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Purification and Properties of Formylglutamate Amidohydrolase from *Pseudomonas putida*†

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Formylglutamate amidohydrolase (FGase) catalyzes the terminal reaction in the five-step pathway for histidine utilization in *Pseudomonas putida*. By this action, *N*-formyl-L-glutamate (FG) is hydrolyzed to produce L-glutamate plus formate. Urocanate, the first product in the pathway, induced all five enzymes, but FG was able to induce FGase alone, although less efficiently than urocanate did. This induction by FG resulted in the formation of an FGase with electrophoretic mobility identical to that of the FGase induced by urocanate. A 9.6-kilobase-pair *Hin*dIII DNA fragment containing the *P. putida* FGase gene was cloned into the corresponding site on plasmid pBEU1 maintained in *Escherichia coli*. Insertion of the fragment in either orientation on the vector resulted in expression, but a higher level was noted in one direction, suggesting that the FGase gene can be expressed from either of two vector promoters with different efficiencies or from a single vector promoter in addition to a less efficient *Pseudomonas* promoter. FGase was purified 1,110-fold from the higher-expression clone in a yield of 10% through six steps. Divalent metal ions stimulated activity, and among those tested (Co, Fe, Zn, Ca, Ni, Cd, Mn, and Mg), Co(II) was the best activator, followed by Fe(II). FGase exhibited a K_m of 14 mM for FG and a specific activity of 100 μmol/min per mg of protein in the presence of 5 mM substrate and 0.8 mM $CoCl_2$ at 30°C. The enzyme was maximally active in the range of pH 7 to 8. FGase was found to be a monomer of molecular weight 50,000. *N*-Acetyl-L-glutamate was not a substrate for the enzyme, but both it and *N*-formyl-L-aspartate were competitive inhibitors of formylglutamate hydrolysis, exhibiting K_i values of 6 and 9 mM, respectively. The absence of FGase activity as an integral part of histidine breakdown in most other organisms and the somewhat uncoordinated regulation of FGase synthesis with that of the other *hut* enzymes in *Pseudomonas* suggest that the gene encoding its synthesis may have evolved separately from the remaining *hut* genes.

Histidine is utilized as both a carbon and nitrogen source by a wide range of microbial species, and this process involves the formation of glutamate plus ammonia and a C-1 unit at the level of formate as key intermediates. The pathway leading from histidine to glutamate is made up of either four or five reactions, depending on the organism studied. The first three steps are common to all and result in the formation of *N*-formimino-L-glutamate (FIGLU). The precise route for the subsequent breakdown of FIGLU differs among organisms. In *Salmonella typhimurium* (21), *Klebsiella aerogenes* (19), and *Bacillus subtilis* (6, 13), FIGLU is hydrolyzed to L-glutamate and formamide by *N*-formimino-L-glutamate formiminohydrolase (EC 3.5.3.8). Mammalian species use tetrahydrofolate as a C-1 acceptor in forming glutamate and formiminotetrahydrofolate from FIGLU (glutamate formiminotransferase, EC 2.1.2.5) (2, 27). However, all species of *Pseudomonas* studied thus far, and also *Streptomyces coelicolor*, employ two enzymes for this process (8, 14, 18, 29). The first enzyme, FIGLU iminohydrolase (EC 3.5.3.13), converts FIGLU to *N*-formyl-L-glutamate (FG) plus ammonia; while a second enzyme, *N*-formyl-L-glutamate amidohydrolase (FGase), degrades FG to glutamate and formate.

Regulation of the genes concerned with histidine utilization (*hut*) has been examined in many of the aforementioned species. These studies revealed that all of the genes are probably under negative control by a single repressor (the *hutC* gene product), but the arrangement of structural genes and regulatory regions is not uniform (3, 15, 25). Investigations with *Pseudomonas putida* have suggested that the *hut* genes are organized into multiple transcriptional units, with *hutU* and *hutH* (urocanase and histidase genes, respectively) in one unit (7, 17) and *hutG* (the FGase gene) being regulated in a somewhat independent fashion (A. T. Phillips and L. M. Mulfinger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, p. 141, abstr. K27). Consevage et al. (7) cloned the entire group of *hut* genes from *P. putida* ATCC 12633 into *Escherichia coli* and demonstrated a polar transcriptional relationship between *hutU* and *hutH* based on Tn*1000* insertional mutagenesis. Restriction endonuclease mapping and subcloning further revealed the following tentative gene arrangement: *hutF hutC hutU hutH hutI hutG*, where *hutF* encodes the fourth enzyme, FIGLU iminohydrolase, and *hutI* is the gene for the third enzyme, imidazolone propionate amidohydrolase (EC 3.5.2.7).

Coote and Hassall (8) obtained evidence that in *P. testosteroni* urocanate induced all of the *hut* enzymes, but FIGLU iminohydrolase and FGase were also induced by their respective substrates. These authors suggested that there could perhaps be two FGase enzymes, one inducible by urocanate and the other by FG.

