Role of the Ortholog and Paralog Amino Acid Invariants in the Active Site of the UDP-MurNAc-L-alanine:D-glutamate Ligase (MurD)†

Ahmed Bouhss,‡ Sébastien Dementin,‡ Claudine Parquet,‡ Dominique Mengin-Lecreulx,‡ Jay A. Bertrand,§ Dominique Le Beller,|| Otto Dideberg,§ Jean van Heijenoort,‡ and Didier Blanot*,‡

EP 1088 du CNRS, Biochimie Structurale et Cellulaire, Bâtiment 430, Université de Paris-Sud, 91405 Orsay, France, Institut de Biologie Structurale Jean-Pierre Ebel (CEA-CNRS), Laboratoire de Cristallographie Macromoléculaire, 38027 Grenoble Cedex 1, France, and Hoechst Marion Roussel, 102 route de Noisy, 93235 Romainville, France

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ABSTRACT: To evaluate their role in the active site of the UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase (MurD) from *Escherichia coli*, 12 residues conserved either in the Mur superfamily [Eveland, S. S., Pompliano, D. L., and Anderson, M. S. (1997) *Biochemistry 36*, 6223−6229; Bouhss, A., Mengin-Lecreulx, D., Blanot, D., van Heijenoort, J., and Parquet, C. (1997) *Biochemistry 36*, 11556−11563] or in the sequences of 26 MurD orthologs were submitted to site-directed mutagenesis. All these residues lay within the cleft of the active site of MurD as defined by its 3D structure [Bertrand, J. A., Auger, D., Fanchon, E., Martin, L., Blanot, D., van Heijenoort, J., and Dideberg, O. (1997) *EMBO J. 16*, 3416−3425]. Fourteen mutant proteins (D35A, K115A, E157A/K, H183A, Y194F, K198A/F, N268A, N271A, H301A, R302A, D317A, and R425A) containing a C-terminal $(His)_6$ extension were prepared and their steady-state kinetic parameters determined. All had a reduced enzymatic activity, which in many cases was very low, but no mutation led to a total loss of activity. Examination of the specificity constants k_{cat}/K_m for the three MurD substrates indicated that most mutations affected both the binding of one substrate and the catalytic process. These kinetic results correlated with the assigned function of the residues based on the X-ray structures.

In the complex process of biosynthesis of bacterial peptidoglycan, the assembly of the peptide moiety of its monomer unit has recently been the topic of numerous investigations. It is performed by a series of enzymes designated as the Mur synthetases (MurC,[1] MurD, MurE, and MurF), which are responsible for the successive additions of L-alanine, D-glutamate, *meso*-diaminopimelate or L-lysine, and D-alanyl-D-alanine to UDP-*N*-acetylmuramic acid (*1*). A fifth synthetase, which catalyzes the addition of tripeptide L-Ala-γ-D-Glu-*meso*-diaminopimelate in the recycling process of peptidoglycan, has also been identified and designated as Mpl (*2*). These enzymes share common properties. They all catalyze the synthesis of an amide or peptide bond with concomitant degradation of ATP into ADP and P_i, and all seem to operate through a similar mechanism involving the formation of acyl phosphate and tetrahedral intermediates (*3*−*10*). The comparison of various amino acid sequences of the *mur* gene products, as well as of other related enzymes, revealed the existence of common invariants: seven amino acids plus the ATP-binding consensus sequence (*11*, *12*). Moreover, the conservation of backbone distances between certain invariants suggested common structural motifs (*12*). Among other ADP-forming peptide synthetases, only folylpoly-γ-L-glutamate synthetase (FolC) and the C-terminal part of cyanophycin synthetase from *Synechocystis* sp. shared the same conserved amino acid residues (*11*−*13*). Site-directed mutagenesis studies were undertaken with MurC (*12*) and MurF (*11*) from *Escherichia coli*. In MurC seven invariant residues (K130, E174, H199, N293, N296, R327, and D351) were replaced by alanine. Analysis of the kinetic parameters of the mutant enzymes suggested that residues K130, E174, and D351 were important for the catalytic process, and that residues K130, H199, N293, N296, and R327 were involved in the structure of the active site and the binding of the substrates (*12*).

Owing to their specific occurrence in eubacteria and their presence in both Gram-positive and Gram-negative pathogens, the Mur synthetases represent potential targets for new antibacterial compounds. Therefore, the identification of amino acid residues involved in the catalytic mechanism or in the binding of the substrates could lead to the rational design of inhibitors. To evaluate the role of the conserved

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* To whom correspondence should be addressed. Phone: +33-1-69-15-61-34. Fax: +33-1-69-85-37-15. E-mail: didier.blanot@ebp.u-psud.fr.

‡ Université de Paris-Sud.

§ Institut de Biologie Structurale Jean-Pierre Ebel (CEA-CNRS).

|| Hoechst Marion Roussel.

[1] Abbreviations: FolC, folylpoly-γ-L-glutamate synthetase; Mpl, UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase; MurC, UDP-*N*-acetylmuramate:L-alanine ligase; MurD, UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase; MurE, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate:*meso*-diaminopimelate ligase; MurF, UDP-*N*-acetylmuramoyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate:D-alanyl-D-alanine ligase; Ni^{2+}-NTA, Ni^{2+}-nitrilotriacetate-agarose; UMA, UDP-*N*-acetylmuramoyl-L-alanine; UMAG, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate.

