Enzymatic Synthesis of D-Alanyl-D-alanine. Two Binding Modes for Product on D-Alanine:D-Alanine Ligase (ADP)†

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ABSTRACT: D-Alanine:D-alanine ligase (ADP) (EC 6.3.2.4) possesses multiple binding sites for D-Ala-D-Ala. Inhibition at these sites by product controls the rate of dipeptide formation (Neuhaus *et al.* (1969), *Biochemistry 8*, 5119). The detection of ligase·[^{14}C]ATP···effector complexes on Sephadex G-100 has been used to study the interaction of product with D-alanine:D-alanine ligase. Observation of these complexes requires the presence of an effector, D-cycloserine, or D-Ala-D-Ala. The concentration of D-cycloserine required to observe one-half the maximal amount of complex is 1×10^{-5} M. Analogs of D-Ala-D-Ala, *e.g.*, D-Ala-D-Nva, D-Ala-D-Val, D-Ala-D-Ser, that inhibit D-alanine:D-alanine ligase do not give a detectable enzyme·[^{14}C]ATP···effector complex on Sephadex G-100. These results suggest the presence of at least two binding modes for dipeptide. Thus, D-Ala-D-Ala in one mode facilitates the binding of [^{14}C]ATP, whereas D-Ala-D-Nva, D-Ala-D-Val, or D-Ala-D-Ser in the second mode does not facilitate binding of [^{14}C]ATP.

D-Alanyl-D-alanine, the terminal dipeptide in the peptidoglycan precursor, UDP-*N*-acetylmuramyl-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala, is synthesized by two cytoplasmic enzymes: (1) alanine racemase (EC 5.1.1.1) (Wood and Gunsalus, 1951) and (2) D-alanine:D-alanine ligase (ADP) (EC 6.3.2.4) (Ito and Strominger, 1960, 1962; Neuhaus, 1960, 1962).

D-Alanine:D-alanine ligase has an absolute specificity for the D-configuration and will catalyze the formation of mixed dipeptides with D-alanine and structurally related D-amino acids (Neuhaus, 1962). The enzyme is inhibited by its product, D-Ala-D-Ala, and by analogs of D-Ala-D-Ala. Both alanine racemase and D-alanine:D-alanine ligase are inhibited by the antibiotic D-cycloserine (Strominger *et al.*, 1960; Neuhaus and Lynch, 1964).

Recent work has demonstrated that D-alanine:D-alanine ligase possesses multiple binding sites for D-Ala-D-Ala (Neuhaus *et al.*, 1969). The evidence suggests that several product binding sites exist and function in the control of D-Ala-D-Ala formation. In the present communication, a new approach has been used to study substrate and product binding with D-alanine:D-alanine ligase. Our results have indicated the presence of more than one binding mode for dipeptide.

Experimental Section

Materials. The DD dipeptides were synthesized by Dr. H. Plaut of Cyclo Chemical Corp. D-Cycloserine was generously donated by Commercial Solvents Corp. Na_2ATP, phosphoenolpyruvate, NADH, and L-lactate:NAD oxidoreductase (EC 1.1.1.27) were obtained from Sigma Chemical Co. ATP:pyruvate phosphotransferase (EC 2.7.1.40) was a product of Boehringer Mannheim Corp. Potassium [^{32}P]phosphate, [^{14}C]ADP, and [^{14}C]ATP were purchased from Amersham/Searle Corp. D-[^{14}C]Alanine was obtained from New England Nuclear.

Enzyme Preparation. D-Alanine:D-alanine ligase was purified through the isoelectric focusing procedure as previously described (Neuhaus *et al.*, 1969). Ampholytes from this preparation were removed by gel filtration on Sephadex G-100. The specific activity of the final preparation was 500 units/mg.

Assay of D-Alanine:D-Alanine Ligase; ADP Assay. D-Alanine:D-alanine ligase activity was determined by the ADP assay as previously described (Neuhaus *et al.*, 1969). Formation of ADP is measured with a coupled assay using ATP:pyruvate phosphotransferase and L-lactate:NAD oxidoreductase.

Dipeptide Assay. The dipeptide assay, performed by the method of Neuhaus (1962), measures the conversion of D-[^{14}C]alanine to labeled D-Ala-D-Ala. The μmoles of dipeptide formed are calculated from the percent conversion of D-[^{14}C]alanine to D-[^{14}C]Ala-D-[^{14}C]Ala.

