Kinetic Evidence for the Formation of D-Alanyl Phosphate in the Mechanism of D-Alanyl-D-alanine Ligase*

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The steady state kinetic mechanism, molecular isotope exchange and the positional isotope exchange (PIX) reactions of D-alanyl-D-alanine ligase from Salmonella typhimurium have been studied. The kinetic mechanism has been determined to be ordered Ter-Ter from initial velocity and product inhibition experiments. The first substrate to bind is ATP followed by the addition of 2 mol of D-alanine, P_i is released, and then D-alanyl-D-alanine and ADP dissociate from the enzyme-substrate complex. In the reverse direction D-alanyl-D-alanine exhibits complete substrate inhibition (K_I = 1.15 ± 0.05 mM) by binding to the enzyme-ATP complex. In the presence of D-alanine, D-alanyl-D-alanine ligase catalyzed the positional exchange of the β,γ-bridge oxygen in [γ-18O]ATP to a β-nonbridge position. Two possible alternate dead-end substrate analogs, D-2-chloropropionic acid and isobutyric acid, did not induce a positional isotope exchange rate. The positional isotope exchange rate is diminished relative to the net substrate turnover as the concentration of D-alanine is increased. This is consistent with the ordered Ter-Ter mechanism as determined by the steady state kinetic experiments. The ratio of the positional isotope exchange rate relative to the net chemical turnover of substrate (V(pos)/V_chem) approaches a value of 1.4 as the concentration of D-alanine becomes very small. This ratio is 100 times larger than the ratio of the maximal reverse and forward chemical reaction velocities (V_R/V_F). This situation is only possible when the reaction mechanism proceeds in two distinct steps and the first step is much faster than the second step. The enzyme was also found to catalyze the molecular isotope exchange of radiolabeled D-alanine with D-alanyl-D-alanine in the presence of phosphate. These results are consistent with the formation of D-alanyl phosphate as a kinetically competent intermediate.

Bacteria are protected from the extracellular environment by the cell wall peptidoglycan layer. Key targets for antibacterial agents are the enzymes found in the peptidoglycan biosynthetic pathway (Neuhaus and Hammes, 1981). Early research with two antibacterial agents, D-cycloserine and O-carbamyl-D-serine, has focused attention on the three enzymes encompassing the D-alanine branch of cell wall biosynthesis (Neuhaus, 1962a, 1962b). The enzymes comprising the D-alanine pathway are alanine racemase, u-alanyl-u-alanine ligase, and the UDP-muramyl tripeptide d-Ala-d-Ala adding enzyme (see Walsh, 1989 for review). Elucidation of the mechanism of action of alanine racemase has contributed to the development of the antibacterial agents β-fluoro-D-alanine (Roise et al., 1985; Badet et al., 1984) and 1-aminoethyl phosphonate (Badet et al., 1986). The details of the kinetic and chemical mechanism of D-alanyl-D-alanine ligase have been largely unknown.

Previous experiments with d-alanyl-D-alanine ligase have resulted in the determination of the substrate specificity (Neuhaus, 1962a, 1962b) and purification of d-alanyl-D-alanine ligase from Streptococcus faecalis (Neuhaus and Lynch, 1964). Recently the gene encoding for d-alanyl-D-alanine ligase in Escherichia coli (Robinson et al., 1986) and Salmonella typhimurium (Daub et al., 1988) have been cloned. The ligase from S. typhimurium has been purified (Daub et al., 1988) and the inhibition by D-alanine phosphate and phosphonate analogs investigated (Duncan and Walsh, 1988).

The reaction catalyzed by D-alanyl-D-alanine ligase is presented below.

\[ \text{ATP} + 2 \text{D-alanine} \rightleftharpoons \text{D-Ala-D-Ala} + \text{ADP} + P_i \quad (1) \]

The reaction has been proposed to proceed via the phosphorylation of the carboxyl group of the first D-alanine by the γ-phosphoryl group of ATP to form an acylphosphate intermediate. The dipeptide is formed after the attack of the amino nitrogen of the second D-alanine on the acylphosphate as illustrated in Scheme I (Duncan and Walsh, 1988).

