Enzymatic Synthesis of D-Alanyl-D-alanine. Control of D-Alanine:D-Alanine Ligase (ADP)*

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ABSTRACT: The incorporation of D-alanine from L-alanine into the peptidoglycan precursor, uridine diphosphate-*N*-acetylmuramyl-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala, is catalyzed by the sequential action of alanine racemase (1); D-alanine:D-alanine ligase (adenosine diphosphate) (2); and uridine diphosphate-*N*-acetylmuramyl-L-Ala-D-γ-Glu-L-Lys:D-Ala-D-Ala ligase (adenosine diphosphate) (3). The product of reaction 2, D-Ala-D-Ala, is a competitive inhibitor (K_i = 1.2×10^{-3} M) of D-alanine:D-alanine ligase. The specificity of product inhibition differs from that predicted from substrate specificity studies; *e.g.*, D-Ala-D-norval, D-Ala-D-α-amino-*n*-butyric acid (K_i = 6.0×10^{-4} M) > D-Ala-D-Ala > D-Ala-D-Ser > D-Ala-D-Thr > D-α-amino-*n*-butyryl-D-α-amino-*n*-butyric acid (K_i = 5.3×10^{-3} M), D-Ala-D-Val D-α-amino-*n*-butyryl-D-Ala. A kinetic analysis of the inhibition is consistent with a model that assumes at least two binding sites for product: *i.e.*, EA, EAA, EP, EAP, and EAP_2, where A = D-alanine and P = D-Ala-D-Ala. An analysis of the inhibition by D-Ala-D-norval with the Hill equation shows a change in the interaction coefficient from $n = 1$ to $n = 3$ as the concentration of D-Ala-D-norval is increased. These results suggest the presence of more than one binding site for D-Ala-D-Ala in D-alanine:D-alanine ligase. Since K_{eq} for alanine racemase is 1, the inhibition of D-alanine:D-alanine ligase (adenosine diphosphate) by D-Ala-D-Ala would, in effect, control the utilization of L-alanine by the racemase.

Further, the level of D-Ala-D-Ala available to enzyme (3) may control the rate of uridine diphosphate-*N*-acetylmuramyl-pentapeptide formation.

The incorporation of D-alanine from L-alanine into the peptidoglycan precursor, UDP-MurNAc-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala, is catalyzed by the sequential action of the following enzymes: (1) alanine racemase (EC 5.1.1.1) (Wood and Gunsalus, 1951), (2) D-alanine:D-alanine ligase (ADP) (EC 6.3.2.4) (Ito and Strominger, 1960, 1962; Neuhaus, 1960, 1962), and (3) UDP-MurNAc-L-Ala-D-γ-Glu-L-Lys:D-Ala-D-Ala ligase (ADP) (Ito and Strominger, 1962; Comb, 1962; Neuhaus and Struve, 1965).

D-Alanine:D-alanine ligase (ADP) has a high specificity for D-amino acids in the N-terminal site (D-alanine, D-α-amino-*n*-butyric acid) and a low specificity for D-amino acids in the C-terminal site (D-alanine, D-α-amino-*n*-butyric acid, D-serine, D-threonine, and D-norvaline) (Neuhaus, 1962). In contrast to D-alanine:D-alanine ligase (ADP), UDP-MurNAc-L-Ala-D-γ-Glu-L-Lys:D-Ala-D-Ala ligase has a low specificity for amino acids in the N-terminal residue and a high specificity for amino acids in the C-terminal residue of the dipeptide (Neuhaus and Struve, 1965). Thus, the two enzymes have complementary specificity profiles and, in effect, cooperate to ensure that D-Ala-D-Ala is the major dipeptide that is added to the nucleotide precursor, UDP-MurNAc-L-Ala-D-γ-Glu-L-Lys.

In the initial experiments on D-alanine:D-alanine ligase, it was observed that D-Ala-D-Ala in the incubation resulted in an inhibition of D-[^{14}C]Ala-D-[^{14}C]Ala formation from D-[^{14}C]-alanine (Neuhaus, 1962). This observation is the result of a pronounced inhibition of D-alanine:D-alanine ligase by its product. It is the purpose of this communication to examine the requirements for the inhibition by D-Ala-D-Ala. The inhibition of the ligase may be a control point in the sequence of enzymes responsible for the incorporation of D-alanine from L-alanine into the peptidoglycan precursor.

