Pure & Appl. Chem., Vol. 68, No. 11, pp. 2029–2036, 1996.
Printed in Great Britain.

The transformylase enzymes of *de novo* purine biosynthesis

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ABSTRACT: Formyl transfer reactions play a key role in the construction of the purine heterocycle during *de novo* purine biosynthesis. Formylation is catalyzed early in the pathway by the *pur*N glycinamide ribonucleotide transformylase (GAR Transformylase, EC 2.1.2.2) in a tetrahydrofolate-dependent manner and also by the *pur*T GAR transformylase in a tetrahydrofolate-independent manner in bacteria. Late in the pathway, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (AICAR Transformylase, EC 2.1.2.3) catalyzes the second and final formylation involved in purine nucleotide biosynthesis. This article summarizes the salient properties and mechanistic knowledge on the transformylases with special emphasis on the mechanism of the *pur*N GAR transformylase as explored by mutagenesis studies.

Introduction

The *de novo* purine biosynthetic pathway produces purine nucleotides that are essential for many processes in the cell. Purines serve as building blocks in DNA and RNA synthesis, are utilized as an energy source for chemical reactions (ATP), are used in cellular redox reactions (NADH, NADPH, FAD, etc.), and also play key roles in regulatory functions (cAMP, ZTP, etc.). Virtually all organisms studied to date utilize this pathway to synthesize purines, with the exception of parasitic protozoa (1) which must scavenge purines from their environment.

The overall *de novo* purine biosynthetic pathway consists of ten enzymatic reactions which serve to convert 5-phosphoribosyl-1-pyrophosphate to inosine monophosphate, which can then be converted to adenosine monophosphate and guanine monophosphate. These reactions are invariant in all organisms synthesizing purines, although the organization and regulation may differ (2). Generally, prokaryotes tend to use smaller single function enzymes, while higher eukaryotic organisms place increased reliance on larger multifunctional enzymes in this pathway (2).

Because cancer cells grow rapidly and require large amounts of purines to maintain such growth, the *de novo* purine biosynthetic pathway has attracted considerable attention as a target for cancer chemotherapy (3). Some of the most successful antiproliferative drugs developed to date have been folate antimetabolites. Two of the enzymes in this pathway require a reduced folate, and are thus natural targets for screening novel antifolates. These enzymes, glycinamide ribonucleotide transformylase (GAR Transformylase, EC 2.1.2.2) and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (AICAR Transformylase, EC 2.1.2.3) catalyze the third and ninth reactions of this pathway. Both of these enzymes are involved in formyl transfer reactions, and both use 10-formyl tetrahydrofolate as a cofactor. These two enzymes are products of the *pur*N and *pur*H genes in *Escherichia coli*.

Recently, a second glycinamide ribonucleotide transformylase enzyme was isolated and characterized from *E. coli* (4, 5). This enzyme is the product of the *pur*T gene and does not utilize a folate cofactor. The *pur*N and *pur*T enzymes both catalyze the formylation of β-glycinamide ribonucleotide (GAR) to produce formyl β-glycinamide ribonucleotide (fGAR), however, they do so using different cofactors and different mechanisms. The reactions catalyzed by the three transformylases of *de novo* purine biosynthesis are shown in Fig. 1.

Physical properties

The *E. coli pur*N GAR transformylase is a monomeric protein of 212 amino acids with a molecular weight of 23,200. The homologous enzyme in humans is a much larger trifunctional polypeptide encoding GAR synthase (EC 6.3.4.13) and aminoimidazole ribonucleotide synthetase (EC 6.3.3.1) activities in addition to a GAR transformylase activity (6). Sequence homology and mutagenesis of catalytic residues suggests that there is a substantial degree of mechanistic similarity between the human and *E. coli* enzymes (6-9). High resolution x-ray crystal structures of the *E. coli pur*N GAR Transformylase have been reported in the absence of ligands (10), in a ternary complex with substrate GAR and a folate inhibitor (11), and in a binary complex with a multisubstrate adduct inhibitor bound (12). These results have shown that the enzyme structure is composed of two domains. The amino terminal domain (residues 1 to 101) contains a