To study the regulation of FGase production and to establish the properties of this enzyme from *P. putida*, we have investigated the inducer specificity for FGase formation in *P. putida*; we also subcloned the *hutG* gene on a multicopy plasmid in *E. coli* to eliminate the *hutC* repressor gene effect and improve expression levels for the *hutG* gene

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in *E. coli*. This construction has permitted the purification of FGase and an evaluation of its metal activator requirements and substrate specificity.

MATERIALS AND METHODS

Bacterial strains. *P. putida* PRS1 (ATCC 12633) was the parental strain used. *E. coli* RDP145 F$^-$ *endA sbcB15 hsdR4 gyrA rpsL recA13* was chosen as recipient for plasmid pBEU1 (5, 29), while RDP210 F$^-$ *lacYI leuB6 thi-1 hsdR hsdM rpsL supE44* (7) was used as the host for pBR322 and related plasmids.

Media and specialty chemicals. LB medium (22) was used as a rich medium for both *E. coli* and *P. putida* strains. Minimal A medium, consisting of 0.1% $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 1.05% K_2HPO_4, and 0.45% KH_2PO_4, with 0.5% sodium succinate or glucose added as a carbon source, was used for growth of *P. putida*. Medium 56 with glucose as a carbon source (22) was used to grow *E. coli* strains. Unless otherwise mentioned, all biochemicals were obtained from Sigma Chemical Co. Restriction enzymes were purchased from either New England Biolabs or International Biotechnologies, Inc.

Labeled FG was synthesized from [^{14}C]glutamate (ICN Corp.) and formic acid by a modification of the procedure of Tabor and Mehler (26). Unlabeled L-glutamic acid (0.2 mmol) was mixed with 60 μCi of [U-^{14}C]glutamic acid in a 10-ml round-bottomed flask to produce a specific radioactivity of 0.3 mCi/mmol, and the sample was lyophilized. This material was then dissolved in 2.4 ml of 97% formic acid, a condenser was affixed to the flask, and 0.8 ml of acetic anhydride was added dropwise with stirring. Heat was gently applied during the addition to maintain the temperature between 50 and 60°C, and the reaction was allowed to proceed for 1 h. The mixture was then dried by rotary evaporation. The oily residue was dissolved in water and applied to a column (4 by 0.7 cm) of Dowex-50 (H$^+$, 200-400 mesh); labeled FG was washed through the column with a small amount of H_2O and collected, while any unreacted [^{14}C]glutamic acid remained bound to the column. The [^{14}C]FG was concentrated and stored refrigerated until used. Acetyl-[^{14}C]glutamic acid was prepared on an identical scale but following the general directions outlined by Greenstein and Winitz (12). Unlabeled FG was synthesized by the original specifications of Tabor and Mehler (26). Other nonlabeled acyl amino acids were obtained commercially.

Mutant isolation. Various *hut* mutants were produced by chemical mutagenesis with ethyl methanesulfonate and subsequent penicillin selection based on inability to utilize histidine as a sole carbon source. Identification of mutants was accomplished by replica plating onto minimal A medium in which succinate had been replaced as carbon source by histidine, urocanate, formiminoglutamate, or formylglutamate; mutant phenotypes were confirmed by direct assays of all *hut* pathway enzymes. Constitutive mutants were isolated by the procedures described by Leidigh and Wheelis (17) and Meiss et al. (21). Mutants of similar phenotype were isolated from different experiments to ensure their independent origins.

Preparation of crude extracts. Cultures of *P. putida* were grown with vigorous aeration to an absorbance of 0.9 at 600 nm (8×10^8 cells per ml) and then centrifuged at 4°C and 12,000 × *g* for 20 min. The pellet was suspended in 10 ml of 1% NaCl, recentrifuged as before, and finally suspended in 2 ml of 50 mM potassium phosphate, pH 7.5. Disruption was by sonic treatment (Branson Sonifier model 140E) with a microprobe operated at half-maximal power for four 30-s treatments. Cell debris was removed after centrifugation at 24,000 × *g* for 30 min, and the supernatant was kept on ice until assays were performed.

Enzyme assays. Histidase was assayed spectrophotometrically at 277 nm by the method of Rechler and Tabor (24). Urocanase was assayed as described by George and Phillips (11), except the assay final volume was increased to 1.0 ml. FIGLU iminohydrolase was determined colorimetrically, and imidazolone propionate (IPA) hydrolase was measured spectrophotometrically by the procedures described by Consevage et al. (7) and Kimhi and Magasanik (15), respectively. Protein was analyzed by the method of Bradford (4). Specific activities are stated as micromoles of product formed per minute per milligram of protein under the conditions of each assay.

In the assay of FGase, each reaction mixture contained 2 μmol of potassium phosphate, pH 7.5, 0.05 μmol of $CoCl_2$, and enzyme. The reaction was initiated by the addition of 0.3 μmol of [^{14}C]FG (0.3 mCi/mmol) to give a final volume of 0.06 ml; the temperature was 30°C. The reaction was terminated by adding 1 ml of 1 M acetic acid, and the sample was loaded on a column of Dowex-50. The column was washed with 5 ml of water to elute unreacted [^{14}C]FG, and then the reaction product, [^{14}C]glutamate, was eluted with 4 ml of 2 M NaOH. The eluate was acidified with 1 ml of 6 M HCl, mixed with 10 ml of Liquiscint solution (National Diagnostics), and counted in a liquid scintillation counter. This assay was used for all data reported herein.

