D-Alanine:D-alanine ligase of *Escherichia coli*

Expression, purification and inhibitory studies on the cloned enzyme

Omar A. M. AL-BAR, C. David O'CONNOR, Ian G. GILES and Muhammed AKHTAR

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, SO9 3TU, U.K.

A 1.2 kb *Bam*HI fragment from pDK30 [Robinson, Kenan, Sweeney & Donachie (1986) *J. Bacteriol.* **167**, 809–817] was cloned in pDOC55 [O'Connor & Timmis (1987) *J. Bacteriol.* **169**, 4457–4482] to give two constructs, pDOC89 and pDOC87, in which the *Escherichia coli* D-alanine:D-alanine ligase (EC 6.3.2.4) gene (*ddl*) was placed under the control of the *lac* and λP_L promoters respectively. Both constructs, when used to transform *E. coli* M72, gave similar levels of expression of the *ddl* gene. The expressed enzyme was purified to homogeneity and the amino acid sequence of its N-terminal region was found to be consistent with that predicted from the gene sequence, except that the N-terminal methionine was not present in the mature protein. [1(S)-Aminoethyl][(2RS)-2-carboxy-1-octyl]phosphinic acid (I), previously shown to bind tightly to *Enterococcus faecalis* and *Salmonella typhimurium* D-alanine:D-alanine ligases following phosphorylation [Parsons, Patchett, Bull, Schoen, Taub, Davidson, Combs, Springer, Gadebusch, Weissberger, Valiant, Mellin & Busch (1988) *J. Med. Chem.* **31**, 1772–1778; Duncan & Walsh (1988) *Biochemistry* **27**, 3709–3714], was found to be a classical slow-binding inhibitor of the *E. coli* ligase.

INTRODUCTION

D-Alanine:D-alanine ligase (EC 6.3.2.4) is involved in the biosynthesis of the peptidoglycan component of the bacterial cell wall, and catalyses the following reaction:

The ligase was first detected in the early 1960s in Gram-negative as well as Gram-positive bacteria, and soon afterwards it was partially purified from *Enterococcus faecalis* (formerly *Streptococcus faecalis*) (Neuhaus, 1962). The gene (*ddl*) for the *Escherichia coli* enzyme has been cloned and sequenced by Robinson *et al.* (1986) and, not unexpectedly, this gene was shown to be essential for the growth of the organism. Subsequently a related gene from *Salmonella typhimurium* was characterized but, since the gene is not essential in this organism, it was designated as *ddlA* (Daub *et al.*, 1988). The ligase encoded by the *S. typhimurium ddlA* gene has been expressed in a *S. typhimurium* strain bearing pDS4, and extensive studies on the purified enzyme have been performed (Daub *et al.*, 1988; Duncan & Walsh, 1988). In the present paper we report the cloning of the *ddl* gene of *E. coli* from the original plasmid pDK30, constructed by Robinson *et al.* (1986), into two different expression vectors, and describe the purification of the expressed enzyme. This work is part of a Ph.D. thesis (Al-Bar, 1990) which was submitted at about the same time as a recent paper that also describes the expression of the *E. coli* ligase (Zawadzke *et al.*, 1991).

MATERIALS AND METHODS

Materials

[1(S)-1-Aminoethyl]2(2RS)-2-carboxy-1-octylphosphinic acid (I), hereafter referred to as heptyl phosphinate, was a gift from Dr. A. A. Patchett, Merck Research, Rahway, NJ, U.S.A., and was prepared as described by Parsons *et al.* (1988).

Strains and plasmids used

E. coli TB1(λ) and M72, which specify wild-type and temperature-sensitive λ repressors respectively, were used as hosts (Bernard *et al.*, 1979; Baldwin, 1984) for plasmids carrying the *ddl* gene. pDOC55, a pBR322 derivative carrying convergent

λP_L and p_{lac} promoters, was used as the expression vector (O'Connor & Timmis, 1987). pDOC87 and pDOC89 were constructed by cloning a 1.2 kb *Bam*HI fragment carrying the *ddl* gene into the corresponding site of pDOC55 so as to place the gene under the control of λP_L and p_{lac} respectively. The source of the *ddl* gene was pDK30 (Robinson *et al.*, 1986).