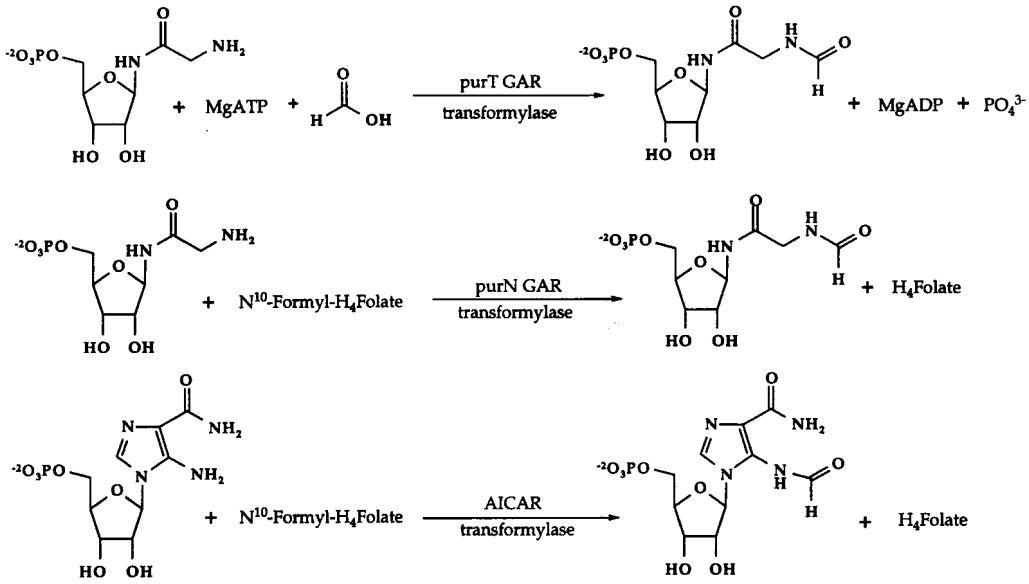


Fig. 1: The formyl transfer reactions of *de novo* purine biosynthesis.

mononucleotide binding fold, consisting of a four stranded parallel β-sheet flanked on both sides by two pairs of α-helixes. The carboxyl terminal domain (residues 102 to 212) initially continues the parallel β-sheet and then adds one antiparallel strand. This mixed β-sheet is shielded on one side by a long α-helix of 27 residues (12).

The *E. coli pur*T GAR transformylase is also a monomeric protein, consisting of 392 amino acids and having a molecular weight of 42,400 (4,5). This *pur*T enzyme is nearly twice the size of the *pur*N GAR transformylase, however, the two GAR transformylase enzymes display no significant sequence homology. The *pur*T enzyme does display a substantial amount of homology to another purine biosynthetic enzyme, the *pur*K-encoded aminoimidazole ribonucleotide carboxylase, with 27% of the residues identical and a total of 55% of the residues conserved between the two enzymes. Currently, no structural information is available for the *pur*T GAR transformylase, however, attempts at crystallization are currently underway.

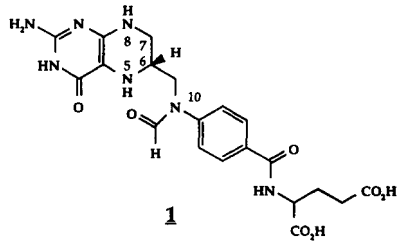
AICAR transformylase has been isolated from a number of eukaryotic sources (13-18), but the protein from chicken liver is the best characterized (19). The avian enzyme is actually a bifunctional protein that contains both the AICAR transformylase and inosine monophosphate cyclohydrolase activities. The cyclohydrolase activity catalyzes the cyclization and dehydration of the formylated product of the transformylase, FAICAR, to yield inosine monophosphate. Inosine monophosphate contains an intact purine nucleus, and is converted in subsequent reactions to adenine and guanine monophosphate. The protein has a molecular weight of about 125,000 and consists of two identical subunits (20). Determination of the crystal structure of AICAR transformylase is currently in progress (21). The amino acid sequence of the avian enzyme is 36% identical to the bacterial *pur*H-encoded enzymes from *B. subtilis* and *E. coli* (22). This indicates significant conservation of structure between the bacterial and chicken proteins.

