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13. ABSTRACT (Maximum 200 words)

Initial studies focused on the purification of the hydrogenase from Methanococcus jannaschii. The hydrogenase was characterized, N-terminal sequenced, and its amino acid content was compared to other hydrogenases.

Researchers then constructed a library of genomic DNA from Methanococcus jannaschii. The genomic library, with over 1 million independent representatives, was cloned into bacteriophage lambda. The DNA from the organism has been found to be methylated, preventing classic enzyme treatments for gene library synthesis. A gene library has been synthesized by mechanical shearing, and the fragment size is approximately 8-15 kb.

Another driving force for the production of the library was the independent discovery of a remarkably thermostable protease which retains activity at 135 °C. The protease was partially purified. The partially purified sample was used for the studies to determine which class of protease it may be.

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**INVESTIGATION OF PRESSURE REGULATION IN AN
ARCHAEBACTERIAL ENZYME**

FINAL REPORT

ALAN J. RUSSELL

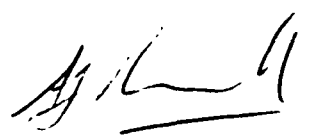
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Introduction / Statement of Problem

As an introduction to this Final Report we include below the Abstract from the initial proposal:

We propose to isolate a library of genomic DNA from the archaeobacter Methanococcus jannaschii. The organism is an extreme thermo- and barophile, its natural habitat being in the vicinity of a deep-sea hydrothermal vent at a pressure of 260 atm. (Methanococcus jannaschii is the most thermophilic organism from which DNA has been isolated with a laboratory growth optimum of 92 °C). The DNA from this extraordinary micro-organism will be cloned into Escherichia coli using standard molecular biology procedures. The genomic library will be screened for the gene encoding a hydrogenase enzyme. Expression of the hydrogenase gene, using an approach which has already proved successful in cloning methanogenic bacterial genes, will enable not only the investigation of the processes controlling extreme thermostability and barophilicity, but also the production of a potentially valuable commercial enzyme. Using the approaches of site-directed and random mutagenesis a family of similar enzymes with differing responses to temperature and pressure will be generated. This series of closely related mutant enzymes will be far easier to compare than a set of related hydrogenases isolated from meso- and thermophiles. Since the enzyme is extremely thermostable, it should be possible to delineate some of the structure-function relationships which give rise to marked stability. Of particular interest is the response of the hydrogenase to increased pressure (especially since there could be a physiological role for such a response in deep sea organisms). The pressure regulation of this enzyme will be investigated in detail. Some enzymes are activated or inactivated by large pressure changes, but, to our knowledge, this hydrogenase is the first enzyme which triples activity over a relatively small change in pressure from 7.5 to 260 atm. The kinetic results of the proposed research will be related to the structure of the enzyme. In addition, enough enzyme will be purified to enable an early attempt at crystallization.

Summary of Results

Our initial studies focused on the purification of the hydrogenase from *Methanococcus jannaschii*. Forty liter of cells were grown at the University of Georgia, and the cell mass was used to optimize a purification procedure for the enzyme. In general we have found that the proteins from this organism agglutinate, without precipitation, under a wide variety of conditions. This phenomenon can only be relaxed by increased temperature or the presence of mild detergents. The final purification protocol, which was developed in collaboration with Professor D. Clark, is given in the attached documentation. The hydrogenase was characterized, N-terminal sequenced, and its amino acid content was compared to other hydrogenases. This comparison indicated that the protein was very homologous to existing hydrogenases. In addition, we have been able to purify and sequence the first 25 amino acids of the methyl-coenzyme C reductase from *Methanococcus jannaschii*.

We then constructed a library of genomic DNA from *Methanococcus jannaschii*. The genomic library, with over 1 million independent representatives, was cloned into bacteriophage lambda. The DNA from the organism has been found to be methylated, preventing classic enzyme treatments for gene library synthesis. A gene library has been synthesized by mechanical shearing, and the fragment size is approximately 8-15 kb.

Another driving force for the production of the library was the independent discovery, in Professor Clark's and our laboratories, of a remarkably thermostable protease which retains activity at 135 °C. In addition, this protease is both activated and stabilized by elevated pressure, as discussed below. To our knowledge, this is the most thermophilic protease,

and the most barophilic enzyme, yet discovered. The purification of this protease has been developed in both laboratories, and while Professor Clark's group has focused on characterizing the protein, Professor Russell has focused on sequencing the protease in order to enable cloning, expression, and further investigation. This protease is expressed at very low levels ($\sim 5\mu\text{g/L}$), which makes purification extremely challenging. A nitrocellulose blot of the most pure sample isolated to date is attached to this report.