Growth of the bacteria and purification of the enzyme

LB medium containing 100 μg of ampicillin/ml was used throughout for the growth of bacteria. A portion (30 ml) of an overnight grown culture of *E. coli* M72 (pDOC87) was added to 1 litre of the medium contained in a 5 litre conical flask, and bacteria were grown at 30 °C with shaking to an absorbance of 0.6 at 600 nm. The culture was then supplemented with 1 litre of a fresh medium preheated to 56 °C and the mixture (2 litres) was transferred to an orbital shaker at 41 °C for 3 h. The contents of four such flasks were pooled and bacteria were harvested by centrifugation at 5000 *g* for 20 min. The cells were then washed with 120 ml of 10 mM-potassium phosphate buffer, pH 6.8, and the recovered bacterial paste (11 g) was stored at –20 °C.

To purify the enzyme, the paste (11 g) was thawed in ice, resuspended in 30 ml of 10 mM-potassium phosphate buffer, pH 7.0, and sonicated using a MSE Soniprep ultrasonic disintegrator in seven 45 s bursts at an amplitude of 8–10 μm (peak to peak). The delay between pulses was 60 s and the sample was cooled in an ice-bath. The extract was centrifuged at 12000 *g* for 20 min and the solution (25 ml) was treated with $(\text{NH}_4)_2\text{SO}_4$ (0–20%, 20–45% and 45–60% saturation). Each precipitate was collected by centrifugation at 17000 *g* for 15 min. The most active fraction, usually the 20–45%-satd. fraction, was dissolved in 8 ml of 10 mM-potassium phosphate buffer, pH 7.0, and dialysed against the same buffer containing 1 mM-GSH for 2 h. The buffer was changed and dialysis was continued overnight.

The resulting solution (12.5 ml) was applied to a 5 cm $\times$ 10 cm DE52-cellulose column which had been previously equilibrated with 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-glutathione. The column was washed with 400 ml of the same buffer and the enzyme was eluted by application of a linear KCl gradient (0–0.5 M-KCl in 500 ml total volume).

The most active fractions (41.5 ml) were pooled and dialysed overnight at 0–4 °C against 20 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-GSH. The dialysed enzyme was concentrated in

an Amicon Ultrafiltration cell fitted with a PM 10 membrane and applied to a high-resolution anion-exchange Mono Q HR 5/5 column attached to a Pharmacia f.p.l.c. system which had been previously equilibrated with 20 mM-Tris/HCl buffer, pH 7.5. The enzyme was eluted using a NaCl gradient by a pre-programmed method (0–0.5 M-NaCl; 30 ml total volume) at a flow rate of 1 ml/min. The absorbance at 280 nm was monitored, and individual peaks were collected and assayed for ligase activity. The activity was eluted at 0.2 M-NaCl and the fractions containing the enzyme were combined. The solution was desalted by gel filtration through a PD10 column which had been equilibrated in 20 mM-sodium acetate buffer, pH 6.0, and the most active fractions were re-applied to a Mono Q HR 5/5 column which had been previously equilibrated with 20 mM-sodium acetate buffer, pH 6.0. The same programme as described above was used and the fractions containing the enzyme were combined (4.6 ml), desalted and rechromatographed at pH 7.5 as above.

Estimation of protein and assay of enzyme activity

Protein was determined by the method of Bradford (1976). The activity of D-alanine:D-alanine ligase was determined by using the coupled spectrophotometric assay, in which ADP formation is linked to oxidation of NADH via pyruvate kinase and lactate dehydrogenase (Neuhaus, 1962). The decrease in absorbance due to oxidation of NADH was measured at 340 nm. The assay mixture (total volume of 1 ml for the experiments of Fig. 3 and 1.2 ml for those of Fig. 4) contained the following components (final concentrations): 50 mM-triethanolamine/HCl buffer (pH 7.4), 10 mM-KCl, 10 mM-MgCl₂, 1.0 mM-ATP (pH 7.4), 0.2 mM-phosphoenolpyruvate, 0.15 mM-NADH, 5 units of pyruvate kinase, 15 units of lactate dehydrogenase and 50 mM-D-alanine. The assay mixture in the cuvette was incubated for 2 min at 30 °C with all components except one; the missing component was the ligase for the experiments of Fig. 3 or D-alanine for those of Fig. 4. The reaction was initiated by the addition of the final component and monitored at 340 nm against a control cuvette containing buffer alone. One unit is defined as the amount of enzyme which releases 1 µmol of ADP/min at 30 °C.