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Table 1: Mutations Introduced in the *murD* Gene Product

plasmid	mutation[a]	oligonucleotide[b]	new restriction site[c]
pABDD35A	D35A	5′-CGCCGCGCGTTATGG**C**TAC**C**CGTATGACAC-3′	*Afl*III
pABDK115A	K115A	5′-CCGGTTCTAACGGC**GCC**AGCACGGTCACCA-3′	*Ehe*I
pABDE157A	E157A	5′-ACTGTACGTGCTGG**C**ACTGTCGAGCTTCCA-3′	*Afl*III
pABDE157K	E157K	5′-GAACTGTACGTG**T**TG**A**AACTGTCGAGCTTC-3′	*Tsp*RI
pABDH183A	H183A	5′-CGTGACTGAAGAT**GCC**ATGGATCGCTATCC-3′	*Nco*I
pABDY194F	Y194F	5′-GGTTTACAACA**ATT**TCGTGCAGCAAAACTG-3′	*Sse*9I
pABDK198A	K198A	5′-TATCGTGCAGC**GGCC**CTGCGCATTTACGAA-3′	*Bsi*ZI
pABDK198F	K198F	5′-TATCGTGCAGCA**TTC**CTGCGCATTTACGAA-3′	*Bsm*I
pABDN268A	N268A	5′-TTCCGGGCAGCA**CGCG**TACACCAATGCGCT-3′	*Afl*III
pABDN271A	N271A	5′-GCATAACTACAC**GGCC**GCGCTGGCGCGCGT-3′	*Bgl*II
pABDH301A	H301A	5′-CTGGTCTGCCG**GCC**CGCTTTGAAGTTGTGC-3′	*Ngo*MI
pABDR302A	R302A	5′-TGGTCTGCCGCA**CGCG**TTTGAAGTTGTGCT-3′	*Afl*III
pABDD317A	D317A	5′-ACGTTGGATTAACG**C**TTCGAAAGCGACCAA-3′	*Hin*fI
pABDR425A	R425A	5′-GAACTTTGAACAA**GCC**GGCAATGAGTTTGCC-3′	*Ngo*MI

[a] Amino acids are represented by their one-letter abbreviation, and the number indicates the localization of the mutated residue in the amino acid sequence of MurD; the numbering does not take into account the N-terminal methionine residue, which was shown to be absent in the isolated protein (*16*, *26*). [b] Mutations of the *murD* gene sequence that have been introduced in the oligonucleotides are indicated in bold. [c] The confirmation of mutated plasmids was facilitated by choosing mutations which, in addition, generated new restriction sites in the gene sequence.

residues in the Mur superfamily, we performed their site-directed mutagenesis in *E. coli* MurD. MurD is a logical choice for this study since its 3D structure in the presence of UMA (*14*) or other ligands (*15*) has recently been solved. The steady-state kinetic parameters of the mutant enzymes were determined; the results were compared with the data of the 3D structure of MurD (*14*, *15*) and with those previously obtained with MurC (*12*). Furthermore, a number of residues conserved in all 26 presently accessible sequences of MurD, but not in all Mur synthetases, were also submitted to site-directed mutagenesis.

MATERIALS AND METHODS

Chemicals. D-[1-^{14}C]Glutamic acid (2.03 GBq mmol^{-1}) was purchased from Isotopchim (Ganagobie-Peyruis, France). UMA and [^{14}C-Ala]UMA were prepared according to Auger et al. (*16*) and Pratviel-Sosa et al. (*17*), respectively.

Strains and Growth Conditions. E. coli strain DH5α (*supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Φ80 d*lacZ* ΔM15) (Bethesda Research Laboratories) was used as a host for plasmids as well as for the overproduction of mutant MurD proteins. *E. coli* strain BMH71−18 *mutS* defective in mismatch repair was used in site-directed mutagenesis experiments (*18*). 2YT (*19*) was used as a rich medium for growing cells, and growth was monitored by measuring the culture absorbance at 600 nm. For strains carrying resistance genes, antibiotics were used at the following concentrations: ampicillin (100 μg mL^{-1}) and tetracycline (15 μg mL^{-1}).

General DNA Techniques and E. coli Cell Transformation. Small- and large-scale plasmid isolations were carried out by the alkaline lysis method (*20*). Standard procedures for endonuclease digestion, ligation, and agarose electrophoresis were used (*20*, *21*). DNA sequencing was performed according to Sanger et al. (*22*), using the T7 sequencing kit from Pharmacia. *E. coli* cells were made competent for transformation by the method of Dagert and Ehrlich (*23*) or by electroporation.

Plasmid Construction. A plasmid suitable for the overproduction of wild-type MurD containing a C-terminal $(His)_6$ extension was constructed as follows: PCR primers were designed to incorporate a *Nco*I site (in boldface type) 5′ to the initiation codon (underlined) of *murD*, 5′-TTAA**C-CATGG**CTGATTATCAGGGT-3′, and a *Bam*HI site (in boldface) 3′ to the gene without its stop codon, 5′-CGCA**G-GATCC**TAACTCCTTCGCCAG-3′. These primers were used to amplify the *murD* gene from the *E. coli* chromosome. The resulting material was treated with *Nco*I and *Bam*HI and was ligated between the same sites of vector p*Trc*His60 (*24*). This plasmid was designated pABD16. Its *murD* gene codes for an enzyme tagged by a C-terminal Ser-Arg-Ser-$(His)_6$ extension.

