# Purification and Characterization of the D-Alanyl-D-alanine-Adding Enzyme from *Escherichia coli*†

Kenneth Duncan,‡,§ Jean van Heijenoort,‖ and Christopher T. Walsh*,‡

*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115, and Unité Associeé du Centre National de la Recherche Scientifique, Biochimie Moléculaire et Cellulaire, Université de Paris-Sud, Orsay, France*

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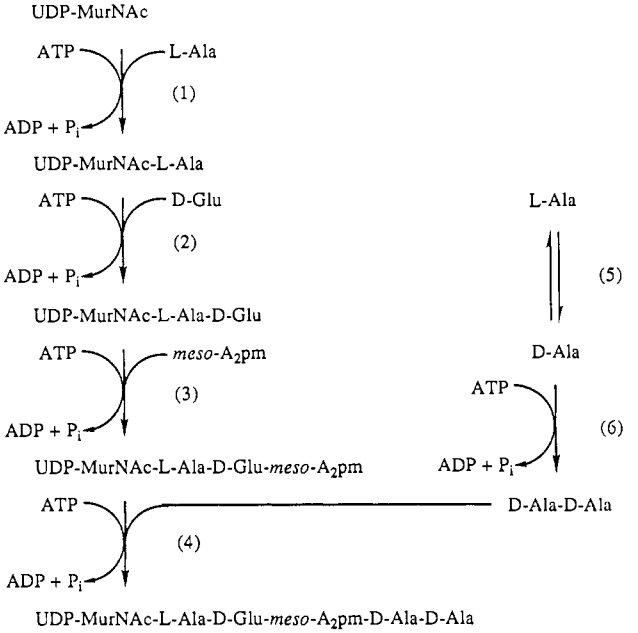
ABSTRACT: The *Escherichia coli* D-alanyl-D-alanine-adding enzyme, which catalyzes the final cytoplasmic step in the biosynthesis of the bacterial peptidoglycan precursor UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-diaminopimelyl-D-Ala-D-Ala, has been purified to homogeneity from an *E. coli* strain that harbors a recombinant plasmid bearing the structural gene for this enzyme, *murF*. The enzyme is a monomer of molecular weight 49 000, and it has a turnover number of 784 $min^{-1}$ for ATP-driven amide bond formation. Experiments monitoring the fate of radiolabeled UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-2,6-diaminopimelate and D-trifluoroalanine proved that the preceding enzyme in the D-alanine branch pathway, D-alanine:D-alanine ligase (ADP), is capable of synthesizing fluorinated dipeptides, which the D-Ala-D-Ala-adding enzyme can then incorporate to form UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-2,6-diaminopimelyl-D-trifluoroAla-D-trifluoroAla.

The pathway for the formation of the bacterial peptidoglycan precursor UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala[1] (UDP-MurNAc-pentapeptide) begins with UDP-MurNAc, which is modified by the sequential addition of the amino acids L-Ala, D-Glu, *meso*-$A_2$pm, and two D-Ala molecules (Rogers et al., 1980; Walsh, 1989) (Scheme I). The terminal D-alanine residues are added as a dipeptide unit by the D-Ala-D-Ala-adding enzyme[2] (reaction 4 in Scheme I). D-Ala-D-Ala is synthesized from L-alanine by the sequential action of alanine racemase and D-Ala-D-Ala ligase. A number of the enzymes which catalyze these reactions are interesting in that they are able to bring about nonribosomal peptide bond formation and, in three of the five cases shown in Scheme I, exclusively utilize D-amino acids. Each of these enzymes fragments ATP to ADP and $P_i$, and it is therefore reasonable to predict that all will operate by an essentially similar mechanism. This would most likely entail carboxyl activation of the first amino acid to an acyl-phosphate intermediate, followed by nucleophilic attack by the amino group of the second condensing amino acid, with elimination of phosphate and subsequent peptide bond formation. These enzymes have for many years been the subject of investigation as possible target sites for antibacterials [for review see Neuhaus and Hammes (1981)], but to date success has been limited. Until our recent efforts with D-Ala-D-Ala ligase (Daub et al., 1988), none of these enzymes involved in peptide bond construction had been purified to homogeneity. We have shown that an (aminoalkyl)phosphinate D-Ala-D-Ala transition-state analogue (Parsons et al., 1988) is a very potent inhibitor of the D-Ala-D-Ala ligase isolated from *Salmonella typhimurium* (Duncan & Walsh, 1988), and although this compound shows poor antibacterial activity in vivo (Parsons et al., 1988), its effectiveness in vitro points toward the possibility to inhibit enzymes that operate with similar mechanisms by a similar strategy. However, a greater knowledge and understanding of the properties of these enzymes is first necessary in order

Scheme I: Enzymes in the Biogenesis of UDP-MurNAc-pentapeptide in *E. coli*[a]




[a] (1) UDP-MurNAc:L-Ala ligase (ADP forming); (2) UDP-MurNAc-L-Ala:D-Glu ligase (ADP forming); (3) UDP-MurNAc-L-Ala-γ-D-Glu:*meso*-$A_2$pm ligase (ADP forming); (4) D-Ala-D-Ala-adding enzyme; (5) alanine racemase; (6) D-Ala:D-Ala ligase (ADP forming).

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* To whom correspondence should be addressed.

‡ Harvard Medical School.

§ Present address: Glaxo Group Research Ltd., Greenford, Middlesex, U.K.

‖ Université de Paris-Sud.

[1] Abbreviations: *meso*-$A_2$pm, *meso*-2,6-diaminopimelic acid; MurNAc, *N*-acetylmuramic acid; UDP-MurNAc-tripeptide, UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-$A_2$pm; UDP-MurNAc-pentapeptide, UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala; PK-LDH, pyruvate kinase–lactate dehydrogenase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] Enzymes: UDP-*N*-acetylmuramyl-L-alanine-D-glutamate-*meso*-diaminopimelate:D-alanyl-D-alanine ligase (ADP forming), or D-Ala-D-Ala-adding enzyme (EC 6.3.2.15); D-alanine:D-alanine ligase (ADP forming), or D-Ala-D-Ala ligase (EC 6.3.2.4); alanine racemase (EC 5.1.1.1).