Experimental Section

Materials. The DD dipeptides were synthesized by Dr. H. Plaut of Cyclo Chemical Corp. The analyses and characterization of these compounds have been previously described (Neuhaus, 1962; Neuhaus and Struve, 1965). ATP:pyruvate phosphotransferase (EC 2.7.1.40) and L-lactate:NAD oxidoreductase (EC 1.1.1.27) were the products of Boehringer Mannheim Corp. and Sigma Chemical Corp., respectively. [^{14}C]ADP and potassium [^{32}P]phosphate were purchased from Nuclear-Chicago (Amersham/Searle). D-[^{14}C]Ala-D-[^{14}C]Ala was prepared by the procedure described by Neuhaus and Struve (1965). The sources of amino acids and other nucleotides have been previously described (Neuhaus, 1962).

Assays. The P_i assay contained: 0.05 M KCl, 0.2 M choline chloride, 0.01 M $MgCl_2$, 0.05 M Tris-HCl (pH 7.8), 0.01 M Na_2-ATP neutralized with NaOH, 2.5 mM glutathione, and enzyme, substrate, and additions as specified. The assay tubes were

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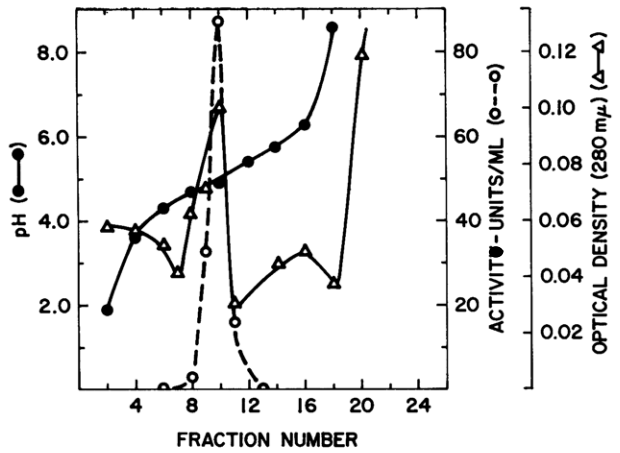


FIGURE 1: Isoelectric pattern and plot of pH against effluent volume. D-Alanine:D-alanine ligase (1400 units) was focused for 30 hr in a pH gradient from 3 to 7. The procedure is described in the Experimental Section. The optical density was measured after dialysis against 0.01 M KCl followed by dialysis against 0.02 M Tris-HCl (pH 7.8).

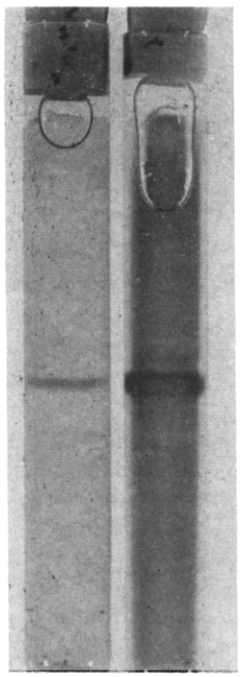


FIGURE 2: Analysis of enzyme preparation by disc gel electrophoresis. The gels are as follows: (1) electrofocused fraction stained with coomassie blue; (2) electrofocused fraction showing activity. The procedures are described in the Experimental Section. Track 1 is on the left, track 2, the right.

incubated at 37°, and aliquots were removed at 0, 5, 10, and 15 min and added to 4.3 ml of 0.1 N HCl for subsequent P_i analyses by the method of Marsh (1959). Unless specified, all velocities are reported as micromoles of P_i per milliliter liberated in 1-hr incubation.

The ADP assay measures ADP with the coupled assay using ATP:pyruvate phosphotransferase (reaction I) and L-lactate:NAD oxidoreductase (reaction II). The reaction mixture contained: 0.01 M $MgCl_2$, 0.005 M Na_2ATP neutralized with NaOH, 1×10^{-4} M NADH, 25 μg of ATP:pyruvate phosphotransferase/ml, 62 μg of L-lactate:NAD oxidoreductase/ml, 0.05 M KCl, 0.2 M choline chloride, 0.05 M Tris-HCl buffer (pH 7.8), 2.5 mM phosphoenolpyruvate, enzyme, and D-alanine as indicated in a total volume of 0.5 ml. The rate of NADH oxidation was measured in a Zeiss spectrophotometer with a Gilford Model 220 absorbance indicator.