Data analysis

Data from each individual progress curve in the presence of

various amounts of slow-binding inhibitor were analysed (Longstaff *et al.*, 1990) using the integrated rate equation:

$$A = v_s + (v_0 - v_s)(1 - e^{-k't})/k' + A_0 \quad (2)$$

In this equation A is the absorbance at 340 nm and A_0 is the initial absorbance. v_0 and v_s are the initial and final steady-state rates respectively. The absorbance data obtained for each progress curve as a function of time were fitted directly to this equation, using non-linear regression analysis, to yield values for the four parameters v_0 , v_s , k' and A_0 .

RESULTS

Sub-cloning of the *ddl* gene

pDK30, a gift from Dr. W. Donachie, was digested with *Bam*HI to release a 1.2 kb fragment carrying the entire *ddl* gene. Following its purification from an agarose gel and ligation to *Bam*HI-cut and phosphatase-treated pDOC55, the DNA was transformed into *E. coli* host strain TB1 (λ). Plasmids containing the 1.2 kb insert were isolated by a rapid method and cut with *Xho*I + *Hind*III to determine the orientation of the cloned fragment relative to the flanking convergent λP_L and *lac* promoters (O'Connor & Timmis, 1987).

A representative plasmid in which the *ddl* gene was under the control of λP_L was designated pDOC87. Similarly, a plasmid with the gene under the control of the *lac* promoter was designated pDOC89. A schematic representation of the two plasmids constructed as described above is shown in Fig. 1. The plasmids were transferred by transformation to *E. coli* M72, a host strain that specifies a temperature-sensitive λcI repressor protein, to allow the expression of the *ddl* gene. Following induction of synthesis of the *ddl* gene product by a temperature shift of an *E. coli* M72 (pDOC87) culture from 30 °C to 42 °C (or addition of isopropyl β-D-thiogalactopyranoside to 2 mM, in the case of the cells containing pDOC89), the culture was incubated for 3 h. SDS/PAGE of the total cell extracts showed the presence of a new protein band with an M_r of about 32 000 (Fig. 2a).

Fig. 1. Structures of the two plasmids containing the essential *ddl* gene of *E. coli*

In pDOC87 the gene is regulated by the heat-inducible promoter λP_L, while in pDOC89 *ddl* it is in the opposite orientation to that illustrated above and is inducible with isopropyl β-D-thiogalactopyranoside. *E. coli* M27 transformed with the former plasmid, i.e. pDOC87, was used for the purification of the ligase.

Fig. 2. SDS/PAGE of (a) total cell proteins of bacteria and (b) fractions obtained during the purification of the ligase

The polyacrylamide concentration was 12% (w/v). (a) Lane 1, SDS-solubilized *E. coli* M72 (pDOC55) following induction; lane 2, SDS-solubilized *E. coli* M72 (pDOC87) following induction. (b) Lane 1, sonication supernatant; lane 2, 20–45% satd. (NH₄)₂SO₄ fraction; lane 3, DEAE-cellulose fractions (pooled); lane 4, f.p.l.c. (pH 7.5) fractions; lane 5, M_r standards (carbonic anhydrase, M_r 29 000, is the fourth band from the top); lane 6, f.p.l.c. (pH 6.0) fractions; lane 7, f.p.l.c. (pH 7.5) fractions.

Table 1. Purification of D-alanine:D-alanine ligase from *E. coli* M72 (pDOC87)

The ligase was isolated from 11 g wet wt. of bacteria by sonication. The cell-free extract was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation. The 20–45 % satd. fraction was collected and purified on DE-52 cellulose column followed by Mono Q columns. 1 unit of enzyme activity represents production of 1 μmol of ADP/min.

