Lysine Acetylation and Moonlighting Functions of IMP Dehydrogenase CBS Domain

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By

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Abstract:

The Bateman domain of IMP dehydrogenase (IMPDH), a rate-limiting enzyme within the guanine nucleotide synthesis pathway, is a subdomain crucial in the regulatory moonlighting functions of the enzyme. In *Escherichia coli* during the stationary phase of growth, the IMPDH Bateman domain is readily acetylated on certain lysine amino acids, a modification that has no described effect on the enzyme or cellular metabolism. We report that acetylation of site K203 of the Bateman subdomain interferes with RNA polymerase interactions of IMPDH. *In vivo* deletion of the Bateman domain (guaBΔCBS) was found to have many of the same metabolic defects in the adenylate nucleotide biosynthesis pathway, caused by growth arrest by adenosine and inosine. We conclude that acetylation of the Bateman subdomain is involved in secondary functions of IMPDH such as protein pathway regulation and protein-protein interaction.

Introduction:

Inosine 5’-monophosphate dehydrogenase (IMPDH) is an essential protein in the guanine nucleotide biosynthetic pathway. IMPDH catalyzes the formation of XMP from IMP through NAD-dependent oxidation. This is the first and rate-limiting step in biosynthesis of guanine nucleotides. \[5\][6][7] Inhibition of IMPDH decreases the cellular guanine pools, which prevents cellular proliferation. Due to the importance of IMPDH in supplying guanine nucleotides, it is a target for antibacterial, anticancer, immuno-suppressive, and anti-viral research. \[8\]
Besides its catalytic role, IMPDH has other moonlighting functions in cellular metabolism. Human IMPDH associates with polyribosomes.\textsuperscript{[14]} In yeast, IMPDH is known to associate with proteins involved in transcription, translation, and as well as with proteins that process and splice RNA.\textsuperscript{[11][4]} While in \textit{Escherichia coli}, IMPDH regulates adenylate synthesis.\textsuperscript{[8]} There are also suggestions that IMPDH is linked with the function of tumor suppressor P53.\textsuperscript{[6]}

While there are many roles of IMDPH, not all are well understood. We especially lack understanding in how these functions are regulated within the cell. As protein acetylation is known to play a crucial regulatory role in eukaryote and prokaryote metabolism\textsuperscript{[12]}, the reversible modification of certain amino acids in the protein is a possible mechanism by which IMPDH controls functions such as catalytic conversion of IMP to XMP and other secondary protein functions know as moonlighting functions.

IMPDH is a homotetramer. Each subunit contains two distinct domains. One is the $(\beta/\alpha)$ barrel catalytic domain, which is responsible for enzymatic activity. The second is a subdomain consisting of a pair of repeats called the cystathionine $\beta$-synthetase (CBS) domain and is located at the center of the protein sequence. This subdomain is also known as the Bateman domain. The tetramer has square planar geometry with the catalytic domains forming a square. The CBS domains extend out from the corners of the tetramer. (Fig. 1)\textsuperscript{[2][8][9]} This subdomain structure is conserved in many known IMPDH sequences, including those of \textit{E. coli} and human. The CBS domains are also found in many other unrelated enzymes. There are a variety of physiological roles connected with the subdomain, and that relationship can vary based the organism. There is no all-
encompassing physiology role described for Bateman domain.\textsuperscript{[3]} As a result, the role of the IMPDH CBS domain is of particular interest for study.

Many secondary roles of IMPDH involve the CBS domain. In human IMPDH1 CBS domain is the portion that associates with polyribosomes. This interaction is potentially important, as mutations in the CBS domain of hIMPDH1 are associated with the disease autosomal dominant retinitis pigmentosa.\textsuperscript{[14]} In \textit{E. coli} IMPDH, it has been theorized that IMPDH has an unexplained role in RNA metabolism, and it is possible that the CBS domain could be the factor in that process.\textsuperscript{[8]} The CBS domain is also the site of \textit{E. coli} IMPDH adenylate synthesis regulation.\textsuperscript{[9]} The role of the CBS domain is independent of the main catalytic functions of IMPDH. All reports indicate that the conversion of IMP to XMP is not affected by the deletion of the Bateman domain.\textsuperscript{[2][8][9]}

One known function of the CBS domain of IMPDH is the regulation of purine biosynthesis pathway in \textit{E. coli}. As \textit{E. coli} has only one gene (guaB) that encodes for IMPDH, it has been shown to be an easy model for CBS domain research.\textsuperscript{[9]} Pimkim et. Al. produced the \textit{E. coli} mutant variation (\textit{guaB}\textsuperscript{ΔCBS}). The \textit{guaB}\textsuperscript{ΔCBS} mutant had its CBS subdomain deleted and replaced with a “scar”.\textsuperscript{(Fig.2)} This was done by first replacing the gene sequence encoding CBS with a kanamycin resistant gene. The addition of the \textit{kan}\textsuperscript{R} caused the cell to be auxotrophic for guanine. After selecting for the \textit{kan}\textsuperscript{R} mutant, a second round recombination was performed by the deletion of the \textit{kan}\textsuperscript{R} and the introduction of a twenty-four amino acid “scar”.\textsuperscript{[8][9]}
Both guaB$^+$ wild type and guaB$^{\Delta CBS}$ cell cultures grew at similar rates on rich media, suggesting that, as expected, the main catalytic functions of IMPDH were retained even with the lack of a subdomain. However, it was found that the deletion of the CBS portion of the guaB gene is replaced with a 24 amino acid “scar” in guaB$^{\Delta CBS}$. Side view of IMPDH structure with emphasis on separation of domain and location of CBS subdomain. 