Although the reaction catalyzed by AICAR transformylase is analogous to that catalyzed by the *pur*N GAR transformylase, the two enzymes share no sequence homology (22). In fact, the putative folate-binding sequence of GAR transformylase, which was identified using a dideazafolate affinity label (23), is not present in AICAR transformylase. Therefore, it will be interesting to determine how two active sites, which apparently contain different amino acids, can catalyze very similar reactions. Investigation of the key sequences of AICAR transformylase might allow identification of a new folate-binding motif. The differences between these two folate-requiring enzymes can also be taken advantage of in the design of selective enzyme inhibitors.

Cofactor Requirements

AICAR transformylase and the *pur*N-encoded GAR transformylase both require 10-formyltetrahydrofolate **1** as a cofactor,where the naturally ocurring configuration at C6 is *R* (19,24). GAR transformylase is actually inhibited by the 6(*S*) isomer with a K_i = 0.75 μM (for the 6(*R*) isomer, K_m= 6.8 μM). The unnatural *S* isomer does not serve as a cofactor or an inhibitor for AICAR transformylase. It has been found that the natural tetrahydrofolate cofactor can be replaced by fully oxidized synthetic analogs for both enzymes (25). GAR transformylase was found to efficiently utilize 10-formyl-5,8-dideazafolate **2** as a substitute formyl donor at 77% V_{max} relative to the tetrahydrofolate cofactor. The Michaelis constant is 1.9

μM for this oxidized analog. However, the dideazafolate did not serve as a cofactor for AICAR transformylase and actually behaved as an inhibitor with a K_i = 29 μM relative to the K_m= 68 μM for the natural cofactor. AICAR transformylase will use 10-formyl-8-deazafolate **3** as a cofactor analog at 47% V_{max} relative to the natural cofactor. In contrast, GAR transformylase does not function efficiently with the 8-deazafolate compound. This striking difference in analog preference for the two transformylases can be used to selectively assay one enzyme in the presence of the other or in the design of inhibitors specific for only one of the two transformylases. It should be noted that both of the useful cofactor analogs contain stable aromatic ring structures which are not subject to the aerobic oxidation that must be considered carefully when conducting experiments with the tetrahydrofolate compound. Due to its oxidative instabilty, the natural cofactor must also be prepared immediatley before use whereas the aromatic analogs can be prepared and stored indefinitely. The aromatic analogs also eliminate concern about the stereochemistry at the C6 position. As a result, the aromatic cofactor analogs are useful tools for mechanistic studies involving the two transformylases.



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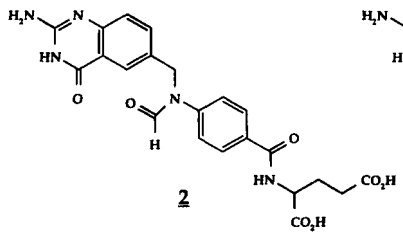

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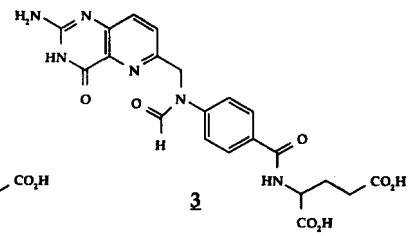

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The *pur*T-encoded GAR transformylase requires GAR, ATP and formate as substrates. GAR is provided by the previous enzymes of the *de novo* pathway and ATP is readily available within the cell. However, at present, the source of the formate for this enzyme has not been established. The recently discovered *pur*U-encoded 10-formyltetrahydrofolate hydrolase produces formate by hydrolysis of formyltetrahydrofolate. The amino acid sequence of this enzyme is 31% similar to the *pur*N GAR transformylase, but lacks the GAR binding site at the N-terminus. (26, 27) It is possible that this enzyme functions with the *pur*T protein as the formate provider and this proposal is presently under investigation.

