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Selection of Amidases with Novel Substrate Specificities from Penicillin Amidase of *Escherichia coli*

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To obtain amidases with novel substrate specificity, the cloned gene for penicillin amidase of *Escherichia coli* ATCC 11105 was mutagenized and mutants were selected for the ability to hydrolyze glutaryl-(L)-leucine and provide leucine to Leu$^-$ host cells. Cells with the wild-type enzyme did not grow in minimal medium containing glutaryl-(L)-leucine as a sole source of leucine. The growth rates of Leu$^-$ cells that expressed these mutant amidases increased as the glutaryl-(L)-leucine concentration increased or as the medium pH decreased. Growth of the mutant strains was restricted by modulation of medium pH and glutaryl-(L)-leucine concentration, and successive generations of mutants that more efficiently hydrolyzed glutaryl-(L)-leucine were isolated. The kinetics of glutaryl-(L)-leucine hydrolysis by purified amidases from two mutants and the respective parental strains were determined. Glutaryl-(L)-leucine hydrolysis by the purified mutant amidases occurred most rapidly between pH 5 and 6, whereas hydrolysis by wild-type penicillin amidase at this pH was negligible. The second-order rate constants for glutaryl-(L)-leucine hydrolysis by two "second-generation" mutant amidases, 48 and 77 M^{-1} s^{-1}, were higher than the rates of hydrolysis by the respective parental amidases. The increased rates of glutaryl-(L)-leucine hydrolysis resulted from both increases in the molecular rate constants and decreases in apparent K_m values. The results show that it is possible to deliberately modify the substrate specificity of penicillin amidase and successively select mutants with amidases that are progressively more efficient at hydrolyzing glutaryl-(L)-leucine.

Previous studies on the substrate specificity of penicillin amidase (EC 3.5.1.11) from *Escherichia coli* have shown the enzyme to be capable of catalyzing the synthesis and hydrolysis of structurally diverse amides of the general form R—CO—NH—R′. The substrate specificity of the wild-type enzyme is largely determined by the characteristics of the acyl moiety, R. Substrates with hydrophobic acyl moieties are preferred to those with functional groups that, depending on the reaction pH, can be polar (ionized) (6, 19, 20, 28). By contrast, R′ can be varied substantially. Substrates with common acyl groups are hydrolyzed at about the same rate regardless of whether R′ is hydrogen, a penam nucleus, a cephem nucleus, or an amino acid (5, 20, 24).

The means for the deliberate modification of the substrate specificity of penicillin amidase are required for the use of penicillin amidase in the semisynthesis of β-lactam antibiotics and other synthetic reactions. We were able to devise a general method for the selection of mutants with amidases having altered substrate specificity. In this method, the cloned gene for penicillin amidase is introduced into an auxotrophic host bacterium, the cells are mutagenized, and mutants are selected by the newly acquired ability to hydrolyze a selection substrate containing the required amino acid as R′. The acyl group of the selection substrate, R, can be varied depending on the substrate specificity of the amidase sought and restrictions of the process being developed.

To demonstrate the feasibility of the approach, we attempted to isolate mutants with amidases that had required the ability to hydrolyze glutaryl-(L)-leucine (Fig. 1). Glutaryl-(L)-leucine and other amides with glutaryl-acyl moieties (R) are not hydrolyzed by the wild-type penicillin amidase of *E. coli* (L. Forney and D. C. L. Wong, unpublished data). The results presented here show that mutant amidases able to hydrolyze glutaryl-(L)-leucine can be isolated readily. Furthermore, culture conditions such as pH and concentration of amide in the medium can be modulated to allow reiterative use of the selection procedure to isolate mutant amidases capable of increased rates of amide hydrolysis.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains RFS740 and RFS817 were obtained from Barbara Bachman (*E. coli* Genetic Stock center), and strains LS1461 and LS1274 were obtained from Larry Soll.