Fig. 2: A) CBS portion of the guaB gene is replaced with a 24 amino acid “scar” in guaB$^{\Delta CBS}$. B) Side view of IMPDH structure with emphasis on separation of domain and location of CBS subdomain.
CBS domain caused major disregulation of the purine nucleotide pools, enzyme activities, ATP, and GTP levels. In minimal media, there was a 1.8-fold increase of ATP in guaB\textsuperscript{ACBS}. AMPs synthetase pool levels also decrease by 2-fold in minimal media. When inosine was added to the media, a 4-fold increase in GTP was observed. ATP and GTP are products of the purine nucleotide pathway and thus measurements of their levels are methods utilized to observe the effects of guaB\textsuperscript{ACBS} on the pathway itself. (Fig. 3) [8] These results suggested to Pimkin et al that the CBS domain had, thus far, an important but yet unexplained role in the regulation of purine nucleotide pathway.

One particular finding in the study was the activity levels of proteins in the purine nucleotide biosynthesis pathway. They found that IMPDH and AMPs synthetase activity levels in the mutant were significantly lower than that of the wild type. [8] The decreased IMPDH activity levels correlated with a decreased total IMDPH concentration found in cell extract probed with anti-IMPDH antibodies. The antibodies also adhered to proteins of lower molecular weight in the same extracts, likely to be proteolytic fragments. [8] This suggests that guaB\textsuperscript{ACBS} mutants were being actively degraded \textit{in vivo} instead of having catalytic defects. Unexpected results were found when AMPs synthetase activity levels were compared to the ATP pool level increase. AMPs synthetase converts IMP into AMPs (Fig. 3), a precursor to ATP. [9] It was expected that the lower activity levels of AMPs synthetase would correlate with lower levels of ATP. Instead, the mutant had higher levels of ATP pools than even that of the wild type, which had higher levels of protein activity than the mutant. Pimkim et al believed that this observation suggested that the deletion of the CBS domain might be having an effect on more specifically the adenylate biosynthesis pathway. [8][9]
Pimkin et. al. observed that the deletion of CBS domain in the *E. coli* sensitizes the bacteria to growth arrest by adenosine. $\text{GuaB}^{\Delta CBS}$ was unable to grow in the presence of adenosine whether or not there was an alternate source of nitrogen.\(^9\) Thus, adenosine toxicity was believed to be preventing cellular growth.

Adenosine has two options in this salvage pathway, it can either be cleaved into adenine or it can be converted into inosine.\(^{\text{Fig. 3}}\) The $\text{deoB}$ gene encodes for purine nucleoside phosphorylase, which catalyzes the reversible conversion of adenosine to

![Figure 3. The de novo and salvage pathways for purine nucleotide biosynthesis. Dashed lines indicate negative regulation. Dash/dotted lines indicate reversible reactions. The highlighted path shows the pathway of adenosine/inosine toxicity. The beginning of the pathway begins with adenosine. IMP is shown to be an intermediate of the adenylate synthesis pathway. Cause of pyrimidine starvation can be traced to AMP and ADP inhibition of the production of PRPP.][7]
adenine. By deleting the \textit{deoB} gene, the cell is forced to convert adenosine into inosine. When grown in adenosine-rich media, Pimkim et. al. found that guaB\textsuperscript{ACBS} mutants without the \textit{deoB} gene (guaB\textsuperscript{ACBS} \textit{deoB}) experienced growth arrest, while guaB\textsuperscript{+} mutants without the \textit{deoB} (guaB\textsuperscript{+} \textit{deoB}) were unperturbed in growth. In contrast, when the \textit{add} gene, which encoded for adenosine deaminase, an enzyme that converts adenosine to inosine, was knocked out for guaB\textsuperscript{+} and guaB\textsuperscript{ACBS}, both mutants retained resistance towards adenosine. The same results were observed for \textit{gsk} gene knock out, which converts inosine to IMP. These results collectively suggest that growth arrest only occurs through the adenosine to IMP pathway. \cite{9}

When guaB\textsuperscript{ACBS} \textit{deoB} was grown in inosine, there was 4-fold increase in ATP compared to that of guaB\textsuperscript{+} \textit{deoB}. The observation was consistent with the idea that the CBS domain regulates the salvage pathway. The absence of the CBS domain caused changes in the level of measurable products in the system. In guaB\textsuperscript{ACBS} \textit{deoB}, pyrimidine nucleotides levels were 3.5 times lower than in guaB\textsuperscript{+} \textit{deoB} when grown on inosine-rich media. \cite{8} This suggested that pyrimidine starvation was the ultimate cause of cellular growth arrest.