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to aid in the rational design of novel inhibitory compounds. Previous attempts at rational design of D-Ala-D-Ala analogues have yielded a number of compounds which show promising in vitro inhibitor activity but again are disappointing antibacterials in vivo (Smissman et al., 1976; Neuhaus et al., 1977; Pelzer & Reuter, 1980; Abo-Ghalia et al., 1988).

Earlier investigations of a number of the enzymes involved in synthesis of the UDP-MurNAc-pentapeptide have been reported from our laboratories. Studies of the D-alanine branch pathway focused on alanine racemase and D-Ala-D-Ala ligase. The alanine racemases of *S. typhimurium* and *Bacillus stearothermophilus* have been purified, and the mode of interaction of these enzymes with inhibitory alanine analogues such as fluoroalanine (Badet et al., 1984; Roise et al., 1984; Faraci & Walsh, 1989), (1-aminoethyl)phosphonic acid (Badet et al., 1986), and (1-aminoethyl)boronic acid (Duncan et al., 1989) has been studied. With the exception of elegant kinetic studies carried out in the 1960s by Neuhaus and his colleagues (Neuhaus, 1962a,b; Neuhaus & Lynch, 1964) on the D-Ala-D-Ala ligase from *Streptococcus faecalis*, little was known about this enzyme until recently, when the cloning and overexpression of the *Salmonella ddlA* gene (Daub et al., 1988) facilitated isolation of homogeneous D-Ala-D-Ala ligase in large quantity. The enzymes responsible for addition of D-Glu, *meso*-$A_2$pm, and D-Ala-D-Ala in *E. coli* have been partially purified and characterized (Abo-Ghalia et al., 1985; Michaud et al., 1987). Studies on partially purified D-Ala-D-Ala-adding enzyme from *E. coli* (Comb, 1962), *Staphylococcus aureus* (Ito & Strominger, 1962), *S. faecalis* (Neuhaus & Struve, 1965), *Bacillus subtilis* (Egan et al., 1973), *Corynebacterium poinsettiae*, and *Corynebacterium insidiosum* (Wyke & Perkins, 1975) have also been reported.

We report in this paper two simple and accurate assays that have facilitated the first purification to homogeneity of a D-Ala-D-Ala-adding enzyme. The enzyme was isolated from an *E. coli* strain which harbors a recombinant plasmid carrying *murF*, the structural gene for the *E. coli* D-Ala-D-Ala-adding enzyme. We report initial characterization of the enzyme and show in vitro that D-Ala-D-Ala ligase is capable of making a fluorinated dipeptide which the D-Ala-D-Ala-adding enzyme can then incorporate into UDP-MurNAc-pentapeptide peptidoglycan precursor.

## Materials and Methods

*Materials.* ATP, D-Ala, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (Hepes), and each of the dipeptides mentioned in the text were purchased from Sigma Chemical Co., St Louis, MO. PK-LDH (1:1 ratio of activities) was from Boehringer Mannheim Biochemicals, Indianapolis, IN. Phenyl-Sepharose, polybuffer exchanger PBE 94 and polybuffer 74, FPLC apparatus, and Mono-Q column were obtained from Pharmacia, Piscataway, NJ. Dyematrex Blue A and ultrafiltration materials were from Amicon, Lexington, MA. [$^{14}$C]-D-Alanine was obtained from Amersham International, Arlington Heights, IL.

UDP-MurNAc-tripeptide and UDP-MurNAc-pentapeptide were prepared as described in Flouret et al. (1981). [$^{14}$C]-UDP-MurNAc-tripeptide (330 $\mu$Ci $\mu$mol$^{-1}$) was prepared by the addition of [$^{14}$C]-*meso*-$A_2$pm to UDP-MurNAc-L-Ala-$\gamma$-D-Glu by incubating with purified *meso*-$A_2$pm-adding enzyme, followed by HPLC separation ($\mu$Bondapack $C_{18}$ and Nucleosil $C_{18}$ reverse phase) of radiolabeled tripeptide product from starting materials. [$^{14}$C]Trifluoroalanine (0.35 Ci M$^{-1}$) was prepared as described in Faraci and Walsh (1989).

*Enzyme Assays.* Two assays for D-Ala-D-Ala-adding enzyme activity were utilized. (*1*) *$P_i$ Release.* The D-Ala-D-Ala-adding enzyme activity was routinely detected during the purification by monitoring the UDP-MurNAc-tripeptide- and D-Ala-D-Ala-dependent release of inorganic phosphate, as ATP is consumed in the reaction. The method described in Lanzetta et al. (1979) was employed to detect nanomole amounts of $P_i$ in the incubation. Fifty-microliter incubations (at 37 °C) contained 100 mM Tris buffer (pH 8.6), 10 mM $MgCl_2$, 5 mM ATP, 100 $\mu$M D-Ala-D-Ala, 100 $\mu$M UDP-MurNAc-tripeptide (5 nmol), and enzyme. The quantity of enzyme in the assay was limited to that which would release 1–5 nmol of $P_i$ in 5 min.

(*2*) *Coupled Spectrophotometric Assay.* Consumption of ATP in the D-Ala-D-Ala-adding enzyme reaction was also detected by coupling ADP formation to the pyruvate kinase and lactate dehydrogenase reactions and monitoring the decrease in $A_{340}$ as NADH is consumed. Assays (0.5 mL) contained 100 mM Tris buffer (pH 8.6), 10 mM $MgCl_2$, 5 mM ATP, 2 mM phosphoenolpyruvate, 100 $\mu$M NADH, 100 $\mu$M D-Ala-D-Ala, 100 $\mu$M UDP-MurNAc-tripeptide, and enzyme.