Plasmid pA1 contains the gene for the wild-type penicillin amidase of *E. coli* ATCC 11105 cloned into the *Pst*I site of pACYC184 (3) and was obtained from Richard Eaton. *E. coli* RFS740 [F′ (F101) Δ(*ara-leu*)*498* Δ(*lac*)*74 relA1 spoT1*? *thi-1*] cured of the F′, was designated *E. coli* C740 and transformed with pA1. *E. coli* Mut was constructed as follows. Bacteriophage P1 *kc* was propagated on *E. coli* LS1461 (*thr*::Tn*10 gal-1 gal-2 strA594 recA* Lac$^-$) and used to transduce *thr*::Tn*10* into *E. coli* RFS817 [Δ(*ara-leu*)*498* Δ(*lac*)*3 rpsL220 thi-1 relA1*). P1 *kc* was then used to cotransduce *thr*::Tn*10* and Δ(*ara-leu*)*498* into *E. coli* LS1274 (*galU95* Str^r Azi^r *mutD5*). Tet^r Ara$^-$ colonies were identified on MacConkey agar plus 2% arabinose. Cotransduction of Thr$^-$ and Ara$^-$ occurred at a frequency of 2%. All Tet^r Ara$^-$ colonies obtained were also found to be Leu$^-$. One colony with the proper genotype [Δ(*ara-leu*)*498 mutD5*] was designated *E. coli* Mut and used for mutagenesis of plasmids encoding amidase genes.

Chemicals. Glutaryl-(L)-leucine and phenylacetyl-(L)-leucine were purchased from Bachem, Inc. (Torrance, Calif.). Chloramphenicol (Cam), MES (2-[*N*-morpholino]

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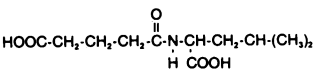

FIG. 1. Structure of glutaryl-(L)-leucine.

ethanesulfonic acid), MOPS (3-[*N*-morpholino]propanesulfonic acid), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, Mo.). 6-Nitro-3-phenylacetamidobenzoic acid (PACNA) was synthesized as described previously (17).

Mutagenesis and selection of mutants. *E. coli* C740(pA1) was mutagenized with UV light or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as previously described (21).

To obtain second-generation mutants, the plasmid DNA from parental strains was mutagenized by passage through *E. coli* Mut. Cells transformed with plasmids that encode first-generation mutant amidases were grown overnight at 30°C in 5 ml of Luria broth with Cam at 20 μg/ml (LBCam) and serially diluted in phosphate-buffered saline (PBS). Tubes containing 2 ml of LBCam were each inoculated with 1 drop of the 10^{-4} dilution and incubated for 48 h at 30°C. The cells from 1-ml samples of culture were collected by centrifugation for 30 s in a microfuge (model 235A; Fisher Scientific), suspended in 1 ml of PBS, and incubated overnight at 30°C to allow the depletion of intracellular leucine pools. Mutants with an improved ability to hydrolyze glutaryl-(L)-leucinewere selected on minimal medium containing glutaryl-(L)-leucine as a sole source of leucine. Plates of selective medium containing M9 basal medium (21) with 0.2% glucose that contained Cam (20 μg/ml), 0.5 mM glutaryl-(L)-leucine, 50 mM MES, pH 5.5, and were solidified with 1.5% agar were spread with 100 μl of the mutagenized, leucine-starved cells and incubated at 30°C in sealed plastic bags. Mutant colonies which appeared were streaked on LBCam.

Plasmid DNA from strains with mutant and wild-type amidases were used to transform naive cells of strain C740 (14). The phenotype of each mutant was confirmed by streaking the transformants onto minimal medium with and without glutaryl-(L)-leucine as a sole source of leucine.

Growth studies. The effect of pH and glutaryl-(L)-leucine concentration on the growth rate of the mutants in M9 basal medium was determined as follows. M9 basal medium buffered with MMS buffer (200 mM MES, 200 mM MOPS, and 200 mM succinate) was adjusted to the indicated pH, supplemented with glutaryl-(L)-leucine and Cam (20 μg/ml), and filter sterilized. The inocula used were prepared by suspending cells from 3 ml of stationary-phase cultures grown in M9 with 0.2% glucose, 0.3 mM phenylacetyl-(L)-leucine, and 20 μg of Cam per ml in 0.5 ml of PBS. Wild-type penicillin amidase readily hydrolyzes phenylacetyl-(L)-leucine (12). Triplicate 16-mm screw-cap tubes each containing 5 ml of medium were inoculated with about 100 μl of these cell suspensions and incubated on a tube rotator (model TC-7; New Brunswick Scientific, Edison, N.J.) at 30°C. Growth of the cultures was determined periodically by measuring turbidity at 600 nm with a Spectronic 21 (Bausch & Lomb, Rochester, N.Y.), with uninoculated medium used as a blank.