ADP and AMP inhibit PRPP synthetase, which produces PRPP, a precursor to pyrimidine nucleotides. \cite{10} Pimkim et al constructed the guaB\textsuperscript{ACBS} \textit{deoB} \textit{prsA1} mutant, which included a PRPP synthetase gene (\textit{prsA1}) resistant to ADP and AMP inhibition. When guaB\textsuperscript{ACBS} \textit{deoB} \textit{prsA1} mutant and the guaB\textsuperscript{ACBS} \textit{deoB} were both grown on inosine, it was found guaB\textsuperscript{ACBS} \textit{deoB} \textit{prsA1} was insensitive to inosine unlike guaB\textsuperscript{ACBS} \textit{deoB}. These results showed that the deletion of the CBS domain has caused an increase in ADP and AMP levels, which in turn resulted in depletion of pyrimidine nucleotides via
the inhibition of PRPP synthetase. These observations led to the conclusion that the CBS domain downregulates the conversion of IMP to AMP in order to prevent such a starvation.

When the genes encoding for proteins AMPs synthetase and AMPs lyase, \textit{purA} and \textit{purB} respectively, were deleted from both \textit{guaB^{ACBS} deoD} and \textit{guaB^{+} deoD}, researchers found both \textit{E. coli} strains to be insensitive to inosine as well as dependent on outside sources of adenine for survival. Since AMPs synthetase and AMPs lyase convert IMP to AMP, this confirmed Pimkim et al’s suspicion that the deregulation of IMP to AMP conversion was the cause of mass increase in AMP and ADP levels in \textit{guaB^{ACBS}}. AMPs synthetase is the first enzyme in the IMP to AMP process, thus it was likely the target of IMPDH mediated regulation. In inosine-rich media, they found that the activity of AMPs synthetase doubled in \textit{guaB^{ACBS} deoD} compared to that of \textit{guaB^{ACBS} deoD} in minimal media. In \textit{guaB^{+} deoD}; the increase in inosine rich media was only 20%. In \textit{guaB^{ACBS} deoD}, the influx of inosine led to increase quantities of substrates produced for AMPs synthetase, leading to increased activity and eventual growth arrest. However, in the “wild-type”, the increase of substrate was countered with the suppression of AMPs synthetase activity by the presence of the Bateman domain.

Pimkim et al. proposed a model by which the EcIMPDH CBS subdomain is a negative regulator of AMPs synthetase. However, it is unknown precisely how IMPDH controls the regulation of the pathway. The exact nature of this regulation is still unclear.

A potentially important lysine acetylation site (K203) was found on the CBS domain of \textit{E. coli} IMPDH. Lysine acetylation of proteins can often be critical in regulating fundamental cellular processes such as transcription, translation, pathways
associated with central metabolism, subcellular localization, and mediated protein-protein interaction.\textsuperscript{[12]} The acetylation of the K203 amino acid site in \textit{E. coli} occurs only during the stationary phase of cell growth.\textsuperscript{[14]} The same site is deacetylated during the exponential growth phase of cell growth. As the stationary phase limits cell growth and reproduction\textsuperscript{[15]}, it is theorized that reversible lysine acetylation on the CBS domain controls IMPDH’s metabolic and regulatory functions.

Acetylation and deacetylation are usually post-translational modifications and mediated by acetylase and deacetylase respectively\textsuperscript{[7]}. However, \textit{in vitro} post-translational modification methods for acetylation are insufficient for producing homogenous products. The reported yield for successful acetylation at desired sites rarely exceed 30\%.\textsuperscript{[10]} Neumann et al developed a novel method to insert acetylated lysine during translation and produce large quantities of homogenous acetylated protein.\textsuperscript{[7]}

Amber codon (UAG) is a three-nucleotide sequence within messenger RNA that signals translational termination for proteins. Certain methanogenic bacteria, such as \textit{Methanosarcina barkeri}, incorporate pyrrolysine in response to an amber stop codon.

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\textbf{Fig. 1.} Amino acid substrate and ATP first binds to aminoacyl-tRNA. The enzyme next joins amino acid to AMP by cleaving off two phosphate. The appropriate tRNA then binds to the enzyme, and the synthetase mediates the attachment of the amino acid to the tRNA. AMP is released in the process. \textsuperscript{[17]}
Pyrrolysyl-tRNA synthetase (MbPylRS), a type of aminoacyl-tRNA synthetase (Fig. 1), mediates the binding of a pyrrolysine onto the amber codon suppressor MbtRNA_{CUA}. MbtRNA_{CUA} recognizes the amber codon sequence on mRNA during translation and inserts the pyrolysine onto the polypeptide chain instead of terminating translation. [7]

Neumann et al created a library of mutant MbPylRS/MbtRNA_{CUA} pairs and isolated a pair (acetyl-lysyl-tRNA synthetase (AckRS)/tRNA_{CUA}), which successfully incorporated N^ε-acetyllysine instead of pyrrolysine into a polypeptide. The resulting pair was found to have high fidelity and efficiency when expressed in *E. coli*. [7] With this method of genetically encoding acetylated protein, we would be able to produce large quantities of homogenously acetylated IMPDH protein for protein regulation research.