The positional isotope exchange technique of Midelfort and Rose (1976) can be used to determine the kinetic competence of the proposed D-alanyl phosphate intermediate. If D-alanyl-D-alanine ligase catalyzes the cleavage of the γ-phosphate of ATP to form the intermediate, then positional exchange of the β,γ-bridge oxygen-18 within [γ-18O]ATP to the β-nonbridge position in the presence of D-alanine would provide evidence for the proposed mechanism. The PIX reaction is illustrated in Scheme II. The PIX experiments can also be used to determine the order of substrate binding by observing the kinetic effect of varying the concentration of D-alanine on the ratio of the net chemical rate relative to the net positional isotope exchange rate (Hester and Raushel, 1987). In this report, the kinetic mechanism in addition to the molecular and positional isotope exchange reactions are described for the ligase from S. typhimurium.

* The abbreviations used are: PIX, positional isotope exchange; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; TAPS, 3-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid.
the inhibitor were as indicated in Table I. The kinetics of inhibition of NADH, and 33 cg of pyruvate kinase and lactate dehydrogenase in a phosphate (Meek and Vallfranca, 1980; Woolfolk et al., 1989) by ADP were monitored with a calorimetric assay for inorganic phosphate (Risley and Van Etten, 1976). Preparation of [γ-32P]ATP was completed as described by Rauschel and Villafranca (1980). d-alanyl-d-alanine ligase was purified by the method of Dashi et al. (1988) as modified in Knox et al. (1980). The D-[U-14C]alanine was purchased from Amersham Corp. at 40 mCi/mmol. Cellulose thin layer chromatograms and XAR-5 film were from Eastman Kodak. All other reagents were purchased from Sigma.

Kinetic Measurements—Initial velocity and product inhibition patterns in the forward direction were monitored spectrophotometrically using the hexokinase and glucose-6-phosphate dehydrogenase system. Each cuvette contained 100 mM HEPES, pH 7.5, 7.5 mM MgCl₂, 0.2 mM NADP, 100 mM KCl, 2.0 mM glucose, 25 units of hexokinase, and 5.0 units of glucose-6-P dehydrogenase in a total volume of 3.0 ml. Various concentrations of P₃, ADP, and d-alanyl-d-alanine were used as described in Table I.

\[ v = \frac{V_{AB}}{K_{Ka} + K_{a} + K_{b} + AB} \]  
\[ v = \frac{V_{AB}}{K_{Ka} + K_{a} + K_{b} + AB} \]
\[ v = \frac{K(1 + (1/K_{Ka})) + A}{K + A(1 + (1/K_{Ka}))} \]
\[ v = \frac{K(1 + (1/K_{Ka})) + A}{K + A(1 + (1/K_{Ka}))} \]
\[ v = \frac{K(1 + (1/K_{Ka})) + A}{K + A(1 + (1/K_{Ka}))} \]
\[ v = \frac{K + A + (A^{2}/K)}{K + A + (A^{2}/K)} \]

Molecular Isotope Exchange—The molecular isotope exchange reactions included 10 mM KCl, 10 mM MgCl₂, 100 mM HEPES, pH 7.5, 0.5 mM d-alanyl-d-alanine, 0.2 mM d-[U-14C]alanine (40 Ci/mmol), 35 μg of D-Ala-D-Ala ligase, and 20 mM KH₂PO₄. Control reactions excluded either KH₂PO₄ or d-alanyl-d-alanine from the mixture above. The exchange and control reaction volumes of 25 μl were incubated at 37°C with 4-μl aliquots removed at hourly time points. Reactions were stopped by heat treatment at 90°C for 3 min. Each time point aliquot was centrifuged to pellet denatured protein before being spotted onto a cellulose thin layer chromatogram (TLC). The TLC plates were developed in a 2-butanol:water:acetic acid (12:5:3), dried, and exposed to film. An autoradiogram allowed the location of the corresponding spots on the TLC plate to be counted.

Exchange velocities for d-[14C]alanine exchange into the C terminus of the d-alanyl-d-alanine dipeptide were calculated by Equation 8 (Segel, 1975),

\[ v^*= \left( \frac{[A][P]}{[A] + [P]} \right)^{1/2} \ln(1 - F) \]

where \( [A] \) = d-alanine, \( [P] \) = d-alanyl-d-alanine, and \( F \) = the fraction of equilibrium value obtained at time \( t \). Plots of ln (1 - F) versus \( t \) were linear to isotope equilibrium, and the slopes were used to determine the velocity.
RESULTS

Variation of D-Alanine—The rate of the forward reaction was monitored as a function of the D-alanine concentration at a fixed concentration of ATP (0.33 mM). The double-reciprocal plot yields a straight line (data not shown) from 0.05 to 20 mM D-alanine giving an apparent $K_m$ of 0.61 ± 0.04 mM.