A colorimetric assay based on glutamate release was also developed for use when interference by amino acids or proteins was not a serious problem and large numbers of samples needed to be assayed rapidly, as in column fraction scanning. This assay mixture contained 10 μmol of potassium phosphate, pH 7.5, 0.5 μmol of $CoCl_2$, and enzyme sample; the reaction was started with 10 μmol of FG, to give a final volume of 0.5 ml. After incubating for the desired period at 30°C, reactions were stopped with 0.5 ml of 10% trichloroacetic acid and then centrifuged for 2 min in a microcentrifuge; 0.5 ml was removed for analysis of the glutamate formed. For color formation, 0.5 ml of a borate-KOH solution (61.1 g of $K_2B_4O_7$ plus 17.2 g of KOH diluted to 1 liter) was mixed with 0.5 ml of sample, and 0.1 ml of 0.03 M trinitrobenzene sulfonic acid was added. The color was allowed to develop at 40°C for 10 min and then stopped with 2.0 ml of ice-cold methanol. The absorbance of the solution was measured at 420 nm with a 1-cm-path cuvette. Under the assay conditions described, an absorbance of 0.5 corresponded to 0.12 μmol of glutamate formed.

Induction of enzyme activity. Cells were inoculated in 200 ml of succinate minimal medium and grown to a cell density of 0.3 at 600 nm. Inducer test compounds were added to a final concentration of 15 mM, and the cultures were then grown for another 4 h. Crude extracts made from these cultures were assayed as described above.

Cloning of *hutG*. Plasmid pMC1, which contains all six *hut* genes from *P. putida* PRS1, was used in the initial construction of plasmid pLH2, which contains the *hutG* and the *hutI* genes in a pBR322 vector; these plasmids were described by Consevage et al. (7). The expression of *P. putida hut* genes can be detected directly in *E. coli*, since this organism lacks its own *hut* operon (28). The DNA fragment containing the *hutG* gene was obtained by digesting pLH2 with *Hin*dIII and was then inserted into the corresponding site on pBEUI (Fig. 1), a temperature-sensitive high-copy-number plasmid (29), to give pLH4 (and pLH4a, in which the insert is oppositely

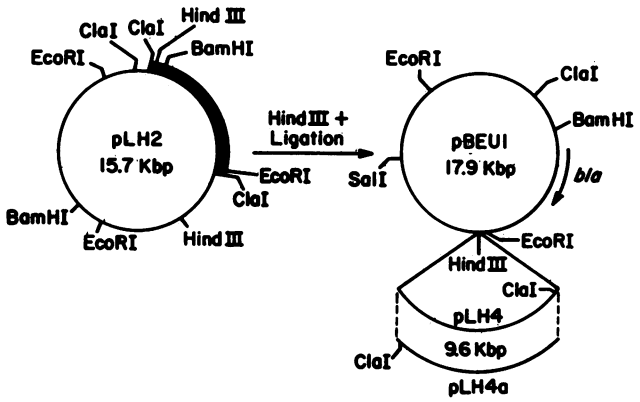

FIG. 1. Construction of plasmids pLH4 and pLH4a. The larger *Hin*dIII fragment from pLH2 was ligated into the corresponding site on pBEU1. Plasmids pLH4 and pLH4a differ only in the orientation of the inserted portion. The portion of pLH2 represented by the heavy line contains sequences from the pBR322 vector.

oriented). The recombinant plasmids were each transformed into *E. coli* RDP145. Copy number control was released by shifting the culture to a temperature of 39°C. Plasmid DNA was purified and analyzed by the method of Maniatis et al. (20).

Purification of FGase. *E. coli* RDP145(pLH4) was grown overnight in 2 liters of LB medium at 30°C and then transferred to 90 liters of the same medium in a New Brunswick F-130 fermentor. Ampicillin was added to a concentration of 50 μg/ml, and growth was allowed to proceed at 30°C. When the cells reached an optical density of 0.8 (600 nm), the temperature was shifted rapidly to 39°C for 1.5 h and then returned to 30°C for an additional hour before harvesting. Approximately 350 g (wet weight) of cells was obtained and stored at −70°C until needed.