Active Site of *pur*N

The crystal structures of the *E. coli pur*N GAR transformylase have revealed a mostly hydrophobic binding site, with a few polar residues interacting with bound ligands. As shown in Fig. 2, three of these residues (N106, H108, and D144) are positioned such that they may assist in the formyl transfer event. In order to investigate the roles of these three amino acids, saturation site directed mutagenesis was used in order to change each one of these residues to a replacement set of amino acids (9). Between 14-17 different mutations of each residue were obtained, encompassing a range of conservative and non-conservative alterations. Limiting mutagenesis studies to only a single mutation may give misleading results about its mechanistic importance (28). By investigating an extensive range of mutations at each position, the shortcomings of studying a single mutation at any position can be avoided. However, investigating the effects each of nearly 50 different single mutants would confer upon protein function would be prohibitively time consuming. In order to overcome this challenge, a rapid screen using functional complementation of auxotrophic cells was used (9). Plasmids containing each of the mutant genes were introduced into *E. coli* cells in which both the native *pur*N and *pur*T genes had been inactivated. By plating these cells on purine deficient selective media, only those cells containing a functional GAR transformylase will be able to grow. This screen allows the mutants to be placed into three broad categories: (1) substantial activity, with growth rates comparable to that of cells containing the wild type gene; (2) significantly decreased activity, with much slower growth rates than cells containing the wild type gene; and (3) inactive, in which the mutant gene confers insufficient activity to allow enough purine biosynthesis for the cells to grow.

Complementation results show that no mutants at any of these three positions (N106, H108, D144) have substantial activity (9). However, a few mutations at each position do yield somewhat active proteins and most mutations at these positions result in inactive enzymes. Partially active mutants include not only conservative substitutions, but also some unexpectedly substantial alterations in size and polarity of the side

chains. One conclusion that can be drawn from the mutagenesis results is that none of the active site polar residues are absolutely essential for activity, although all three are important for full activity. These results are consistent with a direct transfer of the formyl group that would not require a key catalytic residue.

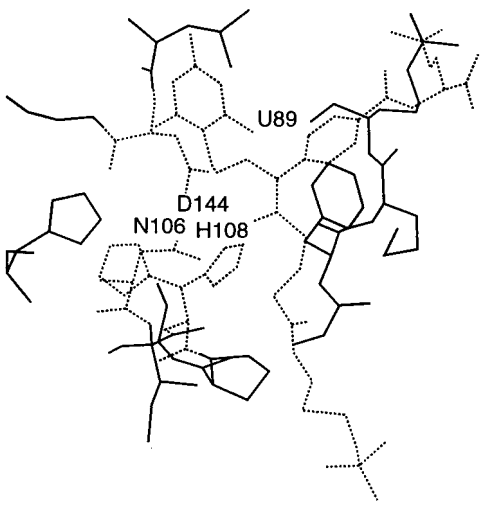

Fig. 2: Active site of PurN GAR Transformylase complexed with multisubstrate adduct inhibitor BW1476U89 (12). H108 resides in two distinct conformations, both are shown.

Mechanism of the Folate-Dependent Transformylases

The formylation reactions catalyzed by AICAR transformylase and the *pur*N-encoded GAR transformylase are believed to proceed via a direct transfer mechanism. This would implicate the involvement of a negatively-charged tetrahedral intermediate produced by attack of the amine group of GAR or AICAR on the formyl group of 10-formyltetrahydrofolate. There are several lines of experimental evidence that support this idea (19, 29). The obligatory formation of a cofactor amidine species (5,10-methenyltetrahydrofolate) in the transfer reaction is ruled out because aromatic cofactor analogs that cannot form amidine-type structures are still efficiently utilized by the enzymes. An enzyme-cofactor based amidinium intermediate, such as could arise from the nucleophilic attack of a side chain amine on the cofactor, also does not seem to be involved. Such a species would require the incorporation of solvent water to produce the final products (FGAR or FAICAR). Solvent incorporation into the products was monitored by attempting to observe an ^{18}O-induced isotope shift of the ^{13}C NMR resonance of the formyl carbon,which was enriched in ^{13}C. No ^{18}O was incorporated into either the FGAR or FAICAR produced. Kinetic analysis indicates a sequential reaction pattern for both enzymes (29). This is in contrast to other acyl-transfer processes which typically exhibit ping-pong kinetics and does not support a mechanism involving formylation of the enzyme by the cofactor followed by cofactor dissociation. However, these kinetic studies do not rule out the formation of a formylated enzyme species in which the cofactor is still bound. Such a formylated enzyme species was probed for using a hydroxylamine trap. No conditions were found in which the trapping reagent disrupted the formylations. A mechanism involving direct transfer of the formyl group seems to be the most reasonable based on the above experimental data. These findings are also consistent with mutagenesis studies on GAR transformylase that indicate that there are no absolutely essential catalytic residues.