Initial Velocity Patterns—The results of the initial velocity kinetic studies in the forward and reverse reaction are presented in Table I. In both the forward and reverse directions all initial velocity patterns are intersecting. In the reverse reaction, all three combinations (ADP versus D-Ala-D-Ala, P, versus D-Ala-D-Ala or P, versus ADP) were tried at saturating concentrations of the third substrate except for the P, versus ADP pattern. D-Ala-D-Ala is a strong substrate inhibitor ($K_i = 1.15 ± 0.05$ mM, $K_m = 0.03 ± 0.01$ mM) exhibiting complete substrate inhibition at the concentrations required for saturation as seen in Fig. 1.

Product Inhibition—Product inhibition data for the forward reaction are presented in Table II. $P_i$ is a noncompetitive inhibitor of ATP and D-alanine at low and saturating levels of D-alanine and ATP, respectively. A noncompetitive pattern is observed when D-alanyl-D-alanine inhibits D-alanine at a saturating concentration of ATP. ATP versus D-alanyl-D-alanine gives an uncompetitive pattern at low and saturating D-alanine. ADP is a competitive inhibitor of ATP.

Molecular Isotope Exchange—Given the demonstrated reversibility of the overall reaction described above, we sought to assess reversible formation of the anticipated reaction intermediate, D-alanyl-PO$_2^-$·. In the absence of ATP or ADP, the enzyme may be able to carry out reversible phosphorylisis of D-alanyl-D-alanine. As shown in Scheme III, this would lead to bound D-alanyl-PO$_2^-$· and D-alanine in the active site. If enzyme-bound D-alanine could exchange with D-[t$^{13}$C]alanine in the medium, the reversible formation of D-alanyl-PO$_2^-$· could be monitored by a molecular isotope exchange partial reaction. This enzyme-catalyzed movement of the $^{13}$C label into D-alanyl-D-alanine should be dependent on PO$_2^-$·.

In the absence of inorganic phosphate or D-alanyl-D-alanine, no detectable incorporation of radioactivity from D-[t$^{13}$C]alanine into the C terminus of D-alanyl-D-alanine was observed. Upon the addition of both phosphate and D-alanyl-D-alanine, exchange proceeded to isotopic equilibrium over the course of 18 h. An exchange velocity of 1.5 (±0.3) $× 10^{-4}$ μmol·min$^{-1}$·mg$^{-1}$ was measured, and thus a turnover number of 5.9 $× 10^{-3}$ $× 10^{12}$ min$^{-1}$ can be calculated for the molecular isotope exchange. This exchange reaction, which was carried out in the absence of ADP, is 1000-fold slower than the overall maximal velocity of the reverse reaction for the dipeptide cleavage by D-Ala-D-Ala ligase in the presence of ADP.

Positional Isotope Exchange Reactions—The PIX reactions were monitored by following the exchange of the $\gamma$-bridge oxygen-18 with a $\beta$-nonbridge oxygen-16. In the absence of D-alanine there was no detectable PIX when ATP alone was incubated with the ligase at a concentration of 0.05, 1.0, and 5.0 mM [y-$^{18}$O]ATP for 6, 8, or 10 h. Variation of the pH of the reaction from pH 7 to 9 did not enhance the PIX reaction in the absence of any added D-alanine. The upper limit for the PIX reaction in the absence of D-alanine relative to the $V_{max}$ for the forward reaction is 0.5%. Labeled ATP was incubated with the ligase in the presence of possible dead-end analogs for D-alanine. Isobutyric acid and D-2-chloropropionic acid did not induce any detectable PIX reaction within the oxygen-18-labeled ATP.

In the presence of D-alanine, a positional isotope exchange reaction was observed. The rate constants for the PIX reaction were calculated from the equation of Litwin and Wimmer (1979),

$$v_{ex} = \left( \frac{X}{\ln(1 - X)} \right) \left( \frac{A_i}{t} \right) \ln(1 - f)$$

where $X = \text{the fractional change of the original nucleotide pool}$, $F = \text{the fraction of equilibrium value obtained at time } t$, and $A_i = \text{the concentration of the original nucleotide pool}$.

