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Site-Directed Mutagenesis Of Conserved Amino Acids Residues In N5-Carboxyaminoimidazole Ribonucleotide Mutase: Converting N5-Cair Mutase Into Aminoimidazole Ribonnucleotide Carboxylase & Developing A High-Throughput Screening Assay For The Discovery Of N5-Carboxyaminoimidazole Ribonucleotide Mutase

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Site-Directed Mutagenesis of Conserved Amino Acids Residues in N^5-Carboxyaminoimidazole Ribonucleotide Mutase: converting N^5-CAIR mutase into Aminoimidazole Ribonnucleotide carboxylase
&
Developing a High-Throughput Screening Assay for the Discovery of N^5-Carboxyaminoimidazole Ribonucleotide Mutase Inhibitors

by

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_____

Advisor Date

DEDICATION

To my parents and friends

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I want thank many people for helping me to complete my master degree. First, I would like thank my advisor Dr. Steven Firestine, who gave me a lot help during the past two years and allowed me to do man stupid things. I also want to thank my lab mates, Dr. Shiv Sharma, Cale Streeter, Qian Lin and Mingyang Zhao, who helped me to solve many problems in the lab.

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Site-Directed Mutagenesis of Conserved Amino Acids Residues in N^5-Carboxyaminoimidazole Ribonucleotide Mutase: converting N^5-CAIR into Aminoinmidazole Ribonnucleotide carboxylase

1.1. Introduction

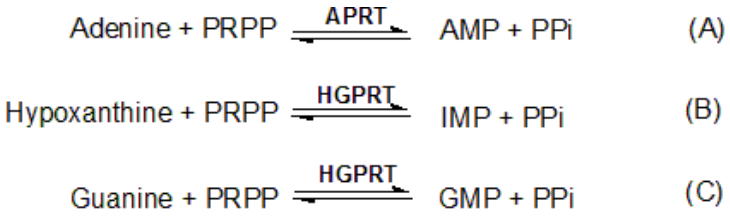
1.1.1. The role of purine nucleotides in cellular activity

Purine nucleotides play an important cellular role. They are utilized in energy storage (ATP), the synthesis of cofactors (NADH, B-12) and in the synthesis of amino acids and secondary natural products. Given the importance of purines to the cell, it is not surprising that two pathways exist for their synthesis, namely the salvage and *de novo* biosynthetic pathways.[1,2]

1.1.2. Salvage pathway

The salvage pathway is a “recycle” pathway, where free bases generated from the degradation of nucleic acids are used to prepare new nucleotides through multiple enzymes. There are two critical enzymes which are commonly used. Adenine phosphoribosyltransferase (APRT) catalyzes the attack of free adenine onto 5-phosphoribosyl-α-pyrophosphate (PRPP) to yield AMP and PP_i (***Scheme 1*** **Salvage pathway A**). The other enzyme is hypoxanthine-guanine phosphoribosyltransferase (HGPRT) which catalyzes a similar reaction to transfer guanine to PRPP to generate GMP (***Scheme 1*** **Salvage pathway C**). This enzyme also catalyzes the reaction of hypoxanthine with PRPP to give IMP (***Scheme 1*** **Salvage pathway B**).[3]

Scheme 1 Salvage pathway



The amount of purine nucleotides prepared by the salvage pathway is about 1%, so the pathway is utilized predominately for cellular stasis. For cells undergoing division, new purine nucleotides must be created. They are synthesized from the de novo purine biosynthetic pathway.[4]

1.1.3. *De novo* purine biosynthetic pathway

Unlike the salvage pathway, the *de novo* purine pathway synthesizes the purine heterocycle from basic building blocks（***Figure 1***）. In this pathway, PRPP is the starting substrate that is ultimately converted to IMP. The pathway is intriguing because of the interesting chemistry which occurs in the pathway and also because it is one of the few primary metabolic pathway which is different between microbes and humans.[5] The difference is focused on the conversion of aminoimidazole ribonucleotides (AIR) to 4-carboxy-5-aminoimidazole ribonucleotide (CAIR). In humans, AIR is directly converted to CAIR by the enzyme AIR carboxylase (Class II PurE), requiring CO_2 as the one carbon substrate. In most bacteria, two steps and two enzymes are needed to convert AIR to CAIR. Step one is catalyzed by N^5-carboxyaminoimidazole ribonucleotide (N^5-CAIR) synthetase (PurK) which converts AIR to N^5-CAIR

with support of ATP and HCO_3^-. The second step is the conversion of N^5-CAIR to CAIR catalyzed by N^5-CAIR mutase (Class I PurE). [6,7]

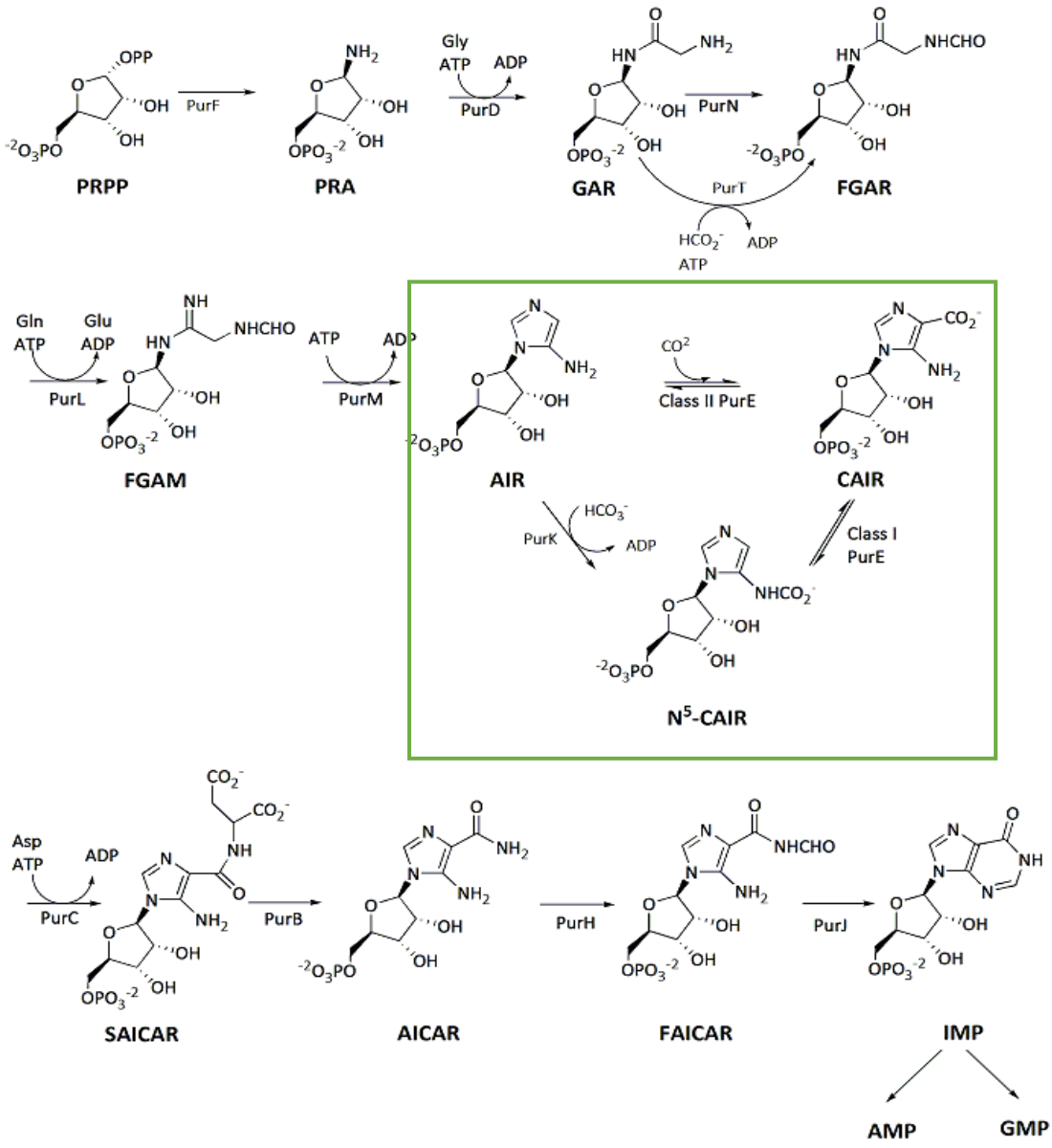


Figure 1. The *de novo* purine biosynthetic pathway. The green square hights the difference between Class I and Class II PurEs found in microbes versus higher eukaryotes. The enzyme names and abbreviations of substrates can be found in Tables 1 and 2

Table 1. Enzymes in de novo purine biosynthetic pathway.

Gene	Enzyme name
PurF	PRPP amidotransferase
PurD	GAR synthetase
PurN	GAR transformylase
PurT	FGAR synthetase
PurL	FGAM synthetase
PurM	AIR synthetase
Class II PurE	AIR carboxylase
PurK	N^5-CAIR synthetase
Class I PurE	N^5-CAIR mutase
PurC	SAICAR synthetase
PurB	Adenylosuccinate lyase
PurH	AICAR transformylase
PurJ	IMP cyclohydrolase

Table 2. Abbreviation of substrates

Abbreviation of intermediate	Full Name
PRPP	5-Phosphoribosyl-α-pyrophosphate
PRA	5-Phosphoribosylamine
GAR	Glycineamide ribonucleotide
FGAR	N-formylglycineamide ribonucleotide
FARM	N-formylglycineamidine ribonucleotide
AIR	Aminoimidazole ribonucleotide
N^5-CAIR	N^5-Carboxyaminoimidazole ribonucleotide
CAIR	4-Carboxy-5-aminoimidazole ribonculotide
SAICAR	Succino 5-aminoimidazole-4-carboxaminde ribonucleotide
AICAR	Aminoimidazole-4-carboxamide ribonucleotide
FAICAR	5-Formamido-4-imidazolecarboxamide ribonucleotide
IMP	Inosine monophosphate

1.1.4. PurK

PurK is one of the ATP-grasp enzymes and its active site includes ATP, AIR and bicarbonate subsites.[8] As mentioned above, PurK catalyzes the turnover of AIR to N^5-CAIR. This is an ATP-dependent process and links CO_2 to the N5 amino group on AIR to generate N^5-CAIR. However, PurK is not required under the condition of high CO_2 concentration as AIR can be non-enzymatically converted to N^5-CAIR without the support of ATP.[9]

1.1.5. N^5-CAIR

N^5-CAIR had not been known as an intermediate in the pathway until the 1990s. This is likely due to the fact that it is a highly unstable compound. It is sensitive to pH and temperature. At 30 ºC and pH 7.8, the half-life of N^5-CAIR is about 0.9 minutes, where it ready breaks down to AIR.[10] The low stability has important implication for how bacteria handle this metabolic intermediate in the middle of a biosynthetic pathway.

1.1.6. Class I and Class II PurEs

Previous studies have shown that the Class I and Class II PurEs are evolutionarily related to each other. Each catalyzes a reversible reaction to generate the same product, CAIR. In addition, both have extremely similar protein structures (**Figure 2**), and shared about 20-30% sequence identity with each other.[11] Despite these similarities, there exist critical differences between these two classes of PurEs.