Purification of amidases. Wild-type and mutant amidases were purified by ion-exchange column chromatography (L. Forney and D. C. L. Wong, manuscript in preparation). Briefly, cells were grown at 30°C in 10 liters of LBCam to early stationary phase, harvested by centrifugation, washed once with 100 mM phosphate buffer (pH 7.5)–10 mM $MgCl_2$, and resuspended in 1 liter of the same buffer. The cells were disrupted by passage through a Gaulin homogenizer (model 15M; Gaulin Corp., Everett, Mass.) two times. Cell debris was removed by centrifugation, and the supernatant was fractionated with ammonium sulfate. The protein that precipitated between 40 and 60% ammonium sulfate was collected and dialyzed against 50 mM MES, pH 5.0. Insoluble material was removed from the dialyzed protein by centrifugation, and the supernatant was applied to an S-Sepharose column (Pharmacia, Piscataway, N.J.) equilibrated with 50 mM MES, pH 5.0. Unretained protein was washed from the column with the same buffer. Amidases were eluted with buffer that contained 50 mM NaCl, and the remaining protein was eluted with buffer that contained 1 M NaCl. Fractions were assayed for amidase activity by using PACNA as described previously (17). Fractions which contained appreciable activity were pooled, dialyzed against 20 mM Tris, pH 7.8, and applied to a Q-Sepharose column (Pharmacia) equilibrated with the same buffer. Unretained protein was eluted with 20 mM Tris, pH 7.8, and retained protein was eluted with a linear NaCl gradient from 0 to 200 mM NaCl. Fractions were assayed as described above, and those with amidase activity were pooled and dialyzed against 20 mM Tris, pH 7.8.

The purity of each enzyme preparation was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18), and the amidase was quantified by titration of the active site with PMSF as described previously (27) with PACNA as a substrate.

Kinetics of glutaryl-(L)-leucine hydrolysis. The kinetics of glutaryl-(L)-leucine by wild-type and mutant amidases was determined in reaction mixes containing purified amidase, various amounts of substrate, and either 100 mM phosphate buffer, pH 7.5, or 100 mM MES, pH 5.0. The reaction mixes were incubated at 37°C for up to 4 h. Reactions were terminated by heating for 10 min at 90°C, and reaction mixes were stored at −20°C. The leucine produced was quantified as follows. After the samples were thawed, 0.2 μmol of phenylalanine was added as an internal standard, and the samples were dried under vacuum in a Speed-Vac concentrator (Savant, Farmingdale, N.Y.). The phenylthiocarbamyl derivatives of the amino acids were formed as described previously (15) and chromatographed by gradient elution from an Ultrasphere ODS column (25 cm by 4.6 mm; Beckman Instruments, San Ramon, Calif.) on an HP 1090 high-pressure liquid chromatograph (HPLC) with a 3390A integrator (Hewlett-Packard, Avondale, Pa.).

The effect of pH on the rates of glutaryl-(L)-leucine and phenylacetyl-(L)-leucine hydrolysis was determined as described above except that MMS buffer adjusted to a pH ranging from 4.0 to 8.0 was used. MMS buffer contains 200 mM MOPS, 200 mM MES, and 200 mM succinate adjusted with either HCl or NaOH to the appropriate pH.

Kinetics of phenylacetyl-(L)-leucine hydrolysis. The kinetics of phenylacetyl-(L)-leucine hydrolysis was determined by measuring the rate of leucine release spectrophotometrically with (L)-amino acid oxidase coupled to the oxidation of *o*-dianisidine by horseradish peroxidase. The reactions contained (per milliliter): purified amidase, between 13.5 and 51 nmol of phenylacetyl-(L)-leucine, 1 U of (L)-amino acid oxidase, 2 U of horseradish peroxidase, 0.3 μmol of *o*-dianisidine, and 100 μmol of phosphate buffer, pH 7.5. The reaction mixes were incubated at 37°C, and reactions were initiated by the addition of amidase. The oxidation of *o*-dianisidine was determined by measuring the change in A_{460}

TABLE 1. Kinetics of glutaryl-(L)-leucine hydrolysis by amidases from *E. coli* C740(pA251) and C740(pA324) compared with those of phenylacetyl-(L)-leucine hydrolysis by wild-type penicillin amidase[a]