For purification, 80 g of cells was suspended in 200 ml of 50 mM potassium phosphate, pH 7.5, and 15 mg of phenylmethylsulfonyl fluoride added from a 5% solution in isopropyl alcohol. The suspension was subjected to sonic treatment (Branson Sonifier) at maximum power for four 1-min intervals; the sample was maintained on ice throughout the disruption. Cell debris was removed by centrifugation in the cold at 13,000 rpm for 30 min in a Sorvall GSA rotor. The crude extract was diluted to give a protein concentration of 20 mg/ml, and $(NH_4)_2SO_4$ was added to 0.1 M. A solution of 2% protamine sulfate dissolved in 50 mM potassium phosphate, pH 7.5, was added dropwise with stirring to a final concentration of 0.33%. Approximately 10 min after the addition was complete, the mixture was centrifuged as before, and the supernatant was retained. Fractionation with $(NH_4)_2SO_4$ was conducted by adding 243 g of $(NH_4)_2SO_4$ per liter of solution (40% saturation) and then centrifuging the mixture as described above. The supernatant fraction was next treated with 168 g of $(NH_4)_2SO_4$ per liter of solution (65% saturation), and the precipitate was collected by centrifugation. This 40 to 65% fraction material was suspended in 100 ml of 20 mM phosphate buffer, pH 7.3, containing 50 μM dithiothreitol and dialyzed against the same solution overnight. After dialysis, any precipitate which formed was removed and discarded.

The dialyzed sample (130 ml) was applied to a DE52 column (3 cm diameter by 22 cm high) equilibrated with the same buffer used for dialysis. The column was eluted with an 800-ml linear gradient of 20 to 100 mM potassium phosphate, pH 7.3, at a flow rate of 0.8 ml/min. About 80 fractions of 10 ml each were collected. Fractions 58 to 65, which contained FGase with a specific activity greater than 2 U/mg, were pooled and concentrated to 10 ml on an Amicon PM10 ultrafilter. The enzyme solution was next loaded on a 200-ml Sephacryl S-200 column (2.5 by 40 cm) equilibrated with 20 mM potassium phosphate, pH 7.3. The column was eluted with 300 ml of the same buffer at a flow rate of 0.2 ml/min, and 5-ml samples were collected. Fractions 27 to 33 were pooled and applied to a 100-ml hydroxyapatite column (2.8 by 16 cm). Samples were eluted with a 300-ml linear gradient from 20 to 100 mM phosphate, pH 7.3, at a flow rate of 0.35 ml/min; 10-ml fractions were collected. Fractions 26 to 33, with an average specific activity of 65, were pooled. Further purification was conducted with a 40-ml DEAE-Sepharose column (1.6 by 20 cm) equilibrated with 40 mM potassium phosphate buffer, pH 7.5. The sample was eluted with a 300-ml linear phosphate gradient from 40 to 100 mM, pH 7.5, at a flow rate of 0.5 ml/min, and 5-ml fractions were collected. Fractions 37 to 46, with an average specific activity of 100, were pooled and concentrated with the ultrafilter. The purity of FGase was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (16). The purified enzyme was stored at −70°C.

RESULTS

Inducibility of FGase. Cells of *P. putida* PRS1 were grown in the presence of various potential inducers and then assayed for four of the *hut* enzymes. The results (Table 1) illustrate that either histidine or urocanate could induce all four of the enzymes examined, whereas FG was able to induce only FGase. Further examination of inducibility by these compounds used several mutants with defects in one of the *hut* genes. When induction was studied with SP54, a mutant lacking histidase and thus unable to synthesize urocanate, histidine could not induce any of the enzymes (Table 1), indicating that histidine itself is not the physiologically important inducer. When a *hutU* mutant (PS15, lacking urocanase) was tested, it was found that either histidine or urocanate could induce all of the remaining enzymes. This confirms that urocanate is the principal inducer, as had been proposed by Newell and Lessie (23) for *Pseudomonas aeruginosa* and Leidigh and Wheelis (17) for *P. putida*. Histidine appears to be an inducer by virtue of its conversion to

TABLE 1. Induction of *hut* enzymes in mutant and wild-type strains of *P. putida*

Strain	Inducer[a]	Sp act (μmol of product formed/min per mg of protein)			
		Histidase	Urocanase	FIGLUase	FGase
PRS1 (wild type)	None	0.002	0.000	0.005	0.001
	His	0.168	0.048	0.066	0.042
	Uro	0.040	0.010	0.015	0.015
	FIGLU	0.002	0.000	0.003	0.005
	FG	0.000	0.000	0.005	0.021
PS15 (*hutU*)	His	0.110	0.000	0.060	0.040
SP54 (*hutH*)	His	0.000	0.000	0.000	0.000
	FG	0.000	0.000	0.000	0.021
LH11 (*hutC*)	None	0.170	0.065	0.093	0.053
LH12 (*hutC*)	None	0.165	0.060	0.090	0.040
LH13 (*hutC*)	None	0.170	0.060	0.086	0.045

[a] Inducers were added to a final concentration of 15 mM in cultures growing on succinate minimal A medium, and cells were harvested 4 h later. His, Histidine; Uro, urocanate.