Detection of Enzyme·[^{14}C]ATP···Effector Complex. In this paper the amount of enzyme·[^{14}C]ATP···effector complex is defined by the amount of [^{14}C]ATP which cochromatographs with D-alanine:D-alanine ligase. To form such complexes, D-alanine:D-alanine ligase (2.5 units) was mixed with 0.04 M Tris-HCl (pH 7.2), 0.04 M KCl, 8 mM $MnCl_2$, 2.4×10^{-5} M [^{14}C]ATP (neutralized with NaOH; 3.3×10^7 cpm/μmole), and effector in a total volume of 0.13 ml. The mixture was maintained at 37° for 15 min and was then applied to a column of Sephadex G-100 (30.5 cm × 5 mm) previously equilibrated with 0.04 M Tris-HCl (pH 7.2), 0.04 M KCl, 8 mM $MnCl_2$, and effector. The column was eluted at 25° with the same buffer solution (salts plus effector) that was used for equilibration. Fractions of the eluate (0.14 ml) were collected, chilled to 0°, and assayed for D-alanine:D-alanine ligase and [^{14}C]ATP.

[^{32}P]HPO_4^{2-}–ATP and [^{14}C]ADP–ATP-Exchange Studies. To test for [^{32}P]HPO_4^{2-}–ATP-exchange activity, D-alanine:D-alanine ligase was mixed with 0.025 M Tris-HCl (pH 7.8), 0.025 M KCl, 5 mM $MgCl_2$, 2.5×10^{-3} M [^{32}P]HPO_4^{2-} ($2.2–2.4 \times 10^5$ cpm/μmole), 5 mM ADP (neutralized with NaOH), and 0.1 M D-Ala-D-Ala in a total volume of 0.25 ml. The mixture was incubated for 20 min at 37°. The reaction was terminated by the addition of 1 ml of 5% trichloroacetic acid.

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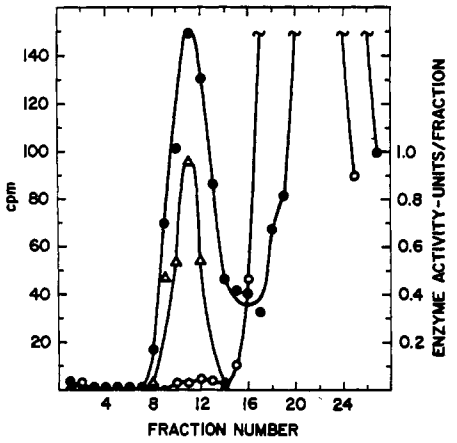

FIGURE 1: Binding of ATP by D-alanine:D-alanine ligase. The reaction mixture (0.13 ml) contained: 0.04 M Tris-HCl (pH 7.2), 0.04 M KCl, 8 mM $MnCl_2$, 2.4×10^{-5} M [^{14}C]ATP (3.3×10^7 cpm/μmole), 2.5 units of D-alanine:D-alanine ligase, and 2 mM D-cycloserine as indicated. The mixture was maintained at 37° for 15 min and was then applied to a Sephadex G-100 column. The column was eluted as described in the Experimental Section. (●) 2 mM D-cycloserine was added to the reaction mixture and the eluting buffer; (○) D-cycloserine was omitted in the reaction mixture and eluting buffer. (△) D-Alanine:D-alanine ligase activity.

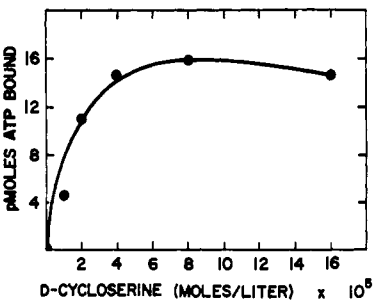

FIGURE 2: Binding of ATP by D-alanine:D-alanine ligase as a function of D-cycloserine concentration. Conditions for the binding of ATP are described in Figure 1.

[^{32}P]HPO_4^{2-} and [^{32}P]ATP were separated by the method of Crane and Lipmann (1953).