Amidase[b]	Substrate	pH	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
pA1	PAL	7.5	62.5 ± 4.8	0.0197 ± 0.0037	3×10^6
pA251	GL	7.5	0.04 ± 0.01	27.0 ± 3.7	1.5
	GL	5.0	0.45 ± 0.03	13.1 ± 1.2	34
pA324	GL	7.5	0.07 ± 0.01	37.7 ± 2.5	1.9
	GL	5.0	0.75 ± 0.04	22.5 ± 1.3	33

[a] The reaction mixes contained purified amidase and either glutaryl-(L)-leucine (GL) or phenylacetyl-(L)-leucine (PAL) and were buffered with either 100 mM phosphate, pH 7.5, or 100 mM MES, pH 5.0. The reaction mixes were incubated for up to 4 h, and the accumulated leucine was quantified by HPLC. The amount of enzyme used was determined by titration of enzyme activity with PMSF. The kinetic constants were calculated by nonlinear least-squares regression analysis (9) of the data and are expressed as the mean ± standard deviation.

[b] The amidases are designated by the plasmids that encode the genes for the enzymes.

with a Beckman DU-7 spectrophotometer (Beckman Instruments, San Ramon, Calif.).

Calculation of kinetic parameters. The kinetic constants K_m and k_{cat} (26) were determined by nonlinear least-squares regression analysis (9) of the data.

RESULTS

Isolation of mutants that hydrolyze glutaryl-(L)-leucine. Mutants were selected by their ability to grow on minimal medium containing glutaryl-(L)-leucine as a sole source of leucine. *E. coli* C740 with a plasmid encoding wild-type penicillin amidase was mutagenized with either *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or UV light and plated on M9 medium that contained 0.3 mM glutaryl-(L)-leucine as a sole source of leucine. Mutant colonies grew very slowly and were picked after 2 to 3 weeks of incubation. Plasmid DNA was isolated from each mutant and used to transform naive cells of *E. coli* C740. Cells transformed with mutant plasmids grew slowly on the selective medium, whereas cells with the wild-type amidase (pA1) did not grow. Cells with either mutant or wild-type amidases failed to grow when glutaryl-(L)-leucine was omitted from the medium, whereas all grew when the medium contained leucine. These observations suggest that mutations within the plasmid DNA, and probably in the gene encoding penicillin amidase, have altered the substrate specificity of penicillin amidase and conferred the ability to hydrolyze glutaryl-(L)-leucine and provide leucine to the Leu^- host cells.

Kinetics of glutaryl-(L)-leucine hydrolysis by mutant amidases. The kinetics of glutaryl-(L)-leucine hydrolysis at pH 7.5 by the amidases encoded by mutant plasmids pA251 and pA324 were determined following purification of the enzymes to near homogeneity. Both mutant amidases slowly hydrolyzed glutaryl-(L)-leucine (Table 1) and retained the ability to hydrolyze phenylacetyl-(L)-leucine at rates similar to that of the wild-type enzyme (data not shown). The low rate of substrate hydrolysis is consistent with the low growth rates on selective medium of strains that expressed these amidases. Glutaryl-(L)-leucine hydrolysis was not detected (k_{cat}, ≤ 0.01 s^{-1}) in reaction mixes containing purified wild-type penicillin amidase or when no amidase was added to the reaction mix.

TABLE 2. Effect of glutaryl-(L)-leucine concentration on the growth of *E. coli* C740 with either pA1, pA251, or pA324 in M9 buffered at pH 5.5[a]

Glutaryl-(L)-leucine concn (mM)	Growth (A_{600}) of *E. coli* C740 carrying:		
	pA1	pA251	pA324
100	0.03	0.48	0.98
5	0.03	0.24	0.62
3	0.04	0.15	0.48
2	0.03	0.07	0.27
1	0.03	0.05	0.14

[a] Triplicate cultures of each strain were grown in M9 plus Cam (20 μg/ml) and 50 mM MES, pH 5.5, and glutaryl-(L)-leucine at the concentration indicated. The initial A_{600} was 0.03. The cultures were incubated at 30°C, and culture density was measured periodically. The culture density shown was measured after 47 h of incubation.