$$\text{ADP} + \text{phosphoenolpyruvate} \longrightarrow \text{ATP} + \text{pyruvate} \quad \text{(I)}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \longrightarrow \text{lactate} + \text{NAD}^+ \quad \text{(II)}$$

Enzyme Preparations. Two preparations of ligase have been used for the experiments. Preparation 1 has been purified through the DEAE-Sephadex column previously described (Neuhaus, 1962) (specific activity 120 units/mg). For preparation 2 the purification procedure has been modified in the following manner. The enzyme from the DEAE-Sephadex column was dialyzed for 4 hr against 1% glycine. Isoelectric focusing (Vesterberg and Svensson, 1966) was performed in the 110-ml column (LKB Instruments, Inc.) with carrier ampholytes selected to give a pH gradient between 3 and 6. The enzyme was focused for 24 hr with the potential gradually increased to 550 V during the first 3 hr. Fractions were collected from the column and analyzed for enzyme activity, absorbance at 280 mμ, and H^+ ion concentration (Figure 1). The enzyme was dialyzed against 0.02 M Tris-HCl (pH 7.8) and then concentrated with Carbowax. After complete removal of the ampholytes on Sephadex G-100, the specific activity of this preparation was 496 units/mg.

Analytical disc gel electrophoresis was performed according to the procedure described by Davis (1964). The standard small pore gel (15%) was operated at pH 9.4. In Figure 2 the electrophoretic pattern of the enzyme preparation from the electrofocused fraction is shown. In this fraction, a single band was observed when the gel was stained with 0.05% coomassie blue in 12% trichloroacetic acid (track 1). The enzymatic activity of the ligase (track 2) was visualized by replacing the $MgHPO_4$ with PbS by the method of Allen and Hyncik (1963). The gel was incubated in the following mixture: 0.1 M Tris-HCl (pH 7.8), 0.05 M KCl, 0.01 M $MgCl_2$, 0.01 M ATP neutralized with NaOH, and 0.04 M D-alanine. The gel was incubated in the mixture at 25° for 15 min.

Results

Specificity of Inhibition. A series of D-Ala-D-Ala analogs were tested as potential inhibitors of the ligase. From the data shown in Table I, it is apparent that the inhibition is specific for dipeptides with the DD configuration. D-Ala-L-Ala, L-Ala-D-Ala, and L-Ala-L-Ala have no inhibitory activity. Furthermore, the inhibition is specific for a dipeptide. The addition of substituents to the N-terminal residue decreases the effectiveness of the dipeptide as an inhibitor. However, there are certain additions to the C-terminal residue which enhance the effectiveness of the dipeptide as an inhibitor. For example, D-Ala-D-norval and D-Ala-D-butyr are more effective than D-Ala-D-Ala as inhibitors of the ligase. Since the specificity profile for the ligase has been extensively studied for D-alanine and D-α-amino-*n*-butyric acid (Neuhaus, 1962), values for K_i of D-Ala-D-Ala, D-Ala-D-butyr, D-butyr-D-Ala, D-butyr-D-butyr, and D-Ala-D-norval have been established from Dixon

plots. These values are summarized in Table I. From Lineweaver–Burk plots, it has been established that D-Ala-D-Ala, D-Ala-D-butyr, D-Ala-D-Val, and D-butyr-D-Ala are competitive inhibitors. In contrast, D-Ala-D-Ser and D-Ala-D-Thr are noncompetitive inhibitors.

Kinetic Analysis of Inhibition. A Lineweaver–Burk (1934) analysis of the inhibition by D-Ala-D-Ala demonstrates that the dipeptide is a competitive inhibitor of the ligase (Figure 3A). Previous kinetic studies (Neuhaus, 1962) were consistent with reactions 1–3, where EA and EAA are binary and ternary

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \tag{1}$$

$$EA + A \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EAA \tag{2}$$

$$EAA \xrightarrow{k_3} EP \tag{3}$$

complexes of enzyme (E) and D-alanine (A), respectively.[1] The reciprocal velocity expression is

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_{AA}}{V_{max}(A)} + \frac{K_A K_{AA}}{V_{max}(A)^2} \tag{4}$$

where $K_A = k_{-1}/k_1$ and $K_{AA} = (k_{-2} + k_3)/k_2$. Equation 4 is rearranged in the following manner in order to evaluate K_A and K_{AA}

$$(A)\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}} + \frac{K_A K_{AA}}{V_{max}(A)} \tag{5}$$

From this expression, values for K_A and K_{AA} have been established to be 6.6×10^{-4} and 0.01 M, respectively.