Site-Directed Mutagenesis. Site-directed mutagenesis of the *E. coli* His-tagged MurD enzyme was performed by using the Transformer site-directed mutagenesis kit purchased from Clontech (Palo Alto, CA) based on the site-directed mutagenesis method of Deng and Nickoloff (*18*). This method works by simultaneous annealing of two oligonucleotide primers to one strand of a denatured plasmid; one primer introduces the desired mutation in the gene and the other primer mutates a unique restriction site in the plasmid vector sequence for purpose of selection. The latter primer which is common to all mutagenesis experiments (5′-CGAAGGC-GAAGCGGCAAGCATTTACGTTGACAC-3′) was defined for suppression of the unique *Nsi*I site lying within the target plasmid pABD16. Table 1 shows the oligonucleotide sequences used for introduction of specific mutations within the *murD* gene. In each case, after annealing of both primers, standard DNA elongation, and ligation steps, the resulting mixture of mutated and wild-type plasmids was transformed into *E. coli mutS* strain BMH71-18. Transformants selected in 2YT-ampicillin medium were pooled (more than 10^3), and their plasmid DNA was prepared and digested by *Nsi*I to eliminate wild-type plasmids carrying an intact *Nsi*I site. The reaction mixture was used to transform strain DH5α. As predicted, DNA sequencing of plasmids resistant to *Nsi*I digestion showed that most of them also carried the expected mutation in the *murD* gene. In each case, one of the mutated clones was chosen and used for further investigation.

Expression and Purification of His-Tagged Wild-Type and Mutant MurD Proteins. DH5α cells carrying either the pABD16 plasmid (His-tagged wild-type MurD) or one of the plasmids listed in Table 1 (His-tagged mutated MurD) were grown exponentially at 37 °C in 2YT-ampicillin

medium (50 mL cultures). When the optical density of the culture reached 0.2, isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 1 mM, and growth was continued for 8 h. Cells were harvested in the cold and washed in cold 20 mM potassium phosphate buffer, 1 mM dithiothreitol, 20 mM imidazole, and 0.3 M NaCl, pH 7.2 (buffer A). The cell pellet was suspended in 1 mL of buffer A and sonicated in the cold. The resulting suspension was centrifuged at 4 °C for 20 min at 200000*g* in a Beckman TL100 centrifuge, and the pellet was discarded. SDS−PAGE analysis revealed that these crude extracts contained 20−30% MurD (data not shown). The supernatant was mixed with 0.5 mL of Ni^{2+}-nitrilotriacetate-agarose (Ni^{2+}-NTA) resin previously equilibrated with buffer A for 2 h at 4 °C. The resin was recovered by centrifugation and washed with increasing concentrations of imidazole in buffer A (20, 25, 30, 40, 50, 60, 80, and 100 mM; from 50 mM imidazole, NaCl was omitted in the buffer). Elution of the MurD protein started at 40 mM imidazole; however, the protein was homogeneous in SDS−PAGE (data not shown) only in the 100 mM washings, which were pooled and dialyzed against 20 mM potassium phosphate, 1 mM dithiothreitol, pH 7.2 (buffer B). In the standard MurD assay (5 mM ATP, 25 μM UMA, and 25 μM D-Glu, pH 8.6; ref *16*), the activity of the His-tagged wild-type MurD was 1500 nmol min^{-1} mg^{-1}, a value which was similar to that of the non His-tagged enzyme (1770 nmol min^{-1} mg^{-1}; ref *16*). Therefore, the C-terminal His-tag had no influence on the activity of the protein.

Determination of the Kinetic Constants. The D-glutamate-adding activity was assayed by following the formation of radioactive UMAG in a mixture containing 0.1 M Tris-HCl buffer, pH 9.4, 5 mM $MgCl_2$, ATP, UMA, D-Glu, and enzyme (25 μL of an appropriate dilution in buffer B) in a final volume of 50 μL. Generally, D-[^{14}C]Glu (0.88 kBq) was used as the labeled substrate; however, when the UMA concentration was much lower than that of D-Glu owing to the great difference in their respective K_m values, [^{14}C-Ala]-UMA (0.88 kBq) was used instead. The mixture was incubated for 30 min at 37 °C, and the reaction was stopped by addition of 10 μL of glacial acetic acid. The radioactive substrate and product were separated by reversed-phase HPLC on a Nucleosil $5C_{18}$ column (4.6 × 150 mm; Alltech France, Templemars, France) using 50 mM ammonium formate buffer, pH 4.7, at 0.6 mL min^{-1}. Detection was performed with a radioactive flow detector (model LB506-C1, EG&G Wallac/Berthold, Evry, France) using the Quicksafe Flow 2 scintillator (Zinsser Analytic, Maidenhead, U.K.) at 0.6 mL min^{-1}. Quantification was carried out with the Winflow software (EG&G Wallac/Berthold). The K_m values for ATP, UMA, and D-Glu were determined by varying the concentration of one of the substrates while maintaining the others at saturating concentrations.

RESULTS

Alignment of the MurD Sequences. The sequences of 26 MurD orthologs from Gram-negative and Gram-positive bacteria were aligned using the CLUSTAL W program (*25*), and the final alignment was optimized manually (Figure 1). The invariant residues which appeared (in bold in Figure 1) could be classified in two groups: (i) residues common to the Mur family (*11*, *12*). In the numbering of *E. coli* MurD, they were D35 (found as E in one species), K115 (belonging to the ATP-binding motif GXXGKST), E157, H183, N271 (sometimes found as D or Q; found as P in one species), R302 (found as K in one species), and D317. On the other hand, N268, which had been previously considered as invariant upon analysis of the Mur and FolC sequences available at the time (*11*, *12*), was not conserved among the MurD sequences examined here. (ii) Residues conserved in all MurD orthologs, but not in all the other members of the Mur superfamily. In view of subsequent site-directed mutagenesis studies, we decided to consider only those having a ionizable side chain: Y194, K198, H301, K348 (sometimes found as R), D417 (found as E in two species), and R425 in the *E. coli* enzyme. It was noteworthy that all the amino acid invariants of both groups lay within the active-site cleft of MurD as observed in its 3D structure (*14*) (Figure 2).