Protein concentrations were determined by the method of Bradford (1976).

*Purification of the D-Ala-D-Ala-Adding Enzyme.* The enzyme was purified from *E. coli* strain JM83 harboring the recombinant plasmid pHE5 (Ayala et al., 1988). pHE5 is a pUC9 derivative and carries a 7.2 kb *Hin*dIII–*Eco*RI fragment subcloned from pLC26-6 of the Clarke and Carbon *E. coli* gene bank. Cells were grown in shake flasks on LB medium containing ampicillin (50 $\mu$g mL$^{-1}$), harvested by centrifugation when the $A_{650}$ was 0.8–1.0, and stored at −80 °C.

The buffers used during the purification were buffer A [50 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT] and buffer B [25 mM imidazole (pH 7.4), 1 mM DTT]. All steps were carried out at 4 °C.

(*1*) *Preparation of Crude Extract.* Ninety-five grams (wet weight) of *E. coli* JM83/pHE5 paste was thawed overnight on ice and resuspended in 150 mL of buffer A before disruption by two passages through a French press (operating at 10000 psi at the orifice). Cell debris was removed by centrifugation at 10000 rpm for 30 min. Forty milliliters of protamine sulfate solution (2% w/v) was added dropwise to the supernatant (200 mL), and after stirring for 20 min, precipitated nucleic acids were removed by centrifugation (10000 rpm, 30 min).

(*2*) *Phenyl-Sepharose Chromatography.* The cleared lysate (233 mL) was brought to 35% saturation by the slow addition of 48.5 g of powdered ammonium sulfate and again centrifuged to remove any precipitated material (15000 rpm, 30 min). This was loaded onto a column (2.5 × 40 cm), which had previously been equilibrated with buffer A containing ammonium sulfate (35% saturation), at 72 mL h$^{-1}$. After loading, a further 100 mL of buffer A/35% ammonium sulfate was pumped through the column, followed by a gradient from 35% to 0% saturated ammonium sulfate in buffer A (800 mL total gradient volume). Fractions containing the D-Ala-D-Ala-adding enzyme activity were pooled (250 mL) and dialyzed against buffer A (2 × 6 L).

(*3*) *Anion-Exchange Chromatography.* The strong anion-exchange resin Mono-Q (Pharmacia FPLC) was employed at this stage, on a preparative HR 16/10 column. In four identical runs, the phenyl-Sepharose pool was loaded onto a column equilibrated with buffer A at 8 mL min$^{-1}$, washed with 100 mL of buffer A, and eluted in a 600-mL gradient of 0–200 mM KCl in buffer A, at 4 mL min$^{-1}$. The D-Ala-D-Ala-adding enzyme activity eluted at approximately 160 mM KCl; ac-

Table I: Purification of the *E. coli* D-Ala-D-Ala-Adding Enzyme

| step | vol (mL) | activity (units $mL^{-1}$) | [protein] (mg $mL^{-1}$) | specific activity (units $mg^{-1}$) | purification | yield (%) |
|---|---|---|---|---|---|---|
| crude extract | 200 | nd[a] | 66 | | | |
| protamine sulfate | 233 | nd[a] | 54 | | | |
| phenyl-Sepharose | 250 | 3.46 | 6.7 | 0.516 | 1 | 100 |
| Mono-Q (pH 7.2) | 231 | 1.18 | 0.57 | 2.07 | 4 | 31 |
| Cibacron Blue | 50 | 4.22 | 0.91 | 4.64 | 9 | 24 |
| Sephadex G-100 | 76 | 5.3 | 0.36 | 14.72 | 28.5 | 46 |
| chromatofocusing[b] | 26 | 1.6 | 0.10 | 16.0 | 31 | 5 |

[a] nd indicates activity not detectable due to high background $P_i$ production. [b] Only 50% of the gel filtration pool was loaded onto this column.

tivity-containing fractions from each run were pooled; the total volume of the final pool was 231 mL.

(*4*) *Dye–Ligand Chromatography.* A column of Dyematrex Blue A (1.0 × 6.5 cm) was equilibrated with buffer A, containing 150 mM KCl. The Mono-Q pool was loaded at a flow rate of 12 mL $h^{-1}$ and washed with 30 mL of buffer A/150 mM KCl; proteins were eluted in a 100-mL gradient of 150 mM–2 M KCl. D-Ala-D-Ala-adding enzyme activity was found in a broad peak and pooled in a volume of 50 mL. The pooled material was concentrated 5-fold by ultrafiltration through an Amicon PM-30 membrane.

(*5*) *Gel Filtration Chromatography.* The pooled material from step 4 was applied to a column of Sephadex G-100 equilibrated in buffer B, and eluted with buffer B at a flow rate of 12.5 mL $h^{-1}$. Activity-containing fractions (pooled volume 76 mL) were concentrated 10-fold as in the previous step.

(*6*) *Chromatofocusing.* Half of the concentrated gel filtration pool (4 mL) was loaded onto a column (1 × 45 cm) of polybuffer exchanger PBE 94, which had been equilibrated with buffer B. Proteins were eluted by washing with 8-fold diluted polybuffer 74 (pH 4.0), which creates a linear gradient of pH 7–4 in approximately 500 mL. In a trial run, it was found that the enzyme eluted in a sharp peak at pH 4.4. Therefore, during the preparative run, 5-mL fractions were collected into tubes containing 0.5 mL of 1 M Hepes, pH 7.2, which was found to increase the pH of the activity-containing fractions to around 6, thereby reducing the length of time that the enzyme was exposed to the low pH. With heavier loading, however, the D-Ala-D-Ala-adding enzyme eluted in a broad peak and was in a very large volume (165 mL). The enzyme was concentrated and separated from polybuffer by ammonium sulfate precipitation. Powdered ammonium sulfate was added to 90% saturation and the enzyme recovered by centrifugation (15 000 rpm, 30 min); the pellet was washed with saturated ammonium sulfate and resuspended in 10 mL of buffer A. This final pool was dialyzed against two 6-L volumes of buffer A, and the sample volume after dialysis was 26 mL. For long-term storage, this enzyme was dialyzed against buffer A containing 50% (v/v) glycerol and stored at −20 °C.