Effect of pH on substrate hydrolysis. Because the hydrophobicity of glutaryl-(L)-leucine increases as the glutaryl-carboxyl group becomes protonated, we reasoned that the rate of glutaryl-leucine hydrolysis would increase as the pH of the reaction mixes was decreased. As anticipated, the rate of glutaryl-(L)-leucine hydrolysis by the mutant amidases increased as the pH of the reaction decreased and occurred most rapidly between pH 5 and 6. Wild-type penicillin amidase hydrolyzed glutaryl-(L)-leucine at a negligible rate in reaction mixes of low pH. The catalytic efficiency (k_{cat}/K_m) of glutaryl-(L)-leucine hydrolysis by the amidases of pA251 and pA324 was about 20-fold greater at pH 5.0 than at pH 7.5 (Table 1). For both enzymes, the increased rate of substrate hydrolysis at low pH was the consequence of both an increased affinity for glutaryl-(L)-leucine (decreased apparent K_m) and an increased molecular rate constant (k_{cat}).

Effect of pH and amide concentration on growth rate. Because penicillin amidase is a periplasmic enzyme (2, 25), there is no effective diffusion barrier between the enzyme and small molecules in the bulk medium. Accordingly, factors expected to influence the rate of substrate hydrolysis by the enzyme, such as pH and glutaryl-(L)-leucine concentration, can be varied in the medium and affect the rate of substrate hydrolysis in vivo. Differences in the rate of glutaryl-(L)-leucine hydrolysis would in turn be reflected in the growth rate of cells, because the growth rate of the Leu^- host is determined by the rate of leucine release through hydrolysis of glutaryl-(L)-leucine.

The growth rate of *E. coli* C740 with either pA251 or pA324 increased as the pH of medium containing glutaryl-(L)-leucine was decreased from pH 7.5 to 5.5. The doubling time of these strains was approximately 6 h in M9 plus 5 mM glutaryl-(L)-leucine buffered at pH 5.5 but exceeded 24 h when the medium was buffered at pH 7.5. By contrast, essentially no growth occurred when the strains were inoculated into M9 plus 0.3 mM glutaryl-(L)-leucine buffered at pH 7.5. The growth rates were unaffected by medium pH when leucine or phenylacetyl-(L)-leucine (the leucine analog of penicillin G; the preferred substrate for penicillin amidase) was substituted for glutaryl-(L)-leucine. Similarly, the growth rate of *E. coli* C740 with either pA251 or pA324 increased as the concentration of glutaryl-(L)-leucine was increased (Table 2) and was correlated with the rate of glutaryl-(L)-leucine hydrolysis by the purified amidase. These results indicate that the pH and glutaryl-(L)-leucine concentration of the medium can be modulated to restrict the growth rate of parental strains and allow the reiterative use of the selection methods to isolate mutants with improved abilities to hydrolyze glutaryl-(L)-leucine.

TABLE 3. Kinetic constants for glutaryl-(L)-leucine hydrolysis at pH 5.0 by amidases from *E. coli* strains C740(pA251-47) and C740(pA324-9)[a]

Amidase[b]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
pA251-47	0.72 ± 0.01	9.4 ± 0.4	77
pA324-9	0.91 ± 0.03	18.8 ± 0.8	48

[a] See Table 1, footnote *a*, for methods used to determine kinetic constants.
[b] The amidases are designated by the plasmids that encode the enzymes.

Isolation of mutants with an improved ability to hydrolyze glutaryl-(L)-leucine. Because *E. coli* C740 with either pA251 and pA324 grew slowly on M9 medium containing 0.5 mM glutaryl-(L)-leucine and buffered at pH 5.5, this medium could be used in experiments to isolate second-generation mutants. This was done after mutagenesis of the penicillin amidase genes of pA251 and pA324 by using *E. coli* Mut. This strain has a defective DNA polymerase 3'-5' exonucleolytic editing activity (8) that results in an increased mutation frequency when the strain is grown in rich medium (10). All possible base pair substitutions occur during DNA replication and cell growth (10). Accordingly, *E. coli* Mut with pA251 or pA324 was grown from a low inoculum in Luria broth, starved to deplete intracellular leucine pools, and plated on selective medium. Mutant colonies appeared following 3 to 4 days of incubation. Of the mutants obtained, pA251-47 and pA324-9 were among those which grew most rapidly on selective medium following transformation of naive *E. coli* C740.