In the rearranged plots (Figure 3B) a slope and intercept change is characteristic of the inhibition by D-Ala-D-Ala.

A number of ordered reaction sequences have been considered in the interpretation of these data. The first model (case A) includes a simple EP complex in the reaction pathway

$$\begin{array}{ccc} E & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & EA \\ {\scriptstyle k_4}\uparrow\downarrow{\scriptstyle k_{-4}} & & {\scriptstyle k_{-2}}\uparrow\downarrow{\scriptstyle k_2} \\ EP & \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} & EAA \end{array} \tag{6}$$

of eq 6, where P is product, k_{-3} is zero, and k_3 is the rate-limiting step. The reciprocal expression is

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_{AA}}{V_{max}(A)} + \frac{K_A K_{AA}}{V_{max}(A)^2}\left[1 + \frac{(P)}{K_P}\right] \tag{7}$$

[1] Kinetic studies with MgATP (L. G. Howell, C. V. Carpenter, and F. C. Neuhaus, unpublished results) show that D-alanine has no effect on the Michaelis constant of MgATP. On the basis of these results MgATP, MgADP, and P_i have not been considered in the models presented in this paper or in previous work (Neuhaus, 1962; Neuhaus and Lynch, 1964). An additional assumption that is implicit in this model concerns the sequence of amino acid addition. Rate expressions are derived that assume ordered binding. Models that involve random binding of the two molecules of D-alanine result in reciprocal rate expressions that cannot be analyzed (Neuhaus 1962).

TABLE I: Inhibition of D-Alanine:D-Alanine Ligase by Analogs of D-Ala-D-Ala.

Addition[a]	D-Ala-D-Ala Formed (nmoles of NADH Oxidized/min)	K_i (moles/l. $\times$ 10^3)
None	2.90	
D-Ala-D-norval	0.63	0.55
D-Ala-D-butyr	0.73	0.60
D-Ala-D-Ala	0.83	1.2
D-Ala-D-Ser	1.35	
D-Ala-D-Thr	1.68	
D-Butyr-D-butyr	1.92	5.3
D-Ala-D-Val	2.14	
D-Butyr-D-Ala	2.22	6.0
D-Norval-D-Ala	2.78	

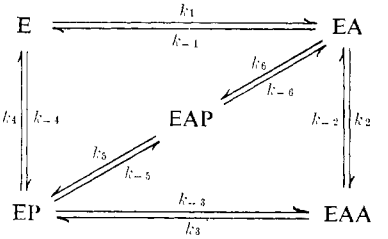
[a] The dipeptides were tested in the ADP assay at 0.001 M D-alanine and 3×10^{-3} M inhibitor. The following dipeptides did not inhibit the reaction under the conditions tested: D-Ala-Gly, D-Val-D-Ala, D-Ser-D-Ala, D-Ala-L-Ala, L-Ala-L-norval, L-Ala-D-Ala, L-Ala-L-Ala, D-Ala-D-Ala-D-Ala, and Gly-DL-Ala. The values for K_i were established from Dixon plots. Except for D-Ala-D-norval the P_i assay was used for establishing the values for K_i of D-Ala-D-butyr, D-Ala-D-Ala, D-butyr-D-butyr, and D-butyr-D-Ala.

where K_{AA} is $(k_{-2} + k_3)/k_2$, $K_A = k_{-1}/k_1$, and $K_P = k_4/k_{-4}$. Equation 7 is rearranged as follows

$$(A)\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}} + \frac{K_A K_{AA}}{V_{max}}\frac{1}{(A)}\left[1 + \frac{(P)}{K_P}\right] \tag{8}$$

From eq 8 a slope change is predicted for each concentration of dipeptide. However, all concentration levels of dipeptide will yield a common intercept. Since the experimental data show both a slope and intercept change, it is concluded that this model is not consistent with the results in Figure 3B.