*Polyacrylamide Gel Electrophoresis.* The subunit molecular weight of the D-Ala-D-Ala-adding enzyme was determined by polyacrylamide gel electrophoresis in the presence of SDS, as described in Laemmli (1970). Proteins were detected by Coomassie blue staining or silver staining, as necessary.

*Enzymatic Synthesis of UDP-MurNAc-pentapeptides with Alternative Dipeptides.* A series of incubations were prepared in 100 mM Tris buffer, pH 8.6, containing 10 mM $MgCl_2$, D-Ala-D-Ala-adding enzyme (200 pmol), D-Ala-D-Ala ligase (180 pmol), ATP (5 mM), and the D-Ala-D-Ala ligase substrates (2 mM) detailed in Table II. D-Ala-D-Ala ligase was omitted from incubations A and C, and thus these acted as negative controls. Incubations were carried out at 37 °C for 1 h (D-alanine incubations) or overnight (fluoroalanine incubations) before analysis by HPLC.

*HPLC Analysis of Pentapeptides.* Reverse-phase HPLC chromatography was employed to separate UDP-MurNAc-tripeptide substrate from reaction products in the above-mentioned incubations. Separation methods have been described previously (Flouret et al., 1981) and were performed on an Alltech $C_{18}$ column linked to a Waters Associates HPLC system, with isocratic elution in 100 mM ammonium phosphate (pH 4.4) buffer, at a flow rate of 1 mL $min^{-1}$. UDP-MurNAc-peptides were detected by absorbance at 262 nm. Fractions (1 mL) were collected and radioactivity was detected by scintillation counting.

## Results

*Detection of D-Ala-D-Ala-Adding Enzyme Activity.* In previous studies of the D-Ala-D-Ala-adding enzyme, activity was monitored by following the incorporation of radiolabeled D-Ala-D-Ala into UDP-MurNAc-pentapeptide (Michaud et al., 1987). This assay necessitates the use of a radioactive substrate and a paper chromatography step to separate product from substrate. Although accurate, it is inconvenient both for the large number of assays in an enzyme purification and for detailed kinetic studies. In order to overcome these problems, assays were developed based on those already in use in these laboratories for the preceding enzyme in the peptidoglycan biosynthetic pathway, D-Ala-D-Ala ligase (Daub et al., 1988) since both enzymes catalyze mechanistically similar reactions with the same net hydrolysis of ATP. The $P_i$ production assay, dependent on both D-Ala-D-Ala and UDP-MurNAc-tripeptide, was used during enzyme purification, with the exception of the crude extract, where a high background of $P_i$ precluded the detection of activity. In our hands this assay is both rapid and reproducible and uses only a small quantity of UDP-MurNAc-tripeptide in each reaction. The PK-LDH coupled spectrophotometric assay provides a means of continuously monitoring activity and is thus the method of choice for kinetic investigations. However, its use is limited by the unavailability of UDP-MurNAc-tripeptide substrate in large quantity.

*Purification of the D-Ala-D-Ala-Adding Enzyme.* The D-Ala-D-Ala-adding enzyme was isolated by a six-step procedure from the *E. coli* strain JM83/pHE5 which overproduces this enzyme approximately 10-fold over the level of the enzyme in a nontransformed *E. coli* JM83 strain (de Roubin, 1987). The procedure consisted of chromatography of a crude cell extract on phenyl-Sepharose, followed by Mono-Q anion exchanger, Dyematrex Blue affinity matrix, Sephadex G-100, and chromatofocusing. Table I details a typical purification. The protein was purified 31-fold from the first stage at which activity was detected, the phenyl-Sepharose pool, with 10% overall yield compared with the same step (5% in Table I represents only half the material chromatographed through this final step). As the total protein in the phenyl-Sepharose pool is approximately one-tenth the total protein in the crude extract, the overall purification from crude is greater than

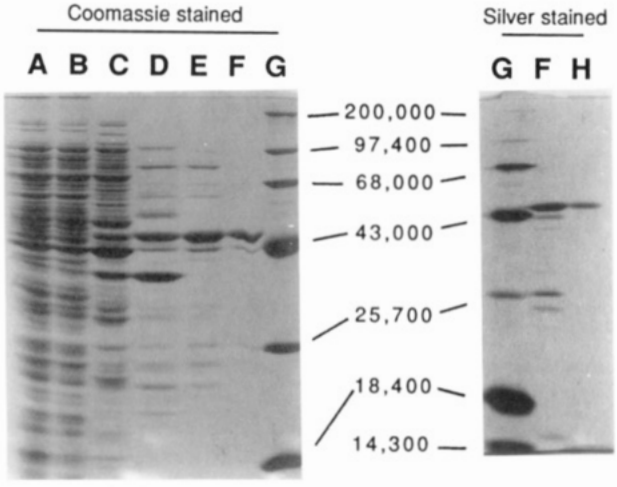


FIGURE 1: SDS–PAGE (10%) monitoring purification of the D-Ala-D-Ala-adding enzyme. Tracks contain samples from the pooled material from the following chromatographic steps: A, crude extract (66 μg); B, protamine sulfate extract (54 μg); C, phenyl-Sepharose (34 μg); D, Mono-Q (17 μg); E, Dyematrex Blue (8 μg); F, gel filtration (4 μg); G, markers (20 μg); H, chromatofocusing (3 μg). Marker proteins are myosin (H chain) ($M_r$ 200 000), phosphorylase B ($M_r$ 97 400), bovine serum albumin ($M_r$ 68 000), ovalbumin ($M_r$ 43 000), α-chymotrypsin ($M_r$ 25 700), β-lactoglobulin ($M_r$ 18 400), and lysozyme ($M_r$ 14 300).