Fig. 2 shows the plot of the ratio of the net chemical rate relative to the positional isotope exchange rate ($v_{chem}/v_{ex}$) versus the initial concentration of D-alanine. As the concentration of D-alanine is increased the rate of the chemical rate is increased relative to the exchange rate. The line $f$ is an equation of the type $y = a + bx + cx^2$ due to the binding of the two D-alanine molecules in the overall mechanism. At low levels of D-alanine the ratio extrapolates to 0.7.

DISCUSSION

Kinetic Mechanism—The kinetic mechanism that is most consistent with the data obtained is an ordered Ter-Ter mechanism with dead-end binding of D-Ala-D-Ala to the E-ATP complex as shown in Scheme IV.

The mechanism is supported by the following observations. 1) The initial velocity patterns are intersecting indicating sequential addition of all substrates in both directions. Therefore, in the forward reaction no products are released prior to the binding of all substrates. 2) The competitive product inhibition pattern of ADP versus ATP sets the first substrate to bind and the last product to be released as ATP and ADP, respectively. This pattern also eliminates the possible release...
of ADP after the binding of the first D-alanine and is thus consistent with the initial velocity patterns. 3) The noncompetitive pattern obtained when Pi inhibits either ATP or D-alanine indicates that Pi is the first product to be released. 4) D-Ala-D-Ala is uncompetitive with respect to ATP as would be expected for the middle product in an ordered Ter-Ter mechanism (Viola and Cleland, 1982). Scheme IV would also predict that the pattern for D-Ala versus D-Ala-D-Ala would be uncompetitive. However, the observed pattern is noncompetitive. The added slope effect versus D-alanine is due to the dead-end binding of n-Ala-n-Ala to the E-ATP complex (as shown in Scheme IV). The substrate inhibition exhibited by D-Ala-D-Ala is caused by the binding of D-Ala-D-Ala to the E-ATP complex as it is formed in the reverse reaction. The formation of the dead-end complex prevents reformation of free enzyme thereby inhibiting the reaction.

The analysis of the kinetic mechanism is complicated by having two identical substrates. In the ligase from S. faecalis, Neuhaus (1962b) observed curved double-reciprocal plots as the activity varied with D-alanine. This is expected for the ordered addition of D-alanine due to the squared term for D-alanine in the rate equation, but a linear relationship is observed (Neuhaus, 1962b) or there is an irreversible step between the second and third substrates are identical. This poses a problem because the reaction cannot be halted after addition of the first two substrates to probe for the formation of the intermediate.

The positional isotope exchange of [γ-18O]ATP to [β-18O, γ-18O, γ-18O]ATP is observed only in the presence of D-alanine. Incubation of [γ-18O]ATP with the ligase at various concentrations and pH values in the absence of D-alanine showed no significant exchange. This observation is consistent with the direct attack of the carboxyl group of one D-alanine on the γ-phosphate of ATP to give the acylphosphate intermediate. The cleavage of the γ-phosphate of ATP could occur either after the first D-alanine binds or only after all three substrates bind. To address this question, two dead-end substrate analogs of D-alanine (Structure I) were incubated with [γ-18O]ATP and the ligase. Both D-2-chloropropionic