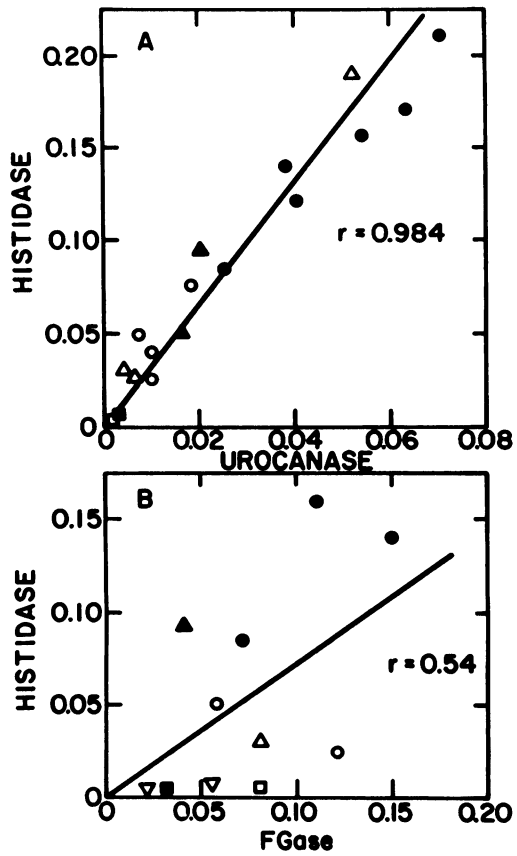

FIG. 2. Inducibility comparisons for urocanase and FGase with histidase. Specific activity of each enzyme was determined after growth of *P. putida* PRS1 for various times. The medium was minimal A salts with either glucose or succinate as a carbon source and 15 mM additive. Symbols: ○, glucose plus urocanate; △, succinate plus urocanate; ●, glucose plus histidine; □, glucose plus FG; ■, glucose plus FIGLU; ▲, succinate plus histidine; ▽, succinate plus FG. The correlation coefficient calculated from linear regression analysis of the data is indicated in each panel.

urocanate and is more efficient than exogenously added urocanate, probably due to its rapid transport. FG was able to induce FGase in all strains, but histidine or urocanate could induce FGase even in *hutU* mutants, which are unable to interconvert urocanate and FG, allowing the conclusion that FGase is induced by either urocanate or FG.

The independence of the control for synthesis of FGase from that of other *hut* enzymes is illustrated by the generally noncoordinate synthesis of FGase and histidase, contrasted with the strongly coordinate synthesis of histidase and urocanase (Fig. 2). These results were all obtained from growth of the wild-type strain (PRS1) of *P. putida* with various medium supplements. In addition, crude extracts prepared from cells which had been induced with either urocanate or FG were examined by polyacrylamide gel electrophoresis at pH 8.3 for the migration of FGase activity (9). These gels revealed a single FGase band in both cases which migrated to the same extent on these native gels; mixed extracts likewise revealed no evidence for two electrophoretically distinct species (Fig. 3). This suggests that only one FGase is present to an appreciable extent in *P. putida* and is induced by either urocanate or FG.

Three constitutive mutants isolated had normal levels of activity for all of the *hut* enzymes which were examined (Table 1), supporting the idea that a single regulator (repressor) gene is controlling induced expression of the *hut* genes despite the existence of two different inducers.

Subcloning of the *hutG* gene into *E. coli*. To obtain greater quantities of the *P. putida hutG* gene product in *E. coli*, a 9.6-kilobase-pair (kbp) *Hin*dIII fragment was removed from plasmid pLH2 and ligated into the corresponding site of pBEU1. The recombinant plasmid was then transformed into *E. coli* RDP145. The two orientations of the insert resulted from the *hutG* cloning (Fig. 1). Enzyme assays conducted after temperature induction of cells growing on LB medium revealed that the clone containing pLH4 had 13 times more FGase than the clone containing pLH4a, in which the *hutG* gene was in the opposite orientation. These results were interpreted as indicating that the cloned *hutG* gene was probably being expressed from a vector promoter (perhaps the *bla* gene promoter) in pLH4 and from a less efficient *Pseudomonas* promoter in the case of pLH4a, but the possibility that vector promoters were used for expression in both directions cannot be dismissed on the basis of our data. The presence of a promoter associated with the *hutG* gene would be consistent with the somewhat independent regulation observed for that gene, and the existence of a separate *hutG* promoter is currently being examined in more detail.

Purification of FGase. When *E. coli* RDP145(pLH4) was grown on LB medium and then shifted from 30 to 39°C, this strain produced FGase to a specific activity four times that observed for FGase from *P. putida* PRS1 grown under similar conditions (but without the temperature shift) and nine times that noted for the FGase from *E. coli* RDP210 containing pMC1, the parent plasmid encoding the entire *hut* operon.

