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Formyltetrahydrofolate Hydrolase, a Regulatory Enzyme That Functions To Balance Pools of Tetrahydrofolate and One-Carbon Tetrahydrofolate Adducts in *Escherichia coli*†

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The enzyme encoded by *Escherichia coli purU* has been overproduced, purified, and characterized. The enzyme catalyzes the hydrolysis of 10-formyltetrahydrofolate (formyl-FH_4) to FH_4 and formate. Formyl-FH_4 hydrolase thus generates the formate that is used by *purT*-encoded 5′-phosphoribosylglycinamide transformylase for step three of de novo purine nucleotide synthesis. Formyl-FH_4 hydrolase, a hexamer with 32-kDa subunits, is activated by methionine and inhibited by glycine. Heterotropic cooperativity is observed for activation by methionine in the presence of glycine and for inhibition by glycine in the presence of methionine. These results, along with previous mutant analyses, lead to the conclusion that formyl-FH_4 hydrolase is a regulatory enzyme whose main function is to balance the pools of FH_4 and C_1-FH_4 in response to changing growth conditions. The enzyme uses methionine and glycine to sense the pools of C_1-FH_4 and FH_4, respectively.

There are two transformylation steps in the pathway for de novo purine nucleotide synthesis. The enzymes catalyzing these reactions in *Escherichia coli* are *purN*-encoded 5′-phosphoribosylglycinamide (GAR) transformylase and *purH*-encoded 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole (AICAR) transformylase (20). Both enzymes use 10-formyltetrahydrofolate (formyl-FH_4) as the formyl donor. *E. coli* also has an alternative GAR transformylase (13, 21) that is coded for by *purT* (21). This enzyme enables *E. coli* to incorporate formate into the purine ring, even though it has no formyl-FH_4 synthetase (8). *pfl*-encoded pyruvate formate lyase is utilized for the production of formate under anaerobic growth conditions (23), and a gene designated *purU* is required for the aerobic production of formate (18). PurU encodes a protein of 280 amino acids. The sequence from residues 85 to 280 exhibits 27% amino acid identity with GAR transformylase N (*purN* encoded), including 10 of 11 residues that are thought to interact with formyl-FH_4 (1). This similarity as well as mutant analysis prompted the suggestion that *purU* encodes an enzyme that binds formyl-FH_4 and catalyzes its hydrolysis to formate and FH_4 (18).

Two observations suggested that PurU-dependent formate production is regulated (18). First, growth of an *E. coli purN* mutant that relies on GAR transformylase T (*purT* encoded) and PurU for purine synthesis was strongly inhibited by glycine. This inhibition was reversed by either formate or purines. Second, methionine had a positive effect on the growth of the *purN* mutant, although when supplied in equimolar concentration with glycine, it did not reverse the growth inhibition. In this paper, we describe the purification and characterization of PurU and show that it is a formyl-FH_4 hydrolase whose activity is regulated by glycine and methionine, in accord with the phenotype of the *purN* mutant.

The importance of PurU extends beyond its role in supplying formate for the GAR transformylase T reaction. It has been noted that a *purU* mutant required glycine supplementation to grow at the wild-type rate (18). In the presence of either adenine and methionine or adenine and histidine, the starvation for glycine was so severe that growth of the *purU* strain was virtually blocked unless glycine was supplied. In *E. coli*, glycine is synthesized in a reaction that is catalyzed by *glyA*-encoded serine hydroxymethyltransferase as follows: serine + $FH_4 \leftrightarrows$ glycine + 5,10-methylene-FH_4 (27). The effect of adenine plus methionine or adenine plus histidine could starve a *purU* mutant for glycine by repressing pathways that regenerate FH_4 from C_1-FH_4. The pertinent reactions that regenerate FH_4 are catalyzed by two homocysteine methylases (homocysteine + methyl-$FH_4 \rightarrow$ methionine + FH_4) (6), by GAR transformylase N (GAR + formyl-$FH_4 \rightarrow$ 5′-phosphoribosyl-*N*-formylglycinamide [FGAR]+FH_4) (11), and by AICAR transformylase (AICAR + formyl-$FH_4 \rightarrow$ 5′-phosphoribosyl-4-carboxamide-5-formamidoimidazole [FAICAR] + FH_4) (20). AICAR produced during histidine synthesis also generates FH_4 in the AICAR transformylase reaction (34). Since all of the compounds having an effect on the *purU* and *purN* mutant strains are involved in the interconversions of FH_4 and C_1-FH_4, it is proposed that *purU*-encoded formyl-FH_4 hydrolase has a general role in one-carbon metabolism, in addition to supplying formate for purine synthesis through GAR transformylase T. The data suggest that PurU regulates the ratio of C_1-FH_4:FH_4 pools in the cell.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Minimal medium contained salts (33), 0.5% glucose, 1 μg of thiamine per ml, and other additions as noted. Unless specified otherwise, amino acids, formate, and adenine were added at 0.5 and 1 mM and 100 μg/ml, respectively. Luria broth was used as rich medium (15). Solid media contained 1.5% agar.