In addition to EA, EAA, and EP, the complex EAP can be considered in the above model (case B), *i.e.*, eq 9, where k_{-3}



(9)

is zero and k_3 is rate limiting. The following rearranged expression is derived for case B

$$(A)\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}}\left[1 + \frac{(P)}{K_{AP}}\right] + \frac{K_A K_{AA}}{V_{max}(A)}\left[1 + \frac{(P)}{K_P}\right] \tag{10}$$

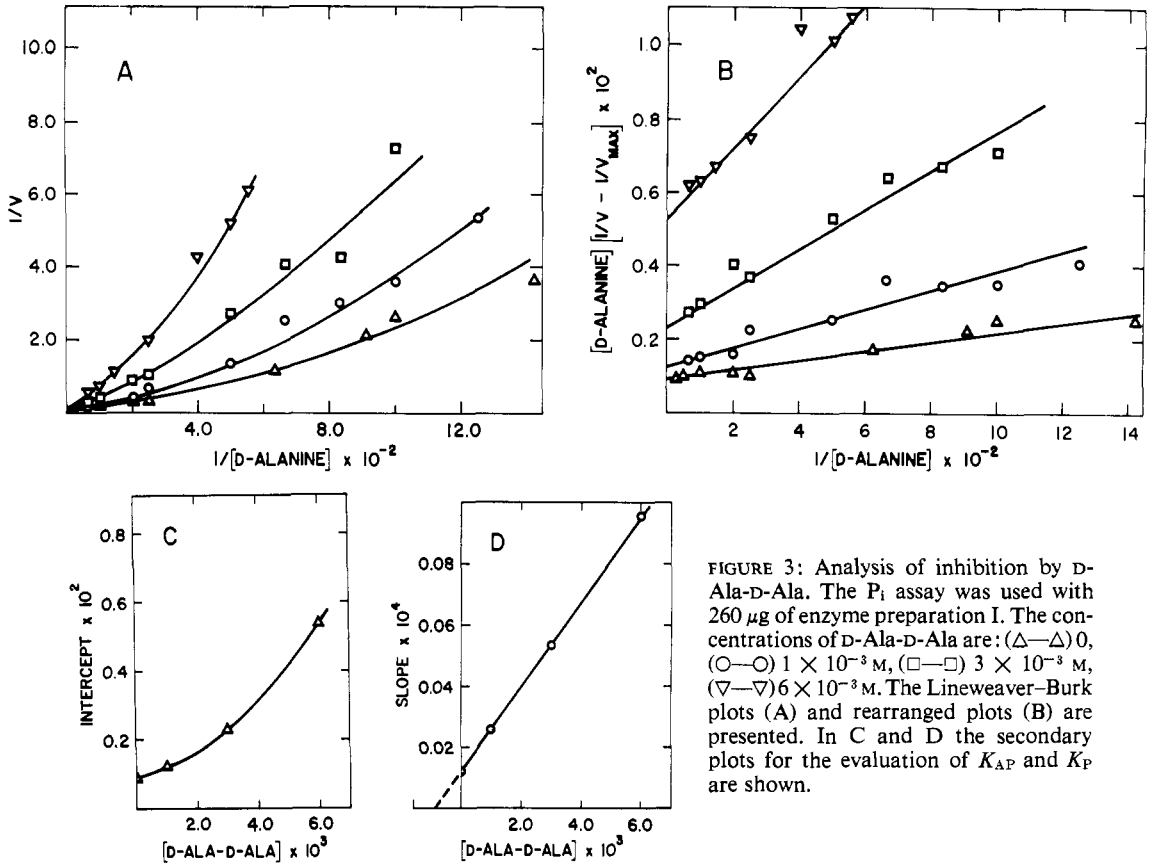


FIGURE 3: Analysis of inhibition by D-Ala-D-Ala. The P_i assay was used with 260 μg of enzyme preparation I. The concentrations of D-Ala-D-Ala are: (△—△) 0, (○—○) 1×10^{-3} M, (□—□) 3×10^{-3} M, (▽—▽) 6×10^{-3} M. The Lineweaver–Burk plots (A) and rearranged plots (B) are presented. In C and D the secondary plots for the evaluation of K_{AP} and K_P are shown.

where $K_{AP} = k_6/k_{-6}$ and $K_P = k_4/k_{-4}$. Case B predicts a change in both the slope and intercept with increasing levels of D-Ala-D-Ala in the rearranged plots. It should be noted that eq 10 predicts secondary plots of slope and intercept that are linear with respect to the concentration of dipeptide. In Figure 3C, D the secondary plots for D-Ala-D-Ala are shown. A linear secondary plot for slope and a nonlinear secondary plot for intercept is observed. From the slope replot in Figure 3D the value of 1.0×10^{-3} M for K_p is established. This is almost identical with that determined from the Dixon plot (1.2×10^{-3} M, Table I).