300-fold. The absolute yield of pure enzyme was thus about 5 mg from 95 g of wet cells. Examination of an SDS–PAGE monitoring the purification (Figure 1) showed that although the Cibacron Blue column did not provide a large increase in specific activity, this step was useful in that it removed one major contaminating band. In the Sephadex gel filtration column pool, the enzyme yield appears to have *increased* relative to the total activity in the previous two pools. The reason for this is unclear, although there are differences in the storage conditions for each pool. Both the Mono-Q pool and the Cibacron Blue pool contain KCl in the buffer as this salt was used to elute the proteins from the column. Also, the gel filtration pool was in buffer B rather than buffer A. Chromatofocusing removed the final contaminating proteins, yielding D-Ala-D-Ala-adding enzyme which was homogeneous as judged by SDS–PAGE (Figure 1). Losses at the final stage reflect the difficulty encountered in recovering protein by precipitation from the chromatofocusing column pool. The subunit molecular weight of the D-Ala-D-Ala-adding enzyme was calculated from a plot of log $M_r$ vs distance migrated for the standard proteins shown in Figure 1. A value of $M_r$ 49 000 was obtained.

The amino-terminal sequence of the purified enzyme was determined by Dr. W. Lane at the Harvard University Microchemistry Facility. The sequence obtained was

M-I-(S)-V-T-L-S-(Q/V)-L-(T/K)-()-I-L-(N/V)-G-K

where the bracketed residues represent uncertainties in the determinations due to the inability to assign peaks unambiguously. No assignment was made at position 11. This set the N-terminus for the protein sequence which has now been predicted from the nucleotide sequence of the *murF* gene (Parquet et al., 1989).

*Characterization of the* D-*Ala*-D-*Ala-Adding Enzyme.* From the final specific activity of the enzyme reported in Table I, 16 μmol min$^{-1}$ mg$^{-1}$, and by use of the subunit molecular weight obtained above, a turnover number ($k_{cat}$) of 784 min$^{-1}$ can be calculated. This number can be compared with the turnover numbers of the two enzymes preceding the D-Ala-D-Ala-adding enzyme in the D-alanine branch pathway, which have been determined in these laboratories for enzymes isolated from *Salmonella*. The *Salmonella* biosynthetic alanine racemase (the *alr* gene product) has a $k_{cat}$ of 860 min$^{-1}$ (Esaki & Walsh, 1986), while the $k_{cat}$ for D-Ala-D-Ala ligase is 644 min$^{-1}$ (Daub et al., 1988). Thus, all three enzymes in this pathway have very similar turnover numbers.

Table II: D-Ala-D-Ala-Adding Enzyme Dipeptide Substrate Profile

| substrate | nonsubstrate |
|---|---|
| D-Ala-D-Ala | L-Ala-L-Ala |
| D,L-Ala-D,L-Ser | D,L-Ala-D,L-Val |
| D,L-Leu-D,L-Ala | D,L-Ala-D,L-Phe |
| Gly-D-Ala | |
| D-Ala-Gly | (D-Ala-D-Ala-D-Ala) |
| Gly-D-aminobutyrate | |
| Gly-Gly | |

In a previous study of partially purified D-Ala-D-Ala-adding enzyme (Michaud et al., 1987) values of 11 μM and 100 μM for the $K_m$'s for the UDP-MurNAc-tripeptide substrate and the D-Ala-D-Ala substrates, respectively, were reported. Using the coupled spectrophotometric assay, we have obtained a $K_m$ with the purified enzyme for the UDP-MurNAc-tripeptide substrate (data not shown). The value obtained (70 μM) is substantially higher than that reported previously. The reason for this discrepancy is not clear to us. This $K_m$ creates a practical difficulty in coupled assays in that inclusion of UDP-MurNAc-tripeptide at severalfold $K_m$ rapidly depletes stocks of this substrate. We attempted to obtain a $K_m$ for ATP, but the low UDP-MurNAc-tripeptide concentration in the assays, in combination with ATP concentrations near to $K_m$, caused a great deal of scatter in the reaction rates, and we can only report a value in the range 50–100 μM. With D-Ala-D-Ala as the variable concentration substrate, a value of 220 μM was obtained for this $K_m$.

A simple dipeptide substrate profile was obtained by addition of dipeptides that contain D-alanine, or a closely related amino acid, to PK-LDH coupled assays containing each of the assay components (including enzyme) except the dipeptide substrate and monitoring the stimulation of ADP release (data not shown). Several dipeptides were capable of substituting for D-Ala-D-Ala in the reaction (Table II). The tripeptide D-Ala-D-Ala-D-Ala was not a substrate.

Incubations were also carried out in the presence of the D-Ala-D-Ala ligase inhibitors D-3-[(1-aminoethyl)phosphinyl]-2-heptylpropionic acid and 3-[[1(*S*)-aminoethyl]-hydroxyphosphinyl]-2(*R*)-methylpropionic acid (Duncan & Walsh, 1988; Parsons et al., 1988) at 1 mM final concentrations. These inhibitors are thought to act as transition-state analogues, mimicking the structure of D-Ala-D-Ala at the D-Ala-D-Ala ligase active site. In the absence of the normal substrate D-Ala-D-Ala, no stimulation of ATP hydrolysis was observed, leading to the conclusion that neither compound can be utilized by the D-Ala-D-Ala-adding enzyme as a substrate. In the presence of D-Ala-D-Ala, neither acted to inhibit the D-Ala-D-Ala-adding enzyme.