A mechanism for GAR transformylase in which the active site polar residues are involved only in assisting formyl transfer and the accompanying proton switch (9) has been proposed. The progression of this mechanism is presented in Fig. 3. Nucleophilic attack by the amino group of GAR upon the formyl carbon of 10-formyl tetrahydrofolate would lead to the initial formation of structure **4**. Protonation of the alcohol group in **4** gives **5**. The required proton switch from the amino group of GAR probably proceeds through **6** and **7**, resulting in protonation of N^{10} to yield **8** and **9**. Finally, the tetrahedral intermediate **8/9** collapses, with cleavage of the formyl carbon - N^{10} bond, yielding the products formyl GAR and tetrahydrofolate.

In order to further delineate the role of the enzymic residues in the catalytic mechanism, pK_a estimates of these intermediates have been made using the methods of Fox & Jencks (30). These authors have estimated acidities of alcohols and aliphatic ammonium ions using $\rho\sigma$ structure reactivity relationships (31). Model compounds with known pK_a values are used for base values, and the alterations caused by the substituent groups are calculated. For the dissociation of alcohols and ammonium ions, $\rho_I = -8.4 \pm 1.0$ (30). The values of σ_I are 0.25 for hydroxyl groups, 0.10 for amine and 0.25 for amide substituents, with σ_I = 0.18 taken as an intermediate for methoxyamine groups (30). With these values available, we can calculate

the individual pK_a values in Fig. 3 to an accuracy of within approximately 1 pK unit. Details on the calculations of the pK_a values shown in Fig. 3 have been previously reported (9).

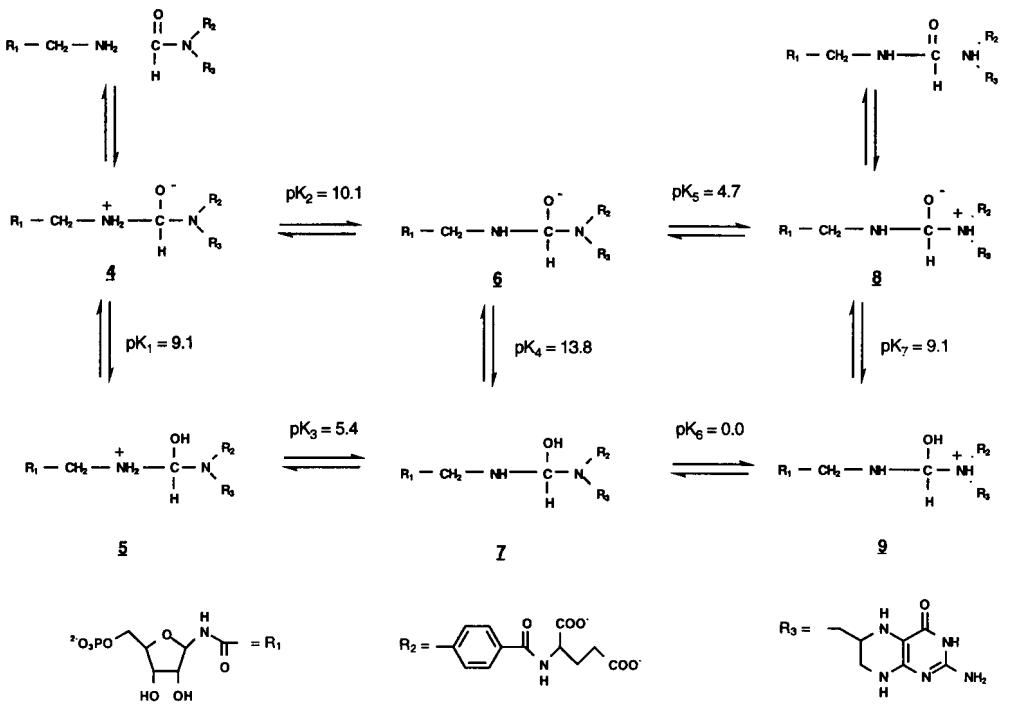

Fig. 3: Putative Mechanism of *pur*N GAR Transformylase

Although the overall transfer reaction is thermodynamically favorable, the relative levels of the key protonated intermediates (**8**/**9**) are unfavorable. The above analysis suggests that the proton switch from **4** would strongly favor the formation of **7**. At neutral pH, the levels of **6** and **9** relative to **7** are both disfavored by approximately a factor of 10^7. Therefore, after formation of **7**, very little of the desired intermediate **8** would be expected to exist under equilibrium conditions. In effect, **7** acts as a sort of "trap" in this scheme, impeding completion of the transfer process.