With the ability to produce acetylated *E. coli* IMPDH, we asked the question of what role acetylation had in the regulation of IMPDH functions such as protein-protein interactions, the conversion of IMP to XMP, and purine biosynthesis pathway regulation. The regulatory role of IMPDH may allow the cell to survive in potentially toxicity environments. How roles such as those are controlled is unknown. Due to the heavily acetylated nature of IMPDH CBS domain during stationary phase in contrast to that of the exponential phase, we theorize that control over certain moonlighting functions of IMPDH is dependent on the acetylation of the subdomain, specifically that of site K203.

We employed cell growth measurements and pull down assays to identify the effects of acetylation. Via comparison between acetylated mutants, *guaB_4CBS* mutant, and wild type, we were able to conclude that acetylation does affect interactions IMPDH has with RNA polymerase and the regulation the adenylate biosynthesis pathway.
Experimental Procedure:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF-1</td>
<td>AGCCACCCGCAGTTCGAAAAGATGCTACGTATCGCTAAAGAAGGCTC</td>
</tr>
<tr>
<td>EGF-2</td>
<td>TTTTGTTCAGGACCCTGGAGGCCACCCGCAGTTCGAAAAGATGCTACGT</td>
</tr>
<tr>
<td>EGF-3</td>
<td>CATCACACAGCCAGGATCCGTTGGAAGTTTTGTTTCAAGGACCGTGAG</td>
</tr>
<tr>
<td>EGF-4</td>
<td>GATATACCATGGGCAGCCATCACCATCATCACACAGCCAGGATCG</td>
</tr>
<tr>
<td>EGR-1</td>
<td>TGGCGAGGCAGGCTCGAATTCAGGAGCGCCAGACGGTATT</td>
</tr>
<tr>
<td>EGR-2</td>
<td>TATATCAGCGCCGCAAGCTTTCGACCTGCAGGCAGGAGCGCTCGAATT</td>
</tr>
<tr>
<td>EGK-1</td>
<td>CTGCTGGTGATGATCTCTACAGGCGACCTTTATAGGC</td>
</tr>
</tbody>
</table>

Fig. 3. Primers sequences used. Produced via IDTDNA.

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Parent</th>
<th>Source</th>
<th>Resistance</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCDF-GuaBHS</td>
<td>pCDFDuet-1</td>
<td>Novagen</td>
<td>Spectinomycin</td>
<td>wt IMPDH with N-terminus His-Strep tag</td>
</tr>
<tr>
<td>pCDF-AC203HS</td>
<td>pCDFDuet-1</td>
<td>Novagen</td>
<td>Spectinomycin and Kanamycin</td>
<td>Ack203 IMPDH with N-terminus His-Strep tag</td>
</tr>
<tr>
<td>pBAckRS</td>
<td>pBAcKRS</td>
<td>[6]</td>
<td>Kanamycin</td>
<td>acetyl-lysyl-tRNA synthetase and tRNA&lt;sub&gt;CUA&lt;/sub&gt;</td>
</tr>
<tr>
<td>pETΔGuaBHS</td>
<td>pET-His6-StrepII-AMP</td>
<td>Addgene</td>
<td>Ampicillin</td>
<td>gua&lt;sub&gt;B&lt;/sub&gt;ΔCBS IMPDH with His-Strep tag</td>
</tr>
<tr>
<td>pETBK203QHS</td>
<td>pET-His6-StrepII-KAN</td>
<td>Addgene</td>
<td>Kanamycin</td>
<td>K203Q IMPDH with N-terminus His-Strep tag</td>
</tr>
</tbody>
</table>

Fig. 4 Plasmids information including type of IMPDH it codes for.
Strains and Plasmids:

The antibiotic concentrations used throughout follows: kanamycin, 50 µg/ml; spectinomycin, 20 µg/ml; ampicilin, 50 µg/ml. Cell procedures were carried out at 37°C and 25°C. LB media supplemented with appropriate antibiotic and minimal media supplemented with 0.5% glucose were used as growth media and plates.