The D-Ala-D-Ala-adding enzyme is not sensitive to product inhibition. The presence of purified UDP-MurNAc-pentapeptide at approximately 1 mM final concentration had no effect on rates of ATP turnover in pyruvate kinase–lactate dehydrogenase coupled assays. In contrast, *S. faecalis* (Neuhaus et al., 1969), *E. coli* (Lugtenberg, 1972), and *Salmonella* (K. Duncan and C. T. Walsh, unpublished results) D-Ala-D-Ala ligases are very sensitive to product inhibition, implying that it is the D-Ala-D-Ala ligase catalyzed step that regulates the flux of intermediates through the pathway.

*Incorporation of Fluorinated Alanines into UDP-MurNAc-pentapeptide.* It has been observed that low concentrations of the alanine analogue D-fluoroalanine are bactericidal whereas high concentrations are autoantagonistic (Kahan & Kropp, 1975). The inhibition stems from the fact that D-fluoroalanine is a potent alanine racemase irreversible inhibitor (Wang & Walsh, 1978), thus preventing racemization of L-alanine to D-alanine in the cell, and depriving D-Ala-D-Ala ligase of substrate. However, at high concentration, cell growth takes place, leading to the proposal that D-Ala-D-Ala ligase makes fluoro-D-Ala-fluoro-D-Ala and that the D-Ala-D-Ala-adding enzyme can incorporate this into UDP-MurNAc-pentapeptide. We have shown recently (Walsh et al., 1988) that both D-fluoroalanine and D-trifluoroalanine are D-Ala-D-Ala ligase substrates, though with elevated $K_m$ and lowered $V_{max}$ values compared with D-alanine. In a preliminary experiment, pure D-Ala-D-Ala ligase was incubated with MgATP and either D-alanine, D-aminobutyrate (a good ligase substrate), or D-fluoroalanine for 1 h at 37 °C. The mixture was boiled and then filtered through an ultrafiltration unit (30K cutoff) to ensure complete inactivation and removal of this enzyme. Any ADP formed by the D-Ala-D-Ala ligase reaction was removed from the filtrate by the addition of phosphoenolpyruvate, NADH, and PK-LDH. An aliquot was then added to a D-Ala-D-Ala-adding enzyme assay containing enzyme and all of the components except dipeptide substrate. In each case there was an immediate stimulation of ADP release. In a control experiment, D-Ala-D-Ala ligase was omitted from a D-alanine incubation and no stimulation of ADP release was observed in the subsequent D-Ala-D-Ala-adding enzyme assay. We were thus encouraged to prove definitively that fluorinated D-alanines can be incorporated into UDP-MurNAc-pentapeptide.

A series of incubations was prepared containing D-Ala-D-Ala ligase, D-Ala-D-Ala-adding enzyme, ATP, UDP-MurNAc-tripeptide, and a ligase substrate (Figure 2). Radiolabel was included in either the UDP-MurNAc-tripeptide, the D-alanine, or the trifluoroalanine. Incubation mixtures were analyzed by HPLC. With the exception of negative controls A and C in Figure 2, incubation resulted in the loss of an HPLC absorbance peak characteristic of the UDP-MurNAc-tripeptide. However, it was found, that under the conditions used, purified UDP-MurNAc-pentapeptide eluted from the column with the same retention time as ATP/ADP and therefore in the experimental runs the product absorbance peak was masked by the higher concentration of ATP/ADP. It was therefore necessary to rely on the disappearance of the UDP-MurNAc-tripeptide peak and the new position of the radiolabeled peak to show that pentapeptide formation had taken place. The incubations contained a total of 10 nmol of UDP-MurNAc-tripeptide and an excess of D-Ala-D-Ala ligase substrate, and so conversion of tripeptide to pentapeptide resulted in a shift of all the counts in experiments with [$^{14}$C]UDP-MurNAc-tripeptide, but only a fraction of the counts when the label was in D-alanine ($^{14}$C) or trifluoroalanine ($^{14}$C). The results are displayed in Figure 2 in the form of histograms showing the number of counts in each fraction from the column. Figure 2a illustrates that, in the absence of D-Ala-D-Ala ligase in the incubation (incubation A), the counts are in fractions 6 and 7, which corresponds to the location of the UDP-MurNAc-tripeptide $A_{262}$ peak. When D-Ala-D-Ala ligase is included (incubation B), the counts are shifted to fractions 12 and 13. This new radiolabeled peak had the same retention time as an authentic UDP-MurNAc-pentapeptide sample. Incubations C and D show that when radiolabeled D-alanine is used in the incubations, a similar shift in the distribution of counts takes place (Figure 2b). In Figure 2c, comparison is made between incubations A and E, where E included D-fluoroalanine as the D-Ala-D-Ala ligase substrate. Again the radiolabeled UDP-MurNAc-tripeptide peak is moved as pentapeptide formation takes place. Finally, Figure 2d represents the incorporation of trifluoroalanine into pentapeptide. Incubation F was with [$^{14}$C]UDP-tripeptide and cold trifluoroalanine, and the counts were found in fractions 13 and 14. Incubation G was with cold UDP-MurNAc-tripeptide and [$^{14}$C]trifluoroalanine; again the counts were found in fractions 11 and 12, providing definitive proof that pentapeptide formation indeed takes place with the fluoroalanines.

## Discussion

In this paper we describe the first purification to homogeneity of a D-Ala-D-Ala-adding enzyme involved in bacterial peptidoglycan biosynthesis. The subunit molecular weight of the purified protein is 49 000, thus implying that it is a monomer since Michaud et al. (1987) determined that the native molecular weight of partially purified D-Ala-D-Ala-adding enzyme was 51 000. Also, Maruyama et al. (1988) have shown by in vivo labeling that the *murF* product is a polypeptide of 52 000 molecular weight. The DNA sequence of *murF* has recently been investigated (Parquet et al., 1989). Knowledge of the location of the 5′-terminus of the enzyme coding region as determined by direct amino acid sequencing of purified protein allowed correct assignment of the *murF* start site and open reading frame. The reading frame encodes a 452 amino acid protein with calculated molecular weight of 47 492.