1.1.6.1. AIR is the substrate of Class II PurEs but not Class I PurEs

When AIR is incubated with Class II PurEs in the presence of CO_2, rapid CAIR formation is observed.[12] In contrast, addition of N^5-CAIR or PurK (to produce N^5-CAIR in situ) fail to give a burst of CAIR formation.[9] NMR studies on the conversion of CAIR by the Class II PurEs fail to detect any N^5-CAIR and instead yield AIR.[13]

1.1.6.2. N^5-CAIR is the substrate of Class I PurE but not Class II PurE

NMR spectroscopy was applied to investigate and analyze the reverse reaction of CAIR with Class I PurE. NMR analysis showed that CAIR is first converted into N^5-CAIR which is then non-enzymatically converted to AIR.[10] This is a strong evidence that Class I PurE catalyzes N^5-CAIR rather than AIR to form CAIR. Addition of CO_2 to AIR and Class I PurE gives a decrease in absorbance (due to N^5-CAIR formation) followed by an increase in absorbance due to CAIR formation. This study indicates that N^5-CAIR, not AIR and CO_2, are substrates for Class I PurEs. [13]

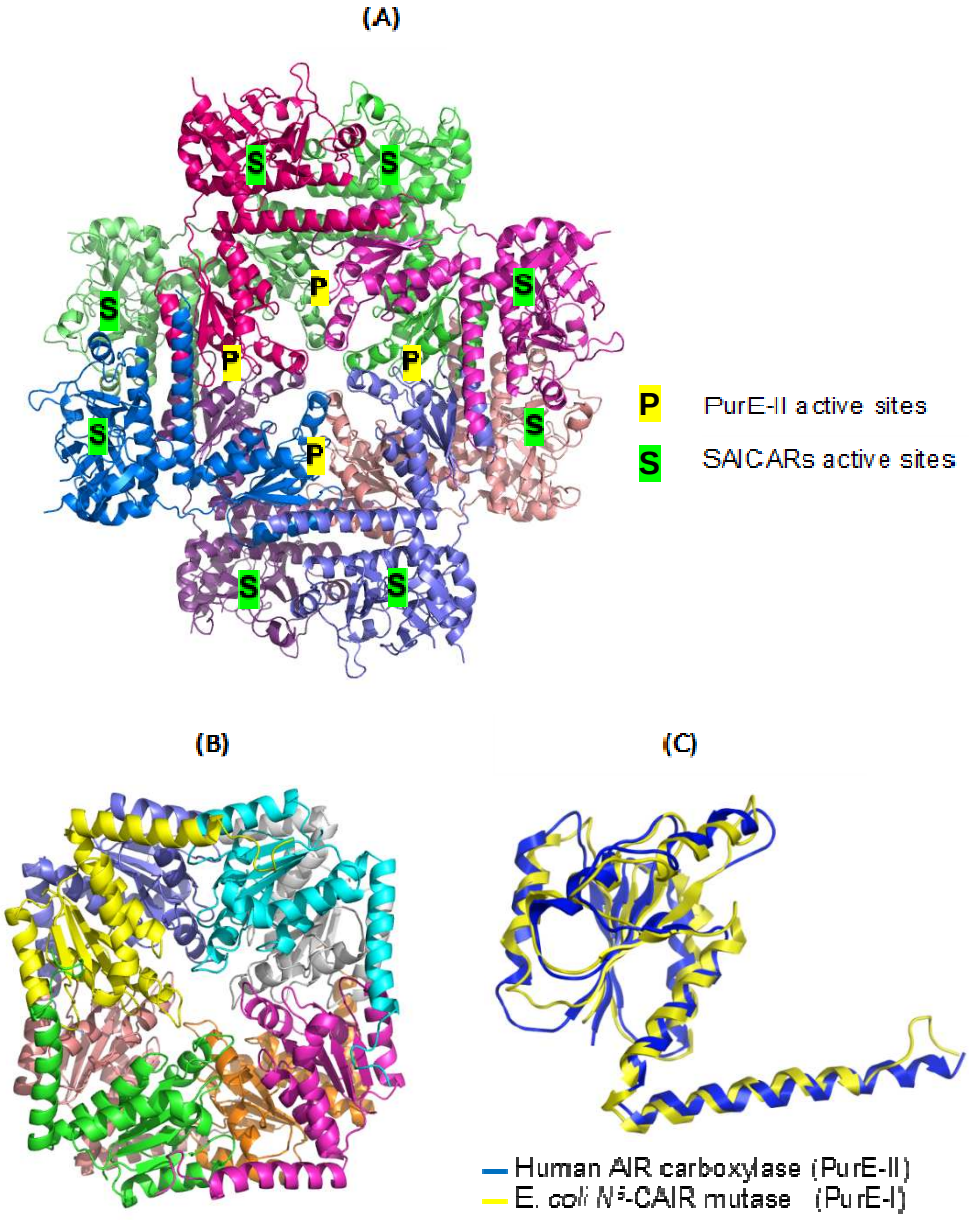


Figure 2 Crystal structure of Class I and Class II PurEs structures. (A)Human PAICS (PBD 2H31) contains Class II PurE active sites and SAICAR synthetase active sites; (B) E.coli PurE (PBD 2ATE).(C) Superposed monomer structures of Class I (yellow) Class II (blue)PurE

1.1.7. Sequence and structural studies

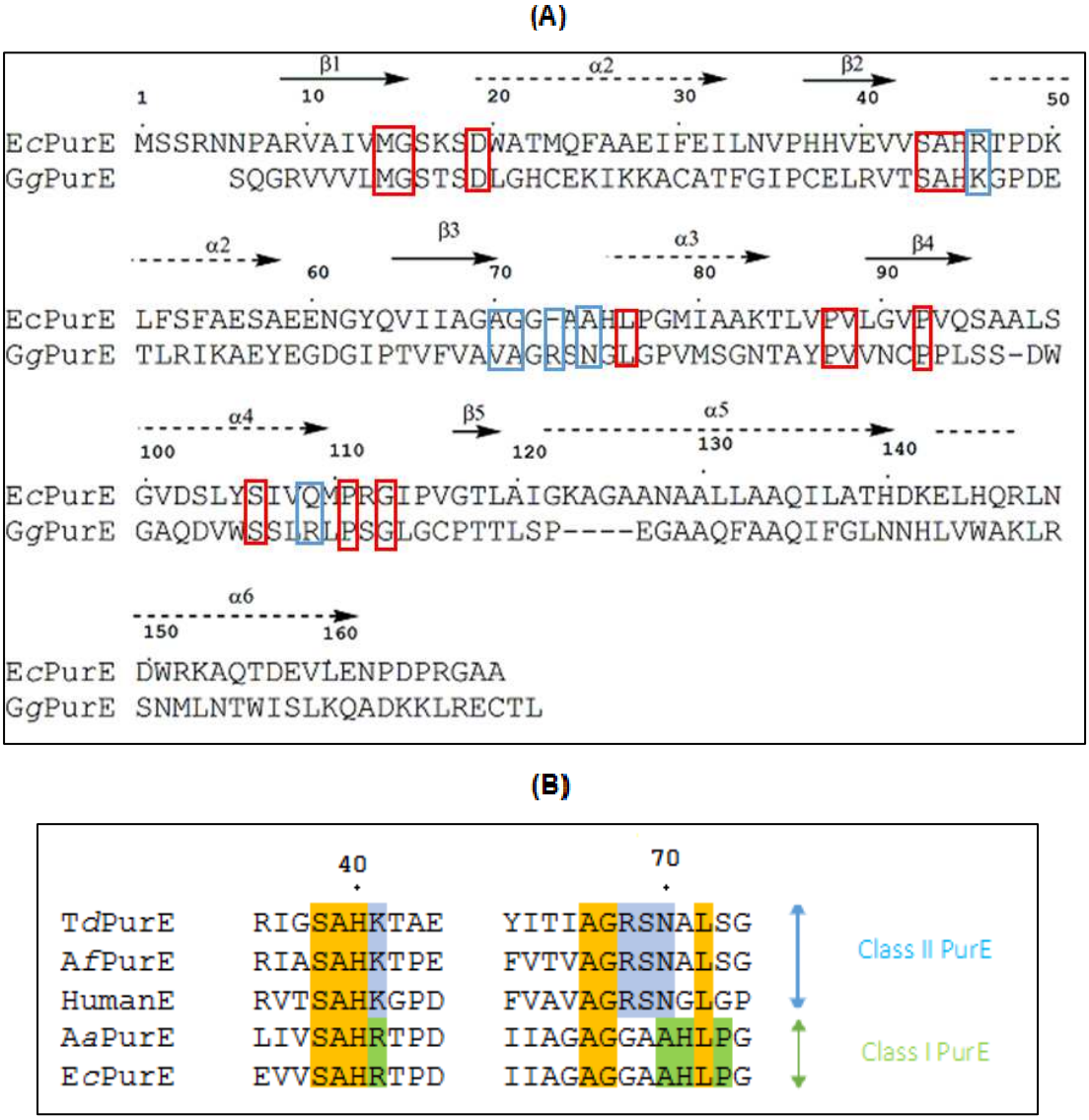


Figure 3. Sequence alignments between Class I and Class II PurEs. (A) Alignments between E.coli PurE and G.gallus PurE. Red cubes indicate universal conserved residues, and blue cubes indicates class specific residues. Sequence numbered after E.coli PurE. (B) Sequence alignments in 40S and 70S loop numbered after T*d* PurE. Orange represent the universal conserved residues, green is Class I specific conserved residues, and blue is Class II specific conserved residues. T*d* is *Treponema denticola*; A*f* is *Archaeoglobus, fulgidus*; A*a* is *Acetobacter aceti*; E*c* is *Escherichia coli*

Previous studies of Class I and Class II PurEs have shown how similar these two enzymes are to one another. The crystal structures (**Figure2**) show that they are both symmetrical octamers formed by similar 17KD subunits.[14,15] In humans, Class II PurEs are fused with SAICAR synthetase (PurC), while bacterial Class I PurEs are usually expressed alone. For yeast and fungi, Class I PurEs are usually fused with PurK. The sequence alignment of Class I and II from different species indicates that both have several conserved regions (***Figure 3A***). P-loop, which is between the $\beta 1$ and $\alpha 1$, is responsible for phosphate group. 40s loop is located between $\beta 2$ and $\alpha 2$, and 70s loop is located between $\beta 3$ and $\alpha 3$.[14,16] Sequences alignments of Class I and II PurEs in the 40s and 70s loops indicates that S38, A39, H40, A66, G67, and L72 are highly conserved amino acid residues for all PurEs (***Figure3 B***). However, there are residues which are only conserved in each class. In the 40s and 70s loop, Class I PurEs' residues are conserved in R41, G68, A69, A70, H71,P73, while most Class II PurEs have K41, G68, S69, N70, G71,S73 at the same residues.[11,15] One of the special case of Class II PurEs is *Treponema denticola* (T*d*) PurE, which is the only known bacterial AIR carboxylase.[16]

The question of how these two similar enzymes catalyze different reactions is still unknown. The hypothesis is that the class specific conserved residues might be the key to determining the functional differences between these two classes of enzymes. However, few studies have been done on these residues and we don't know which residues are important for the specificity of

the reaction. To investigate this issue, we conducted site-direct mutagenesis to convert conserved amino residues (41, 68, 69, 70, 71, and 73) of wild type Escherichia coli into residues which mimic those found in Class II PurEs. Mutants were then investigated to determine, which, if any, mutation produced a change in the specificity of the enzyme reaction.

1.2. Results

The previous study of crystal structures of PurE shows that the active sites of PurEs are located in the P, 40s and 70s loops. Since the conserved residues on the P-loop are universally conserved, we did not alter these residues.[15] We focused on the class-specific conserved residues found in the 40s and 70s loops to investigate whether these residues affected the functional divergence between the two classes of PurEs.[16]

Arg41, Gly68, Ala69, Ala70, His71 and Pro73, (numbered by Td PurE, (***Fiugre 3B***), are Class I PurE conserved residues and we mutated each of these residues to that found in the human region.[a] This gave mutants R41K, G68R, A70N, H71G and P73S. Since T.*denticola* PurE is a bacterial Class II PurE we also mutated His 71 to alanine.