Site-Directed Mutagenesis of the Conserved Residues. The mutation of 12 residues of *E. coli* MurD was undertaken. The mutated residues were the seven invariants of the Mur family (D35, K115, E157, H183, N271, R302, and D317), the asparagine residue previously considered as conserved (N268), and four residues having a ionizable side chain and conserved in the MurD orthologs (Y194, K198, H301, and R425). In most cases, these amino acids were replaced by alanine. E157 was also replaced by lysine in order to reverse the electrical charge of the side chain. Y194F was constructed in order to suppress the phenolic group while maintaining the benzene ring. One residue, K198 in the MurD orthologs group, deserves a special explanation. In the crystal structures of the MurD·UMA complex (Protein Data Bank code: 1uag; ref *14*) and of three other MurD complexes (*15*), K198 appears as a carbamate derivative. Moreover, this residue is not conserved in all the enzymes of the Mur superfamily; the exceptions include MurC and Mpl, where it is replaced by tyrosine or phenylalanine. Therefore, to better understand the role of this modified residue, we prepared the K198A and K198F mutants.

The *murD* gene from *E. coli* was cloned in plasmid p*Trc*His60 under the control of the *trc* promoter. Six histidine codons were added to the part of the gene corresponding to the C-terminus of the protein. This allowed easy purification of His-tagged wild-type and mutant proteins by affinity chromatography. An important consequence of this strategy was the preparation of mutant enzymes devoid of any trace of non-His-tagged wild-type enzyme originating from the chromosomal *murD* gene, which could interfere with mutant proteins endowed with very low enzymatic activity. It was checked that no D-glutamate-adding activity was eluted in the 100-mM imidazole washings of the Ni^{2+}-NTA resin when an extract from plasmidless strain DH5α was loaded.

Enzymatic Properties of the MurD Mutants. The kinetic parameters k_{cat} and K_m of the mutant enzymes (Table 2) were determined at the optimal pH value of 9.4 (*26*). All mutant enzymes had lower k_{cat} values than the wild-type enzyme, but none were totally devoid of activity. Some of them (D35A, N268A, and R302A) were only slightly affected, whereas others (K115A, E157A/K, K198A/F, and D317A) underwent a dramatic decrease of activity. In these latter cases, the very low k_{cat} values (300−20000-fold decreases relative to wild-type) could not be ascribed to a contamination with chromosomal MurD for two reasons: (i) non-His-tagged MurD was not retained on the Ni^{2+}-NTA resin; (ii) the Michaelis constants of these mutant proteins (Table 2)