Construction of *purU* expression vectors. A 949-bp fragment containing the complete coding sequence of *purU* with *Nde*I and *Hin*dIII sites at the 5′ end and the 3′ end, respectively, was amplified by PCR using sense primer 789, (5′-TTC CA**CAT***ATG*CATTCACTCCAACGTAAAG-3′) and antisense primer 1738, (5′-ATGACGCGCCGCTGTA**AAGCTT**TACGTTGA-3′). Primers are named according to the positions of their 5′ nucleotides in the *purU* sequence (GenBank accession number L20251). Nucleotides that were replaced to introduce restriction sites are indicated by boldface letters, restriction sites are underlined, and

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TABLE 1. Bacteria, bacteriophage, and plasmids used in this work

Strain, phage, or plasmid	Genotype or description	Comment or source
E. coli		
MC4100	F⁻*araD139* Δ(*argF-lac*)*U169 rpsL150 thiA1 relA1*	Laboratory stock[a]
PLN100	MC4100 *purU*::*kan*	Laboratory stock
R320	*purR300*	Laboratory stock
PLN103	R320 *purU*::*kan*	This work
NK5526	F⁻ *hisG213*::Tn*10* λ⁻ IN(*rrD-rrE1*)1	Laboratory stock
TX267	*ara*Δ*lac* Δ*purD*	Laboratory stock
BL21(DE3)	F⁻ *ompT* r_B^- m_B^- *lon ompT*	31
Bacteriophage		
P1 *vir*	Bacteriophage P1; lytic	Laboratory stock
Plasmids		
pMLB1034	*lacZY* fusion vector, Ap^r	25
pPLN3	*glyA-lacZ* fusion	This work
pT7-7	Vector for overexpression	32
pT7-PU1	*purU* cloned into pT7-7	This work
pGS1	*glyA* plasmid	28
Bluescript SK$^+$		Laboratory stock
Bluescript SK$^-$		Laboratory stock

[a] Laboratory stocks were from Purdue University.

the start codon is italicized. After digestion with *Nde*I and *Hin*dIII, *purU* was ligated into the corresponding sites of pT7-7. This vector contains the T7 promoter ϕ10 and the translation start site of T7 gene 10 protein along with the *bla* gene required for ampicillin resistance. The resulting construct was named pT7-PU1.

Overexpression of PurU. A single colony of strain BL21(DE3)/pT7-PU1 was grown overnight in 10 ml of Luria broth medium supplemented with 100 μg of ampicillin per ml. Six 2-liter flasks, each containing 0.5 liter of Luria broth with ampicillin were each inoculated with 1 ml of the overnight culture and grown at 37°C to a turbidity of 180 measured with a Klett colorimeter with a 660-nm filter. At this point, lactose was added to a final concentration of 1% for induction of T7 polymerase, and the flasks were incubated at 30°C with shaking for 24 h. Cells were harvested by centrifugation and were frozen at −20°C.

Enzyme purification. All purification steps were performed at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.5)–1 mM EDTA, unless otherwise noted. Frozen cells were resuspended in 4 ml of buffer containing 1 mM phenylmethanesulfonyl fluoride per g and were broken by two passages through a French press at 20,000 lb/in^2. To the broken cell suspension, a 0.1-volume of 10% streptomycin sulfate was added slowly with stirring. After the last addition, stirring was continued for 15 min, which was followed by centrifugation at 18,000 × *g* for 30 min. Ammonium sulfate was added slowly to 30% saturation (0.176 g/ml) with stirring. Stirring was continued for 15 min following the last addition. The precipitate was collected by centrifugation for 30 min at 18,000 × *g*. The pellet was dissolved in buffer and dialyzed overnight against 100 volumes of the same buffer. After centrifugation at 18,000 × *g* for 30 min to remove precipitated protein, the solution was loaded on a DEAE-Sepharose column (1.5 by 5 cm) equilibrated with the same buffer. The column was washed with 50 ml of buffer, and the proteins were eluted with a 300-ml linear gradient of 0 to 0.5 M NaCl in the buffer. Fractions were checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12), and fractions containing the enzyme were pooled. After concentration by ammonium sulfate precipitation, the protein was dissolved in buffer, dialyzed, and adjusted to approximately 10 mg/ml. The protein was stored at −20°C.

Gel filtration chromatography. Native molecular weights of the enzyme in the crude extract and after purification were estimated by gel filtration. A column (1.1 by 90 cm) of Sephacryl S-300 equilibrated with 50 mM Tris-HCl (pH 7.5) at 4°C was calibrated with thyroglobin (669,000), ferritin (440,000), catalase (232,000), and aldolase (158,000). Enzyme (1 ml containing either 1.6 mg of purified hydrolase or 6.8 mg of extract protein) was applied to the column, and 1-ml fractions were collected at a flow rate of 10 ml/h. The crude extract was treated with DNase I and centrifuged at 130,000 × *g* for 2 h prior to gel filtration. In some experiments, the column was equilibrated with buffer containing 2 mM methionine or 100 μM glycine. Log molecular weights were plotted against K_{av} ($K_{av} = V_e - V_o/V_t - V_o$, where V_e is the elution volume for the protein, V_o is the void volume, and V_t is the total bed volume).

Measurements of protein concentrations. Protein concentrations were determined by the Bradford method (3) with bovine serum albumin as the standard.