The addition of an EAP_2 complex to the above model (eq 9) (case C) gives the following equation for the intercept

$$\text{intercept} = \frac{K_{AA}}{V_{max}}\left[1 + \frac{(P)}{K_{AP}} + \frac{(P)^2}{K_{AP}K_{PAP}}\right] \quad (11)$$

where K_{PAP} is the dissociation constant for the reaction

$$EAP_2 \rightleftharpoons EAP + P \quad (12)$$

At low (P) the intercept reduces to $K_{AA}/V_{max}(1 + (P)/K_{AP})$, and if the intercept = 0, $K_{AP} = -(P)$. From the secondary plot (Figure 3C), the value for $K_{AP} = 3 \times 10^{-3}$ M. In addition, K_{PAP} is estimated to be 5×10^{-3} M.

Inhibition by D-Ala-D-norval. Since D-Ala-D-norval is a more effective inhibitor of the ligase than D-Ala-D-Ala, it was decided to analyze the inhibition by D-Ala-D-norval at low concentrations of D-alanine. Dixon (1953) plots are shown in Figure 4 for increasing concentrations of D-Ala-D-norval. If one inhibitor molecule binds per catalytic center, we should expect a linear Dixon plot. However, significant deviations from linearity are observed. A nonlinear Dixon plot is consistent with a model in which two or more inhibitor molecules bind to the enzyme at the catalytic center.

The data in Figure 4 have been analyzed with the empirical Hill equation (eq 13). In this equation, v is the velocity in the

$$\log \frac{v}{V_0 - v} = \log K - n \log (P) \quad (13)$$

presence of inhibitor (P), and V_0 is the velocity in the absence of inhibitor at a fixed substrate concentration. The slope n is an interaction coefficient that is a function of the number of interacting binding sites and the strength of the interaction (Atkinson *et al.*, 1965). It is recognized that this treatment does not provide the number of binding sites as such, but it does provide an indication of the degree of interaction and the cooperativity between the binding sites. As shown in Figure 5, the slope increases from 1 to approximately 3 for each concentration of D-alanine tested.

Reversal of Reaction. Product inhibition can result in two different ways. It may cause reversal of the over-all reaction or it may result only in the formation of an EP complex. In the second case, k_{-3} would be small when compared with k_{+3} (Walter and Frieden, 1963; Koch, 1967). Attempts to demonstrate a significant reversal of the reaction have been unsuccessful, *i.e.*

$$\text{D-Ala-D-Ala} + \text{ADP} + P_i \longrightarrow \text{ATP} + 2\text{D-alanine} \quad (14)$$

TABLE II: Reversal of Reaction Catalyzed by D-Alanine: D-Alanine Ligase.[a]

Reaction	Product (mμ moles)
(1) D-Ala-D-Ala + $[^{32}P]HPO_4^{2-}$ + ADP ⟶ 2D-alanine + $[^{32}P]$ATP	81
(2) D-Ala-D-Ala + HPO_4^{2-} + $[^{14}C]$ADP ⟶ 2D-alanine + $[^{14}C]$ATP	<2
(3) D-$[^{14}C]$Ala-D-$[^{14}C]$Ala + HPO_4^{2-} + ADP ⟶ 2D-$[^{14}C]$alanine + ATP	<5

[a] In expt 1, the reaction mixture contained: 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.05 M KCl, 0.20 M choline chloride, 0.10 M D-Ala-D-Ala, 0.005 M ADP, 0.005 M $[^{32}P]HPO_4^{2-}$ (5.6×10^4 cpm/μmole), and 1 unit of D-alanine:D-alanine ligase (preparation 2) in a total volume of 0.5 ml. The mixture was incubated at 37° for 60 min, and the reaction was terminated by the addition of 0.5 ml of 10% trichloroacetic acid containing 0.05 M KH_2PO_4. The $[^{32}P]HPO_4^{2-}$ and $[^{32}P]$ATP were separated by the method of Crane and Lipmann (1953) and the $[^{32}P]$ATP was assayed for radioactivity. In expt 2, the reaction mixture contained: 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.05 M KCl, 0.2 M choline chloride, 0.01 M $[^{14}C]$ADP (3.5×10^4 cpm/μmole), 0.01 M HPO_4^{2-}, 0.04 M D-Ala-D-Ala, and 1 unit of D-alanine:D-alanine ligase (preparation 2) in a total volume of 0.25 ml. The mixture was incubated at 37° for 60 min, and the reaction was terminated by placing the tube in a boiling water bath for 2 min. Carrier ATP (2.5 μmoles) was added to the reaction mixture, and the ATP was separated from ADP by descending chromatography on Whatman No. 3MM in isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v). The appropriate areas were excised and assayed for radioactivity. In expt 3, the reaction mixture contained: 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.05 M KCl, 0.2 M choline chloride, 0.005 M ADP, 0.005 M KH_2PO_4, 0.04 M D-$[^{14}C]$Ala-D-$[^{14}C]$Ala (5.6×10^4 cpm/μmole), and 1 unit of D-alanine:D-alanine ligase (preparation 2) in a total volume of 0.25 ml. The mixture was incubated for 60 min at 37°, and the reaction was terminated by placing the tubes in a boiling-water bath for 2 min. Carrier D-alanine (25 μmoles) was added to each tube, and the D-alanine was separated from D-Ala-D-Ala by descending chromatography on Whatman No. 3MM in butanol–acetic acid–water (4:1:5, v/v) (organic phase). The appropriate areas were excised and assayed for radioactivity. Values are corrected for a control tube containing boiled enzyme.