Using wild type *E. coli* IMPDH *guaB* genomic DNA, a His-Strep-3C tag was introduced to the sequence and amplified via standard PCR techniques using forward and reverse primers pairs in consecutive sequences: EGF-1/EGR-1, EGF-2/EGR-1, EGF-3/EGR-1. EGF-4/EGR-2. The process is conducted using vector pCDFDuet-1. The resulting pCDFGuaBHS is inserted into BL-21 *E. coli* cells that overexpresses for IMPDH. The resulting purified protein used as wild type IMPDH in these experiments. pETΔGuaBHS and pETK203QHS were plasmid constructs obtained from previous experiments in Hedstrom laboratory. pETΔGuaBHS codes for *E. coli* IMPDH sans CBS domain but also contains a His-Strep tag. pETK203QHS codes for *E. coli* IMPDH with a His-Strep tag and lysine 203 replaced with a glutamine. (Fig. 4, 5)

Using procedure described by Neumann et al.,[7] The amber codon sequence of pCDF-AC203HS is introduced to the pCDFFGuaBHS sequence with forward and reverse primers EGK-1 and EGR-2 via standard PCR procedures. The resulting pCDF-AC203HS plasmid was inserted into electrocompetent *wt* BL-21 ΔGuaB *E. coli* cells along with pBAcK plasmid, giving the new acetylated *AcK203 E. coli* strain both kanamycin and spectinomycin resistance. The new strain now overexpresses *AcK203* IMPDH. Colonies containing both plasmids were selected for on LB medium plates supplemented with both antibiotics. Single colonies of *E. coli* containing pCDF-AC203HS/pBAcKRS were
selected and shaken in 500 mL of LB with kanamycin and spectinomycin at 37°C. At absorbance OD\textsubscript{600} measurement, 0.25 mM IPTG is added to induce growth. For pCDF-AC203HS, before induction, 50mM of Nicotinamide (NAM, a decacetylase inhibitor) and 10 mM of N-Acetyl-L-Lysine were filtered and added. After 20 minutes, 0.25mM IPTG was added to induce growth during exponential phase. The mix was shaken for 3 hours at 25°C. The cells were then centrifuged at 6000 g and harvested in PBS NAM. The resulting pellets are frozen at –80°C for storage. \cite{7}

The same insertion in BL-21 cells is done for pET\textDeltaGuaBHS (Ampilicin resistant), pETK203QHS (kanamycin resistant), and pCDFGuaBHS (spectinomycin resistant), creating strains respectively: \textit{guaB}\textsuperscript{ACBS}, \textit{K203Q}, wild-type (wt). Only IPTG induction is conducted at absorbance OD\textsubscript{600} measured at 0.5, no nicotinimide or N-acetyl-L-lysine is added.

Cell pellets of \textit{AcK203} are sonicated 6 times for 45-second durations in 20mL PBS NAM (50 mM KH\textsubscript{2}PO\textsubscript{4}, 500mM KCl, 1mM TCEP, 5mM Imidazole, 20mM Nicotinimide, pH 8) supplemented with appropriate protease inhibitor. The resulting cell lysate collected after centrifuge at 6000 g was run through a 5mL Ni-NTA His-tag column. Elution was conducted with phosphate buffer saline (50 mM KH\textsubscript{2}PO\textsubscript{4}, 500mM KCl, 1mM TCEP, 5mM Imidazole, pH 8). Imidazole concentration was increased incrementally starting from 5mM, 30mM, 100mM, 150mM, to 200mM. Absorbance/fluorescence assay of 200mM imidazole elution fractions with substrates were conducted to determine presence of protein in elute (1µL of elute fraction, 1mM IMP, NAD\textsuperscript{+}, and DTT). Changes in absorbance and fluorescence are detected when IMPDH enzymatic activity. The resulting positive activity slope is recorded used as
presence of IMPDH activity. Fractions with high protein enzyme activity levels compared to that of lysate were collected. After concentration of protein, dialysis was performed with buffer containing 20mM HEPES, 100mM KCl, 1mM TCEP, pH 8 with 3 buffer changes. The third iteration replaced TCEP with 1mM DTT.

Concentration of *E. coli* AcK203 IMPDH protein was found via Bradford assay. Protein kinetics via spectrometer was performed to determine Michaelis-Menton kinetic values. Using antibodies that recognize acetylated lysine, western blot was conducted to determine presence of acetylation on IMPDH.

**Protein Purification Under Low Salt Concentration:** *EclIMPDH wt* along with *K203Q, AcK203*, and *guaB^{ACBS}* mutants were grown in LB liquid media with appropriate antibiotic (5mL starter culture in 250mL) and allow to culture until absorbance OD$_{600}$ of 0.5 (~2 hours for *wt*, ~4 hours for *K203Q, AcK203*, and *guaB^{ACBS}*). 50mM Nicotinamide, 10mM N-acetyl-L-lysine, and 0.25mM IPTG are then added in order to *AcK203* cells. Only IPTG is added for *K203Q, guaB^{ACBS}* and *wt*. All cell cultures were sonicated with lysate run through Ni-NTA columns under the low salt concentrations, with phosphate buffer using sodium as opposed to potassium. PBS Low Salt contained 100mM NaCl, 150mM KH$_2$PO$_4$, 5mM Imidazole, 10% glycerol, and 1mM TCEP. Elution was again conducted with with PBS Low Salt with imidazole concentrations respectively: 5mM, 30mM, 100mM, 150mM, and 200mM. Presence of protein was confirmed via spectrometer was performed to determine Michaelis-Menton kinetic values