Synthesis of folate derivatives. 6(*R*)-5,10-Methenyl-FH_4 was synthesized from dihydrofolate in two steps. Briefly, dihydrofolate (Sigma Chemical Co.) was reduced enzymatically to FH_4 using dihydrofolate reductase, NADPH, and an NADPH-regenerating system (14). The purified dihydrofolate reductase was a gift from Rowena Matthews, University of Michigan. The FH_4 was purified by chromatography on DEAE-Sepharose and lyophilized. 6(*R*)-5,10-Methenyl-FH_4 was obtained from 6(*S*)-FH_4 by treatment with formic acid (22). 6(*R*)-5,10-Methenyl-FH_4 was isolated by chromatography on a column of Whatman cellulose (CF 12), lyophilized, and crystallized from 0.1 N HCl–0.1 M mercaptoethanol. 6(*R*)-5,10-Methenyl-FH_4 was washed with ethanol and ether and stored under vacuum at −20°C. 6-(*R*)-10-Formyl-FH_4 was prepared fresh daily by dissolving about 1 mg of 6(*R*)-5,10-methenyl-FH_4 in 1 ml of 40 mM potassium phosphate (pH 6.8)–0.1 mM EDTA–10 mM mercaptoethanol and by incubation for 2 h in the dark under argon (26).

Formyl-5,8-dideazafolate (fDDF) was made as described by Inglese et al. (11). The concentration of fDDF was determined spectrophotometrically at 310 nm ($\varepsilon = 3.8\ mM^{-1}\ cm^{-1}$), and that of formyl-$FH_4$ was determined spectrophotometrically at 298 nm ($\varepsilon = 9.54\ mM^{-1}\ cm^{-1}$).

Enzyme assay. Routine assays were conducted with fDDF, and some results were verified with formyl-FH_4. The standard reaction with fDDF was carried out in a volume of 100 μl containing 50 mM Tris-HCl (pH 7.5), 60 μM fDDF, 2 mM methionine, and enzyme. The reaction at 23°C was started by the addition of enzyme, and the initial rate of hydrolysis of fDDF was recorded at 295 nm ($\Delta\varepsilon = 18.9\ mM^{-1}\ cm^{-1}$) (26). The standard reaction mixture with formyl-FH_4 contained 50 mM Tris-HCl (pH 7.5), 80 μM formyl-FH_4, 10 mM mercaptoethanol, 2 mM methionine, and enzyme in a volume of 500 μl. All solutions except the enzyme were degassed and saturated with argon six times. Cuvettes were rinsed with argon and sealed with Parafilm. All additions were made with syringes. Assays at 23°C were started by the addition of substrate. The initial rate of hydrolysis was recorded at 298 nm ($\Delta\varepsilon = 19.7\ mM^{-1}\ cm^{-1}$) (2).

Formate production assay. The production of formate was coupled to the oxidation of NADH using the GAR transformylase T (formate + GAR + ATP → 5′-phosphoribosyl-*N*-formylglycinamide + ADP + P_i), pyruvate kinase (phosphoenolpyruvate + ADP → pyruvate + ATP), and lactate dehydrogenase (pyruvate + NADH + H^+ ⇆ lactate + NAD^+) reactions. The reaction mixtures contained 10 mM $MgCl_2$, 1 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 100 μM β-GAR, 10 U of pyruvate kinase, 10 U of lactate dehydrogenase, 1.3 μM GAR transformylase T, 100 μM fDDF, 2 mM methionine, 5.9 μM formyl-FH_4 hydrolase, and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.0) in a volume of 1.0 ml. The rate of NADH oxidation was measured at 340 nm at 25°C.

Kinetics studies. K_m values for fDDF and formyl-FH_4 were determined by varying the fDDF concentrations from 1.0 to 60 μM at an enzyme concentration of 500 nM (1.6 μg of protein per 100 μl) and varying the formyl-FH_4 concentrations from 5.0 to 80 μM at an enzyme concentration of 280 nM (4.5 μg of protein per 500 μl). The data were fit to the Michaelis-Menten equation, from which K_m was calculated. The activation constant, K_a, for methionine was determined by varying the methionine concentrations from 50 to 2,000 μM at a concentration of 60 μM fDDF or 60 μM formyl-FH_4. The data were fit to the Michaelis-Menten equation. Inhibition by glycine was characterized by varying the fDDF concentrations from 1.0 to 60 μM at four fixed concentrations of glycine (none and 50, 100, and 120 μM) and two fixed concentrations of methionine (400 and 2,000 μM). The data were fit to the Lineweaver-Burk equation (9), and the inhibition constant for glycine was calculated by computer. The Hill coefficient (10) for methionine was determined from measurements at methionine concentrations of 50 to 2,000 μM at 60 μM fDDF and three fixed concentrations of glycine (none and 20 and 40 μM). The Hill coefficient for glycine was determined from measurements in which the glycine concentration was varied from 5 to 250 μM at 60 μM fDDF and four fixed concentrations of methionine (200, 400, 800, and 1,600 μM). The data were fit to the respective equations, and kinetic parameters were derived using UltraFit software (Biosoft, Cambridge, United Kingdom).