Based upon the high resolution (1.96Å) crystal structure of GAR Transformylase complexed with the multisubstrate adduct inhibitor BW1476U89 (12), enzymic groups N106, H108, and D144 are available to alter these pK_a values and shift the equilibrium in Fig. 3 towards products. This crystal structure presents two distinct active site conformations, either or both of which may be crucial for our mechanism. Overall, the mechanism requires two key events: Some enzymic group to stabilize **6**, shifting the equilibrium away from **7**, and, another residue to help shift the equilibrium from **6**/**7** towards **8**/**9**.

We have proposed that an uncharged H108, or N106, or perhaps both, are responsible for hydrogen bonding to the oxyanion in **6** (9). The two conformations seen in the crystal structure each show one of these two residues forming a hydrogen bond with the hydroxyl group of the inhibitor, analogous to the location of the formyl oxygen. It is possible that in the actual transition state of the reaction, both H108 and N106 hydrogen bond with the oxyanion in **6**. We have proposed that the neutral imidazole of H108, with a pK_a of ~14.5 (32), bonding to the oxyanion, would favor **6** over **7**. Similarly, the N106 side chain amide (pK_a~15), could also shift the equilibrium away from **7** towards **6**. Although an uncharged histidine serving as an acid is unusual, it is not unprecedented. In triosephosphate isomerase, the imidazole side chain of H95 acts as an acid, transferring a proton to an oxyanion intermediate (33). Asparagine sidechains have also been previously reported to stabilize oxyanions in subtilisin Carlsberg (34) and in subtilisin BPN' (35). In the case of GAR Transformylase, it is crucial that the catalytic species of H108 be the neutral imidazole and not the cationic imidazolium (pK_a~6). The imidazolium form would simply transfer a proton to species **6**, producing **7**.

The other enzymic group which plays a role in this pK_a scheme is D144. Although D144 does not hydrogen bond directly to N^{10} of the folate cofactor, it does interact through a bridging water molecule. This interaction would be expected to raise pK_5, stabilizing the protonated form of N^{10} (species **8**/**9**). Finally, to shift the equilibrium from **4**/**5** towards **6**/**7**, the lack of any group to hydrogen bond with the terminal amine of GAR may be important. The hydrophobic pocket may destabilize a charged species at this position, decreasing pK_2.

Our mutagenesis results support the mechanism shown in Fig. 3. Mutants of D144 would not be expected to stabilize a protonated N^{10} of the folate cofactor, shifting the equilibrium away from products. Mutants of either H108 or N106 would lead to decreased stabilization of the oxyanion of **6**, substantially shifting this equilibrium towards **7**. This shift would dramatically increase the difficulty of reaching **8**/**9**, as the reaction would have to overcome nearly a 10^5-fold higher barrier by going from **7** to **9** as compared to going from **6** to **8**.

Multiple Mutant Library of *pur*N GAR Transformylase

In order to further investigate these mutants, we have also constructed and screened a library of random mutants at all three positions N106, H108, and D144. This library was constructed in two steps. First, the PCR overlap extension method (36) was used to construct a library of D144 mutants using oligonucleotide primers having the 32-fold degenerate codon NNS (N = a mixture of all bases, S = a mixture of C and G only) in place of the wild type D144 codon. These 32 potential codons will encode all 20 possible amino acids as well as the amber termination codon. Upon completion of this library, sequencing revealed that the D144 codon had indeed been randomized. The plasmid mixture resulting from this library was then used as templates for a second round of mutagenesis. This time, oligonucleotide primers were designed to simultaneously randomize both N106 and H108 codons using NNS degenerate codons.