We investigated each of these mutants using developed assays, and compared them to the same kinetic studies of WT E.*coli* PurE. These studies were done to determine which amino acid(s) will decide the functional divergence between the two classes of PurEs.

1.2.1. Enzyme expression and purification

The cloning of PurE and plasmid purification was done by Aiko Hirata. The single mutants made are R41K, G68R, A70N, H71A, H71G and P73S. All plasmids were transformed into BL21/(DE3) competent cell for protein expression. All proteins were expressed and purified according to previously

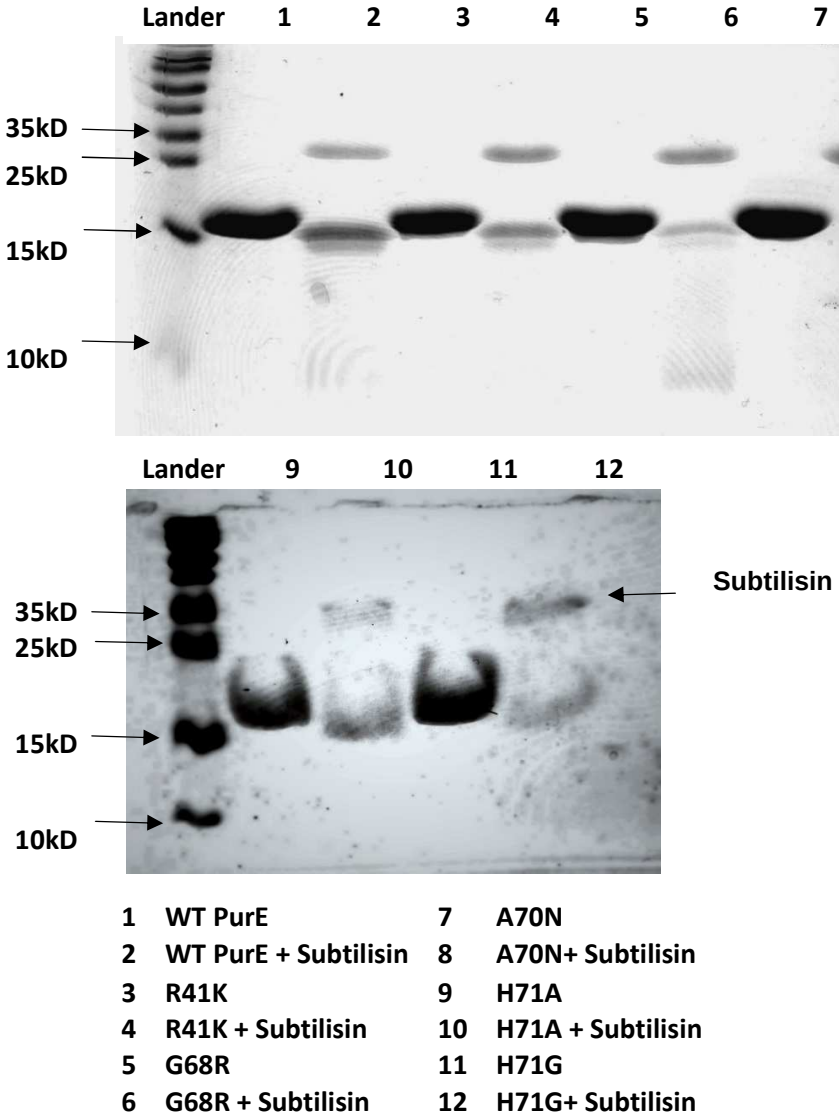
[a] All the mutants cloning was done by Aiko Hirata from Dr. Fusao Hirata's group

developed procedures.[17] SDS-PAGE was utilized to check the protein purification of mutants. We obtained purified mutants R41K, G68R, A70N, H71A, and H71G. The P73S PurE, however, couldn't be expressed and was therefore not purified.

1.2.2. Protein folding of mutants

1.2.2.1. Limited proteolysis for WT and mutant PurEs

In order to check whether the mutants are folding properly, limited proteolysis assays were performed. It is expected that a correctly folded enzyme will be relatively stable to the serine protease, subtilisin, whereas an unfolded protein would be degraded completely.[18,19] As showed in Figure 4, incubation of WT PurE with subtilisin for 1 hour leads to only a modest digestion as indicated by bands below the WT protein. This indicates that the protease only partly digests wild-type PurE. Analysis of mutants R41K, G68R, H71G and H71A with protease showed a pattern qualitatively similarity to the WT PurEs. However, A70N gave complete digestion indicating that this protein is partially unfolded or adopts an alternative conformation.



1	WT PurE	7	A70N
2	WT PurE + Subtilisin	8	A70N+ Subtilisin
3	R41K	9	H71A
4	R41K + Subtilisin	10	H71A + Subtilisin
5	G68R	11	H71G
6	G68R + Subtilisin	12	H71G+ Subtilisin

Figure 4 SDS-PAGE (15%) of 18 µg of wild-type or mutant (17kD) PurEs incubated with 18 µg subtilisin (27kD) for 1 hour. The reaction were stopped by addition of PMSF. Gel were photographed and the contrast was adjusted to provide the image seen here

1.2.2.2. Circular dichroism spectrum for folding of mutants

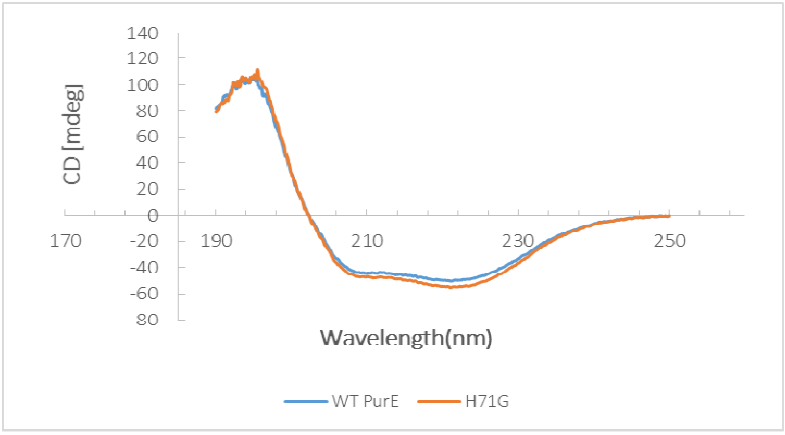


Figure 5 Far-UV CD spectra of H71G and Wild Type PurE in 100 mM Na_3PO_4 (pH, 7.4). The blue line represents 1.24 mg/mL of wild type PurE, while the red line is 1.15 mg/mL H71G.

Circular Dichrosim (CD) can provide information on the structure of a protein. Usually, two UV regions are tested, the far-UV and near-UV. The absorption in the far-UV region (180nm-240nm) are mainly due to protein secondary structure (α-helix, β-sheet, etc.), while the near-UV (250nm-290nm), where aromatic side chains can be detected, usually help to determine the tertiary protein structures.[20,21]

The far-UV CD spectra showed that no significant change of H71G secondary structure compared to WT PurE (**Figure 5**). Near-UV CD spectrum gave, no significant data for either WT PurE or H71G PurE. This result indicates that the H71G mutant is folded properly.

1.2.3. Steady-State kinetic analysis of the PurE mutants

The steady-state kinetic analysis for both WT and mutant PurEs was conducted using the CAIR decarboxylation assay.[12] Kinetic constant were determined by curve fitting a plot of velocity versus CAIR concentration to the Michaelis Menten equation.[22]

Table 3 Steady-State Kinetic Constants for Wild-type PurE and Mutants. V_{max} and K_m was determined by the CAIR decarboxylation assay system; k_{cat} was calculated using the molecular weight of the octamer.

PurE Mutants	V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	relative k_{cat}/K_m (units/mg)	k_{cat}/K_m $min^{-1} \cdot M^{-1}$)
Wild type	54	38	7297	1	$1.9X10^8$
G68R	19	32	2538	0.35	$7.9X10^7$
A70N	0.6	473	75	0.01	$1.6X10^5$
H71G	6	16	800	0.11	$5.0X10^7$
H71A	8	15	1111	0.15	$7.4X10^7$
R41K	1.5	37	204	0.03	$5.5X10^6$

As shown in ***Table 3***, the catalytic proficiency of all mutants, as expected, are decreased about 3-100 fold. Since A70N is not folded properly, it is not surprising to see its low binding affinity and catalytic proficiency. The K_m values are all within 2-fold of WT PurE suggesting that these mutants do not affect CAIR binding. The k_{cat}/K_m of WT and mutant PurEs are again similar. Overall, the kinetic studies indicate that these residues do not play a major role in either substrate binding or catalysis. Such a result may be expected give the fact that we made changes of conserved residues to amino acids that were conserved in the other class of PurE. A key question, though, is whether these mutants catalyze a different reaction from WT Class I PurE.

1.2.4. Comparison of the CO_2 dependence of wild type PurE, human AIR carboxylases and mutant PurE

Previous studies have shown that CO_2 is the substrate only for AIR carboxylase. Class I PurEs are independent of the one-carbon substrate. However, N^5-CAIR synthesis can occur non-enzymatically if enough CO_2 is provided. Therefore, one way to distinguish Class I and Class II PurEs (AIR carboxylase) is by examining their dependence on CO_2.[14]

To determine this, reactions are examined at 10 ºC, where the rate of equilibrium between CO_2 and HCO_3^- is decreased. Thus, at early time points, the predominant one-carbon species is the one added. When CO_2 is added, AIR carboxylases rapidly converts AIR to CAIR.[12] Addition of bicarbonate gives no conversion. In contrast, when AIR is incubated with Class I PurE and CO_2, no burst is observed.[13] In fact, often a decrease in absorbance is seen.[23] This decrease is due to the non-enzymatic turnover of AIR to N^5-CAIR. At later times, CAIR production is seen as an increase in absorbance.

As shown in ***Figure 6***, addition of CO_2 to WT PurE (blue line) generates a curve which shows a lag followed by an increase of CAIR production. Addition of CO_2 to human AIR carboxylase (yellow line) gave a burst in CAIR formation. These results were used as controls for mutants. Incubation with bicarbonate, results in no or modest CAIR formation for either WT PurE, AIR carboxylase or the mutants (***Figure 7***).