To assay for [^{14}C]ADP–ATP-exchange activity, 3 units of D-alanine:D-alanine ligase were mixed with 0.05 M Tris-HCl (pH 7.8), 0.05 M KCl, 0.01 M $MgCl_2$, 0.08 M D-Ala-D-Ala, 0.005 M KH_2PO_4, and 0.004 M [^{14}C]ADP (2.6×10^5 cpm/μmole) in a total volume of 0.12 ml. The mixture was incubated for 60 min at 37°. The reaction was terminated by heating the mixture for 2 min at 100°. Carrier ATP (1 μmole) was added to the reaction mixture, and ATP was separated from ADP by descending chromatography on Whatman No. 3MM paper in isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v). The nucleotide spots were located under ultraviolet light, excised, and counted for radioactivity.

Results

Binding of ATP to D-Alanine:D-Alanine Ligase. When D-alanine:D-alanine ligase is incubated with KCl, $MnCl_2$, ATP, and D-cycloserine, a ligase·[^{14}C]ATP···D-cycloserine complex can be detected by gel filtration (Figure 1). As shown in Table I, binding of [^{14}C]ATP is dependent on the presence of enzyme and D-cycloserine, and is enhanced by the addition of Mn^{2+}. If D-cycloserine is replaced by D-alanine, no complex is observed. The concentration of D-cycloserine required for one-half of the maximal amount of complex is approximately 1×10^{-5} M (Figure 2).

The results with D-cycloserine suggest that other inhibitors of the ligase may also promote binding of ATP. Recent work (Neuhaus *et al.*, 1969) has shown that D-Ala-D-Ala and analogs of D-Ala-D-Ala are effective inhibitors of D-alanine:D-alanine ligase. Product inhibition was observed with D-Ala-D-Ala, D-Ala-D-Nva, D-Ala-D-Val, and D-Ala-D-Ser (Table II). A series of dipeptide inhibitors were tested for their ability to promote the binding of ATP. Of the four dipeptides, only D-Ala-D-Ala facilitated binding of ATP to the ligase. As shown in Table II, a higher concentration is required to equal the effectiveness of D-cycloserine. No binding was detected when the enzyme was incubated with either 0.01 M D-Ala-D-Nva, 0.01 M D-Ala-D-Val, or 0.01 M D-Ala-D-Ser (Table II). It is evident from Table II that the action of these dipeptides on the binding of ATP does not parallel their inhibitory effect on the catalytic activity of the ligase.

Heat Stability of D-Alanine:D-Alanine Ligase in the Presence of Substrates. Additional evidence for the binding of ATP may be obtained from experiments demonstrating the increase in heat stability of D-alanine:D-alanine ligase in the presence of substrate. As illustrated in Figure 3, the $t_{1/2}$ at 65° in the presence of 0.01 M ATP or 0.01 M D-alanine are 43 and 30 min, respectively, whereas the $t_{1/2}$ in the absence of substrate is 15 min. Although the detection of enzyme-bound nucleotide on Sephadex G-100 required the presence of certain effector molecules, *i.e.*, either D-cycloserine or D-Ala-D-Ala, the interaction between enzyme and ATP during heat treatment does not. The effect of several inhibitors on the heat stability of the ligase was also examined. Neither 0.002 M D-cycloserine nor 0.01 M D-Ala-D-Ala protected the enzyme during heat treatment. Moreover, no effect was observed during heat treatment with either 0.01 M D-Ala-D-Nva, 0.01 M D-Ala-D-Val, or 0.01 M D-Ala-D-Ser.

Further Analysis of the Binding of D-Alanine to D-Alanine: D-Alanine Ligase. The effect of D-alanine on the heat stability of D-alanine:D-alanine ligase indicates a specific interaction between D-alanine and enzyme in the absence of ATP. Additional evidence for the specific interaction of D-alanine with

TABLE I: Requirements for Binding of ATP by D-Alanine: D-Alanine Ligase.

Reaction Mixture[a]	ATP Bound (pmoles)
Complete	16.4
$-Mn^{2+}$	4.3
$-Mn^{2+}$, $+Mg^{2+}$	2.7
−Enzyme	<1.0
−D-Cycloserine	<1.0
−D-Cycloserine, +D-alanine	<1.0

[a] The reaction mixture (0.13 ml) contained: 0.04 M Tris-HCl (pH 7.2), 0.04 M KCl, 8 mM $MnCl_2$, 2 mM D-cycloserine, 2.4×10^{-5} M [^{14}C]ATP (3.3×10^7 cpm/μmole), and 2.5 units of D-alanine:D-alanine ligase. In the last experiment, D-cycloserine was replaced by 0.01 M D-alanine. The procedure for detecting the ligase·[^{14}C]ATP complex is described in the Experimental Section.