This work validates earlier studies with impure D-Ala-D-Ala-adding enzyme on alternate dipeptides as substrates (Neuhaus & Struve, 1965; Pelzer & Reuter, 1980). The results obtained indicate that the *E. coli* enzyme is capable of discriminating between different dipeptides on the basis of the amino acid found at the carboxyl end of the dipeptide. It will utilize dipeptides ending in D-alanine, or a structurally related amino acid such as D-aminobutyrate, and with various amino acids on the N-terminus, but will not incorporate those dipeptides that have D-alanine as the amino terminus and a variable amino acid at the carboxy terminus. Thus, as Neuhaus pointed out (Neuhaus & Struve, 1965), D-Ala-D-Ala ligase, which has a high degree of specificity for the amino-terminal amino acid, and the D-Ala-D-Ala-adding enzyme with a complementary specificity profile act with a kind of "double sieving" binding specificity ensuring that the major product of these two reactions is UDP-MurNAc-pentapeptide ending in D-Ala-D-Ala.

Experiments with the purified D-Ala-D-Ala-adding enzyme and purified D-Ala-D-Ala ligase have shown here that it was possible to synthesize UDP-MurNAc-pentapeptide beginning with UDP-MurNAc-tripeptide and either D-fluoroalanine or D-trifluoroalanine in incubations containing both enzymes. The structures of these fluorinated D-alanine containing UDP-MurNAc-pentapeptide products are shown in Figure 3, in comparison to physiological UDP-MurNAc-pentapeptide, terminating in D-Ala-D-Ala. This also validates the notion that the preceding enzyme, D-Ala-D-Ala ligase, will take fluoro-D-Ala (and trifluoro-D-Ala) and make a dipeptide and that the D-Ala-D-Ala-adding enzyme will take this as a substrate and incorporate it into the peptidoglycan precursor. In all probability, this precursor (UDP-MurNAc-pentapeptide) would then be assembled into fully structured peptidoglycan and engage in wall extension and cross-linking, explaining the observed "self-reversal" of antibacterial action with elevated levels of fluoroalanine (Kahan & Kropp, 1975).