AICAR accumulation. The methods described by Bratton and Marshall (4) and Stetten and Fox (30) were used with slight modifications. Cells were initially grown in minimal medium supplemented with histidine or adenine to satisfy specific growth requirements. Following overnight growth, 100 μl was used to inoculate 10 ml of minimal medium in a 200-ml flask supplemented with 10 μg of sulfadiazine per ml and additions specified in Table 5. After 18 h of incubation at 37°C without shaking, cell density was determined at 600 nm, and 1.6 ml was removed for determination of accumulated AICAR. All subsequent steps were carried out at room temperature. Cells were sedimented by centrifugation for 5 min in a microcentrifuge, and 30 μl of acetic anhydride was added to 1.5 ml of supernatant, which was followed by incubation for 30 min to block the amino group of sulfadiazine. The concentration of AICAR was determined in triplicate 500-μl samples by the addition of 500 μl of 0.4 N HCl and 10 μl of 0.1% $NaNO_2$. After incubation for 3 min, 10 μl of 0.5% ammonium sulfamate was added, and the mixture was incubated for 2 min, after which 10 μl of 0.1% *N*-(1-naphthyl) ethylenediamine was added. The diazotized product was measured at 545 nm after 5 min, when color development was maximal. AICAR concentrations were calculated using an ε of $4.5 \times 10^4\ M^{-1}\ cm^{-1}$. The efficiency of acetylation of sulfadiazine was determined by assaying sulfonamide-containing culture medium that was treated in the same way as the culture supernatant. This background value was subtracted from the other measurements. The values in Table 5 are the

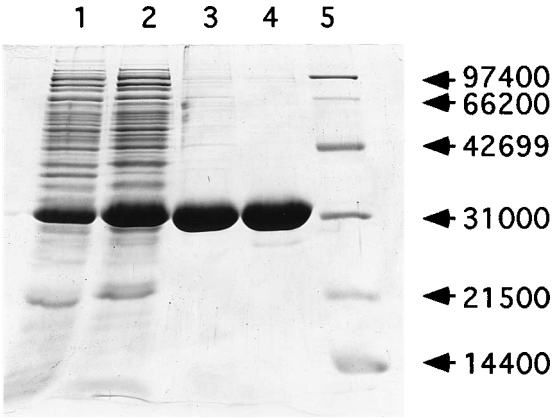

FIG. 1. SDS-polyacrylamide gel electrophoresis to monitor purification of formyl-FH_4 hydrolase. Samples (20 μg) were electrophoresed on 15% polyacrylamide gels containing 1% SDS. The gels were stained with Coomassie blue. The protein samples are as follows: crude extract (lane 1), streptomycin sulfate fraction (lane 2), ammonium sulfate fraction (lane 3), DEAE-Sepharose fraction (lane 4), and molecular weight markers (lane 5). The molecular weight markers are phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (top to bottom).

averages of triplicate parallel determinations normalized for cell density (turbidity at 600 nm). The maximal acceptable difference in cell density was twofold. The values reported in Table 5 are from a single representative experiment.

Regulation of *glyA* expression. To examine regulation, the 5′ end of *glyA* was fused to a *lacZ* reporter. A fragment containing 340 nucleotides of 5′ flanking sequence and the first 55 nucleotides of the *glyA* coding sequence was amplified by PCR from plasmid pGS1 (28) using primers that provided 5′ and 3′ *Eco*RI and *Bam*HI sites, respectively. The resulting fragment was inserted into the *Eco*RI and *Bam*HI sites of plasmid pMBL1034 to give a *glyA-lacZ* translational fusion in which codon 18 of *glyA* was joined in frame to the 9th codon of *lacZ*. The resulting *glyA-lacZ* fusion in plasmid pPLN3 was transformed into strains MC4100 (wild type), R320 (*purR*), PLN100 (*purU*), and PLN103 (*purR purU*). Cells were grown overnight with supplements (indicated in Table 4), and 100-μl samples were used to inoculate 2-ml cultures in the same medium. Cells were grown to mid-log phase, and β-galactosidase activity was assayed using the chloroform-SDS lysis procedure (16).

P1 transduction. The *purU*::*kan* marker was transferred from strain PLN100 to strain R320 as described elsewhere (15).

RESULTS

Overexpression of *purU* and enzyme purification. The *purU* gene was cloned into overexpression vector pT7-7, and the resulting plasmid pT7-PU1 was transformed into *E. coli* BL21(DE3). This strain contains the gene for T7 RNA polymerase in the chromosome under the control of the inducible *lacUV5* promoter. Upon induction by lactose, T7 polymerase is synthesized, resulting in synthesis of the target gene cloned downstream of the T7 promoter in the multicopy pT7-7 plasmid. Following induction, a protein subunit with a molecular mass of approximately 32 kDa was overproduced (Fig. 1). This subunit mass is in good agreement with the value of 31,800 Da calculated from the sequence of the cloned gene. The enzyme was purified threefold to virtual homogeneity in two steps following removal of DNA by precipitation with streptomycin sulfate (Table 2). After ammonium sulfate fractionation, the enzyme was more than 90% pure by SDS-polyacrylamide gel electrophoresis (Fig. 1, lane 3). Chromatography on DEAE-Sepharose removed small amounts of higher-molecular-weight impurities and resulted in a protein that was more than 95% pure (Fig. 1, lane 4). The small amount of contaminating protein (approximately 28 kDa), which is visible in lane 4 but not in lane 3, is assumed to result from proteolytic degradation.