Kinetics of glutaryl-(L)-leucine hydrolysis by second-generation mutants. The molecular and second-order rate constants, k_{cat} and k_{cat}/K_m, respectively, for the hydrolysis of glutaryl-(L)-leucine by the amidases from these strains are shown in Table 3. The second-order rate constants for glutaryl-(L)-leucine hydrolysis by the amidases of pA251-47 and pA324-9 were 2.3- and 1.5-fold greater than those for the respective parental amidases. For the amidases of both pA251-47 and pA324-9, the increased second-order rate constants resulted from increases in the molecular rate constants and increased affinities for the substrate. The second-generation mutants grew more rapidly than the parental strains in medium containing glutaryl-(L)-leucine (data not shown), which is consistent with the observed increases in the second-order rate constants.

The effect of pH on the rates of glutaryl-(L)-leucine and phenylacetyl-(L)-leucine hydrolysis by wild-type penicillin amidase and the amidases encoded by pA251 and pA251-47 was determined. The optimum pHs for glutaryl-(L)-leucine hydrolysis by the amidases of pA251 and pA251-47 appeared to be similar, about 5.5 (Fig. 2a). Both mutant enzymes retained the ability to hydrolyze phenylacetyl-(L)-leucine and did so at high rates that were similar to that of wild-type penicillin amidase and optimal at near pH 7.5 to 8.0 (Fig. 2b). Similar results were obtained with the amidases of pA324 and pA324-9.

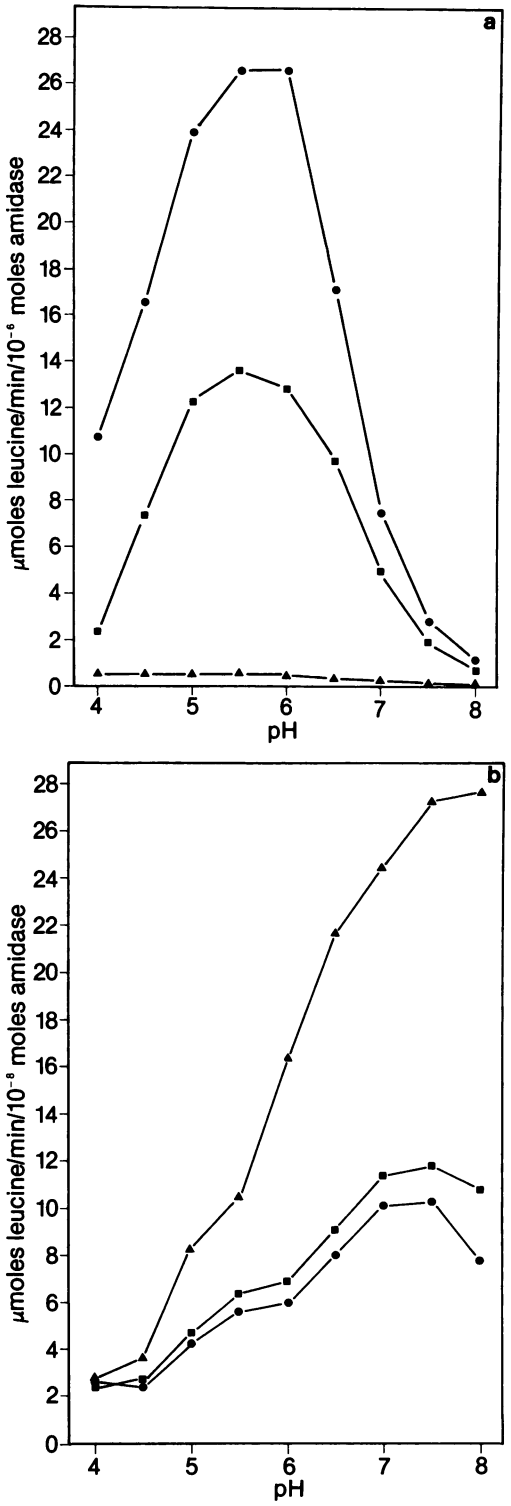

FIG. 2. Effect of pH on (a) glutaryl-(L)-leucine and (b) phenylacetyl-(L)-leucine hydrolysis by the amidases from *E. coli* strains C740(pA1) (▲), C740(pA251) (■), and C740(pA251-47) (●).