Purification stage	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Sonication	25	187.5	710	0.264	1	100
$(\text{NH}_4)_2\text{SO}_4$ (20–45 %)	12.5	173.3	322.5	0.537	2.03	92.4
Ion-exchange (DE-52)	41.5	112.3	137	0.819	3.10	59.9
F.p.l.c. (Mono Q, pH 7.5)	14.9	76.2	20.3	3.76	14.2	40.7
F.p.l.c. (Mono Q, pH 6.0)	4.62	38.3	3.0	12.77	48.4	20.4
F.p.l.c. (Mono Q, pH 7.5)	5.94	17.4	0.92	18.90	71.6	9.3

Purification of the ligase

Although both constructs, *E. coli* M72 (pDOC87) and *E. coli* M72 (pDOC89), gave similar levels of expression of the *ddl* gene, the former strain, in which the gene was under the control of λP_L , was used for the purification of the enzyme because of economy and convenience. Wet cell paste (11.0 g) obtained from an 8 litre culture was subjected to sonication to release the enzyme activity and the supernatant was then carried through the operations shown in Table 1 to give around 80 % pure enzyme after the second f.p.l.c. step performed at pH 6.0. However, a homogeneous enzyme was only obtained after a further chromatographic step. The SDS/PAGE analysis of the enzyme at various stages of purification is shown in Fig. 2(b).

N-Terminal sequence determination of the ligase from *E. coli*

Sequence analysis of the purified ligase was performed using an Applied Biosystems 477A pulsed liquid phase protein sequencer. The N-terminal sequence was: $\text{H}_2\text{N-Xaa-Xaa-Xaa-Ile-Ala-Val-Leu-Leu-Gly-Gly-Thr-Ser-Ala}$. It was not possible to unambiguously identify the first three residues, but the sequence of the next ten residues was found to be completely consistent with that predicted from the gene sequence data reported by Robinson *et al.* (1986). The comparison of the two sequences, however, allowed the deduction that the N-terminal methionine was removed during enzyme maturation.

Inhibition of *E. coli* ligase with the heptyl phosphinate (I)

The heptyl phosphinate (see Fig. 6, structure I) has been reported to be a novel slow-binding inhibitor of the ligases from *Ent. faecalis* and *S. typhimurium* (Parsons *et al.*, 1988; Duncan & Walsh, 1988). In order to evaluate the interaction of the inhibitor with the *E. coli* ligase, the enzyme was assayed in the presence of a constant concentration of the substrate, D-Ala-D-Ala, at various concentrations of the inhibitor heptyl phosphinate. The reaction was initiated by the addition of enzyme. This experiment gave a family of non-linear progress curves in which the degree of inhibition increased with time (Fig. 3). The data were analysed as a slow-binding inhibitor (Morrison & Walsh, 1988) by fitting to eqn. (2) (see the Materials and methods section) to obtain values for v_0 , v_s , k' and A_0 for each inhibitor concentration used.

Morrison & Walsh (1988) described two mechanisms (A and B) for the possible origin of slow-binding inhibition (Scheme 1). Our data clearly indicate that mechanism A can be eliminated for

Fig. 3. Progress curve of the *E. coli* ligase reaction in the presence of the heptyl phosphinate

The abscissa and ordinate represent the time and decrease in absorbance at 340 nm respectively. The composition of the assay mixture was as described in the Materials and methods section containing 0.05 units of ligase and the inhibitor (I) at the final concentrations in μM shown adjacent to each curve.

the *E. coli* ligase, since v_0 is not constant as the inhibitor concentration varies. Mechanism B, however, predicts that both v_0 and v_s vary as a function of inhibitor concentration. Indeed, the equations relating either to inhibitor concentration are analogous to that for classical linear competitive inhibition. Consequently, a plot of the reciprocal of v_0 (or v_s) against inhibitor concentration should yield a straight line as in the Dixon plot (Dixon, 1953). This was the case for our data, from which K_i and K_i^* , the two dissociation constants, were calculated to be 11 μM and 2.7 μM respectively.