TABLE 2. Summary of enzyme purification

Step	Vol (ml)	Amt of protein (mg)	Sp act (nmol/min/mg)	Total activity (nmol/min)
Disruption of cells	135	2,160	30	64,800
Streptomycin sulfate Precipitation	135	850	55	46,800
Ammonium sulfate precipitation	10	280	72	20,200
Ion-exchange chromatography	22	205	85	17,400

TABLE 3. Summary of kinetic constants

Substrate	Concn of Met (mM)	k_{cat} (S^{-})	K_m (μM)	k_{cat}/K_m	K_a Met (μM)	K_{ii} Gly (μM)[a]
fDDF	0	0.001	<10	0.0001	ND[b]	<20
fDDF	0.4	0.019	6.5 ± 0.5	0.0029	194 ± 41	29
fDDF	2.0	0.026	7.0 ± 0.3	0.0037	194 ± 41	40
Formyl-FH_4	2.0	0.10	49 ± 17	0.0020	238 ± 43	ND

[a] K_{ii}, inhibition constant for interaction of uncompetitive inhibitor with the enzyme-substrate complex.
[b] ND, no data.

Kinetics characterization. fDDF, a stable formyl-FH_4 analog, was tested as a substrate to determine whether the purified *purU* gene product had the predicted formyl-FH_4 hydrolase activity. The data summarized in Table 3 established that the enzyme catalyzed the hydrolysis of fDDF as well as of formyl-FH_4. A low rate of fDDF catalysis was stimulated about 25-fold by methionine. The results of two experiments indicated that methionine activated the enzyme instead of participating directly in the reaction. First, the amount of DDF formed, which was measured by a change in the A_{295}, exceeded by sixfold the concentration of methionine in the assay mixture. Second, the rate of formate production, which was determined by coupled enzymatic assay, relative to the rate of deformylation of fDDF was 0.8:1, indicating that formyl methionine is not a product of the reaction. Activation by methionine exhibited saturation kinetics with a K_a of 200 μM (Fig. 2) (Table 3). Therefore, 2 mM methionine was included for the routine hydrolase assay. Because of the low turnover rate of the nonactivated enzyme, the K_m for fDDF was not determined accurately but was esti-

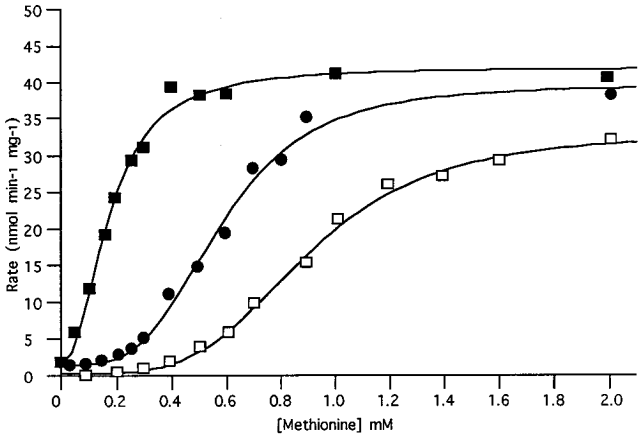

FIG. 2. Activation of formyl-FH_4 hydrolase by methionine and inhibition by glycine. The initial rate of fDDF hydrolysis was assayed at three fixed concentrations of glycine (none [■], 20 μM [●], and 40 μM [□]).

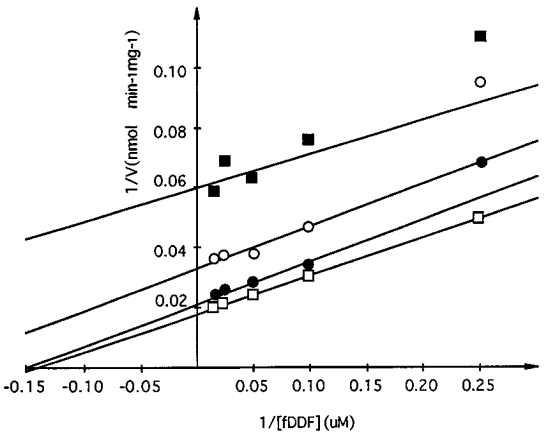

FIG. 3. Double reciprocal plot for inhibition by glycine. Initial rates of fDDF hydrolysis at 2 mM methionine were determined at four fixed concentrations of glycine (none [□], 50 μM [●], 100 μM [○], and 120 μM [■]).

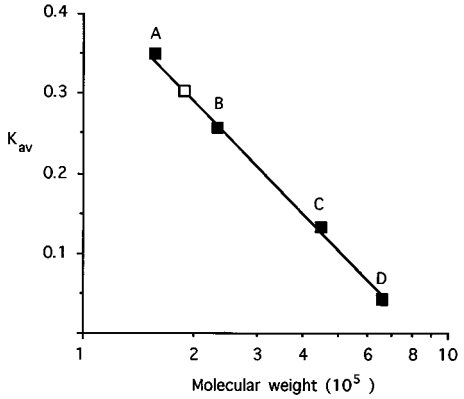

FIG. 5. Molecular weight estimated by gel filtration. The protein standards were thyroglobulin (A), ferritin (B), catalase (C), and aldolase (D). □, the position of formyl-FH_4 hydrolase.

mated to be <10 μM. An fDDF K_m of 7.0 μM was calculated for the activated enzyme. The k_{cat} for hydrolysis of formyl-FH_4 was about four times faster than that for the substrate analog; however, because of a sevenfold higher K_m, the value of k_{cat}/K_m with formyl-FH_4 was about half of that with fDDF. These results establish that the *purU* gene product is a formyl-FH_4 hydrolase.

