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ACADEMIC RANK and DEPARTMENT: Professor, Department of Chemistry

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EDUCATION and EXPERIENCE:

B.A. in Chemistry (1966) St. Olaf College, Northfield, Minnesota
Ph.D. in Biochemistry (1971) Purdue University, West Lafayette, Indiana
NIH Postdoctoral Fellow (1971-73) Cornell University, Ithaca, NY
Sabbatical Leave (1995) Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware

TITLE of PROPOSED WORK:

Elucidation of Amino Acids at the XMP Binding Site of E. coli GMP Synthetase

ABSTRACT:

The recently-determined crystal structure of GMP synthetase from E. coli provides important information about active and substrate binding sites located on each of the two structural domains of this key biosynthetic enzyme. The crystal structure, however, does not define the binding site for the XMP substrate/GMP product. This proposal requests preliminary funding to cover the first two years of a multiyear project to use nucleotide-based affinity labels to determine amino acid residues at the XMP binding site of E. coli GMP synthetase. Elucidating the active site topography of the E. coli enzyme will assist in understanding the homologous human GMP synthetase, a target enzyme for chemotherapeutic and immunosuppressive therapies.

PUBLICATIONS:


REQUESTED MATCHING FUNDS from INSTITUTION $11,500
REQUESTED from RESEARCH CORPORATION $38,470

This proposal seeks funding for an entirely new project that arose out of my sabbatical experience at the University of Delaware. The sabbatical research, which gave me the expertise necessary to carry out the project proposed here, was supported by Professor R. F. Colman at Delaware, by a $15,000 ROA summer grant from NSF, and by the sabbatical salary granted by Lebanon Valley College. This proposal is currently my only request for support of the GMP synthetase project, but I plan to submit proposals to NSF-RUI and NIH-AREA in 1997 or 1998.

I have only one other ongoing research project: a project involving cyclic voltammetric studies of the solvent dependence of the reduction of substituted benzo- and naphthoquinones (see list of current publications). This project has been supported by Lebanon Valley College, and also by an interdisciplinary (biology/chemistry) undergraduate research grant from AAAS/Merck (this three-year grant has provided $45,000/yr to support three joint projects in the chemistry and biology departments at Lebanon Valley College). The quinone project will be continued by a single student working during the academic semesters only.

BUDGET RATIONALE (Include the source of other items [equipment, supplies] which are needed for this research beyond those requested from Research Corporation)

This budget requests faculty and student stipends for two ten-week summer research sessions, as well as Lebanon Valley College’s portion of FICA and Workman’s Compensation payments for the student and faculty stipends. Modest support for equipment and supplies is requested to help offset institutional costs for chemicals, biochemicals, chromatographic media and columns, bacterial growth media, micropipets and other miscellaneous laboratory supplies. No major equipment is requested.

Lebanon Valley College will supply other equipment that is needed for this project: high-speed refrigerated centrifuges, cold room, autoclave, incubators, fraction collectors, gradient HPLC system, rotary evaporator, lyophilizer, diode-array spectrophotometers, multinuclear 250 MHz FT-NMR and FT-IR/Raman spectrometers to characterize synthesized affinity labels, a liquid scintillation counter for work with radioactive affinity labels (later years of project), and computer hardware and software required for the molecular modeling of proteins.
PLAN of PROCEDURE and SIGNIFICANCE of RESEARCH (Experimental plan of what is to be done; the significance of the problem, originality of the approach, and feasibility of the method must be clearly stated. Point out innovative features, explain significance and the relationship to previous work, and include pertinent literature references)

Introduction and Background Information

Guanosine-5'-monophosphate (GMP) synthetase (EC 6.3.5.2) catalyzes the glutamine-dependent synthesis of guanosine monophosphate (GMP) from xanthosine monophosphate (XMP) and adenosine triphosphate (ATP):

\[
\text{XMP} + \text{ATP} + \text{glutamine} \quad \underset{\text{Mg}^{2+}}{\rightarrow} \quad \text{GMP} + \text{AMP} + \text{PP}_i + \text{glutamate}
\]