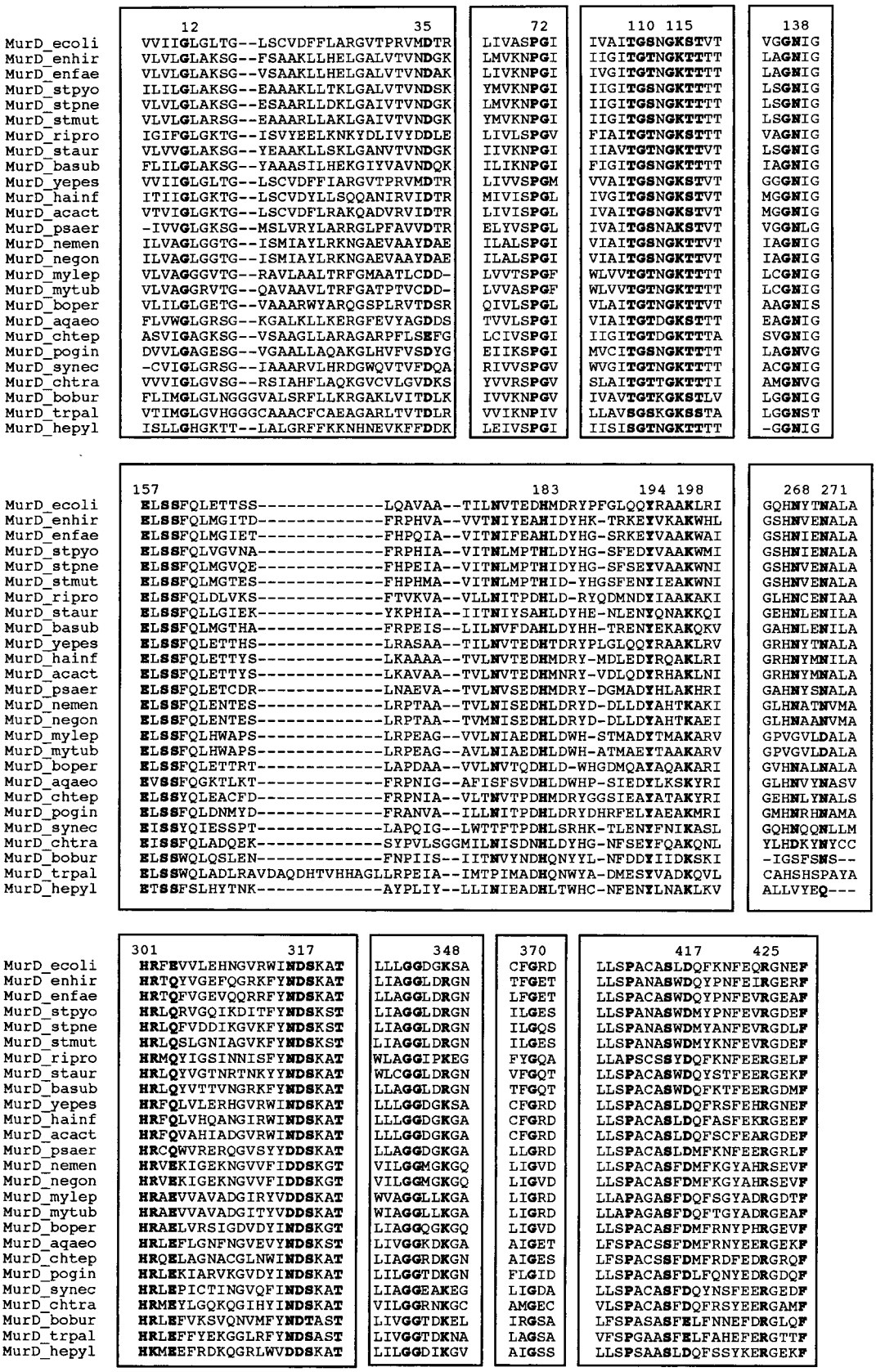

FIGURE 1: Multiple alignment of the amino acid sequences of MurD proteins from *Escherichia coli* (ecoli), *Enterococcus hirae* (enhir), *E. faecalis* (enfae), *Streptococcus pyogenes* (stpyo), *S. pneumoniae* (stpne), *S. mutans* (stmut), *Rickettsia prowazekii* (ripro), *Staphylococcus aureus* (staur), *Bacillus subtilis* (basub), *Yersinia pestis* (yepes), *Haemophilus influenzae* (hainf), *Actinobacillus actinomycetemcommitans* (acact), *Pseudomonas aeruginosa* (psaer), *Neisseria meningitidis* (nemen), *N. gonorrheae* (negon), *Mycobacterium leprae* (mylep), *M. tuberculosis* (mytub), *Bordetella pertussis* (boper), *Aquifex aeolicus* (aqaeo), *Chlorobium tepidum* (chtep), *Porphyromonas gingivalis* (pogin), *Synechocystis sp.* PCC6803 (synec), *Chlamydia trachomatis* (cltra), *Borrelia burgdorferi* (bobur), *Treponema pallidum* (trpal) *and Helicobacter pylori* (hepyl). Only the clusters containing the conserved amino acid residues (in bold) are shown. The numbering refers to *E. coli* MurD.

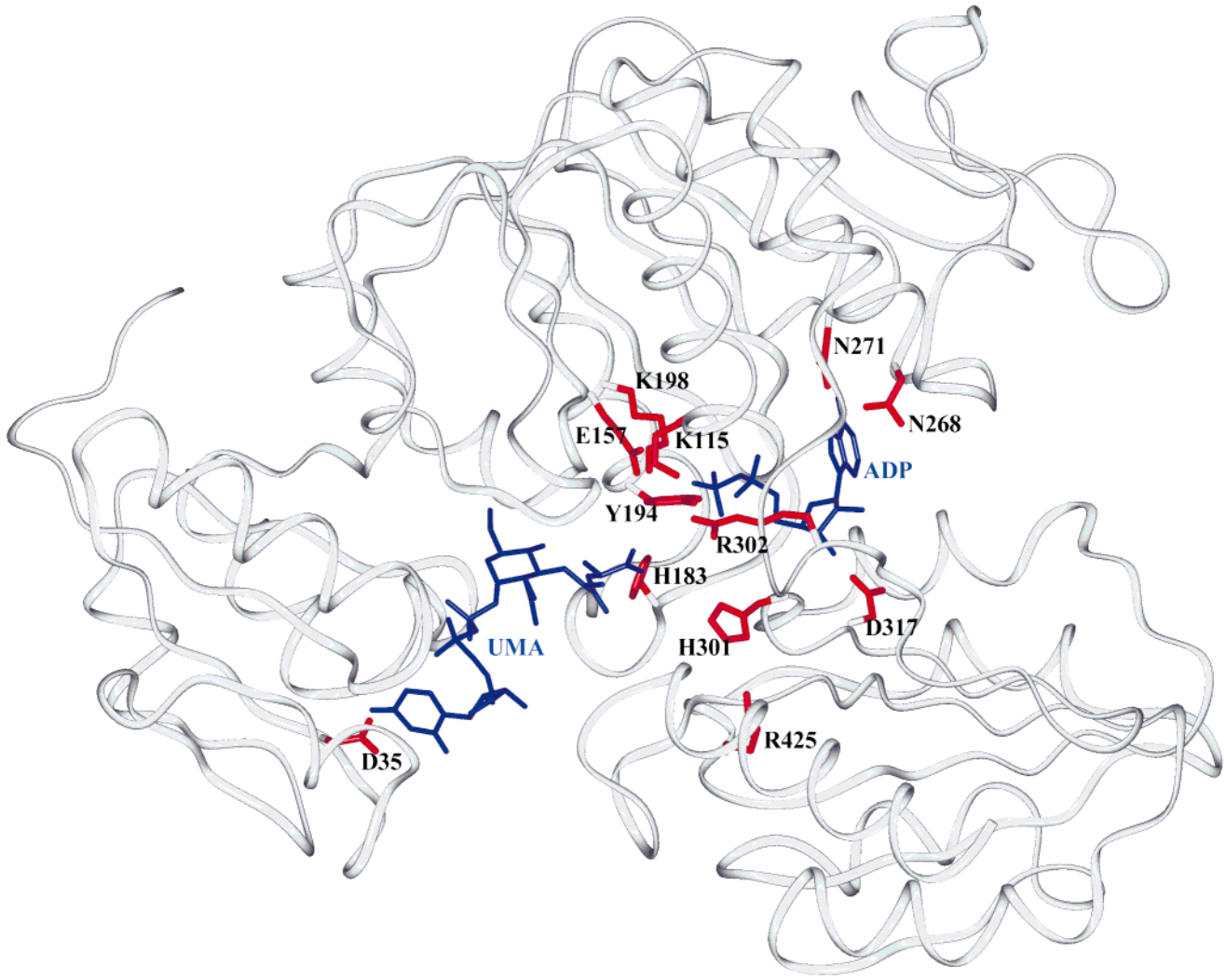

FIGURE 2: Location of the residues submitted to mutagenesis (in red) in the active-site cleft of MurD. Two ligands, UMA and ADP, are shown in blue. The figure was generated with the program Insight II.