For mechanism B, the observed rate constant for the formation of $\text{E} \cdot \text{I}^*$ is given by the expression:

$$k' = k_s + \frac{(k_s I)}{(K_i + I)} \quad (3)$$

This equation predicts a hyperbolic curve on increasing the

Scheme 1. Proposed mechanisms of slow-binding inhibition

See the text for details.

Fig. 4. Analysis of the *E. coli* ligase reaction with heptyl phosphinate

k' was determined at each inhibitor concentration by fitting to eqn. (2). The points are the derived constants, and the line represents the line of best fit to eqn. (3).

concentration of inhibitor, but indicates that there is a finite value of k' (equal to k_6) at zero inhibitor. Consequently we fitted our data to eqn. (3) using non-linear regression. Fig. 4 shows the data points used together with the line of best fit corresponding to the following parameter values: K_1 , 10.5 μM ; k_5 , 0.0717 s^{-1} ($t_{1/2} = 9$ s); k_6 , 0.00155 s^{-1} ($t_{1/2} = 447$ s). This result shows that there is good agreement for the value of K_1 calculated from the effect of inhibitor concentration on both k' and v_0 . The values for both k_5 and k_6 are considerably smaller than 10^6 , as required for an inhibition which involves slowly equilibrating enzyme-inhibitor species. The ratio of k_5/k_6 is 46:1, i.e. within the range found for other slow-binding inhibitors (Morrison & Walsh, 1988).

The K_1 value and the non-linear profiles are similar to those previously described for the *S. typhimurium* ligase; however, with the latter enzyme, the presence of 3.0 μM inhibitor (I) was found to lead to complete abolition of the activity within 60 s of the initiation of the assay. This was not the case with the *E. coli* ligase. The differences between the *S. typhimurium* and *E. coli* enzymes were further signalled by preincubation experiments. Samples of the *E. coli* ligase were incubated for 10 min at room temperature with 36 μM -heptyl phosphinate in the presence or absence of ATP, together with a control lacking the inhibitor.

Fig. 5. Effect of preincubation of the ligase with the heptyl phosphinate (I)

Three 50 μl samples of the stock solution of the ligase (about 1 unit in 50 mM-triethanolamine/HCl buffer, pH 7.4) were diluted with an equal volume of Hepes buffer, pH 7.8, containing 10 mM- Mg_2Cl and 10 mM-KCl (buffer A). One of the samples, without any other addition, served as a control, and the other two contained either 36 μM -heptyl phosphinate or 36 μM -heptyl phosphinate plus 10 mM-ATP. The three samples were incubated at room temperature for 10 min and then each of these was diluted with 100 μl of buffer A, so that the concentration of the inhibitor in the two experimental samples was decreased to 12 μM . Aliquots of 10 μl from the three samples were then analysed with the complete enzyme system in a total volume of 1.2 ml as described in the Materials and methods section. Curve a, control without addition ($\bullet$); curve b, sample incubated with the inhibitor only ($\circ$); curve c, sample preincubated with the inhibitor and ATP ($\blacksquare$). Curve d ($\square$) was obtained when 10 μl aliquots from the sample incubated with the inhibitor were first incubated with 1.05 ml of the assay buffer containing 50 mM-D-alanine for 20 min. The reaction was then initiated by the addition of the remaining components of the coupled assay system in 150 μl of the buffer.