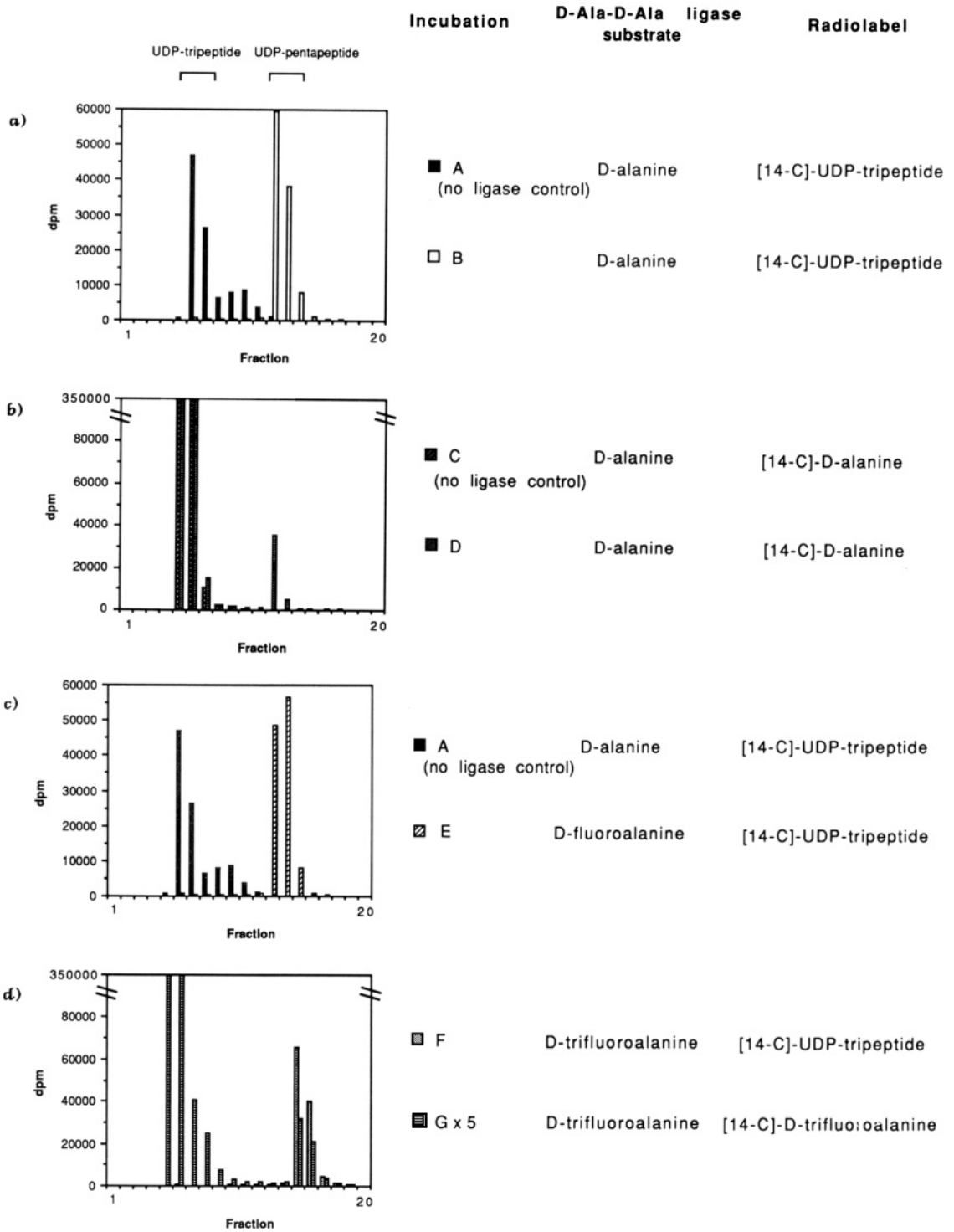


FIGURE 2: Result of incubations leading to UDP-MurNAc-pentapeptide formation with substituted D-alanines. Histograms show the disintegrations $min^{-1}$ (dpm) in fractions from reverse-phase HPLC chromatography of UDP-MurNAc-pentapeptide formation in the incubations. In (a), comparison is made between incubations in the absence (A) and presence (B) of D-Ala-D-Ala ligase. Incubation B results in the counts in UDP-MurNAc-tripeptide being shifted to a new position, with a longer retention time on the column. The counts in incubation B now elute from this column with the same retention time as the $A_{262}$ peak of an authentic UDP-MurNAc-pentapeptide sample chromatographed under the same conditions. A similar comparison is made in (b) between incubations in the absence (C) and presence (D) of D-Ala-D-Ala ligase, but in this case the radiolabel is in D-alanine. Again counts are shifted to a new position corresponding to that of UDP-MurNAc-pentapeptide. Histogram c compares an incubation containing D-fluoroalanine (E) (UDP-MurNAc-pentapeptide label) with the control sample A. In histogram d, incubations with D-trifluoroalanine are compared. All of the counts in incubation F (UDP-MurNAc-pentapeptide label) are shifted to a new position. Counts from incubation G are shifted to the same position. Note that the counts in incubation G have been multiplied by 5 in order to accommodate both sets of data on the same histogram.

Three types of enzyme are known to recognize D-Ala-D-Ala moieties. Two of these are found on the D-alanine branch of peptidoglycan biosynthesis, namely, D-Ala-D-Ala ligase and the D-Ala-D-Ala-adding enzyme. The third class includes the $\beta$-lactam-sensitive D-Ala-D-Ala transpeptidases and carboxypeptidases. It is not yet clear whether the same or different structural motifs are available for recognizing D-Ala-D-Ala in each of the three enzymes. Although no homology has been detected at the primary sequence level between D-Ala-D-Ala ligase and D,D-carboxypeptidase (L. E. Zawadzke and C. T.

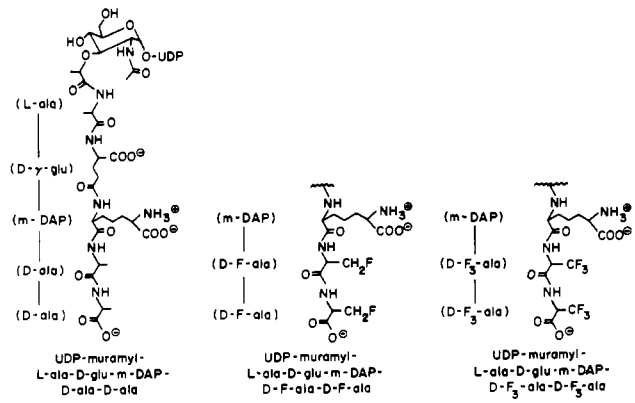


FIGURE 3: Structure of the UDP-MurNAc-pentapeptide. (Left) Physiological structure of UDP-MurNAc-pentapeptide. (Center) UDP-MurNAc-pentapeptide with F-D-Ala-F-D-Ala substitution. (Right) UDP-MurNAc-pentapeptide with $F_3$-D-Ala-$F_3$-D-Ala substitution.

Walsh, unpublished results), it may be that only a few residues throughout the protein may be involved in recognition. Now that all of these D-Ala-D-Ala recognizing enzymes have been cloned, overproduced, and purified, examination of the three-dimensional structures of a number of D-Ala-D-Ala-recognizing enzymes may now be a realistic way to resolve this question. Quite recently, the structure of a soluble D,D-carboxypeptidase from *Streptomyces* R61 has been determined and found to bear homology to $\beta$-lactamases (Kelly et al., 1986; Joris et al., 1988). Thus one mode of enzyme recognition of the D-Ala-D-Ala moiety in the substrate is beginning to be defined at atomic resolution for the $\beta$-lactam-sensitive enzyme class. The second category is exemplified by the D-Ala-D-Ala ligase, and one of our goals in sequencing, expressing, and purifying the *Salmonella* D-Ala-D-Ala ligase was its eventual structural determination. This *Salmonella* D-Ala-D-Ala ligase has been crystallized, and preliminary data showed that the crystals diffract to ~2.2 Å and may be suitable for high-resolution analysis (Knox et al., 1989). This leaves only the third member, the D-Ala-D-Ala-adding enzyme, to begin to have a complete set of these potentially similar enzyme classes. A separate *N*-acyl-D-Ala-D-Ala binding motif, well-defined by NMR and molecular modeling studies (Williams & Waltho, 1988), is that found in the vancomycin or ristocetin class of glycopeptide antibiotics which act by stoichiometric complexation of the D-Ala-D-Ala terminus in un-cross-linked pentapeptide strands on the outer face of the cytoplasmic membrane. It will be of interest to compare the D-Ala-D-Ala binding motifs of all three enzymes and to compare these with the glycopeptide antibiotic domain.

This work on purification and initial characterization of the *murF* gene product sets the stage for further structure/function studies, which when combined with recombinant DNA and better overproduction to provide sufficient quantities of pure D-Ala-D-Ala-adding enzyme for X-ray analysis will lead to a better understanding of the mechanism of this cell wall biosynthetic enzyme. Potential inhibitors including new structural mimics of D-Ala-D-Ala might then be identified, although the difficulty in getting D-Ala-D-Ala-type molecules past the cytoplasmic membrane into bacterial cells must be overcome before an effective antibacterial compound targeted against these enzyme types can be developed.

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**Registry No.** UDP-MurNAc-tripeptide, 17114-33-9; D-alanyl-D-alanine, 923-16-0; D-alanyl-D-alanine adding enzyme, 55354-36-4.

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