TABLE II: Binding of ATP by D-Alanine:D-Alanine Ligase in the Presence of D-Ala-D-Ala and Analogs of D-Ala-D-Ala.

Addition	ATP Bound[a] (pmoles)	Inhibition of Ligase[b] (%)
No inhibitor	0.0	0
D-Ala-D-Ala	7.6 (20.8)	70
D-Ala-D-Nva	<1.0	70
D-Ala-D-Val	<1.0	67
D-Ala-D-Ser	<1.0	37

[a] The reaction mixture (0.13 ml) contained: 0.04 M Tris-HCl (pH 7.2), 0.04 M KCl, 8 mM $MnCl_2$, 2.4×10^{-5} M [^{14}C]-ATP (3.3×10^7 cpm/μmole), 2.5 units of D-alanine:D-alanine ligase, and 0.01 M dipeptide. The procedure for detecting the ligase·[^{14}C]ATP complex is described in the Experimental Section. The value in parenthesis indicates ATP bound in the presence of 0.10 M D-Ala-D-Ala. [b] The dipeptides were tested in the ADP assay in the presence of 1 mM D-alanine, 0.05 M Tris-HCl (pH 7.2), 0.01 M $MnCl_2$, and 0.29 unit of D-alanine:D-alanine ligase. The concentration of dipeptide was 3 mM.

TABLE III: Binding of D-Alanine by D-Alanine:D-Alanine Ligase.[a]

Reaction Mixture	Radioactivity in Dipeptide (cpm)
I. a. (Enzyme + D-[^{14}C]alanine)	
b. Plus (D-[^{12}C]alanine + ATP)	4030
II. a. (Enzyme + D-[^{14}C]alanine + D-[^{12}C]alanine)	
b. Plus (ATP)	1039

[a] The reaction mixture contained the following components in a total volume of 0.25 ml: 0.05 M Tris-HCl (pH 7.2), 0.05 M KCl, 0.01 M $MnCl_2$, 1 mM D-[^{14}C]alanine (1.6×10^6 cpm/μmole), 0.08 M D-[^{12}C]alanine, 0.01 M ATP, and 4.2 units of D-alanine:D-alanine ligase. In expt I, enzyme was incubated with (a) KCl, $MnCl_2$, Tris-HCl, and D-[^{14}C]alanine at 37°; (b) after 2 min, ATP and a 100-fold excess of D-[^{12}C]alanine were added, and the incubation was continued for another 30 sec. In expt II, enzyme was incubated with (a) KCl, $MnCl_2$, Tris-HCl, and D-[^{14}C]alanine, and a 100-fold excess of D-[^{12}C]alanine at 37°; (b) after 2 min, ATP was added and the incubation was continued for another 30 sec. The reactions were terminated by heating the tubes for 2 min at 100°. The incubation mixture was made 5 mM in carrier D-Ala-D-Ala. Dipeptide was isolated by paper chromatography in butanol–acetic acid–water (4:1:5, v/v), organic phase (Neuhaus, 1962). The appropriate areas were excised and assayed for radioactivity. All values are corrected for a control tube containing boiled enzyme.

the ligase in the absence of ATP is given in Table III. In this experiment, the pulse-labeling technique of Krishnaswamy *et al.* (1962) was used to examine binding of D-alanine to the ligase prior to dipeptide synthesis.

D-Alanine:D-alanine ligase was incubated with KCl, $MnCl_2$, and D-[^{14}C]alanine for 2 min at 37°. ATP and a 100-fold excess of D-[^{12}C]alanine were then added to the mixture. The reaction mixture was incubated for another 30 sec and was terminated by heating the mixture for 2 min at 100°. In a second tube, enzyme was incubated with KCl, $MnCl_2$, D-[^{14}C]alanine, and a 100-fold excess of D-[^{12}C]alanine for 2 min at 37°; ATP was added and the incubation continued for another 30 sec.

As illustrated in Table III, an increased amount of radioactivity in D-Ala-D-Ala is observed in expt 1 where the enzyme was preincubated with D-[^{14}C]alanine before the addition of ATP and D-[^{12}C]alanine. The difference in radioactivity between expt 1 and 2 reflects the labeled D-alanine which was bound to the enzyme before the addition of ATP and D-[^{12}C]-alanine. The bound alanine is thus available for dipeptide synthesis and does not readily equilibrate with the D-[^{12}C]-alanine.