This reaction completes the final step in the de novo biosynthesis of guanine nucleotides in both procaryotic and eucaryotic cells. Cellular production of guanine nucleotides provides essential precursors for the synthesis of RNA and DNA, and also supplies GTP for essential cellular processes such as protein biosynthesis, microtubule assembly, synthesis of adenosine nucleotides, and activation of G proteins (1). Because of its critical role in purine nucleotide biosynthesis, GMP synthetase is found at elevated levels in malignant and other rapidly proliferating cells, making it an important target enzyme for anticancer and immunosuppressive therapies (2). Two current research groups, a crystallography group led by Janet Smith at Purdue University (3,4) and a research team directed by Lillian Lou at Syntex Discovery Research (1,2,5,6), have made significant breakthroughs that promise to energize efforts to elucidate the molecular structure, active site topography, and catalytic mechanism of GMP synthetase.

Crystal Structure of E. coli Enzyme. The Purdue group has recently determined at 2.2 Å resolution the crystal structure of the GMP synthetase from E. coli, a structure that reveals important features of the procaryotic enzyme (4). The crystal structure confirms earlier conclusions (8), that the enzyme exists in two distinct structural and catalytic domains: a glutamine amidotransferase (or GAT) domain, and a synthetase (or ATP pyrophosphatase) domain. The GAT domain transfers ammonia from the amide group of glutamine to the synthetase domain, where it adds to an activated \( O^2- \)-adenyl-XMP intermediate that is produced on that domain by a reaction between XMP and MgATP (7). The crystal structure indicates the presence of a catalytic triad of Cys\(^{86}\), His\(^{181}\), and Glu\(^{183}\) at the active site of the GAT domain (4). In the ATP pyrophosphatase domain, the crystal structure reveals a specific hydrophobic binding pocket for the adenine ring of ATP, and a signature nucleotide-binding motif, or P-loop, for the binding of the \( \beta-\gamma \) pyrophosphoryl moiety of ATP (4).

Human GMP Synthetase. The group from Syntex has succeeded in purifying, cloning and expressing human GMP synthetase (1,2). Sequence comparisons indicate a high degree of homology (41%) between the human and E. coli enzymes (1). The Syntex group has used affinity labeling to elucidate an active site residue, cys\(^{104}\), a conserved residue that is homologous with cys\(^{86}\) of the catalytic triad of the E. coli enzyme (5). Although the human (M = 76,725; 693 amino acids) and E. coli (M = 58,604; 525 amino acids) enzymes differ in size, the high degree of sequence homology indicates that structural and functional aspects of the bacterial enzyme are likely to apply to the human enzyme.

XMP Binding Site. One important structural aspect of E. coli GMP synthetase that is not revealed by the crystal structure is the nature of the binding site for XMP on the synthetase domain, a site that would also be expected to bind the product GMP that results from amination of XMP. The Purdue group noted that they crystallized GMP synthetase in the presence of ATP and XMP, but that neither nucleotide was seen in the crystal structure. Only AMP and PP\(_i\) were identified suggesting that ATP and XMP had reacted to form PP\(_i\) and the \( O^2- \)-adenyl-XMP intermediate, which subsequently hydrolyzed to form AMP and XMP (4). The authors note that a phosphate residue seen in the crystal structure at the C-terminal end of the molecule is probably the 5'-phosphate of XMP, but that the rest of the XMP molecule, as well as a specific 22 amino acid portion of the ATP pyrophosphatase domain that is also missing from the crystal structure, are disordered and impossible to model (4). The authors further suggest that the 22-residue disordered peptide may be directly affected by XMP binding since the nucleotide protects the enzyme against trypsin proteolysis at cleavage sites within the 22-residue segment. The crystal structure, therefore, is unable to define the nature of the XMP binding site.
Goal and Overview of Proposed Research

The goal of this research project is to elucidate amino acid residues at the XMP binding site of the GMP synthetase from E. coli through the use of nucleotide-based affinity labels. For this enzyme system, in which the crystal structure is not sufficiently detailed to define the nature of a substrate binding site, the technique of affinity labeling can provide valuable auxiliary information about that binding site. A summary flow chart of the entire project, showing the main experimental stages, is given below. Given the time framework inherent in carrying out undergraduate research, this project will have an estimated duration of four to five years. This proposal seeks support only for the first two years of the project, as shown in red in the flowchart. Subsequent proposals to NSF-RUI and NIH-ROA will solicit support for the remaining years of the project.