In Table II, the results of three reversal experiments are shown. With $[^{14}C]$ADP and D-$[^{14}C]$Ala-D-$[^{14}C]$Ala a significant reaction could not be detected. With $[^{32}P]HPO_4^{2-}$ an apparent reversal was observed. However, this reversal may be the result of an exchange reaction. The results of the experiments presented in Table II indicated that k_{-3} is small in comparison with k_3, and, thus, the inhibition studies presented in this paper result from the formation of one or more enzyme–product complexes that are not involved in the reversal of the over-all reaction.

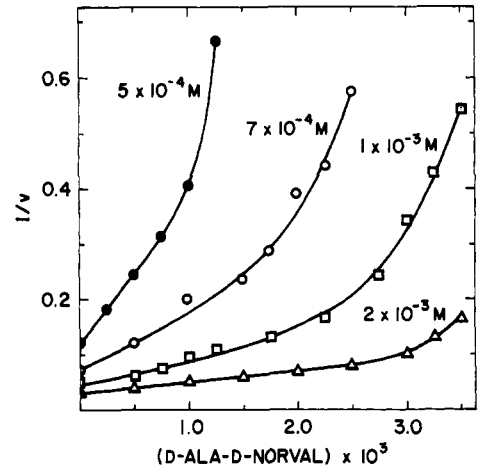


FIGURE 4: Dixon plots of inhibition by D-Ala-D-norval. The ADP assay was used with the following concentrations of D-alanine: (△—△) 2×10^{-3} M, (□—□) 1×10^{-3} M, (○—○) 7×10^{-4} M, and (●—●) 5×10^{-4} M.

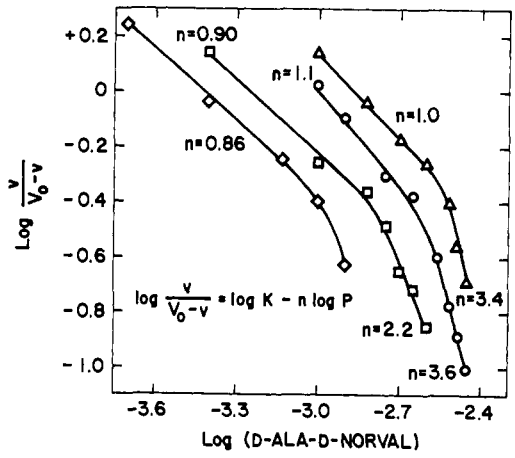


FIGURE 5: Analysis of the inhibition by D-Ala-D-norval with the empirical Hill equation.

Discussion

In the biosynthesis of UDP-MurNAc-pentapeptide, two independent pathways converge at reaction 3 (Figure 6). In the alanine branch, alanine racemase is coupled with D-alanine:D-alanine ligase. Since the equilibrium constant for the racemase is 1 whereas that for D-alanine:D-alanine ligase is estimated at 10^3, lack of control in the alanine branch could lead to the accumulation of D-Ala-D-Ala[2] and, thus, could result in the depletion of the L-alanine pool. D-Ala-D-Ala does not inhibit alanine racemase (F. C. Neuhaus, unpublished experiments). If alanine racemase were inhibited by D-Ala-D-Ala, the cell would be deprived of the necessary D-alanine required for teichoic acid biosynthesis and other systems requiring this amino acid. Thus, the inhibition of D-alanine:D-

[2] Ito and Strominger (1960, 1962) reported that D-Ala-D-Ala accumulates in lysine-deprived cells together with UDP-MurNAc-L-Ala-D-Glu. Since no control experiments were reported, it is difficult to establish whether this represents a significant accumulation of D-Ala-D-Ala.