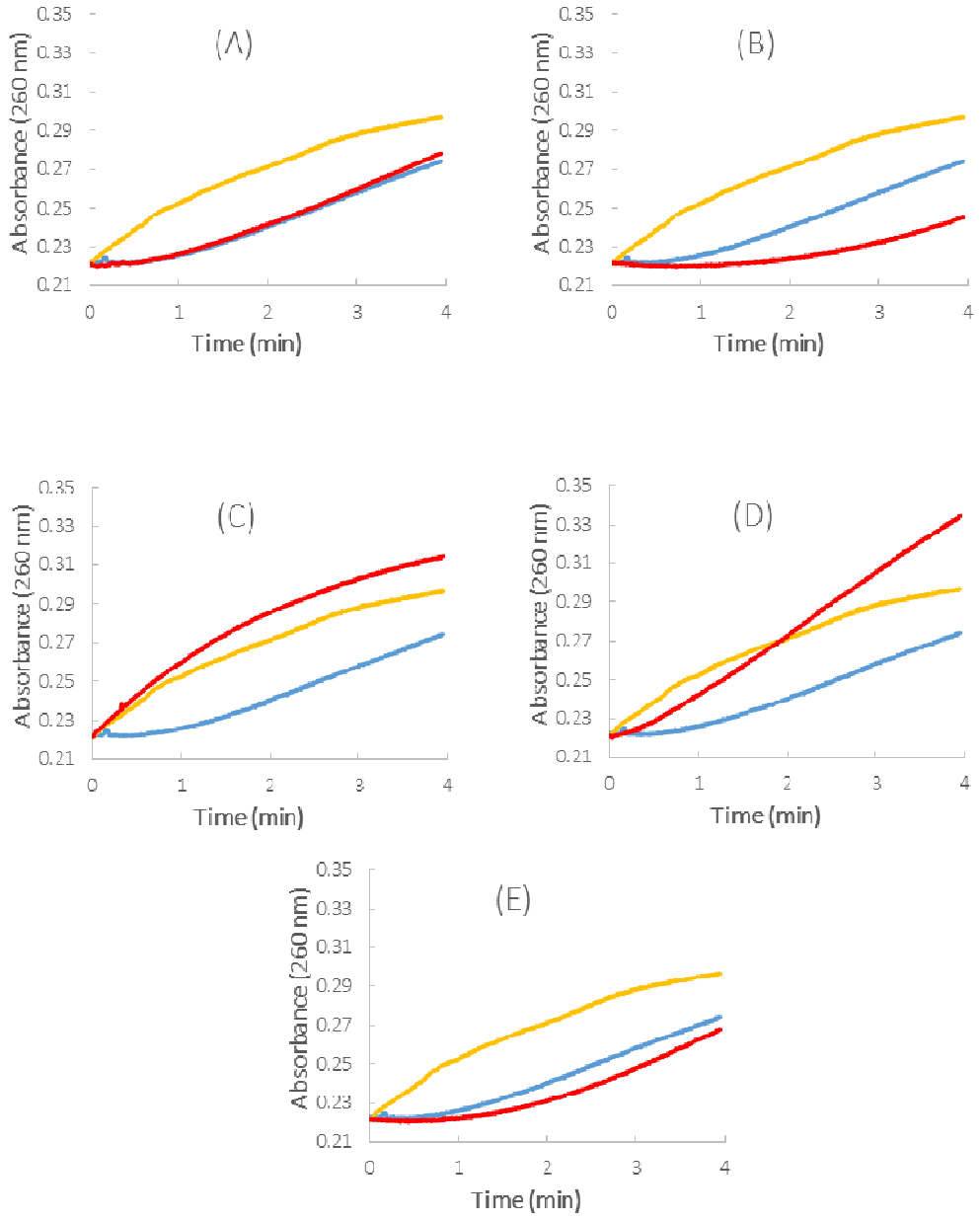


Figure 6. 150 µm AIR incubated with wild type or mutants PurE in the presence of 20 mM CO_2. Yellow line is the absorbance of 600 ng human AIR carboxylase incubated with CO_2, blue line is the 180ng E.*coli* PurE incubate with CO_2. Red line shows each mutants with CO_2 20 mM with same specific activity (0.01µm·min^{-1}). For comparison, the data for WT PurE and AIR carboxylase is shown with each mutant. (A) G68R 800 ng; (B) A70N 27 µg; (C) H71G 1.3 µg (D) H71A 1.2 µg (E) R41K 5.8 µg

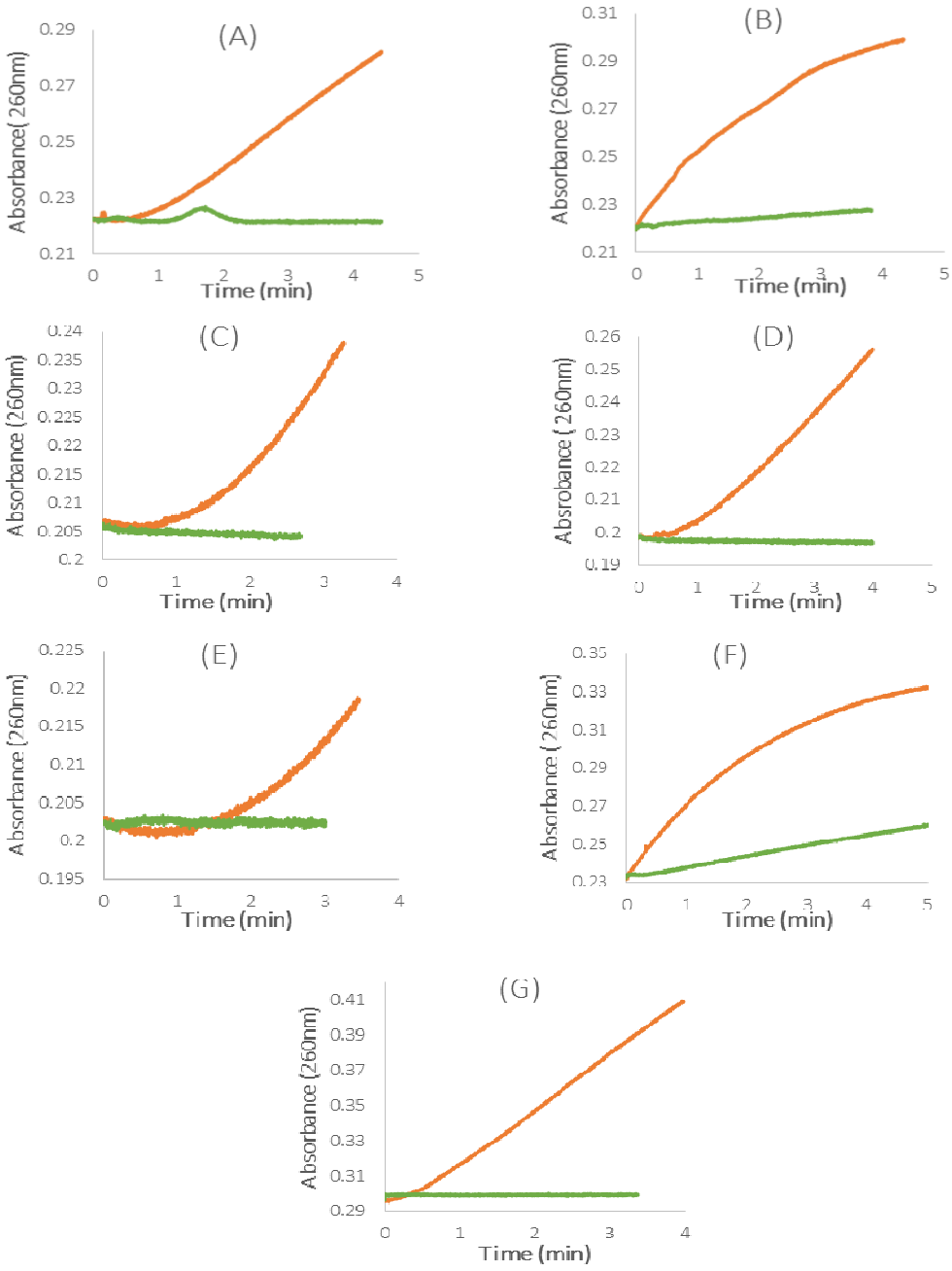


Figure 7. Comparaison of WT and mutant PurEs with CO_2 or HCO_3^-. Orange lines represent enzymes incubated with 20mM CO_2, green lines are enzymes with 20 mM HCO_3^-. (A) Wild-type PurE 180ng (B) Human AIR carboxylase 600 ng ; (C) R41K 5.8 µg; (D) G68R 800 ng; (E) A70N 27 µg; (F) H71G 1.3 µg ;(G) H71A 1.2 µg.

G68R, R41K and A70N all show obvious delays(**Figure 6**)when incubated with CO_2. This indicates that CO_2 is not a substrate for these three mutants.

H71G shows a burst upon the addition of CO_2 and a slow increase when HCO_3^- added. This is very similar to the curve generated with AIR carboxylase (**Figure 7 B&F**) with CO_2. In addition, it is interesting that the rate of CAIR production is greater than for human AIR carboxylase. In order to verify that CO_2 was a substrate for H71G, carbonic anhydrase was introduced into the assay. Carbonic anhydrase rapidly establishes the equilibrium between CO_2 and bicarbonate.[24] As showed in **Figure 8**, in the presence of carbonic anhydrase, the rate of CAIR production incubated with CO_2 decreases due to rapid conversion of CO_2 to bicarbonate. In contrast, incubation of H71G with bicarbonate and carbonic anhydrase leads to an increase in rate of CAIR production. This evidence shows that CO_2, but not HCO_3^-, is the substrate for H71G.

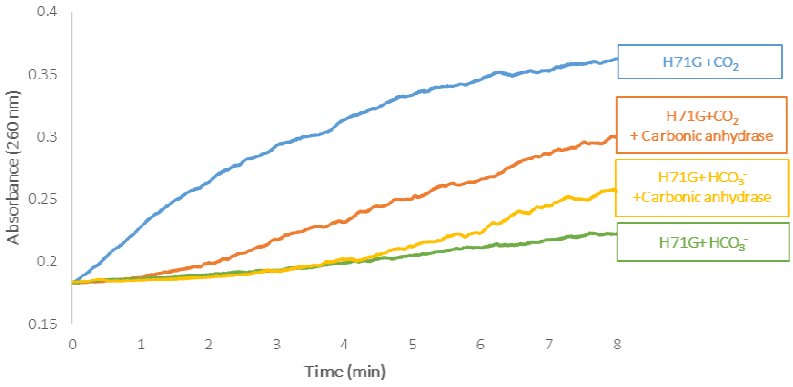


Figure 8 Mutation H71G with one-carbonate substrates incubated with 1 μg carbonic anhydrase.

H71A is another interesting mutant. Incubation with CO_2 does not generate a burst, however CAIR production is much more rapid than for WT PurE. Incubation with HCO_3^- curve indicates that HCO_3^- is not the substrate for H71A. Thus, H71A appears to be a hybrid between Class I and Class II PurEs.The analysis of the use of CO_2 as a substrate revealed that H71G and perhaps H71A could utilize CO_2. This is a hallmark trait of AIR carboxylase and suggests that H71G may be an AIR carboxylase enzyme. However, these experiments do not indicate whether H71G has the ability to still utilize N^5-CAIR as a substrate.

1.2.5. The PurC-coupled assay

In order to determine whether AIR or N^5-CAIR is the substrates for H71A and H71G, we used a PurK/PurC coupled assay (Figure 9). This assay was done to produce N^5-CAIR in situ and to make CAIR production irreversible by converting it to SAICAR. SAICAR production is monitored at 282nm to indicate the production of CAIR.[13] In the absence of PurK (Figure 10 A), almost no SAICAR is produced from AIR by WT E.*coli* PurE. In addition, this demonstrates that the non-enzymatic synthesis of N^5-CAIR is not sufficient for turnover. This indicate that AIR, as expected, is not a substrate for WT PurE. In the contrast, addition of H71G or H71A gave an increase in SAICAR production, showing the AIR converted to CAIR in the absence of its conversion to N^5-CAIR by PurK.