**Anti-IMPDH and Anti-RNA Polymerase Western Blot Analysis:** Antibodies specific for His-strep tag on IMPDH using was used to ascertain presence of IMPDH in blot. Antibodies specific for *E. coli* RNA polymerase are employed to detect the presence
of the respective protein. Antibodies are introduced via standard western blot procedure using TBS-Tween (0.1%). Secondary mouse or rabbit antibody are then introduced for detection. 20µg of protein was used as the standard for each sample.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Anti-Strep tag</th>
<th>Anti-RNA polymerase</th>
<th>Mouse</th>
<th>Rabbit</th>
<th>Anti-AcLysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Primary</td>
<td>Secondary</td>
<td></td>
<td></td>
<td>Primary</td>
</tr>
<tr>
<td>Detects</td>
<td>E. coli IMPDH</td>
<td>E. coli RNA polymerase</td>
<td>Anti- RNA polymerase</td>
<td>Anti-Strep/Anti-AcLysine</td>
<td>Acetylated lysine</td>
</tr>
<tr>
<td>Source</td>
<td>GenScript</td>
<td>GenScript</td>
<td>Abcam</td>
<td>Abcam</td>
<td>GenScript</td>
</tr>
</tbody>
</table>

Fig. 6. Antibodies used in western blots. Primary antibodies detect proteins with E. coli. Mouse secondary antibody is visible at channel 800 while rabbit secondary are scanned at channel 700 for visibility. Standard BioRad protein ladder is used and visible at 700.

**Cell Growth in Minimal Media:** M9 minimal media supplemented with 0.5% glucose and 50µM IPTG were used for cell density observation. Appropriate concentrations of nicotinamide and N-Acetyl-L-Lysine are added for AcK203 BL21 E. coli cells. Plates for spotting use M9 mixed with hot agar.
Results:

*E. coli IMPDH Interacts with RNA polymerase:* In recent experiments conducted in Hedstrom laboratory, when His-Strep tag *E. coli* wt IMPDH was pulled down at low salt concentrations, it was observed that an RNA polymerase protein came out of the assay along with IMPDH. Subsequent western blotting with RNA polymerase antibody confirmed that RNA polymerase was the protein in question. It was believed that we had found a previously not described protein with which *E. coli* IMPDH interacts. Under standard purification salt concentrations, RNA polymerase was not observed in *wt* IMPDH. (Fig. 8)

As all previously characterized moonlighting functions of IMPDH involved the CBS subdomain, it is possible that IMPDH also regulates RNA polymerase via the subdomain, likely through direct protein-protein interactions.

*IMPDH interaction with RNA polymerase is associated with Acetylation of site K203:* Although western blotting reviewed the presence of Acetylated IMPDH in the resulting AcK203 protein solution, it is unclear whether the target lysine at site 203 is acetylated as opposed to other unrelated sites. Comparisons of Michaelis-Menton kinetic values with *wild type* and K203Q show that AcK203 has a turnover rate four times lower than that of the wild type, which was comparable to that of K203Q, a mutant that mimics the acetylated lysine on site 203. The glutamine has been shown to be an effective substitute for acetylated lysine functions in proteins due to similarity of charge and chemical structure. [14] This and the similar inhibition constant for NAD suggest that substantial acetylation occurred in AcK203. The differing turnover rate of acetylated IMPDH with that of *wt* suggest that in contrast to previous statements on the CBS
domain, modification of the subdomain does affect the main catalytic function of IMPDH. (Fig. 7)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild Type</th>
<th>AcK203</th>
<th>K203Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{cat}$ ($s^{-1}$)</td>
<td>14 ± 2</td>
<td>3.25 ± 0.7</td>
<td>3.59 ± 0.9</td>
</tr>
<tr>
<td>$K_m$ IMP (µM)</td>
<td>60 ± 5</td>
<td>61 ± 1.8</td>
<td>78 ± 14</td>
</tr>
<tr>
<td>$K_m$ NAD (mM)</td>
<td>2.1 ± 0.8</td>
<td>2.3 ± 0.002</td>
<td>1.5 ± 0.0002</td>
</tr>
<tr>
<td>$K_i$ NAD (mM)</td>
<td>3 ± 0.8</td>
<td>8.8 ± 1.2</td>
<td>8.0 ± 2.0</td>
</tr>
</tbody>
</table>

Fig. 7. Enzyme kinetics value are found via spectrometry of enzyme with substrate and buffer DTT with numbers calculated via SigmaPlot. Form $K_m$ values for IMP was found using constant 2.5mM NAD. Likewise, $K_m$ and $K_i$ value was found for NAD with 5mM IMP constant. $K_{cat}$ values are calculated from found $V_{max}$.

The His-strep tag on AcK203 IMPDH allows the protein to bind to Ni-NTA solution under low imidazole buffer washes. By trapping IMPDH on to a Ni-NTA column, proteins that have strong interactions with IMPDH will stay in the column as well. When subjected to high imidazole buffer washes, IMPDH and other associated protein be eluted into the same elute fractions. We observed through western blotting the presence of IMPDH in wt, K203Q, and AcK203 samples.