Table 2: Kinetic Constants of the MurD Mutant Proteins

protein	k_{cat} (min^{-1})	K_m^{ATP} (μM)	k_{cat}/K_m^{ATP} ($min^{-1}\,\mu M^{-1}$)	K_m^{UMA} (μM)	k_{cat}/K_m^{UMA} ($min^{-1}\,\mu M^{-1}$)	K_m^{D-Glu} (μM)	k_{cat}/K_m^{D-Glu} ($min^{-1}\,\mu M^{-1}$)
wild-type	403	57	7.07	5	80.6	64	6.30
D35A	256	114	2.25	84	3.05	95	2.69
K115A	0.26	328	0.000 79	46	0.0056	128	0.0020
E157A	0.020	30	0.000 67	304	0.000 066		
E157K	0.022	85	0.000 26	218	0.000 10	56	0.000 39
H183A	5.6	130	0.043	59	0.095	806	0.0069
Y194F	32	144	0.22	111	0.29	53	0.60
K198A	0.96	135	0.0071	120	0.0080	185	0.0052
K198F	0.72	129	0.0056	140	0.0051	216	0.0033
N268A	307	140	2.19	12	25.6	264	1.16
N271A	18	1580	0.011	16	1.12	53	0.34
H301A	46	153	0.30	40	1.15	95	0.48
R302A	188	2000	0.094	65	2.89	74	2.54
D317A	1.3	2180	0.000 60	57	0.023	44	0.029
R425A	32	183	0.17	53	0.60	5160	0.0062

were different from those of wild-type MurD (*26*). From the determined k_{cat} and K_m parameters, the specificity constant k_{cat}/K_m was calculated for each substrate (Table 2 and Figure 3). Here again, all values were lower than those of the wild-type enzyme, in part reflecting the decrease of k_{cat}; nevertheless, a more particular decrease for one or two substrates was observed in several instances (ATP for N271A, R302A, and D317A; UMA for E157A/K, H183A, Y194F, K198A/F, and D317A; D-Glu for H183A and R425A). The mutation of N268 to alanine did not significantly affect the specificity constants, a result similar to the one previously observed with N293 in MurC (*12*). This is not surprising, since N268 is not conserved among the Mur family. The four residues conserved in all MurD orthologs (Y194, K198, H301, and

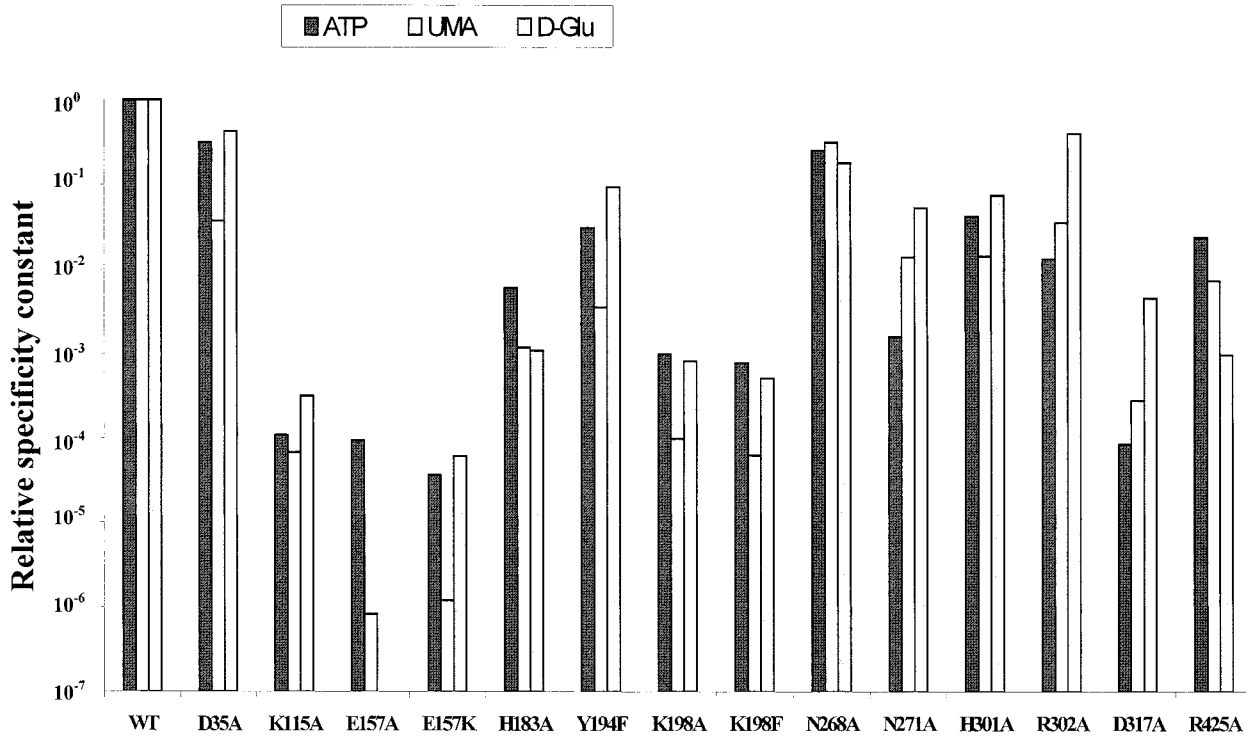

FIGURE 3: Specificity constants of the mutant enzymes for the three MurD substrates. Values are normalized relative to the specificity constants of the wild-type enzyme, taken as 1.