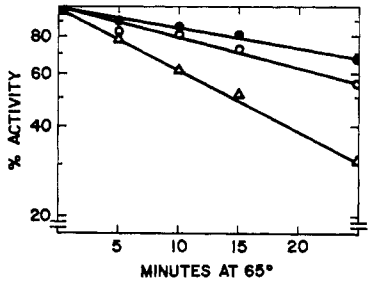

FIGURE 3: Heat stability of D-alanine:D-alanine ligase in the presence and absence of substrate. A reaction mixture (0.30 ml) containing 0.1 M magnesium acetate (pH 5.5), 6 units of D-alanine:D-alanine ligase, and substrate as indicated was maintained at 65°. At the indicated times, aliquots (0.05 ml) were removed and tested for D-alanine:D-alanine ligase activity in the dipeptide assay. Concentration of D-[^{14}C]alanine in the dipeptide assay was 4 mM (3.5×10^6 cpm/μmole.) Concentration of substrate during heat treatment: (●) 10 mM ATP, (○) 10 mM D-alanine, and (△) no substrate present.

Analysis of Substrate and Product Binding by Reverse Reactions. Since D-Ala-D-Ala was effective in promoting a detectable enzyme·[^{14}C]ATP···D-Ala-D-Ala complex, it was proposed that D-Ala-D-Ala might also promote a [^{32}P]HPO_4^{2-}–ATP-exchange reaction. In previous studies (Neuhaus *et al.*, 1969), an apparent [^{32}P]HPO_4^{2-}–ATP-exchange reaction, dependent on D-Ala-D-Ala and ADP, was reported. Further studies, however, revealed that the addition of ATP to the reaction mixture markedly inhibited the amount of [^{32}P]-HPO_4^{2-} incorporated into ATP (Table IV). This additional observation is more consistent with a net reversal of the reaction rather than [^{32}P]HPO_4^{2-}–ATP exchange (reaction 1).

$$\text{D-Ala-D-Ala} + [^{32}\text{P}]HPO_4^{2-} + \text{ADP} \longrightarrow 2\text{D-alanine} + [^{32}\text{P}]\text{ATP} \quad (1)$$

As illustrated in Table IV, the formation of [^{32}P]ATP requires both ADP and D-Ala-D-Ala.

The possibility that the above results are due to contaminating substances in the substrates is unlikely. Analysis of the D-Ala-D-Ala revealed no contamination from other dipeptides or amino acids. ADP was purified by gel filtration on Sephadex G-25 (Stickgold and Neuhaus, 1967) and assayed for trace amounts of ATP in the firefly luciferase system

TABLE IV: Requirements for [^{32}P]ATP Formation.[a]

Expt	Addition (μmoles) ADP	ATP	D-Ala-D-Ala	ATP Formed (nmoles)
1	1.3		25	10.2
	1.3			1.4
			25	0
				0
2	1.3		25	11.5
	1.0	0.3	25	3.6
	0.5	0.8	25	<0.5
		1.3	25	<0.5

[a] In expt 1, the reaction mixture contained the following components in a total volume of 0.25 ml: 0.025 M Tris-HCl (pH 7.8), 0.025 M KCl, 5 mM $MgCl_2$, 2.5×10^{-3} M [^{32}P]-HPO_4^{2-} (2.4×10^5 cpm/μmole), 2 units of D-alanine:D-alanine ligase, ADP, and D-Ala-D-Ala as indicated. In expt 2, the reaction mixture contained the following components in a total volume of 0.25 ml: 0.025 M Tris-HCl buffer (pH 7.8), 0.025 M KCl, 5 mM $MgCl_2$, 2.5×10^{-3} M [^{32}P]HPO_4^{2-} (2.2×10^5 cpm/μmole), 1.6 units of D-alanine:D-alanine ligase, 0.1 M D-Ala-D-Ala, ADP, and ATP as indicated. The reaction mixtures were incubated for 20 min at 37°. The reactions were terminated by the addition of 1 ml of 5% trichloroacetic acid. 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. All values are corrected for a control tube containing boiled enzyme.