The mixtures were then diluted to decrease the inhibitor concentration to 12 μM in the experimental samples. At various time intervals 10 μl aliquots from these samples were removed and added to 1.2 ml of the assay mixture to give a final inhibitor concentration of 0.1 μM , and the ligase activity was monitored through the decrease of absorption at 340 nm. The first set of data points, recorded within 5 min of the dilution of the inhibitor to 12 μM , gave rate profiles of the type shown in curves a, b and c in Fig. 5. The same general rate profile was obtained using samples which were removed periodically during the next 6 h of the preincubation at room temperature, followed by storage at 4 $^\circ\text{C}$ overnight. The most noteworthy feature of these results is that the incubation of the enzyme with the heptyl phosphinate in the absence or the presence of ATP (Fig. 5, curves b and c respectively) gave almost identical non-linear profiles in which the extent of inhibition decreased with time until a new steady-state rate had been established. The rates of reactions at the steady state were the same whether the incubation of the ligase with the inhibitor was performed with or without ATP. Furthermore, in the steady state the rates of the two reactions in which the enzyme had been preincubated with the inhibitor were between 64 and 75 % of that with the uninhibited control enzyme (Fig. 5, compare curve a with curves b and c). The recovery of the activity of the enzyme on a steady-state time scale observed here with the *E. coli* ligase preincubated with the inhibitor and ATP is dramatically different from the behaviour of the *S. typhimurium* ligase reported previously (Duncan & Walsh, 1988). In the latter study the incubation of *S. typhimurium* ligase with

the heptyl phosphinate (I) alone was without any effect, but when a similar incubation was carried out in the presence of inhibitor and ATP only 2.5% of the original enzyme activity was recovered.

The results in Fig. 5 (curves b and c) clearly show that in both cases the enzyme-inhibitor complexes formed during the pre-incubation phase were reversible. The overall kinetic behaviour portrayed in Fig. 3 and Fig. 5 (curves b and c) is characteristic of conventional slow-binding inhibition. In this case a rapidly formed enzyme-inhibitor complex ($E \cdot I$) undergoes slow isomerization to another complex ($E \cdot I^*$) where the inhibitor is bound more tightly (Morrison & Walsh, 1988). Further support for this view was provided by the following experiment.

A 10 μ l aliquot of the sample in which the enzyme had been preincubated with the inhibitor (I) was added to 50 mM-D-alanine, and after 10 min the mixture was supplemented with the remaining component of the coupled-assay system. A normal linear reaction profile was obtained (Fig. 5, curve d), thus showing that the substrate promotes the dissociation of the inhibitor from the enzyme-inhibitor complex. Attention is again drawn to the fact that the steady-state rates for the three curves b, c and d were identical and were 64–75% of the control value. The high recovery of the ligase activity under steady-state conditions, which was achieved by a 370-fold dilution of the inhibitor (from 36 μ M in the original preincubation to 0.1 μ M in the assay mixture), provides additional evidence in support of the conclusion that the heptyl phosphinate is a classical slow-binding inhibitor of the *E. coli* enzyme. It should be noted that when the ligase sample which had been preincubated with the heptyl phosphinate as well as with ATP was further incubated with 50 mM-D-alanine, the presence of all of the components required for the complete reaction in the second incubation produced ADP. The latter mixture, when added to the coupled assay system, resulted in a burst of NADH oxidation in the early phase of the analysis. In this case, therefore, a reliable initial velocity could not be obtained. Notwithstanding this inherent complication, the experiment is testimony to the reversible nature of the inhibitor complex formed between the *E. coli* enzyme, heptyl phosphinate and ATP.

Zawadzke *et al.* (1991) have reported the cloning of the *E. coli* ligase from the same Robinson-Donachie plasmid, pDK30, used in the present study. Although Zawadzke *et al.* (1991) did not study the interaction of their *E. coli* ligase with the heptyl phosphinate, they used an analogue of the latter compound in which the heptyl side chain of (I) was replaced by a methyl group (I , $C_7H_{15}=CH_3$) and showed that the latter was a time-dependent inhibitor of the enzyme. The main feature of the inhibition was the formation of an ATP-dependent complex in which a phosphorylated derivative of the inhibitor was tightly bound to the enzyme. A similar conclusion had previously been drawn for the interaction of heptyl phosphinate with *Ent. faecalis* and *S. typhimurium* ligases (Parsons *et al.*, 1988; Duncan & Walsh, 1988). In view of the failure to demonstrate a similar phenomenon with the *E. coli* ligase expressed by us, the key feature of the original experiments reported by Al-Bar (1990) were repeated and found to be valid in broad terms. The data in Fig. 5 are based on these experiments.