Upon completion of the mutagenesis, a plasmid library containing 32768 potential members was obtained encoding all possible combinations of amino acids at these three positions. Since each wild type residue is represented by only one codon, this library should contain only one possible wild type clone, present at a frequency of 1/32768. This library should also contain each possible "single mutant", in which two positions are represented by wild type codons while the third is a mutant codon. The frequency of single mutant clones should be 93/32768, or approximately 1/352. "Double mutant" clones in which one position has a wild type codon while the other two have mutant codons should occur at a frequency of 2883/32768, or approximately 1/11. Finally, the remaining "triple mutant" clones, in which no wild type codons are present, will occur in almost 91% of all clones.

In order to verify that the library had been successfully constructed, cells were transformed with this plasmid library and plated. Twenty random clones were picked, plasmids were purified from these clones, and each was sequenced through the mutation region. Sequencing results showed that twenty independent clones had been obtained, each having mutant codons at all three positions.

Screening this library by functional complementation and sequencing 120 clones that showed activity has thus far revealed only wild type and single mutants that have sufficient activity to demonstrate growth. No active clones were obtained that had mutations at any two (or three) of these positions. The library constructed only contains 88,000 independent clones, so it is probable that some mutant combinations are not represented in this library. We did not, in fact, obtain all possible single mutant clones known from previous studies (9) to be active. Only 50% of these clones were obtained, indicating that our library probably contains only about half of all possible amino acid combinations at positions 106, 108, and 144. However, from the results we have obtained so far, it seems likely that mutating any two of these residues sufficiently disrupts the stabilizations required during catalysis to eliminate the possibility of enough activity for complementation of auxotrophic cells. Although unlikely, we cannot yet conclusively rule out the possibility that a couple of double mutant combinations at these positions may have sufficient activity to complement auxotrophic cells.

Mechanism of the *pur*T GAR Transformylase

In contrast to the folate dependent *pur*N enzyme, the *pur*T enzyme does not bind any formylated folate cofactors. Instead, it utilizes formate and ATP as cofactors, transferring the formate to GAR, with a stoichiometry of 1:1:1. Studies on the cofactor specificity of the *pur*T enzyme have shown that no other NTP or formyl donors are capable of substituting for ATP or formate. A metal requirement for divalent Mg is somewhat less stringent, as Mn and Co are able to substitute for Mg, although these metals lead to decreased levels of activity (5).

Steady-state kinetic studies of the *pur*T enzyme have shown that the specific activity and $K_{m(GAR)}$ values are comparable to those of the *pur*N enzyme, indicating that both enzymes are biologically competent (5). Indeed, studies on both wild type and serine/glycine auxotrophic *E. coli* showed that formate was incorporated into the C8 position of purines in amounts ranging from 14% to 50% (37). These results demonstrate that both enzymes are likely to be active simultaneously, with the *pur*T enzyme contributing at least 14% and perhaps up to 50% of the total cellular GAR transformylase activity in *E. coli*.

A side reaction of the *pur*T enzyme has also been reported in which ATP is cleaved in the presence of acetate but in the absence of GAR (5). No comparable ATP cleavage in the presence of formate was detected unless GAR was also included. Trapping experiments detected the production of acetyl phosphate during the side reaction, giving some evidence that the catalytic mechanism of the *pur*T is to use formate and ATP to produce a formyl phosphate intermediate, which can then be used to transfer the formyl group to GAR. Similar trapping experiments using formate, ATP, and GAR failed to produce any formyl phosphate. However, several factors may prevent the detection of formyl phosphate even if it is produced by the enzyme. These include probable tighter binding of the kinetically competent formyl phosphate than of acetyl

phosphate, shorter solution half-life of formyl phosphate, and perhaps most importantly, the presence of GAR. Including GAR in the reaction probably causes essentially all of the formyl phosphate to react and produce fGAR before it could be released from the enzyme, while the acetyl phosphate will not react with GAR and is free to be released into solution.