TABLE V: Formation of [^{14}C]ATP from [^{14}C]ADP.[a]

Addition	ATP Formed (nmoles)
I. E + [^{14}C]ADP + HPO_4^{2-}	
+ D-Ala-D-Ala	41
− D-Ala-D-Ala	1
II. E + [^{14}C]ADP + ATP	
+ HPO_4^{2-}	2
− HPO_4^{2-}	2

[a] In expt I, the reaction mixture contained: 0.05 M Tris-HCl (pH 7.8), 0.05 M KCl, 0.01 M $MgCl_2$, 0.08 M D-Ala-D-Ala, 0.005 M KH_2PO_4, 0.004 M [^{14}C]ADP (2.6×10^5 cpm/μmole), and 3 units of D-alanine:D-alanine ligase in a total volume of 0.12 ml. The mixture was incubated for 60 min at 37° and the reaction was terminated by heating the mixture for 2 min at 100°. Conditions for separating [^{14}C]ADP from [^{14}C]ATP are described in the Experimental Section. Experiment II was identical with expt I except for the addition of 0.004 M ATP to the reaction mixture and the omission of KH_2PO_4 and D-Ala-D-Ala.

(Strehler and McElroy, 1957). The purified ADP contained less than 0.03% ATP. Thus, the dependence of [^{32}P]ATP formation on ADP and D-Ala-D-Ala indicates a reversal of the reaction rather than D-Ala-D-Ala, ADP-dependent [^{32}P]-HPO_4^{2-}–ATP exchange.

If the incorporation of [^{32}P]HPO_4^{2-} into [^{32}P]ATP represents a reversal of the reaction, it should be possible to observe formation of [^{14}C]ATP from [^{14}C]ADP. As shown in Table V, formation of [^{14}C]ATP was observed when the ligase was incubated with KCl, $MgCl_2$, [^{14}C]ADP, and D-Ala-D-Ala; omission of D-Ala-D-Ala reduced the radioactivity in ATP to background. No exchange activity could be detected in reaction mixtures containing ligase, KCl, $MgCl_2$, ATP, and [^{14}C]ADP (Table V). The absence of a substrate-independent [^{14}C]ADP–ATP exchange indicates that the formation of [^{14}C]ATP does not result from the action of adenylic kinase in the enzyme preparation.

Discussion

D-Alanine:D-alanine ligase possesses multiple binding sites for the product D-Ala-D-Ala. Inhibition at these sites by product controls the rate of dipeptide formation. With the kinetic analysis that was previously used (Neuhaus *et al.*, 1969), it was not possible to distinguish between different types of product binding sites. In order to define further product binding, an independent approach has been used that involves the detection of enzyme·ATP···D-cycloserine and enzyme·ATP···D-Ala-D-Ala complexes on Sephadex G-100. Observation of enzyme complexes that contain [^{14}C]ATP requires the presence of either D-cycloserine or D-Ala-D-Ala.

The detection of an enzyme·[^{14}C]ATP···D-cycloserine complex is dependent on the presence of enzyme and D-cycloserine, and is enhanced by the addition of Mn^{2+}. The concentration of D-cycloserine required to observe one-half the maximal amount of complex is 1×10^{-5} M. This value is similar to the K_i for D-cycloserine established in kinetic studies on the D-alanine:D-alanine ligase (Neuhaus and Lynch, 1964). If D-cycloserine is replaced with D-alanine, no enzyme·[^{14}C]ATP···D-alanine complex is observed. In contrast to the incubation mixture containing D-cycloserine, the presence of both ATP and D-alanine provides a complete system for the synthesis of D-Ala-D-Ala. Turnover of the enzyme·[^{14}C]-ATP···D-alanine complex with the synthesis of dipeptide results in dissociation of the [^{14}C]nucleotide from the enzyme complex.