High-speed supernatants of sonic extracts were subjected to $(NH_4)_2SO_4$ precipitation from 40 to 65% saturation. After dialysis, the enzyme was purified by a series of column chromatography steps, including DE52 anion-exchange

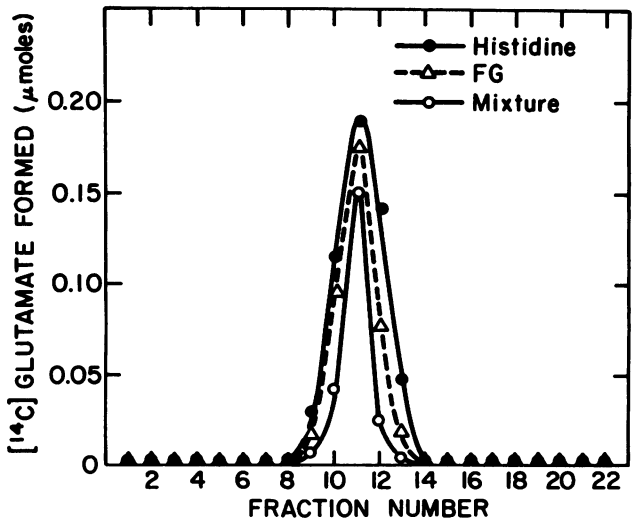

FIG. 3. Polyacrylamide gel electrophoresis of FGase activity present in extracts from cells grown with different inducers. Separations were conducted on a 10% acrylamide gel (1.5 mm thick) in a Hoefer SE600 vertical cell operated at 30 mA. Lanes were divided into 0.5-cm lengths and placed into tubes prior to maceration in 0.5 ml of 50 mM phosphate buffer, pH 7.5. Tracking dye migration was 11 cm (fraction 22).

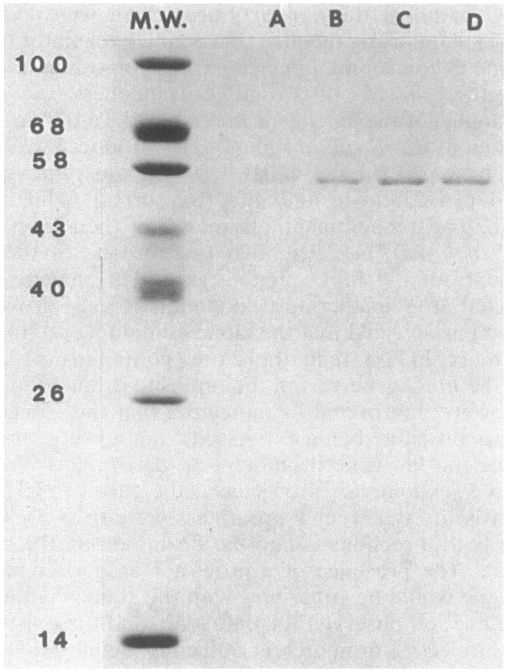

FIG. 4. Electrophoresis of purified FGase on an SDS-polyacrylamide gel. Samples were mixed with an equal volume of solution containing 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.125 M Tris hydrochloride, pH 6.8, and then boiled for 2 min. The left lane contained standard proteins and their molecular weights (10^3) are indicated. Lanes A through D, FGase (0.25, 0.5, 1.0, and 1.5 μg of protein, respectively). The standards were (from top to bottom): rabbit muscle glycogen phosphorylase a, bovine serum albumin, bovine catalase, chicken egg albumin, rabbit muscle fructose bisphosphate aldolase, bovine chymotrypsinogen A, and chicken egg lysozyme.

chromatography and Sephacryl S-200, hydroxyapatite, and DEAE-Sepharose chromatography. The six steps resulted in a 1,110-fold purification of FGase with a yield of 10%. SDS-polyacrylamide gel electrophoresis on a 10% gel showed a major band containing over 90% of the applied protein and having a molecular weight of 50,000 (Fig. 4). Molecular weight determination of the native enzyme by gel filtration on a column of Bio-Gel P-300 (1) resulted in a value of 52,000, indicating that the enzyme exists as a monomeric species under the conditions of the analysis. The purification of FGase is summarized in Table 2.

The initial specific activity for the crude extract presented in Table 2 was not as high as could be obtained in other instances. By more careful adjustment of the treatment time at 39°C, it has been possible to achieve starting specific activities up to 0.34 U/mg of protein; the optimum time at the higher temperature prior to harvesting would appear to be 150 to 180 min, rather than the 90 min used for the purification described in Table 2. To improve the specific activity further, it will probably be necessary to reclone the *hutG* gene under the control of a regulatable promoter, such as the *tac* promoter or the p_L promoter from bacteriophage lambda.

TABLE 2. Summary of FGase purification

Step	Total activity (U)	Sp act (U/mg)	% Recovery	Purification (fold)
Crude extract	1,240	0.0905	100	
Protamine sulfate	1,100	0.181	89	2.0
40–65% $(NH_4)_2SO_4$	912	0.382	74	4.2
Dialysis	1,230	0.589	99	6.5
DE52	399	3.39	32	37
Sephacryl S-200	356	16.5	29	180
Hydroxyapatite	364	65.1	29	720
DEAE-Sepharose	130	100	10	1,100

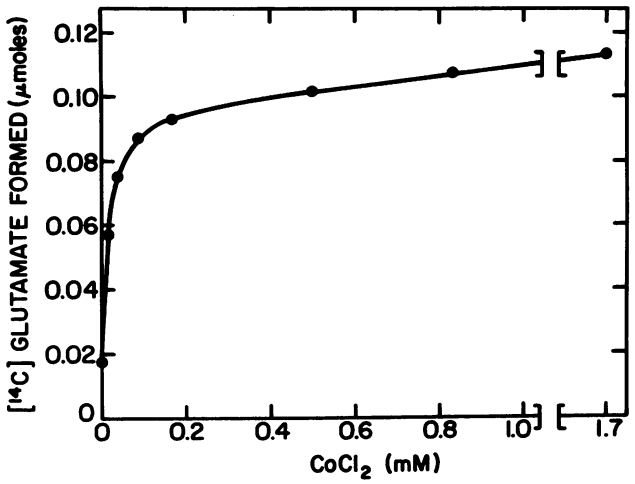

FIG. 5. Concentration dependence of FGase activation by Co(II). Assay conditions were as described in the text except with varied cobalt chloride levels.