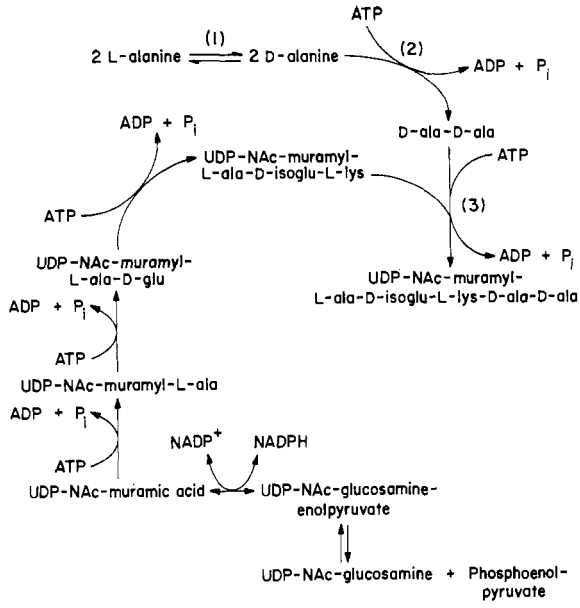


FIGURE 6: Biosynthesis of UDP-MurNAc-pentapeptide.

alanine ligase by its product[3] can serve as an effective control of the alanine branch.

Although the data do not provide unequivocal evidence for a separate control site, there are two types of experiments that suggest the presence of more than one binding site for dipeptide. (1) The kinetic analysis of the inhibition is consistent with a model that assumes at least two binding sites for D-Ala-D-Ala, *i.e.*, EA, EAA, EP, EAP, and EAP_2. (2) The analysis of the inhibition by D-Ala-D-norval with the Hill equation shows a pronounced change in the interaction coefficient. In the kinetic analysis at least two enzyme–product complexes must exist. The equation based on a single EP complex is not consistent with the data. The equation based on the addition of EP, EAP, and EAP_2 complexes to the model is consistent with the data.

The second type of experiment suggests that the product binding sites interact with each other. This was particularly apparent from an analysis of the inhibition of the ligase by D-Ala-D-norval. This type of analysis has been applied to many enzymes and it is generally concluded that those enzymes with strongly interacting binding sites for substrate or product give interaction coefficients that are independent of the substrate or inhibitor concentration (Atkinson *et al.*, 1965; Whiteley and Tahara, 1966) whereas those with weakly interacting binding sites give interaction coefficients that vary with the substrate or inhibitor concentration. For example, aspartyl transcarbamylase shows a change in the interaction coefficient for aspartate from $n = 1.2$ to 4.0 (Gerhart and Pardee, 1964). In addition, with threonine deaminase from *Clostridium tetanomorphum* the interaction coefficient varies from 0.6 to 3.6 (Whiteley and Tahara, 1966). In the case of the ligase described in this paper the interaction coefficient increases from 1 to values approaching 4. This analysis provides additional support for more than one binding site for product and indicates that these binding sites may interact.

It is interesting to note that D-Ala-D-norval and D-Ala-D-butyr are more effective inhibitors than D-Ala-D-Ala of the reaction catalyzed by the ligase. However, as demonstrated previously (Neuhaus, 1962), D-norvaline and D-α-amino-*n*-butyric acid are less effective than D-alanine as substrates on the acceptor site. Incubation of D-alanine and D-valine with the ligase and ATP does not result in the synthesis of D-Ala-D-Val (C. V. Carpenter and F. C. Neuhaus, unpublished observations). In contrast, D-Ala-D-Val will inhibit the ligase as effectively as D-butyr-D-butyr and D-butyr-D-Ala (Table I). Thus, the specificity profile of inhibition is different from that predicted from the substrate specificity profile.

The data presented in this paper suggest that a single enzyme–product complex cannot explain the available data. Thus, we should like to propose that more than one product binding site exists and that these sites function in the control of D-Ala-D-Ala formation.

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[3] UDP-MurNAc-pentapeptide does not inhibit D-alanine:D-alanine ligase (F. C. Neuhaus and M. Gragg, unpublished observation).