The hydrolase activity was subject to inhibition by glycine. Glycine inhibition was uncompetitive with fDDF (Fig. 3), although there was apparent upward curvature of the double reciprocal plot, indicative of cooperativity, for the activated enzyme at the lowest concentration of fDDF. The inhibition constant for glycine was dependent on the methionine concentration, varying between <20 μM with no methionine to 40 μM with saturating methionine (Table 3). Cooperativity for glycine inhibition was likewise dependent on the methionine concentration (Fig. 4). Hill coefficients for glycine, calculated from the data in Fig. 4, were 0.83 ± 0.12 for the unactivated enzyme and 1.68 ± 0.20 with 1.6 mM methionine. In a like manner, the Hill coefficient for methionine was 1.34 ± 0.30 in the absence of glycine, 2.54 ± 0.56 with 20 μM glycine, and 2.74 ± 0.30 with 40 μM glycine (Fig. 2). Thus, methionine and glycine, the regulators of formyl-FH_4 hydrolase, exhibit heterotropic cooperative interactions.

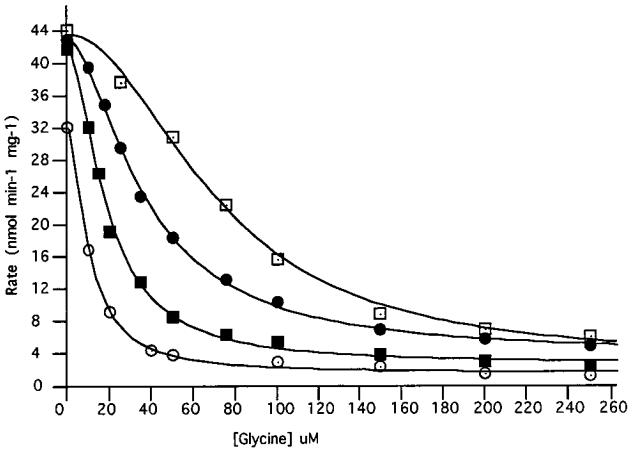

FIG. 4. Relationship between glycine inhibition and methionine activation. Glycine inhibition of fDDF hydrolysis was determined at four fixed concentrations of methionine (200 μM [○], 400 μM [■], 800 μM [●], and 1,600 μM [□]).

Oligomeric state. Gel filtration on Sephacryl S-300 was used to estimate the native molecular weight of formyl-FH_4 hydrolase. A value of approximately 190,000 was obtained (Fig. 5), suggesting that the native enzyme is a hexamer of 32,000 molecular weight subunits. A similar molecular weight was obtained for crude or purified enzyme preparations. The value was unchanged by the regulatory effectors, methionine and glycine (not shown).

Role of formyl-FH_4 hydrolase. Synthesis of glycine is limited in a *purU* mutant, resulting in a decreased growth rate (18). Addition of adenine and methionine or adenine and histidine further restricts glycine synthesis and blocks growth. To determine whether these effects are due to direct repression of *glyA*, which codes for serine hydroxymethyltransferase, or are a result of perturbations of the FH_4 and C_1-FH_4 pools, we constructed a *glyA-lacZ* fusion and examined *glyA* expression in several relevant strains, including PLN100 (*purU*) and MC4100 (wild type). The results of β-galactosidase reporter assays are given in Table 4. Glycine, which was required for growth of strains PLN100 and PLN103 in the presence of adenine and either methionine or histidine, had no effect on *glyA* expression. The data show that adenine plus methionine repressed *glyA* about 3-fold in strain PLN100 (*purU*). This repression, likely mediated by *metR* and *purR*, was partially abolished in strain PLN103 (*purU purR*) in accord with the results of previous work (29). Two key experiments shown in Table 4 indicate that the threefold repression of *glyA* by adenine plus methionine is not responsible for inhibition of glycine synthesis

TABLE 4. Effect of adenine, histidine, and methionine on expression of *glyA*

Strain	Activity[a] in medium supplemented with:						
	None	Gly	Met	His	Ade and Gly	Ade, Met, and Gly[b]	Ade, His, and Gly[b]
MC4100 (wild type)	27	26	18	30	21	9.0	28
R320 (*purR*)	34	34	27	28	35	26	36
PLN100 (*purU*)	21	21	13	21	21	7.3	23
PLN103 (*purU purR*)	33	35	25	15	34	29	32

[a] Expressed in Miller units (16). Values are from a single representative experiment.

[b] Required for growth of a *purU* mutant in medium with adenine plus methionine or adenine plus histidine.