Catalytic properties of FGase. An investigation of the catalytic properties of FGase revealed that the enzyme exhibited an optimum pH of 7.5 in 100 mM potassium phosphate and retained over 65% of its maximum activity between pH 6.6 and 8.5. Eight divalent metals were tested at concentrations of 0.08 and 0.8 mM for their effect on FGase activity, with the finding that Co(II) was the most stimulatory; an analysis of its concentration dependence is presented in Fig. 5. Fe(II) was also a good activator of FGase, particularly at lower concentrations, but it inhibited slightly when used at concentrations over 0.1 mM. Other divalent metals tested (Cd, Ca, Mn, Zn, Ni, and Mg) were generally not effective activators. FGase did not exhibit total dependence on added divalent cation even when purified, and activation with 0.8 mM Co(II) was only fourfold the untreated activity. FGase had a K_m of 14 mM for formylglutamate in the presence of 0.8 mM $CoCl_2$. Based on a specific activity of 100 μmol/min per mg of protein determined at 5 mM FG, it was calculated that the enzyme would have a specific activity of 380 μmol/min per mg with saturating substrate.

Several substrate analogs were tested for their ability to inhibit the action of FGase on formylglutamate. These were the *N*-formylated derivatives of L-aspartic acid, L-methionine, and L-leucine, as well as the formylated dipeptide L-methionyl-L-phenylalanine, and two *N*-acetylated amino acids, glutamate and glutamine. Enzyme assays were conducted with analogs at concentrations of 2.5, 5.0, and 10 mM in the presence of 5 mM FG. Formylaspartic and acetylglutamic acids were inhibitory to FGase, while none of the others showed any effects except for a small inhibition by *N*-acetylglutamine, which may have been due to contaminating *N*-acetylglutamate. Determination of the inhibition constants for acetylglutamate and formylaspartate from Dixon plots gave competitive inhibitions with K_i values of 6 and 9 mM, respectively (Fig. 6). [^{14}C]Acetylglutamate was prepared and tested directly for potential activity as a

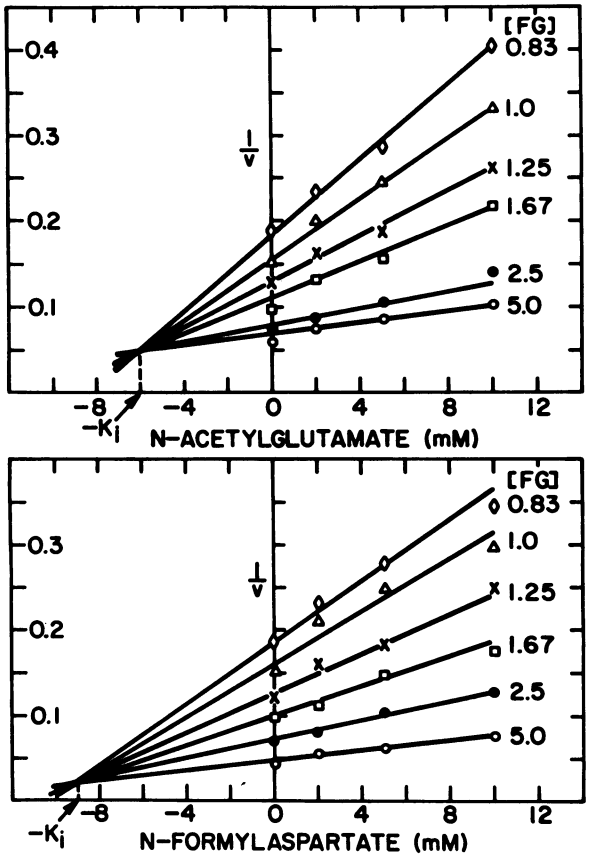

FIG. 6. Dixon plots for the inhibition of FGase by acetylglutamate or formylglutamate. Purified enzyme was used for these analyses.

substrate, but it proved to be inactive (<2% of FG activity at comparable concentrations).

If formate produced by FGase is actively used for building purines during growth of *P. putida* on histidine as a carbon source, then one could argue that hydrolysis of FIGLU to FG and subsequently to glutamate plus formate is wasteful since activation of formate to produce formyltetrahydrofolate requires an energy input. One alternative considered was that FGase could act on FG with tetrahydrofolate as the C-1 acceptor instead of water, conserving ATP and producing formyltetrahydrofolate directly. Incubation of tetrahydrofolate and FG with the enzyme failed to result in the formation of N^5,N^{10}-methenyltetrahydrofolate, as indicated by no increase in absorbance at 350 nm. Thus, we conclude that FGase cannot effectively use tetrahydrofolate as a C-1 acceptor in place of water in the formation of glutamate from FG.