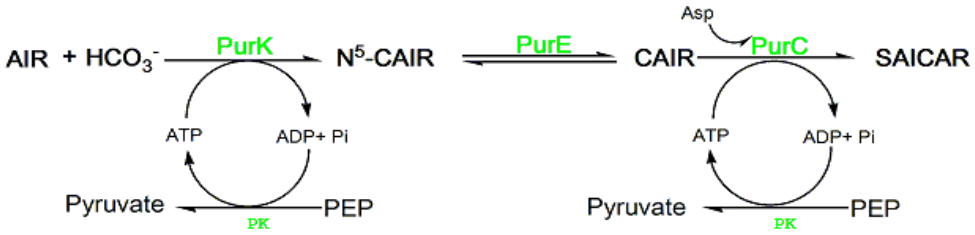


Figure 10 PurC coupled assay

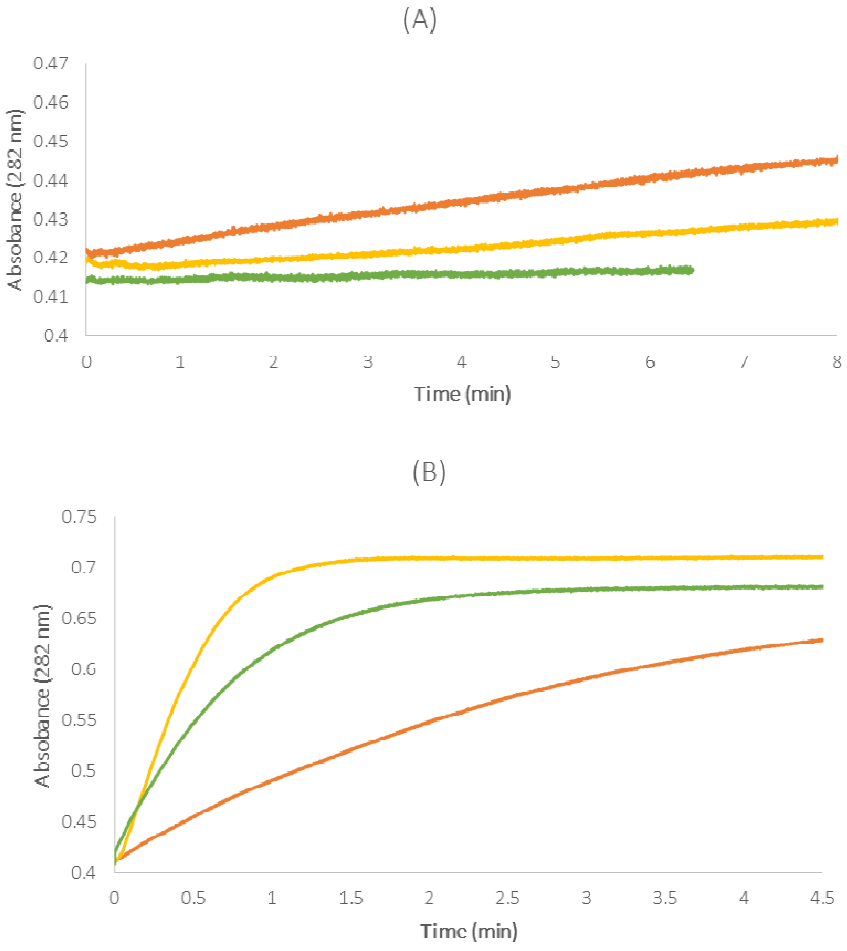


Figure 10 PurC coupled assay to monitor N5-CAIR turnover. Green lines are 325 ng WT PurE; Yellow lines are 2 µg H71A; Red lines are 3 µg H71G. (A) Enzymes incubated without PurK; (B) enzymes incubated with 1.1 µg PurK.

Addition of excess PurK leads to the rapid conversion of AIR in to N^5-CAIR, and consequently, SAICAR is produced rapidly with WT PurE (**Figure 10 *B, green line***). This validates that N^5-CAIR is the substrate of PurE. Interestingly, H71G also gave a bust of SAICAR production in the presence of PurK (**Figure 10*B, red line***). The rate, however is lower than WT PurE. H71A shows a large burst of SAICAR production and appears it be faster than for WT PurE (**Figure 10*B, yellow line***).

These results indicate that AIR is a substrate for both H71A and H71G. However, each protein also appears to utilize N^5-CAIR as a substrate. Thus, H71A and H71G are both AIR carboxylase and N^5-CAIR mutase enzymes. This data suggests that His71 plays a critical role in the reaction specificity of these enzymes.

1.3. Discussion

The purpose of the site-directed mutagenesis of N^5-CAIR mutase is to find possible amino acid residues that might determine the functional divergence between Class I and Class II PurEs. Therefore, the residues chosen in this study are in the active sites of both Class I and II PurEs and, also, are specifically conserved in each type of PurE. We then mutated E.*coli* PurE conserved residues in the 40s and 70s loop to the corresponding conserved residues in human AIR carboxylase. We also mutated His71 to alanine because it is found in the only known bacterial AIR carboxylase, *Treponema denticola* PurE.[16] For these studies, we were able to express and purify, mutants R41K, G68R, A70N, H71G and H71A[b].

We examined the steady-state kinetics using CAIR which is a substrate for both classes of PurE. All mutants, except for A70N, are active with k_{cat} values between 2 to 35 folded lower than WT E.*coli* PurE. Km values are roughly the same as WT PurE. A70N was excluded as it is folded incorrectly and its catalytic ability and binding affinity was very low. The kinetic studies indicated that the enzymes were active, but the assay could not provide information on what product, AIR or N^5-CAIR, is produced from CAIR. Thus, the study could not determine whether the mutants are N^5-CAIR mutases or AIR carboxylase.

[b] All numbered after the T. *denticola* PurE sequences

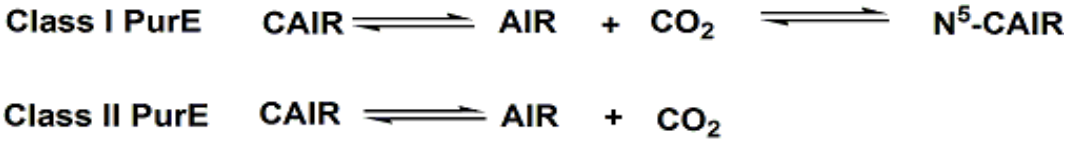
A key difference between N^5-CAIR mutase versus AIR carboxylase is the fact that AIR carboxylase uses CO_2 as a substrate and N^5-CAIR mutase does not. Therefore, we evaluate whether CO_2 was a substrate for the mutants. All mutants were studied at the same specific activity to ensure that any burst of CAIR production was due to CO_2 and not elevated enzymes level. The experiments were also run at 10 ºC to slow the kinetics of the CO_2/HCO_3^- equilibrium. The results indicates that H71G and maybe H71A utilized CO_2 as a substrate. This suggests that these mutants are AIR carboxylase.

The next question is whether N^5-CAIR is still a substrate. To answer that question, we utilized the PurC coupled assay investigating the forward reaction. In this assay, PurC is added, in the presence or absence of PurK, to monitor CAIR synthesis via SAICAR production. In the absence of PurK, H71G and H71A produced SAICAR from AIR and residual CO_2. This confirms the AIR carboxylase activity. However, addition of PurK results in a large burst of SAICAR production indicating that N^5-CAIR is also a substrate. This assay shows that H71G and H71A can utilize both AIR and N^5-CAIR as substrates. This demonstrates that the H71G and H71A mutants are both N^5-CAIR mutase and AIR carboxylase enzymes.

Based on these studies, His 71 seems to be very interesting residue and plays an important role in determine the functional divergence between human and bacteria PurEs.

1.3.1. Possible mechanism the conversion of N^5-CAIR to CAIR.

Scheme 2 Simple mechanistic model for Class I and Class II PurEs



Currently, the most plausible mechanism for Class I PurEs is shown in **Scheme 2.** CAIR is first reversibly decarboxylated to the intermediate AIR·CO_2 complex which is then reversibly converted to N^5-CAIR. Class II PurEs appears to only catalyze the first reversible conversion, CAIR to AIR·CO_2.[23,25] Structure studies on the active site of PurEs show that the aminoimidazole ring of CAIR is in the hydrophobic pocket of Class I PurE formed by conserved residues located in the 40s and 70s loops.[15] The CO_2 generated from CAIR decarboxylation is believed to bind at the bottom of the active site. Therefore, mechanistic and structural studies indicate that Class I PurEs can trap CO_2 in the active site for the attacking AIR to produce N^5-CAIR. Class II PursE allow CO_2 to dissociate from AIR and, therefore, prevent the production of N^5-CAIR.[14] However, Class II PurEs must also facilitate the binding of CO_2 for CAIR formation. How this occurs is unknown.

1.3.2. What role does His71 play in this mechanism?

Structure studies on *Acetobacter aceti* PurE (Class I PurE) indicates that His59 and His89 (T*d*PurE His40 and His71) are in contact with the His59 and His89 from their neighboring subunits to form a twofold histidine chain. This chain is isolated from solvent and it has been speculated that the possible

function of this chain is to transfer protons from one site.[26] Several studies have been done with these two histidine residues. His59 is a universally conserved residues in both Class I and Class II PurEs and is believed to be the general base needed for the reaction to form CAIR.[14] Site-direct mutagenesis studies on this residue indicates that it is essential as mutation to other amino acids abolishes activity.[27] On the other hand, our study on His71 agree with other's studies which show that site-direct mutagenesis of His71 decreases the enzyme activity but the enzyme is still functional.[26,27] However, examination on the reaction catalyzed by this mutant revealed that it has AIR carboxylase activity. Such a result has not been previously reported.

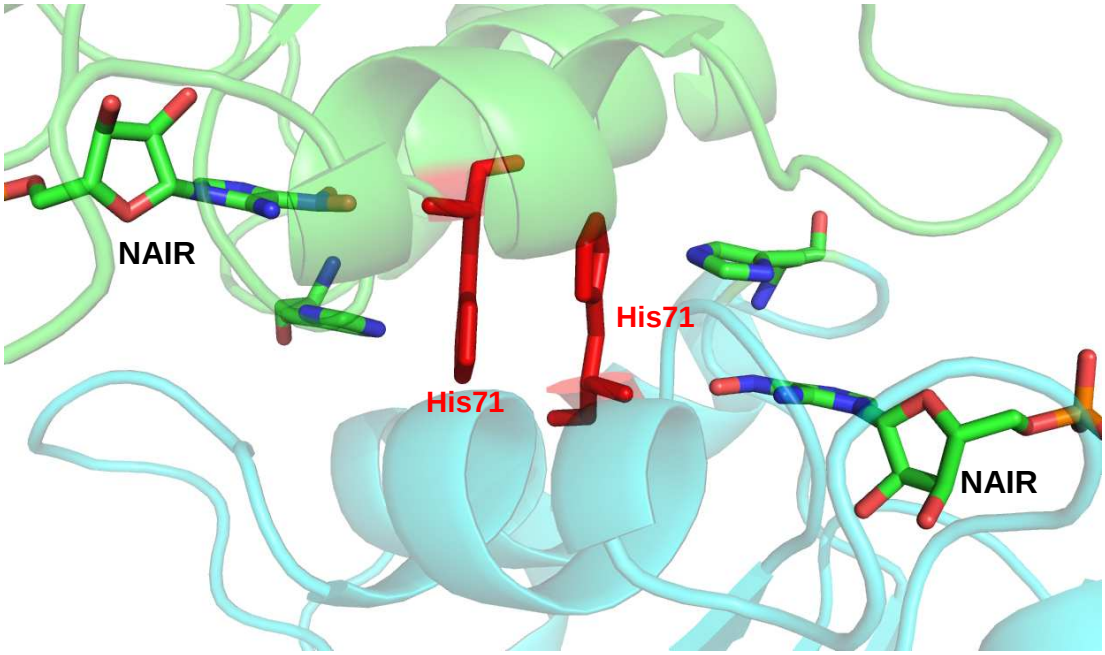
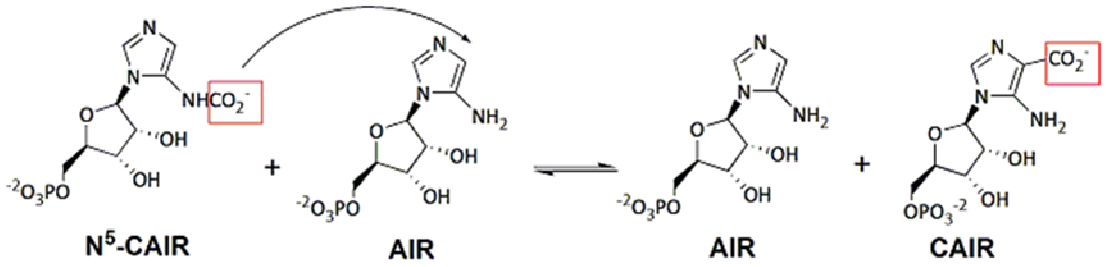


Figure 11. Dimer structure of WT Class I PurE (2ATE). His 75 (same as T*d*PurE His 71) is shown in red. NAIR is an analogue of CAIR and shown to indicate the substrate binding site in the active site position.