TABLE 5. Relative contributions of the histidine and purine pathways to AICAR synthesis

Supplement[a]	AICAR accumulation (μM)[b]		
	MC4100 (wild type)	NK5526 (*hisG*)	TX267 (*purD*)
None	6.6	NG[c]	NG
Ade	5.0	NG	5.4
His	3.6	5.0	NG
Ade + His	0.3	0	0

[a] Minimal medium was supplemented with 0.5 mM histidine, 1 mM adenine (as indicated), and 10 μg of sulfadiazine per ml.
[b] AICAR accumulation was determined colorimetrically by the amount of secreted 5-amino-4-imidazole carboxamide.
[c] NG, no growth.

TABLE 6. Comparison of kinetic constants for *E. coli* GAR transformylase N and formyl-FH_4 hydrolase

Enzyme	Substrate	k_{cat} (S^{-1})	K_m (μM)	k_{cat}/K_m
Formyl-FH_4 hydrolase[a]	fDDF	0.026	7.0 ± 0.3	0.0037
	Formyl-FH_4	0.10	49 ± 17	0.0020
GAR transformylase N	fDDF	16.1 ± 1.3	16.7 ± 3.0	0.96
	Formyl-FH_4	13.5 ± 0.8	84.8 ± 5.9	0.27

[a] Data are from Table 3.
[b] Data are from Inglese et al. (11).

and growth inhibition of the *purU* mutant. First, adenine plus methionine gave a similar threefold repression of *glyA* in the wild-type *purU*$^+$ strain (MC4100); however, these conditions do not limit glycine synthesis in the wild type (18). Second, adenine plus histidine, which also inhibit glycine synthesis and growth of the *purU* mutant, repressed *glyA* only weakly. These results indicate that repression of *glyA* may contribute to a decreased production of glycine but cannot account for the *purU* phenotype. Rather, as explained in the Discussion, repression of the synthesis of purines and methionine or purines and histidine appears to decrease the utilization of formyl-FH_4 and methyl-FH_4, with a corresponding reduction in the regeneration of FH_4, a serious problem in the *purU* mutant lacking formyl-FH_4 hydrolase.

The idea that repression of histidine biosynthesis could have a marked effect on the regeneration of FH_4 was quite unexpected, considering the relatively low abundance of histidine in proteins (19). For this reason, we investigated the relationship between histidine synthesis and C_1-FH_4 utilization. AICAR, a byproduct of histidine biosynthesis, and formyl-FH_4 are utilized by AICAR transformylase for purine synthesis, thus regenerating FH_4. Our approach was to estimate the relative contributions of the histidine and purine biosynthetic pathways for synthesis of AICAR. Sulfonamides inhibit AICAR transformylase, leading to the accumulation of AICAR and the secretion of 5-amino-4-imidazole carboxamide, which can be determined from the growth medium by the method described by Bratton and Marshall (4, 24, 30). The amount of AICAR produced when either purine or histidine biosynthesis is repressed or disrupted will give an estimate of the relative contributions of the two pathways to AICAR production. These data are shown in Table 5.

In the wild type, addition of adenine or histidine to the medium repressed the cognate biosynthetic pathway and decreased AICAR accumulation by 25 to 45%, whereas accumulation was inhibited 95% by repression of both pathways. AICAR accumulation was comparable in mutants blocked at an early step of each pathway. Accumulation of AICAR was completely suppressed by repression of purine biosynthesis in the histidine mutant and repression of the histidine pathway in the purine mutant. Therefore, by this analysis, the histidine and purine pathways contribute comparable amounts of AICAR for IMP synthesis. However, this conclusion must, be qualified, because the extent of sulfonamide inhibition of step three in purine biosynthesis catalyzed by the two GAR transformylases is not known. It is possible that in the absence of sulfonamide, more AICAR is produced by the purine pathway than by histidine biosynthesis. Nevertheless, repression of the histidine and purine pathways decreases the cell's capacity to regenerate FH_4 from formyl-FH_4 and leads to an imbalance in a *purU* mutant lacking formyl-FH_4 hydrolase.

DISCUSSION

The protein encoded by *E. coli purU* has been overproduced, purified approximately threefold to near homogeneity, and characterized. We have demonstrated that PurU is a formyl-FH_4 hydrolase, as had been inferred from its amino acid sequence similarity with GAR transformylase N and from mutant analysis (18). On the basis of SDS-polyacrylamide gel electrophoresis and gel filtration estimates of subunit and native molecular weights, the enzyme is a hexamer with subunits having a molecular weight of 32,000. Formyl-FH_4 hydrolase is a regulatory enzyme that is activated by methionine and inhibited by glycine. Methionine and glycine, which exhibit heterotropic cooperative interactions, may interact with the NH_2-terminal 84-amino-acid polypeptide that is fused to the GAR transformylase-related sequence, residues 85 to the CO_2H terminus at position 280. Thus, the NH_2-terminal 84 amino acids may function as a regulatory domain to modulate catalysis by the transformylase-related protein.

Formyl-FH_4 hydrolase catalytic activity is low compared with that of *E. coli* GAR transformylase N. Values for k_{cat}, K_m, and k_{cat}/K_m are compared in Table 6. It can be seen that the catalytic efficiency of GAR transformylase N (k_{cat}/K_m) is 260 and 135 times higher than those for formyl-FH_4hydrolase with fDDF and formyl-FH_4, respectively. This difference in relative activities may reflect cellular requirements for biosynthesis versus hydrolysis or may indicate that the hydrolase was not assayed under optimal conditions. For example, the true substrate for the hydrolase may be a polyglutamate derivative of formyl-FH_4.