When D-cycloserine is replaced with D-Ala-D-Ala, an enzyme·[^{14}C]ATP···D-Ala-D-Ala complex is detected on Sephadex G-100. In contrast, analogs of D-Ala-D-Ala that inhibit the reaction do not promote the formation of a detectable enzyme·ATP complex. For example, when D-Ala-D-Ala is replaced with D-Ala-D-Nva, an effective inhibitor of D-alanine:D-alanine ligase, no enzyme·[^{14}C]ATP···dipeptide complex is detected. Thus, the specificity profile of product inhibition cannot be used to predict the action of these dipeptides on the binding of [^{14}C]ATP. These results suggest the presence of more than one type of binding mode for dipeptide. Thus, D-Ala-D-Ala in one mode facilitates the binding of [^{14}C]ATP, whereas either D-Ala-D-Nva, D-Ala-D-Val, or D-Ala-D-Ser in the second mode does not facilitate binding of [^{14}C]ATP. It is possible that D-alanine:D-alanine ligase may contain two types of product binding sites; one for D-Ala-D-Ala and one for D-Ala-D-Nva, D-Ala-D-Val, or D-Ala-D-Ser. Alternatively, either D-Ala-D-Nva, D-Ala-D-Val, or D-Ala-D-Ser may bind to the same site as D-Ala-D-Ala but does not induce the correct conformational change to facilitate binding of [^{14}C]ATP. The methods employed in this paper do not distinguish between these two possibilities.

The above results indicate that the affinity of the enzyme for ATP may be influenced by certain effector molecules.

Thus, in the presence of either D-cycloserine or D-Ala-D-Ala, eq 2 may be formulated. If these effectors are omitted, the

$$\text{ligase} + \text{effector} + [^{14}\text{C}]\text{ATP} \rightleftarrows \text{ligase}\cdot[^{14}\text{C}]\text{ATP}\cdots\text{effector} \quad (2)$$

enzyme·nucleotide complex easily dissociates and is not detected on Sephadex G-100 (eq 3).

$$\text{ligase} + [^{14}\text{C}]\text{ATP} \rightleftarrows \text{ligase}\cdot[^{14}\text{C}]\text{ATP} \quad (3)$$

The formation of an enzyme·ATP complex in the absence of an effector is inferred from the stabilization of the enzyme by ATP during heat treatment. For example, when 10 mM ATP is included during heat treatment, the rate of inactivation is 33% of that observed in the absence of ATP. Similarly, the addition of D-alanine during heat treatment significantly reduces the rate of inactivation.

Additional evidence for an enzyme·D-alanine complex was obtained using the pulse-labeling technique of Krishnaswamy *et al.* (1962). In expt I, D-alanine:D-alanine ligase was preincubated with D-[^{14}C]alanine. After 2 min, ATP and excess D-[^{12}C]alanine were added, and the incubation was continued for another 30 sec. In expt II, ligase was preincubated with both D-[^{14}C]alanine and excess D-[^{12}C]alanine. After 2 min, ATP was added, and the incubation was continued for another 30 sec. The increased amount of radioactivity in dipeptide in expt I reflects the labeled D-alanine which was bound to the enzyme before the addition of ATP and D-[^{12}C]alanine. Thus, the bound alanine is available for dipeptide synthesis and does not readily equilibrate with the D-[^{12}C]alanine.

Attempts to detect an E–ADP or E–P intermediate through exchange reactions have been unsuccessful. Neither [^{32}P]-HPO_4^{2-}–ATP nor [^{14}C]ADP–ATP exchange activity has been observed. Incubation of D-alanine:D-alanine ligase with ADP, inorganic phosphate, and D-Ala-D-Ala results in a significant reversal of the reaction. Similar results have been reported for several steps in the biosynthesis of the peptidoglycan precursor, UDP-MurNAc-pentapeptide. Reverse activity has been demonstrated with UDP-MurNAc-L-Ala:D-glutamate ligase (ADP) from *Staphylococcus aureus* (Nathenson *et al.*, 1964) and UDP-MurNAc-L-Ala-D-Glu:*meso*-2,6-diaminopimelate ligase from *Bacillus cereus* (Mizuno and Ito, 1968).

The data reported in this paper suggest that the affinity of D-alanine:D-alanine ligase for ATP may be influenced by certain effector molecules. Detection of enzyme·[^{14}C]ATP complexes on Sephadex G-100 requires the presence of either D-cycloserine or D-Ala-D-Ala. Although analogs of D-Ala-D-Ala inhibit D-alanine:D-alanine ligase, they do not promote the formation of a detectable enzyme·[^{14}C]ATP complex. Thus, we propose the presence of two binding modes for dipeptide. D-Ala-D-Ala in one mode facilitates the binding of [^{14}C]ATP, whereas D-Ala-D-Nva, D-Ala-D-Val, or D-Ala-D-Ser in the second mode does not facilitate binding of [^{14}C]ATP. The presence of two binding modes for product suggests that a precise form of product control may exist.

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