Based on the previous mechanism and the crystal structures, we hypothesize that mutating the His71 residue leads to the opening of a channel between the two active sites from two monomers in the PurE octamer. The open channel between the two active sites may provide an avenue for CO_2 to dissociate from the AIR·CO_2 complex. Thus, H71G can partition between either CO_2 escape, leading to the generation of AIR or reaction with CO_2 to generate N^5-CAIR. It is important to recognize, however, that the CO_2 must also bind to the H71G as the PurC assay in the absence of PurK, indicated turnover from AIR. How CO_2 binds and what is the driving force for this binding is unclear.

1.3.3. The open channel hypothesis for AIR carboxylase

Scheme 3. Transfer model of CO2 from N5-CAIR to AIR



Early studies indicated that N^5-CAIR was utilized as a carboxylase intermediate for transferring CO_2 and AIR to the PurE active site and the use of a carbamate was similar to the biotin-dependent carboxylase mechanisms.[10,28,29] One mechanism for N^5-CAIR mutase was that N^5-CAIR and AIR both bond to the site and CO_2 was transfered from N^5-CAIR to AIR to generate AIR and CAIR (**Scheme 3**). However, structural studies indicate that the active site of Class I PurE does not have enough space for binding both N^5-

CAIR and AIR. The opening channel, as hypnotized above, may provide a CO_2 tunnel between two isolated active sites. Thus, CO_2 dissociated from N^5-CAIR in one active site may migrate through the channel to bind AIR in the connecting active site to produce CAIR. Such a mechanism model indicates that both N^5-CAIR and AIR are substrates for AIR carboxylase. The CO_2-dependence on the reaction could be explained by the rapid production of N^5-CAIR which then deliveries the CO_2 to the enzyme. N^5-CAIR itself would not be turned over until enough AIR was produced to occupy the other active site.

1.4. Future Studies

According to the results and discussion above, we make a hypothesis that His 71 might be the critical conserved residue to determine the functional different and removal of histidine may open a channel between two actives for CO_2 transferring. Therefore much more studies can be applied to prove that.

Some Crystal structures studies should be done to prove whether the open channel exist and whether they can let CO_2 go through. Also, there are some mutants have not been made properly, which might also important to determine or assist His 71 to determine the functional differences.

Further studies on this mechanism will need to be done to validate this proposed mechanism.

Chapter 2.

Developing a High-Throughput Screening Assay for the Discovery of N^5-Carboxyaminoimidazole Ribonucleotide Mutase Inhibitors

2.1. Introduction

2.1.1. Drug Resistance and new antibiotics

Since the 1970s, only two new classes of antibiotics (Linezolid, and Daptomycin) have been introduced to the market.[30] However, infections due to resistant organisms have become much worse.[31,32] Therefore, there is an urgent need for new targets of antibiotics.

2.1.2. PurEs as a new target for antibiotic drug discovery

The *de novo* purine biosynthetic pathway could be a potential target for the development of new antibiotics because the pathway is different between humans and bacteria.[5] As mentioned in the first part of the thesis, Class II PurEs, found in humans, directly catalyze the conversion of AIR to CAIR, while Class I PurE, found in bacteria, turns N^5-CAIR to CAIR.[6] While Class I and Class II PurEs are similar, they each catalyze their own reaction and selective inhibition by NAIR suggests that small molecules can be found which can selectively inhibit one class over other.[33] Therefore, PurEs could be a new antimicrobial drug target.

2.1.3. High-throughput screening

High-throughput screening (HTS) is utilized in drug discover, because it can screen thousands of compounds in a short period and can provide

potential “hits” for follow-up studies.[34] Critical to the success of a HTS is a robust and rapid assay which is capable of detecting enzyme catalysis in a small volume with great sensitivity. A HTS assay must be rapid, sensitive and free from interference by other parts of the assay. A number of techniques have been used for HTS assay mostly fluorescence, absorbance, FACS and even imaging.[35]

2.1.4. Limitation of CAIR decarboxylase assay

Since no small molecule non-nucleotide inhibitors are known for N^5-CAIR mutase, the Firestine lab conducted a HTS based on the CAIR decarboxylase assay. While the assay worked, only one selective inhibitor was found and the assay suffered from false negatives. The possible reason for the unsuccessful HTS for N^5-CAIR mutase inhibitors is the limitations of the CAIR decarboxylase assay.[12] This assay is based on observing an absorbance change of CAIR at 260nm. Unfortunately, many organic small molecules also absorb in this region. To circumvent this problem, the absorbance of the compound was subtracted from each well. However, for highly absorbent compounds, this often lead to concerns over measuring the small change in CAIR absorbance due to catalysis. The potential inhibitors might be missed during the HTS. It is clear that a better HTS assay is needed for screening for inhibitors of N^5-CAIR mutase. In this chapter, we will discuss the assessment of several potential assays for HTS of N^5-CAIR mutase.[36]

2.2. Results and discussion

2.2.1. AIR carboxylation assay

Another classic assay for the study of PurE activity besides CAIR decarboxylation is the coupled N^5-CAIR mutase assay which measures the conversion of N^5-CAIR to CAIR by the production of SAICAR. This assay starts with AIR which is rapidly converted to N^5-CAIR by PurK. Conversion of N^5-CAIR to CAIR is monitored by SAICAR synthetase at 282 nm or at 340 nm for ATP assumption.[10,13] However, this assay not appropriate for HTS as several enzymes (PurK, PurC, PK, LDH and NADH) are involved and, therefore, it is hard to tell if the hits generated from HTS inhibit PurE the coupling enzymes. Thus, extensive deconvolution would be needed limiting its usefulness for HTS.

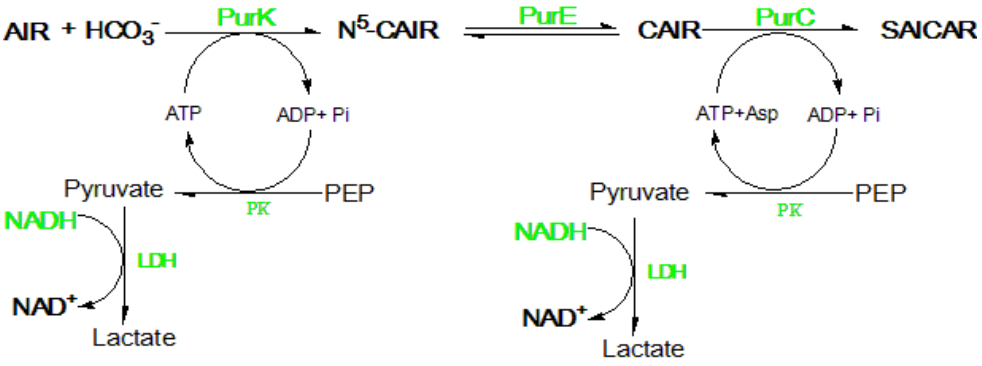


Figure 12 Procedure of PurC coupled assay.

2.2.2. Thermoflour®

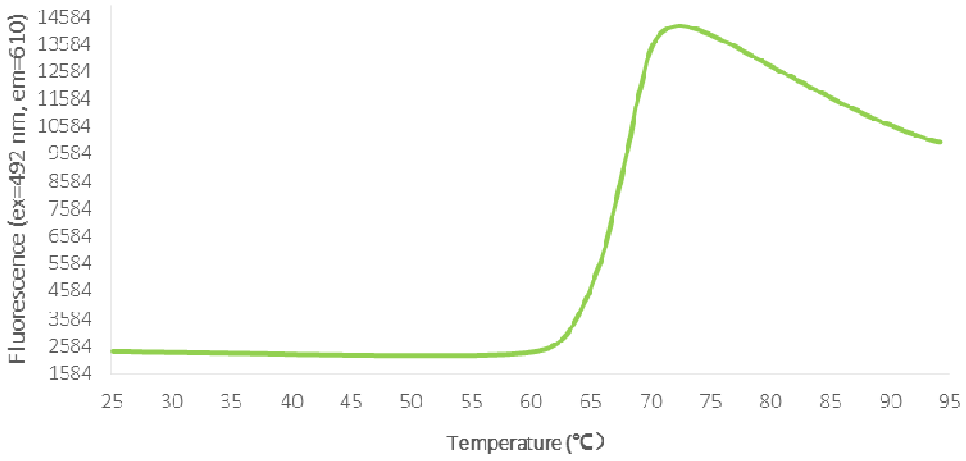


Figure 13 Fluorescence intensity versus temperature for unfolding PurEs in the presence of SYPRO orange (ex=492 nm, em=610 nm).

The thermoflour® assay is based on measuring the changes in the protein melting temperature (Tm) upon the binding of a ligand (inhibitor).[37] Previous studies have shown that binding of small molecules can alter a protein's melting temperature, by either stabilizing or destabilizing the folding of the protein.[38] Therefore, compounds which bind to Class I PurEs could theoretically be screened by this method.

For PurE, we followed the standard assay determined by Nisesn, et al.[39] SYPRO orange (ex=492 nm, em=610 nm) is a fluorescent protein dye whose fluorescence increases when a protein unfolds. We utilized a real-time (RT)-PCR instrument to gradually increase the temperature from 25 to 95ºC, while monitoring the fluorescence. The Tm was determined from the melting curve by calculating the maximum of the first derivative.

The melting curve of E. coli PurE is showed on ***Figure 13***. PurE is stable until approximately 60 ºC at which point the fluorescence intensity increases rapidly due to the unfolding of PurE. The signal decrease from 75 ºC to 95 ºC is due to protein precipitation or aggregation. The calculated Tm of PurE is 68.12± 0.03 ℃, as determined by 8 replicate experiments.

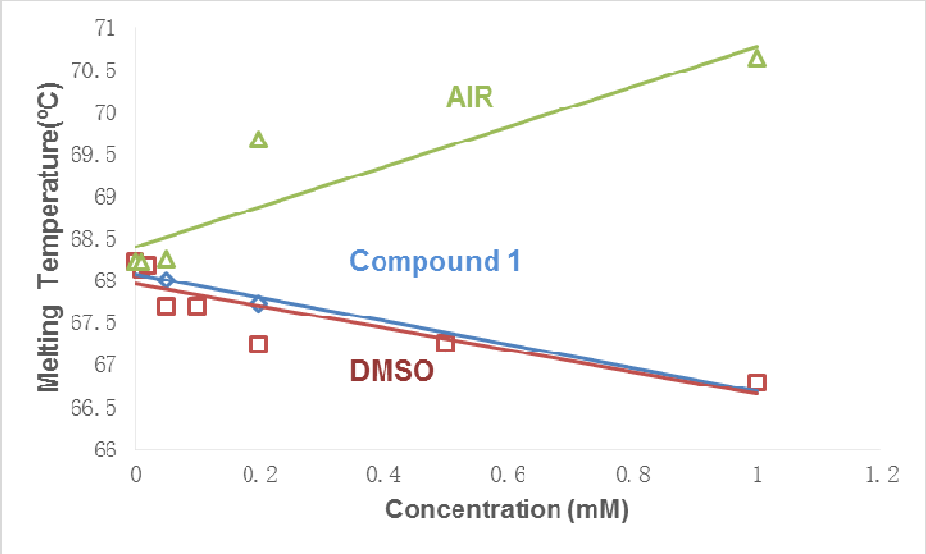


Figure 14 The melting temperature versus the concentration of compounds. 75 µg/ml PurEs incubated with different concentration (0.01 –1 mM) of compounds. Green line is AIR; Blue compound 1 (IC_{50}=150 µM); Red line is DMSO.