**Table II**

**Product inhibition patterns for D-Alanyl-D-alanine ligase**

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>Inhibitor</th>
<th>Fixed substrate</th>
<th>Inhibition type</th>
<th>K_i</th>
<th>K_a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>P_i</td>
<td>D-Ala, 1 mM</td>
<td>NC</td>
<td>37 ± 13</td>
<td>129 ± 24</td>
</tr>
<tr>
<td>ATP</td>
<td>P_i</td>
<td>D-Ala, 25 mM</td>
<td>NC</td>
<td>18 ± 3</td>
<td>105 ± 9</td>
</tr>
<tr>
<td>ATP</td>
<td>P_i</td>
<td>D-Ala, 100 mM</td>
<td>NC</td>
<td>31 ± 8</td>
<td>163 ± 19</td>
</tr>
<tr>
<td>ATP</td>
<td>D-Ala-D-Ala</td>
<td>D-Ala, 1 mM</td>
<td>UC</td>
<td>0.065 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>D-Ala-D-Ala</td>
<td>ATP, 1 mM</td>
<td>UC</td>
<td>0.228 ± 0.016</td>
<td></td>
</tr>
<tr>
<td>d-Ala</td>
<td>P_i</td>
<td>ATP, 1 mM</td>
<td>NC</td>
<td>82 ± 27</td>
<td>113 ± 10</td>
</tr>
<tr>
<td>d-Ala</td>
<td>D-Ala-D-Ala</td>
<td>ATPl, 1 mM</td>
<td>NC</td>
<td>0.017 ± 0.003</td>
<td>0.10 ± 0.012</td>
</tr>
<tr>
<td>ATP</td>
<td>ADP</td>
<td>D-Ala, 100 mM</td>
<td>C</td>
<td>0.009 ± 0.002</td>
<td></td>
</tr>
</tbody>
</table>

Conditions were: at pH 7.6, 25 °C, 100 mM KCl (or constant ionic strength), and 7.5 mM MgCl₂. NC, noncompetitive; C, competitive; UC, uncompetitive.
Mechanism of D-Alanyl-D-alanine Ligase

For the positions of the exchange rate relative to the chemical rate (υ\text{chem}/υ\text{ex}) approaches a value of 1.4. The simplest mechanism that can be written for this enzymatic reaction is one in which all the substrates bind to the enzyme and then the chemistry occurs in a single step and the products dissociate together (Scheme V). The PIX reaction would be initiated from the EP complex.

\[
E \xrightleftharpoons{\kappa_5} ES \xrightleftharpoons{\kappa_0} [EP] \xrightarrow{\kappa_e} P_E
\]

SCHEME V

It can easily be shown that in the above mechanism the ratio of the maximal velocities in the reverse (Vr) and forward (Vf) reactions must be larger than the ratio for υ\text{ex} and υ\text{chem} (Equation 11). Since the observed value of 1.4 for υ\text{ex}/υ\text{chem} is 100 times larger than the observed value of 1/70 for Vr/Vf, this simple model cannot apply.

\[
\frac{V_r}{V_f} \geq \frac{V_{ex}}{V_{chem}}
\]

SCHEME VI

In this two-step mechanism the value for υ\text{ex}/υ\text{chem} can be larger than Vr/Vf when k5 is small relative to k0. Therefore, the minimal mechanism for the D-alanyl-D-alanine ligase reaction must consist of at least two distinct steps with an intermediate complex (E-X) which precedes the formation of the complex from which products dissociate. The molecular isotope exchange experiments clearly demonstrate that D-alanyl phosphate is a component of this complex. The phosphorolysis of the amide bond in D-alanyl-D-alanine (k5) must be much slower than the transfer of the phosphoryl group from this intermediate to ADP (k0). This is as expected for the thermodynamic stability of an amide bond in the dipeptide compared with the mixed anhydride bond in the acylphosphate intermediate.

In summary, the kinetic mechanism for D-alanyl-D-alanine ligase has been determined to be an ordered Ter-Ter reaction with ATP the first substrate to bind and ADP the last product off. A positional isotope exchange reaction within [γ-\text{18O}]ATP occurs in the presence of D-alanine, and a phosphate-dependent molecular isotope exchange of D-alanine and D-alanyl-D-alanine occurs in the absence of ADP. The molecular isotope exchange results and a quantitative analysis of the positional isotope exchange experiments demonstrate that dipeptide formation proceeds through a D-alanyl phosphate intermediate.

REFERENCES


acid (Structure II) and isobutyric acid (Structure III) are similar in structure to D-alanine but the amino group has been replaced with an unreactive functionality. It was anticipated that these compounds would bind to the ligase in a manner similar to D-alanine and be phosphorylated by the γ-phosphoryl group of ATP. Since these compounds do not contain the amino group, the reaction would be forced to stop after the formation of the intermediate. This would then provide evidence that chemistry could occur prior to the binding of all substrates. However, no exchange was detected when [γ-\text{18O}]ATP and either one of the dead-end substrates were incubated with D-alanyl-D-alanine ligase. Therefore, either all three substrates are required before chemistry can occur or n-2-chloropropionic acid and isobutyric acid are not sufficiently good dead-end substrate analogs for D-alanine.
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