DISCUSSION

A procedure for the selection of variant penicillin amidases having novel or improved abilities to hydrolyze substrates with a given acyl group has been developed. Clones that express mutant penicillin amidases can be selected by their ability to hydrolyze a selection substrate and release a nutrient essential for growth of the host bacterium. In this study, Leu^- cells transformed with a plasmid encoding penicillin amidase were mutagenized and plated on selective medium containing glutaryl-(L)-leucine. Amidases purified from mutants that grew on the selective medium hydrolyzed glutaryl-(L)-leucine, whereas purified wild-type penicillin

amidase did not. The lack of glutaryl-(L)-leucine hydrolysis by the wild-type enzyme probably results from the inability of the enzyme to effectively bind substrates with polar acyl groups (6, 19, 20, 28). By contrast, amides with hydrophobic acyl groups, such as hexanoyl-(L)-leucine and phenylacetyl-(L)-leucine, will serve as a source of leucine to Leu$^-$ cells expressing wild-type penicillin amidase (12; L. Forney, unpublished data).

Mutants with amidases able to hydrolyze glutaryl-(L)-leucine were readily isolated. As expected, the growth rates of Leu$^-$ cells that expressed mutant amidases were correlated with the rates of glutaryl-(L)-leucine hydrolysis. Thus, colonies that grew most rapidly on selective medium were likely to express amidases that hydrolyzed glutaryl-(L)-leucine most rapidly. Leucine prototrophs (revertants) were never obtained, probably because the host strains have a deletion in the operon for leucine biosynthesis.

The rates of glutaryl-(L)-leucine hydrolysis by the purified mutant amidases were affected by substrate concentration and the reaction pH. Because penicillin amidase is a periplasmic enzyme, these parameters could be varied in the selection medium and affect the rate of substrate hydrolysis in vivo. We restricted the growth of parental strains by modulating the substrate concentration and pH of the medium and selected mutants which grew more rapidly. The amidases from these second-generation mutants were characterized and shown to have an improved ability to hydrolyze the selection substrate. Preliminary results indicate that similar means can be used to obtain third- and fourth-generation mutants. We expect that the growth of parental strains could also be restricted through incorporation of leucine analog or antagonists of leucine metabolism in selective medium.

The amides used as selection substrates could carry different amino acids or acyl groups. Studies have shown that wild-type penicillin amidase is capable of hydrolyzing amides in which the acyl group is a phenylacetyl moiety and the amino group is any of several amino acids, including glycine, alanine, serine, valine, isoleucine, and phenylalanine (28). Consequently, the selection substrates used could be varied and based on any of a number of different amino acids or acyl groups. For example, D-(−)-α-aminophenylacetyl-(L)-leucine or thienylacetyl-(L)-leucine might be useful to select for enzymes that more efficiently synthesize cephalexin or cephaloridine, respectively. Amidases with novel substrate specificity do not have to be selected in amino acid autotrophs. For example, Daumy et al. (7) showed that variant amidases could be derived from the penicillin amidases of *E. coli* ATCC 9637 and *Proteus rettgeri* ATCC 31052 by serial passage of the strains in medium containing primary amides as a sole nitrogen source. This approach allowed them to obtain select populations of bacteria that hydrolyzed bromocaproamide, cyclohexanamide, 6-bromohexanamide, and [1-(4-hydroxy-1-cyclohexenyl)]-acetamide more efficiently than the wild-type enzymes of the respective strains. However, cells that expressed the mutant amidases obtained through this method were unable to grow on solidified medium, a factor that would limit the usefulness of this approach.

Selective pressures have been used previously to direct the evolution of enzymes (23). This approach has been successfully used to alter the substrate specificity of aliphatic amidase (1), ribitol dehydrogenase (22), β-lactamase (13), penicillin amidase (7), β-galactosidase (3), and alcohol dehydrogenase (29). Whereas modifications to proteins made by site-directed mutagenesis are often either silent or deleterious (16), the use of selective pressure to alter enzyme characteristics offers the advantages that only catalysts with greater effectiveness are obtained and prior knowledge of enzyme structure or enzyme-substrate interactions is not needed. The sequencing of mutant genes and the determination of the kinetic parameters of mutant enzymes would provide information useful in understanding the structure and function of the enzyme. Consequently, this approach provides a useful means to identify amino acid residues and protein domains that affect substrate binding and catalysis, which may facilitate the development of improved enzymes.

Penicillin amidase is used to make 6-aminopenicillanic acid from penicillin G (20). Other potential uses of the enzyme include the semisynthesis of various β-lactam antibiotics (25), selective N-terminal deprotection in peptide synthesis (29), and the enantioselective hydrolysis of esters (11). The development of means to alter the specificity and catalytic properties of the enzyme could lead to its increased use in these or other hydrolytic or condensation reactions.

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