DISCUSSION

Recent advances in molecular biology and genetic engineering have enabled the isolation and sequencing of the genes for D-alanine:D-alanine ligase from *E. coli* (*ddl*) and *S. typhimurium* (*ddlA*). The two protein sequences deduced from the gene sequences when maximally aligned showed only about 30% identity. *ddl* is an essential gene in *E. coli*; however, *ddlA* was

found not to be essential for the growth of *S. typhimurium*, in spite of the fact that the dipeptide D-alanyl-D-alanine is of crucial importance to bacterial cell wall biosynthesis. In view of the non-essential nature of *ddlA*, the prospect of more than one ligase gene in *S. typhimurium* was raised (Daub *et al.*, 1988). Several other differences between the *E. coli* *ddl* and the *S. typhimurium* *ddlA* genes and gene products are highlighted by sequence comparison. The *E. coli* coding sequence consists of 918 nucleotides encoding a 306 residue protein of M_r 32840 (Robinson *et al.*, 1986), whereas that from *S. typhimurium* consists of 1092 nucleotides and encodes a protein of 346 residues with an M_r of 39271 (Daub *et al.*, 1988).

In the original construct (pDK30) the *E. coli* ligase gene (*ddl*), along with its associated promoter, was resident on a 1.2 kb *Bam*HI fragment, but the enzyme was not detected in the cell-free extracts, presumably due to poor expression of the gene. In the present work the *Bam*HI fragment was inserted into expression vector pDOC55 to give two recombinant plasmids, pDOC87 and pDOC89. In pDOC89 the *ddl* gene is controlled by the *lac* promoter, whereas in pDOC87 its expression is under the control of λP_L , a heat-inducible promoter. *E. coli* M72 transformed with both of these plasmids produced a new protein of M_r 32000 in amounts corresponding to about 5% of the total cellular protein. The two cell-free extracts were found to have an activity of 17 μ mol/min per g wet wt. of bacteria, the specific activity of the purified ligase from *E. coli* M72 (pDOC87) was about 19 μ mol/min per mg of protein and the enzyme was purified approx. 72-fold in a 9.3% yield. The enzyme isolated in this way was completely free of phosphatase activity, and on denaturing SDS/PAGE gave an M_r of $31\,000 \pm 1000$, which is in excellent agreement with the M_r of 32840 calculated from the deduced amino acid sequence (Robinson *et al.*, 1986). The *E. coli* ligase showed heat-sensitivity, as is the case for the *S. typhimurium* ligase, thus contrasting these enzymes with that of *Ent. faecalis*, which is stable to 63 °C for 5 min (Nehaus, 1962; Al-Bar, 1990). The *N*-terminal sequence (ten amino acids) of our *E. coli* ligase was determined and found to be consistent with the deduced sequence reported by Robinson *et al.* (1986), except that the *N*-terminal methionine predicted from the DNA sequence was not present in the purified protein. In this respect our results are different from those described by Zawadzke *et al.* (1991), who found that the *N*-terminal amino acid of their purified *E. coli* ligase was blocked. A significant feature of the latter work, however, is the demonstration that *E. coli* also contains a non-essential ligase whose properties are similar to those of the enzyme encoded by the *ddlA* gene of *S. typhimurium*.

Despite marked differences in the sequences and sizes of the ligases from *E. coli* and *S. typhimurium*, the two enzymes are very similar in terms of their catalytic efficiency and other kinetic parameters, but differ with respect to their behaviour towards the inhibitor, heptyl phosphinate (I), originally designed by Patchett and colleagues at Merck (Parsons *et al.*, 1988). In previous studies with the *S. typhimurium* enzyme, the kinetic profile displayed by the heptyl phosphinate was shown to be characteristic of a novel type of slow-binding inhibitor, and the phenomenon was found to depend on the simultaneous presence of enzyme, ATP and the inhibitor (Parsons *et al.*, 1988; Duncan & Walsh, 1988). These were the considerations which led two groups to suggest that, with ligases from *Ent. faecalis* (Parsons *et al.*, 1988) and *S. typhimurium* (Duncan & Walsh, 1988), the inactive complex contains the phosphorylated form of the inhibitor (II) (Fig. 6) and ADP tightly bound to the ligase. This conclusion was supported by n.m.r. studies (McDermott *et al.*, 1990). However, using an isotopic approach it was found that when the inhibitory complex produced from compound (I), following isolation by gel filtration, was subjected to heat denaturation,