RNA polymerase was present in thr wt specimen, suggesting a likely yet not described protein-protein interaction between the two enzymes. However, RNA polymerase is not present in samples of K203Q and AcK203 under the same conditions, suggesting a lack of interaction between the mutants and RNA polymerase observed in wild type specimen. (Fig. 8) However, subsequent Ni-NTA column pull downs using pure wild type IMPDH protein and pure RNA polymerase revealed lack of RNA polymerase in elute.
Fig. 8. Western blots showing presence of A) Acetylated IMPDH blot elute fractions showing presence of acetylation on IMPDH, anti-acetylated lysine antibody used. 10µg of protein from elute used in each lane. B) PBS Low salt buffer elutes from purified E. coli strains indicated. RNA polymerase Antibody used to identify presence of RNA polymerase in samples of elute and lysate. 20µg of protein used for each lane. C) Purified protein elutes (10µg) from E. coli strains obtained from low salt PBS buffer. Anti-Strep tag antibody used to identify IMPDH protein presence. IMPDH size is 55kD D) RNA polymerase blots from protein strains (20µg) obtained from standard PBS buffer. E) Pure protein column pull down using 20µg wt containing His-strep tag and 10µg core E. coli RNA polymerase. wt and RNA polymerase were mixed and then trapped in Ni-NTA solution. PBS buffer used sodium described in procedures. Elution conducted with PBS low salt with 500mM imidazole.
Deacetylation of IMPDH lysine site 203 Occurs as a Natural Process: When AcK203 was grown with nicotinamide and N-acetyl-L-lysine in minimal media, the OD$_{600}$ measured was 27% that of wt. In AcK203 grown with N-acetyl-L-lysine but without nicotinamide saw cell density levels close to IMPDH. The same results are seen for cells grown in media containing inosine. AcK203 grown with nicotinamide are observed to have OD$_{600}$ values closer to that of wt than that of mutants grown with nicotinamide. (Fig. 10) The increase of cellular growth suggests that natural deactylation had occurred within cells without deacetylase inhibitor

**Acetylated K203 Allele Sensitizes a wild-type E. coli strain to Adenosine:** In previously performed studies [8], it has been conclusively shown that *E. coli* strains without the CBS domain of IMPDH are sensitive to adenosine, an intermediate in the purine biosynthesis pathway of IMPDH. These studies proposed a model by which IMPDH regulates and interacts with proteins such as AMPs synthetase via the Bateman domain. In light of the observed loss of interaction with RNA polymerase IMPDH exhibited with acetylated K203, alleles AcK203 and K203Q were tested for adenosine toxicity. BL-21 E. coli strains AcK203, K203Q, wt, and guaB$^{4CBS}$ were grown on M9 minimal salt medium supplemented with 0.5% glucose and 2mM adenosine. Control growth in M9 minimal media without adenosine saw similar growth for all for strains. The guaB$^{4CBS}$, AcK203, and K203Q mutants were unable to grow on minimal media in the presence of adenosine compared to the healthy growth of wt, suggesting adenosine toxicity caused growth arrest. (Fig. 9) Similar results were obtained when cells were grown in liquid M9 cultures. After overnight growth, the cell count observed in mutant culture growth was 25% of the OD$_{600}$ levels of wild type. OD$_{600}$ measured at 10-minute intervals
revealed observable difference in growth curve between the mutants and wt. Cells grown on media supplemented with inosine sees a much smaller drop in cell density, with only a 30% drop. *(Fig 10)* Toxicity on 2mM inosine plates showed less difference between strains, suggesting that toxicity is less strong for mutant strains compared to that seen in 2mM adenosine plates. *(Fig. 9)*

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**Fig. 9.** All M9 minimal media agar plates are supplemented with 0.05 mM IPTG and noted compound. **A)** and **C)** Indicated *E. coli* strain grown on 2mM adenosine plates and 2mM inosine plates. Streaking was performed. **B)** and **D)** Strains spotted on 2mM adenosine plates and 2mM inosine with indicated starting colony number at the bottom gotten via dilution.
Fig. 10. A) OD600 Measurements of *E. coli* IMPDH alleles grown in minimal media supplemented with 50mM nicotinamide, 10mM N-acetyl-L-lysine, 50µM IPTG, and indicated compound. All cells are grown from 10⁸ colonies starter at 37°C for 12-16 hours. Error bars are standard deviation left to right: (wt: ±0.2, ±0.07, ±0.14), (AcK203: ±0.14, ±0.11, ±0.15), (AcK203 w/o Nicotinamide: ±0.07, ±0.073, 0), (K203Q: ±0.07, ±0.07, 0) (guaBΔCBS: ±0.07, ±0.03, ±0.14) B) OD600 measurements of *E. coli* IMPDH alleles grown in minimal media supplemented with only 50µM IPTG, and indicated compound. All cells are grown from 10⁸ colonies starter at 37°C for 12-16 hours. Error bars are standard deviation left to right: (wt: ±0.07, ±0.21, ±0.06), (AcK203: 0, ±0.07, ±0.28), (K203Q: ±0.07, ±0.14, ±0.07) (guaBΔCBS: ±0.07, ±0.1, ±0.14)
Discussion:

We demonstrate that deacetylation of lysine 203 on the CBS subdomain of IMP dehydrogenase is a process, which occurs within *E. coli* cells. In the absence of deacetylase inhibitor nicotinamide, *E. coli* growth grows at *wt* rate, suggest natural deacetylation had occur. This suggests that the acetylation and deacetylation process of the Bateman domain is regulated within the cellular metabolism of *E. coli*.