We next investigated the affect on the Tm as a function of inhibitor concentration. ***Figure 14*** shows the Tm versus compound concentration. AIR was used as a positive control, because previous studies have shown that it binds to PurE.[14] As expected, the fluorescence intensity increase as the concentration of AIR increased. Compound 1 {1-(4-ethoxyphenyl)-3-[(3-

pyridinylmethyl) amino]-2,5-pyrrolidinedione}studied as these were identified as binders in a fragment screening study for PurE inhibitors.[40] Preliminary experiments indicated that DMSO could affect the Tm, and the two compounds used in this experiment were dissolved in 100% DMSO. Therefore, we also measured the change in Tm at DMSO concentrations equivalent to what would be found for these two compounds. As shown in ***Figure 14***, compound 1 and DMSO show an identical change. This indicates that both of the compounds have no significant affect on the PurE Tm change.

Why did we fail to measure a change in Tm for compounds which have been shown to bind to PurE? One possible reason is that the binding between PurE and these two compounds is not tight enough. Another possibility is that the Tm of PurE is fairly high, thus changes in the stability of the protein are small and so difficult to measure. Alternately, at high temperature, binding of the compound to the enzyme is poor due to the deformation of the binding site. Ultimately, despite much effort, we were unable to find a condition that allowed us to detect binding of various compounds by alternation in Tm. Therefore we abandoned this assay.

2.2.3. Metal stopping assay

2.2.3.1. Assay design and hypothesis

Previous studies indicated that metal cations can chelate to CAIR so tightly that it stops PurE catalysis.[41] The tight binding of metals (such as Ni^{+} and Zn^{2+}) to CAIR suggested that this property may be useful to determine the concentration of CAIR, which in turn, could lead to a suitable assay for PurE.

Two questions need to be answered before such an assay can be developed: which metal to use and how to detect the CAIR:metal concentration. Previous studies indicated that Ni^{+} and Zn^{2+} tightly bind to CAIR and, therefore stop the reaction. Zn^{2+} was chosen for our experiments as the previous studies showed that a CAIR:Ni^{+} complex had poor kinetic stability.[41] To measure the concentration of CAIR-Zn^{2+}, we utilized the Zn fluorescent dye, TSQ. When TSQ is incubated with Zn^{2+}, a 1:2 complex is formed which is fluorescent (ex=360nm, em=490nm).[42,43] In the presence of a high CAIR concentration, zinc would bind to CAIR instead of TSQ and the fluorescence would decrease. Such a competition assay between TSQ, CAIR and Zn^{2+} could provide a new assay for PurE. As CAIR decreases, due to PurE activity, more Zn^{2+} would be available to bind to TSQ and the fluorescent signal will increase. Using this method, we can calculate how much CAIR is converted by the enzyme, and, therefore, deduce the kinetic data for the enzyme. Ultimately, this assay may be applicable for HTS.

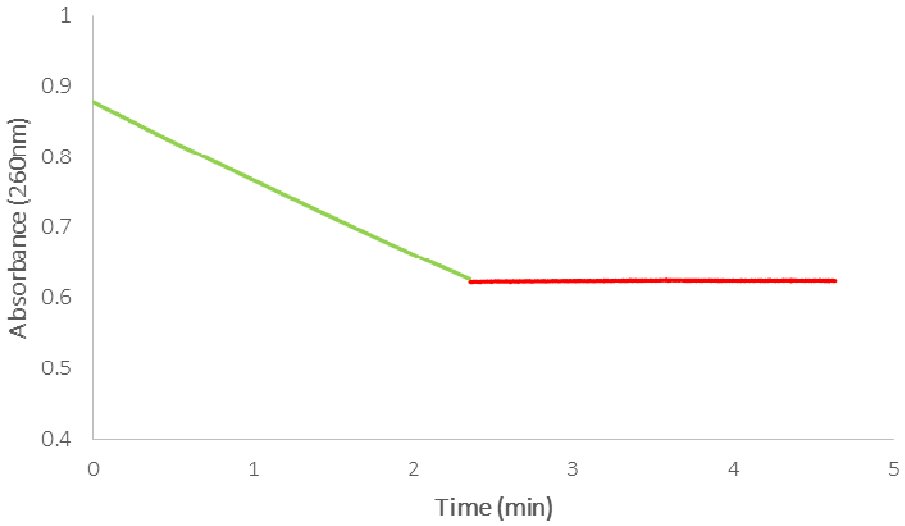


Figure 15. Zn^{2+} stops PurE from converting CAIR to AIR. Green line is 60ng PurE incubated with 130 µM CAIR at room temperature; red Line is when 250 µM ZnSO4 added.

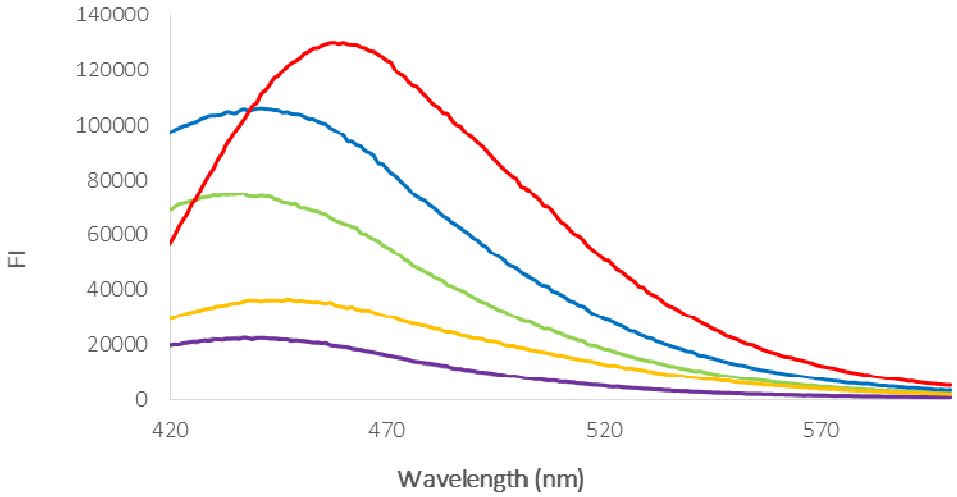


Figure 16. Fluorescent signal scan (ex=360 nm) for Zinc, AIR and CAIR. Grey line is the buffer (50mM Tris-HCl, PH7), Purple line is 250 µM $ZnSO_4$ in buffer; orange line is 250 µM CAIR in buffer; Red Line is 250 µM $ZnSO_4$ with 250 µM CAIR in buffer; green line is 250 µM AIR; and blue line is 250 µM AIR with 250 µM $ZnSO_4$.

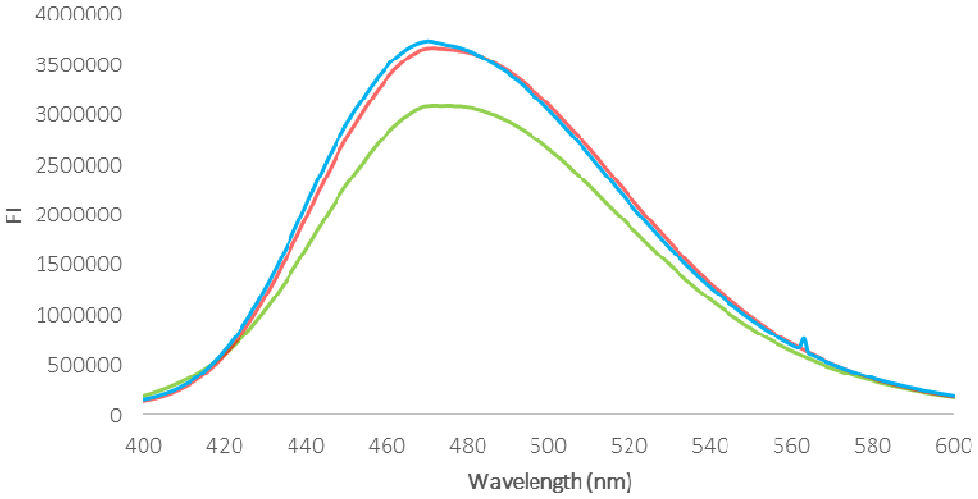


Figure 17. Wavelength versus fluorescence (ex=360 nm). Green line is 100 µM $ZnSO_4$ incubated with 50 µM TSQ; Red line is when 50 µM CAIR was added into solution; Blue line is 550 µM CAIR was added.

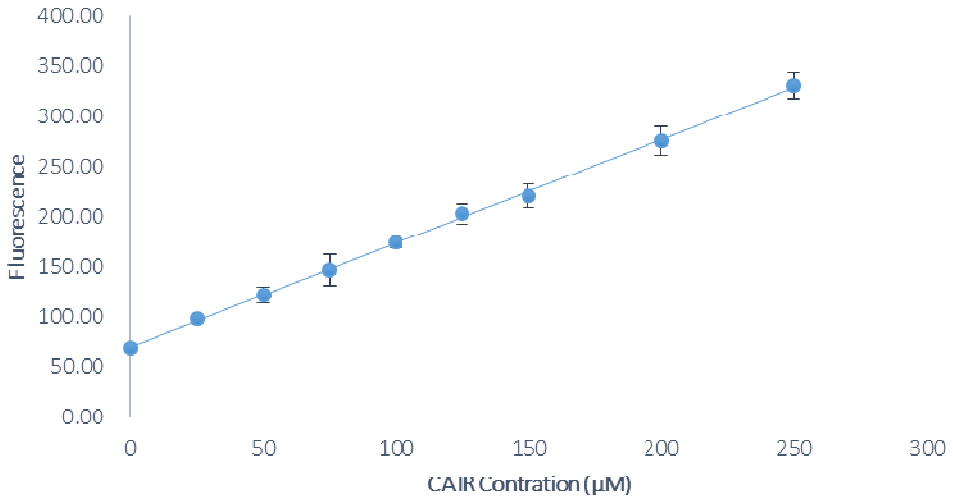


Figure 18. CAIR concentration versus Fluorescence (ex=360 nm, em=485 nm). CAIR from 0 to 250 µM incubated with 250 µM $ZnSO_4$ in 96 wells plate. Each concentration of CAIR has 8 duplicates.

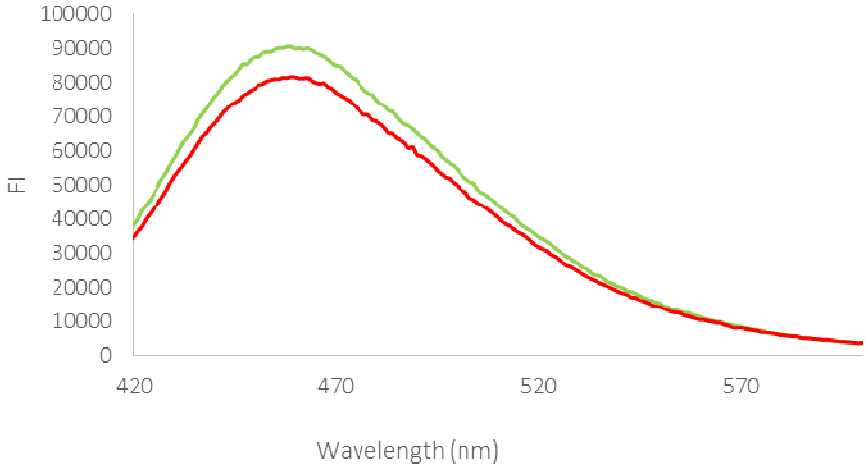


Figure 19 Fluorescent signal scan (ex=360nm) with PurEs. Green line represent that 250 µM CAIR incubated with 250 µM $ZnSO_4$, Red line is when 250 µM CAIR incubated with 3 µg E.*coli* PurE for 5 mins, 250 µM $ZnSO_4$ was added to the mixture.