Fig. 6. Structure of the heptyl phosphinate (I) and its phosphorylated derivative (II)

ADP and ATP were released in a ratio of 1:2. That the predominant nucleotide produced in the experiment was ATP was rationalized by assuming that during the denaturation process the phosphoryl group was transferred from the phosphorylated inhibitor (II) back to ADP. The failure to detect the phosphorylated inhibitor was attributed to the instability of the anhydride bond in (II). The *E. coli* ligase expressed in our laboratory and used in this work behaves differently, however, and interacts with the heptyl phosphinate (I) in a manner that is reminiscent of classical slow-binding inhibitors (Morrison & Walsh, 1988). In this case, either a phosphorylated derivative of the inhibitor is not formed at all, or it is formed but the species rapidly dissociates from the active site. The consequence of this is that the kinetic profile of the *E. coli* ligase is dominated by the presence of an enzyme-inhibitor complex, rather than the enzyme-phosphorylated-inhibitor-ADP complex which has been suggested to be involved with the ligases from *Ent. faecalis* and *S. typhimurium*.

We thank Dr. A. A. Patchett (Merck Sharp & Dohme, Rahway, NJ, U.S.A.) for the gift of the heptyl phosphinate, and are deeply indebted to him that after we obtained the unexpected results described above his

group reanalysed the remnants of the sample used in our study and confirmed its authenticity. We thank Dr. W. Donachie (University of Edinburgh, Scotland, U.K.) for a gift of pDK30 and also for helpful suggestions. O. A. M. A.-B. thanks the Government of Saudi Arabia and King Abdulaziz University, Jeddah, Saudi Arabia, for a scholarship. We also thank Dr. A. Alwan for assistance with the purification of the ligase. This is a contribution from the SERC Centre for Molecular Recognition, University of Southampton, where protein sequencing was performed by Dr. M. G. Gore and Mr. L. Hunt.

REFERENCES

- Al-Bar, O. A. M. (1990) Ph.D. Thesis, University of Southampton
 Baldwin, T. (1984) *BRL Focus* **6**, 7
 Bernard, H. U., Remaut, E., Herschfield, M. V., Das, H. K., Helinski, D. R., Yanofsky, C. & Franklin, N. (1979) *Gene* **5**, 59-75
 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
 Daub, E., Zawadzke, L. E., Botstein, D. & Walsh, C. T. (1988) *Biochemistry* **27**, 3701-3708
 Dixon, M. (1953) *Biochem. J.* **55**, 170-171
 Duncan, K. & Walsh, C. T. (1988) *Biochemistry* **27**, 3709-3714
 Longstaff, C., Campbell, A. F. & Fersht, A. R. (1990) *Biochemistry* **29**, 7339-7347
 McDermott, A. E., Creuzet, F., Griffin, R. G., Zawadzke, L. E., Ye, Q. Z. & Walsh, C. T. (1990) *Biochemistry* **29**, 5767-5775
 Morrison, J. F. & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* **61**, 201-301
 Neuhaus, F. C. (1962) *J. Biol. Chem.* **237**, 778-786
 Robinson, A. C., Kenan, D. J., Sweeney, J. & Donachie, W. D. (1986) *J. Bacteriol.* **167**, 809-817
 O'Connor, C. D. & Timmis, K. N. (1987) *J. Bacteriol.* **169**, 4457-4462
 Parsons, W. H., Patchett, A. A., Bull, H. G., Schoen, W. R., Taub, D., Davidson, J., Combs, P. L., Springer, J. P., Gadebusch, H., Weissberger, B., Valiant, M. E., Mellin, T. N. & Busch, R. D. (1988) *J. Med. Chem.* **31**, 1772-1778
 Zawadzke, L. E., Bugg, T. D. H. & Walsh, C. T. (1991) *Biochemistry* **30**, 1673-1682

Received 14 June 1991/18 October 1991; accepted 28 October 1991