IMPDH associates with RNA polymerase via the presence of acetylation on the subdomain. The *wt* interaction and the *guaB*\(^{ACBS}\) lack of interaction with RNA polymerase suggest a mechanism by which IMPDH interacts with metabolic enzyme through the CBS domain as a novel function of IMPDH. *AcK203* is not observed to have active protein interactions with RNA polymerase, suggesting that interaction is dependent on the state of acetylation on K203. Similarly, *K203Q*, a mutant that mimics the effects acetylation on K203, also exhibits no RNA polymerase interaction. The interaction of IMPDH and RNA polymerase also appears to be one that involves not only the two enzymes. Pure protein column pull down revealed the interaction does not appear *in vitro*, suggesting other factors *in vivo* involved in the interaction.

We propose a model in which acetylation of K203 in IMPDH regulates RNA polymerase interactions with IMPDH to inhibit cell growth. As acetylation of K203 does not occur during exponential growth phase,\(^{[16]}\) it is likely that exponential phase is when RNA polymerase interacts with IMPDH. All cells culture grown for IMPDH purification in low salt conditions were induced and collected before the cell cultures entered stationary phase of cell growth. IMPDH interactions with RNA polymerase may contribute to increased metabolic activity associated with exponential growth phase.
Acetylation on K203 in turn would inhibit such a change. \( AcK203, K203Q, \) and \( guaB^{4CBS} \) took twice as long to reach \( \text{OD}_{500}: 0.5 \) when compared to \( \text{wt} \), which suggests that acetylation could be negatively regulating IMPDH-RNA polymerase interaction, contributing to the processes by which the cells enter stationary phase.

IMPDH’s regulatory role in \( E. coli \) purine biosynthesis pathway also appears to involve acetylation. Adenosine toxicity experiments reveal stark contrasts between growth rates of acetylated strains and non-acetylated. \(^{[6]}\) The cell density measurements derived from acetylated mutants are similar to those without CBS domain, suggesting acetylation of the Bateman domain is connected with regulating the subdomain. The lower decline of cell growth on inosine is most likely due to active alternate pathways in the \( E. coli \) cell. The funneling of inosine into other pathways allows the cell to abate complete pyrimidine nucleotide starvation and cell arrest. Plates grown with starting \( 10^4 \) colonies still exhibit some growth even at 2mM inosine and adenosine. When alternate pathways are cut off, growth arrest is much more evident. In \( E. coli guaB^{4CBS} \) cells without purine nucleoside phosphorylase \( (deoB) \), an enzyme that allows inosine to be converted into hypoxanthine, inosine is seen to be completely toxic at 0.5mM while adenosine is toxic at 0.1mM. \(^{[8]}\)

We hypothesize that acetylation at lysine site 203 is the mechanism by which the CBS subdomain regulates the purine biosynthesis pathway of \( E. coli \). Acetylation at K203 is observed to confer the adenosine toxicity much like in \( guaB^{4CBS} \) cells. \( \text{OD}_{600} \) measurements for \( AcK203 \) grown in inosine are observed to have lower decreases compared to \( \text{wt} \) than the \( \text{OD}_{600} \) found for \( guaB^{4CBS} \). \(^{[6]}\) As acetylation would act in an inhibitory role for an existing CBS domain, it would decrease such a regulation. Unlike
\textit{guaB}^{\Delta CBS}, where the CBS domain is completely missing, acetylation would not have the same inhibitory effectiveness. As all cells strains grown are overexpress for their respective IMPDH type, a decrease in inhibitory effectiveness due to acetylation would be observed.

In wild type \textit{E. coli}, ATP levels, a product of the purine biosynthesis pathway, and other nucleotides universally decrease once the cell enters stationary phase after log growth phase. ATP pool levels steadily decrease to half of the peak observed in growth phase. \textsuperscript{[1]} As \textit{guaB}^{\Delta CBS} is associated with increases in ATP pool levels, \textsuperscript{[8]} acetylation would very well be the method of control over nucleotides production in the purine biosynthesis pathway.

While there is evidence supporting the idea that acetylation is involved in certain moonlighting functions of IMPDH, it is imperative that the exact mechanism by which the modification controls the functions is found. Whether the interaction with RNA polymerase is directly involved or via an intermediate remains to be sought out. The possibility of influencing cellular metabolism via acetylation of the CBS domain IMPDH suggest that it may present itself as a pharmacological target for antibiotic development.


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