2.2.3.2. Experiments and results

Previous studies showed that 250 µm $ZnSO_4$ totally stops E.*coli* PurE from converting CAIR to N^5-CAIR.[41] As shown in ***Figure 15***, once Zn^{2+} is added, there is an immediate end to the PurE catalyzed conversion of CAIR to N^5-CAIR. Therefore, we utilized this condition.

Incubation of TSQ with Zn^{2+} gave the expected fluorescent signal. However, addition of CAIR failed to decrease the signal. Therefore, we checked all compounds used in this assay and interestingly, found that Zn^{2+} plus CAIR generated a strong fluorescent signal at 460nm (***Figure 16***). This signal is close to Zn^{2+}:TSQ complex fluorescent peak. Thus, we found that the Zn^{2+}:CAIR complex interfered with the monitoring of Zn^{2+}:TSQ complex formation.

Based on these results, we redesigned the assay, using the Zn^{2+}:CAIR fluorescent signal to calculate the CAIR concentration. As shown in ***Figure 16*** and **Figure 18**, as the concentration of CAIR increases the fluorescence increases linearly. This provides an easy method to calculate CAIR concentration. In order for this PurE assay to work, AIR must not form a strong complex with Zn^{2+} nor must it generate a strong fluorescent signal. As showed in ***Figure 16***, incubation AIR with Zn^{2+} yielded a fluorescent signal, but one with an intensity much lower than CAIR. In addition, the emission peaks are different between these two complexes; the peak of AIR complex is about 439 nm while the CAIR complex peak shifts to 459 nm.

Despite this preliminary results, experiments with PurE showed that the assay is not sensitive. As shown in ***Figure 19***, the fluorescent signal change can only be observed when a large amount of PurE is added. The amount of PurE needed (3 µg) is about 60 times that used in the decarboxylation assay. This would require a large amount of enzyme for HTS. In addition, the small change in signal could make the assay difficult to use in a screening system where only a modest inhibition is frequently observed. Since the assay is not sensitive, we decided not to continue to work on this method.

Chapter 3

Materials and Methods

3.1 Materials

All chemical compounds come from Fisher, Sigma-Aldrich, or Thermo Scientific. E.coli PurE mutants were prepared in pET-21 with a C-terminal his-tag and contained additional sequences CTG GAG CAC CAC CAC CAC CAC CAC. These plasmids were cloned and amplified by Aiko Hirata. Sequences were verified by the DNA Sequencing Lab at Wayne State University. BL21 (DE3) was obtained from New England Biolab Inc.. E.coli PurC was the kind gift of Dr. T. Joesph Kappock (Purdue University, West Lafayette). Wild-type E. coli PurK was expression and purified by Jordan Rantucci.

All UV assays were conducted on a Cary 100 UV-vis spectrophotometer equipped with a temperature controller. Fluorescence was done using the Fluoro MAX®-3 with FluorEssence™ software. Circular Dichroism (CD） was conducted using Jasco J-1500 high performance CD spectrometer and access to this instrument was kindly provided by Dr. Timothy Stemmler. Thermal shift assays were conducted using the Mx3005P qPCR System and access to this instrument was kindly provided by Dr. Olivia Merkel.

3.2 Expression and purification of WT and mutated PurEs

E.coli BL21/(DE3) was transformed with plasmids containing mutants or wild-type PurEs cloned into pET-21. A single colony was incubated overnight at 37 ºC in 5 ml LB medium with 50 mg/L ampicillin and the overnight culture was

diluted (1:100) into 500 mL LB medium with 50 mg/L ampicillin and incubated until an OD_{600} of 0.5 was obtained. Protein expression was induced by the addition of 0.1 M IPTG and incubated for an additional 4 hours. Cells were centrifuged (20,000 rpm) and stored at -20 ºC until use.

For purification, bacterial cells were lyzed by addition of B-PER reagent (4 mL per gram of pellet, Pierce Biotechnologies) and incubated for 10 min. The resulting suspension was centrifuged at 20,000 rpm for 60 min (Beckman ultra-high-speed centrifuge, rotor JA-20) to remove cell debris. The clear supernatant was treated with streptomycin (5 mg/mL) and incubated for 2 hours before precipitated nucleic acids were removed by centrifugation at 20,000 rpm for 60 min. The supernatant was loaded, at 4 ºC, onto a column (10 mL) containing Cobalt RAPID RUN™ Agarose Beads (Gold Biotechnology) which had previously been pre-conditioned with buffer A (10mM sodium phosphate, 300 mM sodium chloride, and 10 mM imidazole, pH 7.4). The column was successively washed, at 4ºC, with Buffers A-D (Buffer A containing either 50 mM (B), 150mM (C), or 400 mM (D) imidazole). Fractions were collected and the PurE proteins eluted with buffer D. The purity of the protein was checked with 15% denatured SDS-PAGE. Purified PurE was dialyzed against 10 mM Tris-HCl, 200 mM NaCl, pH 8 and concentrated to at least 1 mg/mL using an Amicon® centrifugal concentrator. The protein was stored in -80 ºC. Protein concentrations were determined using the Coommassie

(Bradford) Protein Assay Kit following the manufactures instructions (Thermo Scientific).[36]

3.3 Limited Proteolysis

Limited proteolysis was conducted according to previous methods. (18) Briefly, wild type or mutant PurEs (18 µg) were added to 30 µL of buffer (50 mM Tris-HCl, pH 8.0) and 0.18 µg of subtilisin (from Bacillus licheniformis) was added. The reaction was incubated on ice for 1 hour at which time it was quenched by the addition of 1 mM PMSF. Products of the proteolysis were examined using a 15% denatured SDS-PAGE stained with Commassie Blue.

3.4 Circular Dichroism.

Wild-type or mutant PurE was dissolved in buffer ($NaPO_4$ 100mM, pH 7.4) to a final concentration of 1 mg/ml. The protein sample (70-100 µL) was loaded in a 0.2 mm path length cuvette and the CD spectrum was obtained at room temperature for far UV (190-250nm) and near UV (260-320nm).[44]

3.5 Steady-state kinetic

The assay contained 100 mM Tris-HCl (pH8), 6.25-150 µM CAIR and varied amounts of WT (48ng) or mutant PurEs (0.2-0.6 µg) in final volume of 1mL in a 1cm path length cuvette. (12) The consumption of CAIR was monitored at 260nm at room temperature. A plot of initial velocity versus substrate concentration was generated and the data was fitted to the Michaelis Menten equation **(1)** and kinetic parameters were calculated by Kaleidagraph (Synergy).[22]

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \qquad \textbf{(1)}$$

3.6 CO_2-dependence assay

To a stopper-fitted cuvette containing 100 mM Tris-HCl, pH 8, 3U of WT or mutant PurEs was added along with 125 µM AIR. The reaction was incubated at 10 ºC for a min. In some experiment, 1µg/mL carbonic anhydrase was also added. Reaction was initiated by addition of 20 mM CO_2 or $NaHCO_3$. CO_2 solutions were prepared by continuous bubbling CO_2 into 100mL distilled de-ionized H_2O at 4ºC and the concentration was estimated by the solubility constant of CO_2 (0.2871g/100g of water).[13]

3.7 PurC coupled assay.

A cuvette with a 1mL final volume containing 100 mM HEPES, pH 7.5 buffer, 20 mM KCl, 6 mM $MgCl_2$, 2 mM PEP, 100 µM ATP, 10mM aspartic acid, 4 U of pyruvate kinase, 17 U lactate dehayrogenase and 100 µM NADH was incubated at 37 ºC for 5 minutes. To this, E. *coli* PurC (13.5 µg) was added along with PurK (0 or 11.5 µg) and WT or mutant PurE (6U). The reaction was initiated by adding 60 µM AIR and SAICAR synthesis was monitored at 282 nm.[9,10,45]

3.8 Thermoflour®

In a 0.2 mL PCR tube, 10 mM HEPES (pH7.5) buffer was added along with150 mM NaCL, 5X SYPRO Orange, and 75 µg/ml of E.*coli* PurE, (0-1mM) AIR or, 1-(4-ethoxyphenyl)-3-[(3-pyridinylmethyl)amino]-2,5-pyrrolidinedione

(ChemBridge Corporation). The final volume was 30 µL, Thermal melting was done using a RT-PCR instrument from 25-95 ºC while monitoring fluorescence of SYPRO orange (ex=492 nm, em=610 nm). The melting temperature was calculated from the maximum of first derivate curve.[39] For studies of ligand induced changed in the Tm, the same experiment was repeated in the presence of various concentrations of ligand.

3.9 Metal stopping assay.

In a final volume of 1mL containing 50 mM Tris-HCl (pH 7) buffer, 250 µM CAIR or AIR was added along with E.coli PurE (0-3 µg) and the reaction was incubated at room temperature for 10 minutes. The reaction was quenched by the addition of 250 µM $ZnSO_4$ and fluorescence was determined from 400-600 nm while being excited at 360nm. The same concentration and method was applied to a 96-well microplate using a 100 µL per well final volume. Fluorescence data was collected using the Multi-Mode Microplate Reader (Synergy) with the excitation filter set at 360 nm and the emission filter set at 485 nm.[41]

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ABSTRACT

Site-Directed Mutagenesis of Conserved Amino Acids Residues in N^5-Carboxyaminoimidazole Ribonucleotide Mutase
&
Developing a High-Throughput Screen Assay for the Discovery of N^5-Carboxyaminoimidazole Ribonucleotide Mutase Inhibitors

by

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July 2014

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The *de novo* purine biosynthesis pathway plays a critical role in providing new purines to the cell. Previous studies have shown differences between the human and bacterial pathways which suggests that pathway may be a potential target for antibiotic drug discovery. Three critical enzymes are involved in the pathway divergence. In the bacterial pathway, N^5-CAIR synthetase (PurK) first converts AIR to N^5-CAIR, which is then transformed into CAIR catalyzed by the enzyme N^5-CAIR mutase (Class I PurE). In the human pathway, AIR carboxylase (Class II PurE) catalyzes the direct conversion of AIR to CAIR. Class I and Class II PurEs have structure and sequence similarities but also are functionally different. However, the residues responsible for these differences are unknown. In this study, we hypnotized that the class-specific conserved residues in the active site might be the key to

determining the functional differences between the two PurEs. Site-directed mutagenesis was used to convert residues of Class I PurEs to Class II PurEs. Several mutant PurEs were made and examined for CO_2-dependent conversion of AIR to CAIR. Two mutants H71A and H71G displayed CO_2 dependent that was similar to that observed for AIR carboxylase. Additional studies revealed that these enzymes also were capable of using N^5-CAIR as a substrate. This indicates that the H71A and H71G mutant have AIR carboxylase as well as N^5-CAIR mutase activity. Examination of the crystal structure of N^5-CAIR mutase indicates that His71 is at the bottom of the active site and removal of this residue creates a channel between active sites in two subunits. We speculate that this channel may be key for the utilization of CO_2.

The second project focused on developing an appropriate high-throughput screening (HTS) assay for Class I PurE inhibitor. Existing assays have significant issue for HTS. We examined both a thermo shift assay as well as a metal stopping/fluorescence assay. While both assays had some potential issues for analysis indicated that neither assay was suitable for HTS.

AUTOBIOGRAPHICAL STATEMENT

EDUCATION

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PROJECTS

1. Site-Directed Mutagenesis of Conserved Amino Acids Residues in N^5-Carboxyaminoimidazole Ribonucleotide Mutase: converting N^5-CAIR mutase into Aminoimidazole Ribonnucleotide carboxylase
2. Developing a High-Throughput Screening Assay for the Discovery of N5-Carboxyaminoimidazole Ribonucleotide Mutase Inhibitors