The protease was partially purified using the following procedure. The cell paste was homogenized in a French Press at 2,000 psi. It was then clarified by centrifugation at 10,000 x g for 20 minutes. The protease precipitated in a 40% to 60% ammonium sulfate cut. The resuspended pellet was resuspended, and loaded on to a gel filtration column. This step served mainly to desalt the sample, but did provide some separation. The combined active fractions were then loaded on to a DEAE anion exchange column. The column was run with a pH gradient from 10-8, and a salt gradient from 40 to 80% 1M NaCl.

The partially purified sample was used for the studies to determine in which class of protease may be. The samples were incubated for one hour at 90 °C with the different inhibitors. The volume of the sample was 700 ul. 10 ul of each of the inhibitors was used; 100 mM PMSF in DMSO, .1 mg/ml pepstain in DMSO, 100 mM 1,10 phenanthroline in DMSO, 10 mM E-64 in water. 100 ul of 200 mM EDTA in water were also used. An 11% drop in the rate was seen in the sample with 1,10 phenanthroline, and a 9% drop in the rate was seen with EDTA. The rest of the samples had the same rate as the sample without an inhibitor. This information may suggest a metal binding protein. N-terminal sequencing of the protease appears to indicate homology with chymotrypsin.

Our continued efforts to clone the protease are driven by its remarkable properties as a catalyst:

Exceptional thermophilicity ($T_{min}=65\text{ }^{\circ}\text{C}$, $T_{opt}=115\text{ }^{\circ}\text{C}$, $T_{max}=135\text{ }^{\circ}\text{C}$)

Pressure dependent activity and stability

Non-linear Arrhenius plot

Flexibility (as measured by EPR) insensitive to temperature below T_{min} .

Relatively low molecular weight (29,000)

*Unusually high apparent turnover numbers in amidase reactions ($k_{cat} \approx 300$
/ sec)*

Publications:

- *Rozeiwski, K. and Russell, A.J. (1992) *Biotechnol. Bioengin.* 37, 1171-1175. Effect of hydration on the morphology of enzyme powders.**
- *Rozeiwski, K. (1990) Purification of thermostable enzymes from archaeobacterium. Masters Thesis, University of Pittsburgh.**

Participating Personnel

Russell, A.J.

Rozeiwski, K. (M.S.)

Vierheller, C.Z.

Appendix 1: Purification protocol for hydrogenase

The cells were lysed in a french press at 20000 psi (3 passes). The crude extract was run through a DEAE Sepharose CL 6B column with a radius of 2.5 cm and a length of 10 cm. The 1 gram sample was loaded at a flow rate of 5 ml/min and eluted from column at a flow rate of 10 ml/min. A constant temperature of 4 °C was maintained, and the column was equilibrated with a 50mM Bis-TRIS pH 6.5 buffer. A linear gradient from 0 to 1 M NaCl was used. Each fraction was tested for activity in an anaerobic glove box with benzyl viologen. The fractions containing the active hydrogenase changed color within 15 minutes. The first active peak on the chromatograph contained the F420 (+), and the second active peak was the F420(-). The active fractions for F420(+) were combined and precipitated with 70 % ammonium sulfate. The F420(-) fractions were combined and precipitated with 70% ammonium sulfate. The samples were run through a Sephacryl S-300 at room temperature (D=1.5 cm, L=70 cm) to remove the salt and change the buffer to 34 mM Asp-TRIS pH 7.2. The fractions were tested with benzyl viologen in the glove box for activity. The active fractions were combined and concentrated in an ultrafiltration cell using a 10YM3 Amicon membrane. 10 mg of each sample, in 2.5% glycerol-25µM FAD, were loaded onto a 3.0 mm thick 5% native polyacrylamide gel with 3.1% stacking gel. The gel was run at 30mA for 13 hours at 4 °C. A one inch thick strip was cut from the edge of the gel and stained in the glove box with methyl viologen. A permanent stain, still visible in aerobic environment, was obtained by treatment with triphenyl tetrazolium chloride. This gel strip was used as a guide to excise the active bands from the gel. The bands were diced and placed in six electroelution chambers. The hydrogenase was electroeluted into a TRIS-

glycine buffer (14.4 g glycine, 3.0 g TRIS to 1.0 liter) at 10mA/cell for three hours. The buffer was then changed to include 1% SDS and electroelution was continued for three more hours. These samples are not active due to denaturing by the SDS. The samples were concentrated in ultracentrifugation filters to approximately half their original volume. The samples were loaded on to a 12% polyacrylamide gel and run at 30mA for 5 hours. The gel was then silver stained to determine the sample purity. The samples that were not eluted in SDS were too dilute to see in the gel. The F420(-) sample was the most concentrated sample and we estimated 80% purity.