DISCUSSION

The cloning of *hut* genes from *P. putida* into *E. coli* by Consevage et al. (7) allowed *E. coli* to be induced for the use of histidine as a carbon source, and this regulated expression demonstrated that the *P. putida hut* genes were under the transcriptional control of their own promoter(s) and regulatory gene product (repressor). Expression has been observed in a variety of *E. coli* strains, although levels have varied somewhat with strain. We believe that major transcriptional start sites are located in front of *hutF* and *hutU* and that the latter can generate transcripts which extend through *hutU*, *hutH*, *hutI*, and perhaps into *hutG* (Hu and Phillips, in preparation). Evidence presented here, i.e., the noncoordinate induction of histidase and FGase, points to the presence of another promoter site and an operator region associated with *hutG*. The extent to which this independent control region is used for normal expression of *hutG* is still unknown. We are not aware of a source of FG other than from histidine breakdown, and thus it is not obvious why it would be desirable to have *hutG* transcription independently controllable.

The production of FGase in *P. testosteroni* was reported by Coote and Hassall to be under the control of both urocanate and formylglutamate (8), just as we have observed for the FGase from *P. putida*. They concluded, however, that this might be due to the presence of two enzymes capable of degrading FG and supported this by the fact that cells grown on FG have high activities of FGase but only basal levels of other *hut* pathway enzymes. Furthermore, they were unable to isolate mutants lacking FGase which could still grow on glutamate as a sole carbon source. While our data are similar, we conclude that it is more likely that a single *hut*-related FGase exists which can be induced by either FG or urocanate. This would require the existence of a bifunctional repressor whose binding to the operator region(s) is controlled separately by FG and urocanate. In view of the apparent multiple transcription units which exist for the *hut* genes based on their organization, we propose there are at least two operator regions whose recognition by the single *hutC* repressor protein is affected differently by urocanate or FG. Thus, either FG or urocanate could block repressor interaction with the *hutG* promoter-operator region, whereas only urocanate would affect repressor binding to the other *hut* control regions. Proof of this hypothesis must await isolation of the *hutC* repressor protein and examination of the effect of inducers on its ability to bind to various control regions.

The failure to find *hutG* mutants is perhaps explained by the presence of a related enzyme, an *N*-acetylglutamate hydrolase, believed to be involved in arginine biosynthesis. Früh and Leisinger (10) studied this enzyme in *P. aeruginosa* and found it to be active towards FG, although slightly less so than towards acetylglutamate. It was also Co^{2+} dependent, but interestingly was localized in the periplasmic space. Although *P. putida* mt-2 grew only marginally on acetylglutamate and had undetectable levels of this enzyme in crude extracts (10), we nevertheless attribute the difficulty in isolating *hutG* mutants of *P. putida* to the presence of low levels of this enzyme and its ability to provide sufficient breakdown of FG to obscure the *hutG* phenotype.

The FGase isolated from *P. putida* consists of a single type of subunit based on a molecular weight of 50,000 to 52,000 for both the native and SDS-treated enzyme. Most notable among its properties are its requirements for Fe^{2+} or Co^{2+} as divalent metal ion and its inhibition by *N*-acetylglutamate without being detectably active in hydrolysis of this material. On this basis alone, it is easily distinguished from the periplasmic *N*-acetylglutamate hydrolase described for *P. aeruginosa* (10).

One of the more intriguing questions raised by the existence of FGase in pseudomonads is why the *hut* pathway differs from that seen in most other organisms, even though the end products, a C-1 unit and L-glutamate, are generally the same as found elsewhere. The key to this question may not lie in the FGase but rather in the comparison of FIGLU-hydrolyzing enzymes. For example, the terminal enzyme in

the *Klebsiella hut* sequence hydrolyzes FIGLU to glutamate plus formamide. In contrast, the *Pseudomonas* enzyme converts FIGLU to FG plus ammonia. Thus, a different site of water attack is used in these enzymes, suggesting perhaps that an evolutionary change occurred which altered the catalytic mechanism of the FIGLU hydrolase to produce FG and ammonia instead of glutamate and formamide. The existence of this new intermediate, FG, may have forced the recruitment of a related activity, possibly an *N*-acetylglutamate hydrolase capable of attacking FG, to be used in FG breakdown. In time, gene duplication could have brought FGase synthesis under the control of the *hut* repressor. This scenario would predict that any structural similarity existing among the *hut* enzymes due to their possible origin by duplication of ancestral genes and subsequent coevolution (31) would not be found in the case of FGase, since its origin is predicted to be different from that of the other pathway members. It may also be worth noting that the K_i for *N*-acetylglutamate with FGase is of the same magnitude as the K_m for FG, in keeping with the hypothesis that FGase possesses some ancestral relationship with a protein recognizing and acting on *N*-acetylglutamate.

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