Table 3: Location and Interactions of the Mutated Residues and Enzymatic Effect of the Mutations

residue	domain[a]	X-ray analysis[c]	kinetic analysis[d]
D35	N-terminal	salt bridge with R37; interplane stacking with the uracil ring	UMA (26)
K115	central	interaction with the β-phosphate of ADP	ATP (9000), UMA (14 500), D-Glu (3000)
E157	central	coordinated to Mg^{2+} (site 2)	ATP (10^4), UMA (10^6), D-Glu (10^4)
H183	central	near L-Ala of UMA	ATP (160), UMA (850), D-Glu (900)
Y194	central	H-bonded with the hydroxyl of S112 and with one of the carbamoyl oxygens (K198)	ATP (30), UMA (280)
K198	central	the carbamoyl oxygens are H-bonded with 2 water molecules which are also linked to Mg^{2+} (site 1)	ATP (10^3), UMA (10^4), D-Glu (10^3)
N268	central	H-bonded with the backbone carbonyl of S264 and the side-chain of N271	none
N271	central	H-bonded with the adenine ring	ATP (640), UMA (70), D-Glu (18)
H301	hinge[b]	on a loop between an α-helix and a β-strand	ATP (24), UMA (70), D-Glu (13)
R302	hinge[b]	H-bonded with the α-phosphate of ADP	ATP (75), UMA (28)
D317	C-terminal	H-bonded with ribose hydroxyls	ATP (11 800), UMA (3500), D-Glu (220)
R425	C-terminal	interaction with the carbonyls of S318 and C413; buried in the C-terminal domain	ATP (42), UMA (135), D-Glu (1000)

[a] Location of the amino acid residues in the three previously defined domains of MurD (*14*). [b] Hinge region between the central and C-terminal domains. [c] From ref *14* and *15*. [d] Substrate(s) for which the specificity constant is particularly affected by mutation; the *n*-fold decrease relative to the wild-type enzyme is indicated in parentheses.

R425) displayed specificity constants diminished to various extents according to the substrate.

DISCUSSION

The results obtained in the present work with 14 MurD proteins mutated in the amino acids conserved either among the Mur synthetase family (*11*, *12*) or among 26 MurD orthologs (Figure 1) are summarized in Table 3, in parallel with the possible structural function of each residue as deduced from the 3D structure of MurD. The striking features are that all the residues considered lie within the active-site cleft of the enzyme (Figure 2), that all the corresponding mutant proteins have a reduced enzymatic activity, which in many cases is very low, but that no mutation leads to a total loss of activity. Moreover, a role of the mutated amino acids in the overall structure of the protein can be ruled out since its structural and thermodynamic characteristics, as determined by circular dichroism and microcalorimetry, were not altered by the mutations (data not shown). Therefore, the significant decreases of activity observed suggest that in each case the mutated amino acid is involved in substrate binding and/or in catalysis. It can be speculated that cells carrying such mutations in the chromosome would not be viable; the transfer of the mutated *murD* genes into the chromosome would be necessary to confirm this assertion.

Since the order of substrate binding and product release is not known for MurD, the significance of the steady-state parameters K_m and k_{cat} is not straightforward (*27*). Nevertheless, the specificity constant k_{cat}/K_m is a relevant parameter which, when examined in the light of the X-ray structural

data of four enzyme complexes (MurD·UMA, MurD·UMA·ADP·Mg^{2+}, MurD·UMA·ADP·Mn^{2+} and MurD·UMAG; *14*, *15*), can provide useful information about the role of the invariant amino acids in the enzymatic activity. The fact that most mutations bring about a strong decrease in the specificity constants for two or three substrates (Table 3) indicates that they not only affect the binding of one substrate, as could be expected from a brief examination of the structural data, but they also perturbate the catalytic process.

Mutation of the Invariant Residues of the Mur Family. The mutation of residues K115, E157, H183, N271, and D317 led to strongly reduced specificity constants for the three substrates. This indicates that these residues are of primary importance for the enzymatic activity.

The K115A mutant protein showed dramatic decreases in the specificity constants for all three substrates relative to the wild-type enzyme. The corresponding MurC mutant (K130A) also presented reduced k_{cat}/K_m ratios (*12*). K115 (MurD) and K130 (MurC) belong to a well-known consensus ATP-binding site (*28*). In the 3D structures of the MurD·UMA·ADP·Mg^{2+}/Mn^{2+} complexes, K115 has been shown to interact with the β-phosphate of ADP (*15*). On the basis of the structural results, a role for K115 has been proposed in the formation and stabilization of the acyl phosphate intermediate (*15*). The kinetic results further substantiate the potential role of K115 in the catalytic process.

Mutant proteins E157A/K displayed the lowest specificity constants found in this study. The corresponding MurC mutant protein (E174A) was devoid of enzymatic activity (*12*). Many enzymes which share a common nucleotide-binding fold contain, in the second ATP-binding motif, an acidic residue interacting with a magnesium ion (*28*). Presumably, E157 is this acidic residue for MurD. This is consistent with the observation that, in the MurD·UMA·ADP·Mg^{2+} complex, E157 is coordinated with the Mg^{2+} ion of site 2, which is also linked to the β-phosphoryl oxygen of ADP[2] (*15*). The fact that the specificity constant for UMA is particularly affected is difficult to understand and must await further kinetic studies for a proper explanation.

In mutant protein H183A, the specificity constants for UMA and D-Glu are more particularly altered. A similar pattern was found with the corresponding MurC mutant protein (H199A; ref *12*). In the MurD·UMA complex, H183 is H-bonded with one of the carboxylate oxygens of UMA and probably orients the carboxylate group for the formation of the acyl phosphate intermediate (*14*); in the presence of ADP and Mg^{2+}, the hydrogen bond is lost and H183 interacts indirectly with the carboxylate group of UMA through the Mg^{2+} ion of site 1[2] (*15*). In the MurD·UMAG complex, one of the D-Glu α-carboxylate oxygens is H-bonded with a water molecule, which is one of the Mg^{2+} ligands (*15*). All these structural data explain why an alteration in Mg^{2+} site 1 leads to a correlated effect on the k_{cat}/K_m values for UMA and D-Glu.