Summary Flowchart

Experimental Details

Enzyme Purification. Purification of the E. coli GMP synthetase has been routinely carried out in a number of laboratories, and two standard methods have been published in Methods in Enzymology (9,10). We will grow E. coli strain 396 (ATCC No. 13473), a purine auxotroph which can be grown under conditions that allow derepression of GMP synthetase (10). The purification process involves cell disruption, treatment with streptomycin sulfate, ammonium sulfate precipitation, and three chromatographic steps: DEAE cellulose; DEAE Sepharose, and GPC using BioGel A (10). Enzyme activity will be assayed spectrophotometrically by following the production of GMP at 290 nm (10). Protein will be determined by the method of Bradford (11). Two students working under my supervision will carry out the enzyme purification during the first summer of research. We will freeze the purified enzyme in aliquots until needed in subsequent summers.
**Synthesis of Affinity Labels.** We will synthesize four potential nucleotide-based affinity labels for the XMP/GMP binding site of GMP synthetase. The first two reagents were originally developed by Roberta Colman at the University of Delaware (12,13): guanosine 5′-O-[S-(3-bromo-2-oxopropyl)] thiophosphate (GMPSBOP); and guanosine 5′-O-[S-(4-bromo-2,3-dioxobutyl)] thiophosphate (GMPSBDB). These GMP analogs react with enzyme nucleophiles through displacement of proline; they can also form hemiacetals or thiohemiacetals with alcohols and thiols; and in the case of GMPSBDB, the reagent can react further with arginine residues to produce a cyclic adduct (14). The corresponding bromo-2-oxopropyl and 2,3-dioxobutyl derivatives of xanthosine 5′-O-thiophosphate, XMPSBOP and XMPSBDB, have not been previously reported. We will attempt the synthesis of these xanthosine analogs using procedures for GMPSBOP and GMPSBDB (12,13). Synthetic products will be characterized by UV-vis, 1H-NMR, and 31P-NMR (13). I gained important first-hand experience in the preparation of nucleotide-based affinity labels in Professor Colman’s laboratory in 1995 as I synthesized 3MPSBOP and GMPSBDB, and helped to direct an undergraduate student in the synthesis of the corresponding IMP-based analogs. It is possible that the reagents described above will prove not to be effective as inactivators of E. coli GMP synthetase. In such a case we will synthesize and test other potential affinity labels such as 6-((4-bromo-2,3-dioxobutyl)thio)-6-deaminoadenosine 5′-monophosphate and 5′-p-fluorosulphonylbenzoyl guanosine (15). I will work closely with one student to carry out these syntheses during the first summer of research, with work possibly continuing into the second summer.

