosynthesis, to give it cytotoxic activity. The acyladenylate intermediate is highly reactive and it forms a ketene which will inhibit the biosynthesis. Our goal is to use crystallize the protein after purification in order to understand the structure of the protein with bound inhibitor.



FUTURE WORK

Crystallization and Protein Structure

Once the proteins are purified, crystals can be created over a period of twelve days. Once the crystals are grown to there maximum capacity, the X-Ray data collection and processing are done in order to determine the wavelength, cell dimension, resolution, and more. After doing several imaging tests, the protein structure can be determined. The density of the object was studied closely in order to get an improved version of the structure. Once getting a basic formation of this protein structure, a computer may be used to enhance the clarity of the image. The proposed structure of PPCS is shown at the right. It has three proteins attached together in symmetry.



Testing the Antibiotic, CJ – 15,801

Once the protein is purified, the antibiotic can be tested to see if it inhibits the second step in the biosynthesis of CoA. When the reaction, PPate + ATP + cystine → PPC + AMP + iPP, occurs Mo-malachite is added into the reaction. Mo-malachite is a green dye that shows the continuation of the biosynthesis of CoA. The greener the dye, the more the reaction has progressed. A graph can be made showing the number of reactions occurring. In parallel, another reaction can be inhibited with the antibiotic, CJ- 15,801, which should stop the biosynthesis. Once the Mo-malachite is added, this reaction is hypothesized to stay a light green color. On the graph, it should have a horizontal line, but a normal biosynthesis of CoA should be gradually increasing. After completing this experiment, it could be concluded that CJ – 15801 works as an antibiotic inhibiting the biosynthesis of CoA which is a vital process in organisms.

REFRENCES

1. Structure of Human Phosphopantothenoylcysteine Synthetase at 2.3 Å Resolution Narayanan Manoj, Erick Strauss, Tadhg P Begley, Steven E Ealick Structure - 1 August 2003 (Vol. 11, Issue 8, pp. 927-936)
2. CJ-15,801, a Novel Antibiotic from a Fungus, Seimatosporium sp. Yutaka Sugie*, Koen A. Dekker, Hideo Hirai, Toshio Ichiba, Masaru Ishiguro, Yukio Shiomi, Akemi Sugiura, Lori Brennan1", Joan Duignan1", Liang Hsiung Huang, Joyce Sutcliffe and Yasuhiro Kojima THE JOURNAL OF ANTIBIOTICS VOL. 54 NO. 12, DEC. 2001 pp.1060 - 1065

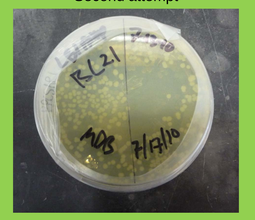
PROCEDURES AND DATA

First attempt

DNA Preparation

The plasmid was used to prepare DNA for B834. The bacteria was grown twice, using E. Coli, to amplify the amount of the plasmid. The plasmid had ampicillin resistance; therefore, all of the E. Coli died on the ampicillin plates while those containing the DNA for B834 survived. When doing the experiment, I had to prepare the plasmid twice because no bacteria colonies grew on the plate overnight. In order to correct this mistake, I redid the experiment, and amplified the bacteria using a miniprep. A miniprep is a small amount of purified sample of plasmid DNA; a series of purification steps are then taken to isolate the plasmid DNA from the rest of the cellular content.

Second attempt



Collecting Cells

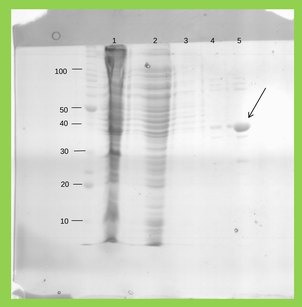
The starter culture, LB, and ampicillin was incubated until an OD600 ~ 0.6. The optical density needs to be at 0.6 because the cells need to be caught in logarithmic phase instead of stationary phase. If the OD is below 0.6 there will not be enough cells therefore less protein. Isopropyl – B – D – thiogalactoside (IPTG) is mixed into the culture in order to stop the growth of the cells. I made 4 liters of cells because after I centrifuged it the first time there wasn't enough cells.

Protein Purification

A polypropylene column filled with Ni-NTA resin (Novagen) and wash buffers were used to purify the protein. Ni-NTA resin caught the protein and discarded the impurities while rinsed with a wash buffer of 20 mM imidazole. After the column was washed with an increased concentration of imidazole, 200 mM, the purified protein was no longer attached to the Ni-NTA resin. Imidazole purifies the protein, but the higher concentration washes the protein through the resin.

Dialysis

Dialysis is the process of separating molecules with a semi permeable membrane by using a dialysis tube. In the experiment, imidazole needs to be removed from the protein.

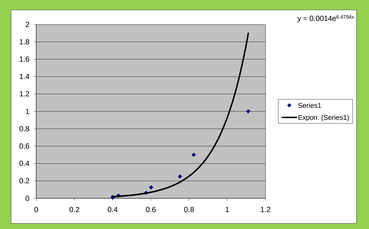


SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, separates proteins based on their molecular weight and function of length of the polypeptide chain. The marker on the far left indicates the kDa of the sample. The kDa of the protein is around 34-kDa, but in the photo it is around 37-kDa.The picture indicates the purified protein with the lightest mark because it contain the other impurities that was previously in the protein.
Symbols on Image: 1- crude cells, 2- flow through, 3- wash with 20 mM Imidazole, 4- wash with 60 mM imidazole, 5- wash with 200 mM imidazole, and ↙ - purified protein

Tobacco etch virus (TEV) protease

Tobacco etch virus (TEV) protease was used to cleave the 6-histidine tag from the protein. TEV protease was put in the dialysis tube with the protein; overnight the TEV protease should have dispersed into the buffer solution. It was then purified using a polypropylene column filled with Ni-NTA resin; the resin caught all of the leftover TEV protease and the cleaved histidine tags.



Bradford Assay

A bradford assay is a spectroscopic analytical procedure to determine the concentration of the protein. To test the protein, a sample of it has to be placed in wells where the concentration is half of the well before. Bradford reagent is also put into the wells which dyes it blue. The more blue the higher the concentration of protein. A standard protein, BSA, is also used in order to create the curve and equation shown at the left. This equation is then able to calculate the concentration of the protein by looking at the absorbance of the sample protein.

1.1104	0.8246	0.7529	0.6019	0.5747	0.4302	0.4001	0.4015
1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.007813
Column Wash							
0.7743	0.6424	0.6126	0.5062				
Cleaved CoaB							
0.8214	0.7076	0.7273	0.5741				

0.138939
0.277877 Concentration of Cleaved CoaB
0.28 mg/mL
0.214307 Concentration of Wash
0.21 mg/ mL