[2] Two sites for Mg^{2+} were revealed in the MurD·UMA·ADP·Mg^{2+} complex: in site 1, Mg^{2+} was coordinated by one of the carboxylate oxygens of UMA, one imidazole nitrogen of H183 and four water molecules; in site 2, it was coordinated by the β-phosphoryl oxygen of ADP, the hydroxyl of S116, one of the side-chain carboxylate oxygens of E157 and three water molecules. In the MurD·UMA·ADP·Mn^{2+} complex, only site 1 was occupied by Mn^{2+}, site 2 being vacant.

Mutant N271A corresponds to MurC N296A (*12*). In both cases, the specificity constant for ATP was the most affected one. In the 3D structures of the MurD·UMA·ADP·Mg^{2+}/Mn^{2+} complexes, N271 plays a key role in providing ATP specificity by forming two hydrogen bonds with adenine atoms N6 and N7 (*15*).

Mutant protein D317A is characterized by a very low k_{cat}/K_m value for ATP, thereby suggesting an important role in the interaction of MurD with ATP. This is substantiated by the structures of the MurD·UMA·ADP·Mg^{2+}/Mn^{2+} complexes, from which hydrogen bonds with the 3′ and 2′ ribose hydroxyls of ADP (the latter through a water molecule) have been deduced (*15*). Owing to its extremely weak activity, no kinetic study had been made for the corresponding MurC mutant protein (D351A; ref *12*).

The mutation of D35 and R302 led to proteins with specificity constants affected only for one or two substrates. Mutant protein D35A was essentially altered in the constant for UMA. This is in accordance with the structure of the MurD·UMA complex. D35, which forms a salt bridge with R37, participates in an interplane stacking with the uracil ring (*14*). Mutant protein R302A was affected in the specificity constant for ATP and, to a lesser extent, that for UMA. This result also agrees with the structural studies, which showed the existence of a hydrogen bond between R302 and the α-phosphate of ADP (*15*). However, site-directed mutagenesis and kinetic studies had led us to propose a role in L-alanine binding for the corresponding residue in MurC (R317; ref *12*). Since R302 is conserved among the Mur and FolC synthetases, a similar role of this residue whatever the enzyme would be expected. Therefore, it is surprising that it apparently belongs to the binding site of different substrates (ATP for MurD, L-Ala for MurC). The 3D structure of MurC, as well as the mutagenesis of the corresponding residue in other synthetases, would perhaps clarify this point.

Mutation of the Residues Conserved in the MurD Orthologs. Their conservation in MurD alone or in some of the members of the Mur family suggest a role in substrate binding rather than in catalysis.

K198 is conserved in all the synthetases except MurC and Mpl, where it is replaced by tyrosine or phenylalanine. The fact that mutant proteins K198F and K198A have similar kinetic constants rules out an identical role for K198 and the corresponding residue (Y or F) in MurC or Mpl. The comparison of the carboxylic substrates of MurD, MurE, MurF, and FolC, on one hand, and MurC and Mpl, on the other hand, shows that the main difference is the absence of the lactoylamide or pteroylamide bond (and of the possible additional peptide bonds) in UDP-*N*-acetylmuramic acid (the substrate of MurC and Mpl). This difference in the carboxylic substrates may explain why this residue has been changed in MurC and Mpl. A potential interaction with the carboxylic substrate makes sense in the case of MurD: indeed, if the specificity constants for the three substrates are altered, that for UMA is particularly affected. In four crystal structures of MurD (*15*), K198 was shown to be present as a carbamate derivative. Although it was close to UMA, no direct interaction was apparent. However, in the MurD·UMA·ADP·Mg^{2+}/Mn^{2+} complexes, the carbamoyl oxygens were in indirect interaction with the Mg^{2+} or Mn^{2+} ion (site 1)[2] through two water molecules. Since the metal ion itself

interacts with the carboxyl group of UMA, it is not surprising that the mutations of K198 affect the k_{cat}/K_m value for this substrate. It is noteworthy that Y194, the mutation of which also brings about an altered k_{cat}/K_m value for UMA, is H-bonded with one of the carbamoyl oxygens of K198.

The conservation of H301 among the MurD synthetases is intriguing. Although this residue is located near the ATP-binding site, its mutation does not greatly affect the specificity constant for this substrate. Interestingly, that for UMA is more significantly decreased. H301 lies in the hinge region between the central and C-terminal domains. Its mutation may affect the proper orientation of the two domains and, as a consequence, that of the reacting substrates, thereby explaining the decrease of catalytic efficiency.

Mutant protein R425A displayed a strongly decreased specificity constant for D-glutamic acid. Since R425 is conserved among the MurD proteins, one could conclude that it belongs to the D-Glu-binding site. In the MurD·UMAG structure (*15*), R425 appears as buried in the C-terminal domain, interacting with the backbone carbonyls of S318 and C413. No interactions are made with the D-Glu moiety of UMAG; however, the residues interacting with this moiety (T321, K348, S415, and F422) belong, as R425, to the C-terminal domain of MurD. As a result, R425 seems to be a "structural" arginine (*29*): its mutation probably destabilizes the C-terminal domain, thereby affecting D-Glu binding.

It can therefore be concluded that residues K198, R425, and possibly Y194, which are conserved in the MurD orthologs but not in all the Mur synthetases, participate in the MurD specificity. In this paper, we limited our investigations to conserved residues having a ionizable side chain. As can be seen in Figure 1, several residues with an unionizable side chain are conserved in the MurD sequences. Their importance in the specificity of the MurD reaction or in the proper conformation of the protein molecule should also be evaluated by site-directed mutagenesis and crystallography.

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