In order to obtain additional evidence that the *pur*T enzyme uses a formyl phosphate intermediate, NMR experiments using ^{18}O labeled formate have been performed. If labeled formate does go through a formyl phosphate intermediate before being transferred to GAR, then one atom of ^{18}O should be transferred to the inorganic phosphate released from ATP. Such ^{18}O transfer can be tracked by monitoring the effect of this isotope on the ^{31}P NMR spectrum of the released inorganic phosphate (38). NMR studies have been performed and confirm that one atom of ^{18}O is indeed transferred to the released inorganic phosphate (5). However, there is another mechanistic possibility that would also be consistent with this result. A concerted reaction mechanism in which no acyl phosphate intermediate exists could also exhibit this type of result. In order to gain additional NMR evidence for the existence of a formyl phosphate intermediate, positional isotope exchange studies using γ-^{18}O labeled ATP are in progress. Exchange of ^{18}O from the β-γ bridge position to the β-nonbridge position would be indicative of the formation of an activated intermediate such as formyl phosphate. The exchange reaction can be monitored using ^{31}P NMR spectroscopy since substitution of ^{18}O for ^{16}O will shift the ^{31}P NMR resonance upfield by 0.02 ppm (38).

Perhaps the best evidence for the use of formyl phosphate as an intermediate in the *pur*T reaction comes from mutagenesis results. A G162I mutant shows slightly weaker binding of ATP and GAR, but substantially weaker binding of formate (39). Because of the weaker binding of formate, and presumably of formyl phosphate, trapping experiments showed that formyl phosphate was indeed being produced by this mutant enzyme. In the absence of GAR, this mutant enzyme readily uses ATP to convert either formate or acetate to the corresponding acyl phosphate (39).

We believe that the *pur*T GAR transformylase mechanism is sequential in nature, with formyl phosphate initially formed from ATP and formate, followed by a nucleophilic reaction between the GAR amine and the formyl group.

GAR Transformylases in other species

Analogs of the *E. coli pur*N enzyme in other species, including humans, are well known. However, until recently, the *E. coli pur*T had no known analogs in other species. We have used a variety of techniques, including functional complementation, PCR, and hybridization on DNA libraries from bacterial sources as well as chicken liver and human tissues (40). We have also assayed cellular homogenates from chicken liver and bacterial sources for the presence of PurT enzyme activity.

Assays of chicken liver homogenates did show an enzymic activity in which GAR was converted to fGAR in a reaction requiring the presence of formate and ATP. Extensive attempts at purifying this PurT activity failed, although size exclusion chromatography implicated an enzyme with molecular weight of approximately 140,000. We now believe that this activity was not due to a PurT type of enzyme, but rather the known PurN analog accompanied by or perhaps complexed with another enzyme, which was either capable of tightly binding 10-formyl tetrahydrofolate or producing it from formate and ATP. Assays of *Bacillus mycoides* and *Bacillus subtilis* lysates also demonstrated an enzyme activity analogous to the *E. coli* PurT.

Screening of human cDNA libraries by functional complementation yielded several clones possessing GAR transformylase activity (40). After checking these by PCR or sequencing, each positive clone was shown to be analogous to the *E. coli pur*N gene, no *pur*T analogs were ever isolated. These libraries were also screened by PCR, using degenerate oligos to portions of the *E. coli pur*N and *pur*T genes. Again, positive results were obtained for *pur*N analogs but no *pur*T analogs were ever isolated.

Screening of a *Bacillus subtilis* library by complementation also yielded several clones demonstrating GAR transformylase activity (40). These clones proved to not be limited to only *pur*N analogs, but also included novel sequence. Simultaneous screening by PCR using degenerate primers to both *E. coli pur*N and *pur*T genes also gave positive results for each. While this work was in progress, a report by Saxild and coworkers (41) appeared in which a *B. subtilis pur*T analog had been isolated and sequenced, which appears to be identical to the gene we were isolating.

It appears that genes analogous to the *E. coli pur*T are probably limited to bacteria, while higher organisms rely exclusively on *pur*N analogs. This may reflect an evolutionary change. It would be interesting to investigate GAR transformylases from the most ancient of organisms. The evolutionary pathway may have been to start with only PurT type of enzyme, eventually adding the folate dependent PurN in eubacteria, and finally discarding the PurT enzyme altogether in favor of the PurN enzyme in higher organisms.

Acknowledgments

This work was supported by the National Institutes of Health through PHS Grant GM24129 (S.J.B.) and postdoctoral fellowship GM16559-02 (K.M.M.).

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