**Inactivation Kinetics and Protection Studies.** Kinetic studies of the E. coli enzyme indicate an ordered mechanism in which XMP binds to the enzyme after MgATP binding (7), although more recent evidence with the human enzyme is not consistent with that order (6). The situation is further complicated by the fact that the enzyme-MgATP-XMP complex reacts to form the O2–adenyl-XMP intermediate, which slowly hydrolyzes in the absence of glutamine to produce enzyme-bound XMP, AMP, and PPi. Piscofururanine, an inhibitor of the enzyme, acts by preventing further reaction of the O2–adenyl-XMP intermediate with glutamine, ammonia, or water (7). Another enzyme inhibitor, 2-iodo-ATP, is postulated to bind with an incorrect orientation of the adenine ring, preventing the reaction with XMP to form the O2–adenyl-XMP intermediate (3). It is our goal to find a set of conditions under which affinity analogs for the XMP/GMP site will bind with high affinity to the active site of the synthetase domain and react to cause inactivation. We will try the following sets of preincubation conditions: (i) affinity label and enzyme alone; (ii) affinity label, Mg2+, and enzyme; (iii) affinity label, Mg2+, AMP, and PPi; and enzyme; (iv) affinity label, Mg2+, AMP, PPi, and enzyme; (v) affinity label, 2-iodo-ATP, Mg2+, and enzyme; and (vi) affinity label, picrofururanine, ATP, Mg2+, and enzyme. Using the enzyme assay described earlier, we will carry out kinetic studies to determine which affinity labels inactivate the enzyme. Pseudo first order rate constants for inactivation will be measured over a range of reagent concentrations. A nonlinear dependence of the rate constant on the reagent concentration (i.e., saturation behavior) will indicate that an affinity label forms a complex with the enzyme prior to the labeling reaction. Analysis of this concentration dependence will yield a dissociation constant for the enzyme-affinity label complex.

We will also study the effects of substrates and products of GMP synthetase on the rate constants for inactivation by the modifying reagents. If a modifying reagent does indeed bind at the enzyme active site, active site ligands such as the GMP product or the XMP substrate are expected to compete with the reagents and “protect” the enzyme by inhibiting the rate of the inactivation reaction. I will work with two to three students on the inactivation kinetics and protection studies during the second summer of research. Once we have identified one or more reagents that behave as affinity labels, I will seek additional grants from NSF-RUI and NIH-AREA to support the remaining portions of the project: radioactively labeling active site residues; determining labeling stoichiometry; identifying the labeled residues by protein digestion, peptide separation, and peptide sequencing; and modeling the labeled protein.

**Principal Investigator and Facilities.** My experience with undergraduate research spans 23 years, during which time 34 different students working with me on four major research projects have been co-authors on 10 scientific publications and 29 presentations at scientific meetings. I have developed a sound basis for organizing and planning undergraduate research, and for judging what can be reasonably accomplished in the summer-session time frame of such a project. Lebanon Valley College has the instrumentation needed to complete most of the first four years of this project (see page 2 for a listing of relevant equipment). We are missing only the instrumentation for peptide sequencing needed during the fourth and fifth years of the project. I will either request funds from the planned NSF and NIH grants to pay for a peptide sequencing service at the core facility of the nearby Hershey Medical Center, or I will establish a collaborative arrangement to that will include peptide sequencing.
Literature Cited


Possible Reviewers

- Dr. Roberta F. Colman, Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716. E-mail: rfcolson@udel.edu (Mentor, 1995 Sabbatical Research and Possible Future Collaborator)
- Dr. Gordon G. Hammes, Duke University Medical Center, P. O. Box 3701, Durham, NC 27710. E-mail: hammes001@duke.edu (Mentor, 1971-73 Postdoctoral Research)
- Dr. Larry G. Butler, Department of Biochemistry, Purdue University, West Lafayette, IN 47907. E-mail: larry.g.butler.1@purdue.edu (Ph.D. Advisor and Mentor, 1981 Postdoctoral Research)
- Dr. Herbert J. Fromm, Department of Biochemistry and Biophysics, Iowa State University, 4110 Molecular Biology Building, Ames, IA 50011. E-mail: hjfromm@aestate.edu (Collaborator with R. F. Colman and myself on a recent publication in Biochemistry, but not personally known to me)

The following individuals are not personally known to me:

- Dr. Janet L. Smith, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.
- Dr. Lillian Lou, Syntex Research, 3401 Hillview Ave., S3-1, Palo Alto, CA 94304.
- Dr. Boyd E. Haley, Medicinal Chemistry, College of Pharmacy, University of Kentucky Medical Center, Lexington, KY 40536-0082. E-mail: behaley@pop.uky.edu
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