The results of enzyme characterization and analyses of the *purU* phenotype indicate two roles for formyl-FH_4 hydrolase. First, this hydrolase provides the major source of formate under aerobic growth conditions. This is shown by the requirement of formate for aerobic growth of a *purN purU* mutant that relies on formate-dependent GAR transformylase T for purine synthesis and growth (18). Even in the wild type containing the two GAR transformylases, formate can provide up to 50% of the carbon for position 8 of the purine ring (8).

The second and apparently major role for formyl-FH_4 hydrolase is to balance the pools of FH_4 and C_1-FH_4 to ensure that synthesis of glycine can be maintained when cells have excess purines, methionine, and histidine and the biosynthetic pathways for these molecules are shut down. Purine biosynthesis regenerates FH_4 as a product of the GAR and AICAR transformylations at steps 3 and 10. Methionine biosynthesis provides FH_4 as a product of the methionine synthase reactions, and the histidine biosynthetic pathway produces AICAR, which is formylated by AICAR transformylase to yield 5′-phosphoribosyl-4-carboxamide-5-formamidoimidazole and FH_4. In a *purU* mutant lacking formyl-FH_4 hydrolase,

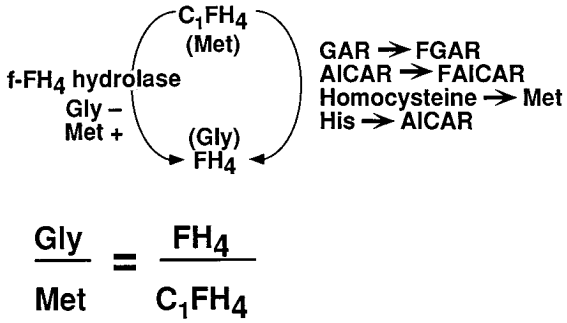

FIG. 6. Model of the role of formyl-FH_4 hydrolase. According to the model, glycine and methionine sense the FH_4 and C_1-FH_4 pools, respectively, and regulate the activity of formyl-FH_4 hydrolase activity. + and −, activation and inhibition, respectively. Biosynthetic reactions that consume C_1-FH_4 and regenerate FH_4 are shown to the right. The enzymes for these steps are GAR transformylase, AICAR transformylase, and methionine synthase. AICAR produced by the histidine pathway mixes with AICAR from de novo purine biosynthesis. FGAR, 5′-phosphoribosyl-*N*-formylglycinamide; FAICAR, 5′-phosphoribosyl-4-carboxamide-5-formamidoimidazole.

repression of purine synthesis by adenine and repression of either the methionine or histidine pathways leads to a growth requirement for glycine (18). This glycine requirement cannot result from the threefold repression of *glyA* by adenine plus methionine (Table 4), as was explained in the preceding section. Therefore, we conclude that repression of the purine and either the methionine or histidine pathways starves the *purU* mutant for FH_4 needed for glycine synthesis. Although formate derived from hydrolysis of formyl-FH_4 is a substrate for GAR transformylase T, the role of this alternative enzyme in step three of purine biosynthesis is not known.

The biochemical characterization of formyl-FH_4 hydrolase explains how this regulatory enzyme functions to balance the FH_4 and C_1-FH_4 pools. We propose that the methionine/glycine ratio monitors the pools of C_1-FH_4 and FH_4. Thus, as shown by Fig. 6, excess methionine can activate the hydrolase to generate FH_4 for glycine synthesis and excess glycine can inhibit the hydrolase to preserve C_1-FH_4 for biosynthesis of methionine, purines, and thymidylate.

Animals contain an enzyme, formyl-FH_4 dehydrogenase, that may be related to *E. coli* formyl-FH_4 hydrolase. Formyl-FH_4 dehydrogenase is a bifunctional enzyme that catalyzes the reaction formyl-FH_4 + $NADP^+ \rightarrow FH_4 + CO_2 + NADPH + H^+$ as well as the $NADP^+$-independent hydrolysis of formyl-FH_4 to FH_4 and formate at 20 to 30% of the oxidative rate (17 and references therein). The sequence of the rat liver enzyme derived from the cloned cDNA has three putative domains: residues 1 to 203 (GAR transformylase N), residues 204 to 416 (unknown), and residues 417 to 900 (aldehyde dehydrogenase) (7). The physiological significance of this enzyme is controversial. Suggestions for the dehydrogenase include disposal of excess one-carbon units and metabolism and detoxification of formate. Min et al. (17) have noted that the hydrolase could provide a mechanism for regenerating FH_4 under conditions in which utilization of substituted folates for biosynthesis is impaired. This hypothesis is supported by the recent finding that in mice lacking the enzyme, there is a marked depletion of the cytoplasmic FH_4 pool (5). However, an important distinction is that the mammalian dehydrogenase/hydrolase is not regulated by methionine and glycine in response to fluctuations in FH_4 and C_1-FH_4 pools and does not contain a domain corresponding to residues 1 to 84 of the *E. coli* formyl-FH_4 hydrolase.

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