Subcloning, Expression, and Purification of the Enterobactin Biosynthetic Enzyme 2,3-Dihydroxybenzoate-AMP Ligase: Demonstration of Enzyme-Bound (2,3-Dihydroxybenzoyl)adenylate Product†

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ABSTRACT: The gene coding for the enzyme 2,3-dihydroxybenzoate-AMP ligase (2,3DHB-AMP ligase), responsible for activating 2,3-dihydroxybenzoic acid in the biosynthesis of the siderophore enterobactin, has been subcloned into the multicopy plasmid pKK223-3 and overproduced in a strain of *Escherichia coli*. The protein is an α_2 dimer with subunit molecular mass of 59 kDa. The enzyme catalyzes the exchange of [^{32}P]pyrophosphate with ATP, dependent upon aromatic substrate with a turnover number of 340 min^{-1}. The enzyme also releases pyrophosphate upon incubation with 2,3-dihydroxybenzoic acid and ATP; an initial burst corresponding to 0.7 nmol of pyrophosphate released per nanomole of enzyme is followed by a slower, continuous release with a turnover number of 0.41 min^{-1}. The 1000-fold difference in rates observed between ATP–pyrophosphate exchange and continuous pyrophosphate release, as well as the close to stoichiometric amount of pyrophosphate released, suggests that intermediates are accumulating on the enzyme surface. Such intermediates have been observed and correspond to enzyme-bound (2,3-dihydroxybenzoyl)adenylate product.

In *Escherichia coli* the iron-chelating compound enterobactin requires a number of genes for its biosynthesis (Scheme I). The *entC*, *entB*, and *entA* genes code for enzymes involved in the biosynthesis of 2,3-dihydroxybenzoic acid from chorismic acid (Young et al., 1969a,b, 1971; Nahlik et al., 1987, 1989; Liu et al., 1989; Ozenberger et al., 1989), the last common intermediate in the biosynthetic pathway of the aromatic amino acids, *p*-aminobenzoic acid, and anthranilic acid. Subsequent condensation of 2,3-dihydroxybenzoate with serine followed be cyclization to yield enterobactin was shown to require at least four additional genes referred to as *entD*, *entE*, *entF*, and *entG* (Luke & Gibson, 1971; Woodrow et al., 1975). The four polypeptides coded for by the *entD–entG* genes have been studied in partially purified form and proposed to function as a complex referred to as enterobactin synthase (Bryce & Brot, 1972; Greenwood & Luke, 1976, 1980; Woodrow et al., 1979). The *entA–entG* genes are known to be distributed into distinct operons and appear to be regulated by iron (Fleming et al., 1983; Pickett et al., 1984; Pettis & McIntosh, 1987; Schmitt & Payne, 1988). Recently, the nucleotide sequence of the *entC*, *entE*, *entB*, and *entA* genes has been determined and shown to constitute a single operon (Elkins & Earhart, 1988; Liu et al., 1989; Nahlik et al., 1989; Ozenberger et al., 1989). These studies have provided the opportunity to express these enzymes separately and to study their properties independent of any of the other enzymes involved in enterobactin biosynthesis. Previous work from this laboratory has succeeded in expressing the *entA* gene product (Liu et al., 1989) while very recent results have indicated that *entB* and *entC* have been successfully subcloned and the respective polypeptides overproduced in *E. coli* strains (Liu, Rusnak, and Walsh, unpublished data) and are currently being studied.

In this paper, we report the subcloning, overproduction, and purification to homogeneity of 2,3-dihydroxybenzoate-AMP (2,3DHB-AMP) ligase, the gene product of *entE*. With the exception of 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (product of the *entA* gene) (Liu et al., 1989), no other enzymes involved in enterobactin biosynthesis have been produced in sufficient quantities for rigorous enzymological studies. Herein, we provide evidence to support the contention that (2,3-dihydroxybenzoyl)adenylate is the product of the reaction of 2,3DHB-AMP ligase with 2,3-dihydroxybenzoate and ATP and that this acyladenylate remains bound to the enzyme for further reactions in the overall biosynthesis of enterobactin.

Materials and Methods

Materials. Disodium hydrogen [^{32}P]pyrophosphate (4 mCi/mmol), adenosine 5′-[γ-^{32}P]triphosphate triethylammonium salt (5000 Ci/mmol), [U-^{14}C]adenosine 5′-triphosphate ammonium salt (543 mCi/mmol), and α-[^{35}S]-thio-dATP were from Amersham, Arlington Heights, IL. [7-^{14}C]Salicylic acid (58.2 mCi/mmol) was from NEN Dupont Inc, Wilmington, DE. Anthranilic acid, thiosalicylic acid, 2,3-, 2,6-, and 3,5-dihydroxybenzoic acid, and 2,3,4- and 2,4,6-trihydroxybenzoic acid were purchased from Aldrich Chemical Co, Milwaukee, WI. Inorganic pyrophosphatase, adenosine 5′-triphosphate, *p*-hydroxybenzoic acid, dithioithreitol, and gentisic acid were purchased from Sigma Chemical Co., St. Louis, MO. *m*-Hydroxybenzoic acid and 2,3-dihydroxybenzoic acid were from Eastman Organic Chemicals, Rochester, NY, and salicylic acid was from Malinckrodt Chemical Co., Paris KY. The oligonucleotide 25-mer primer used for mutagenesis was prepared by Dr. Alexander L. Nussbaum of Harvard Medical School.

Bacterial Strains and Plasmids. E. coli JM101 [supE thi Δ(lac-proAB)/F′[traD36proA$^+$ proB$^+$ laqI^q lacZΔM15]] and JM105 [Δ(lac-pro)$_{XIII}$ thi rpsL (str^r) endA sbcB supE hsdR/F′[traD36 proA$^+$ proB$^+$ laqI^q lacZΔM15]] and the replicative form of M13mp19 were obtained from New England Biolabs, Beverly, MA. pKK223-3 was from Pharmacia, Piscataway, NJ. pMS111, a pBR322 derivative harboring the *entA*, *entB*, and *entE* genes on an *Eco*RI fragment was pro-

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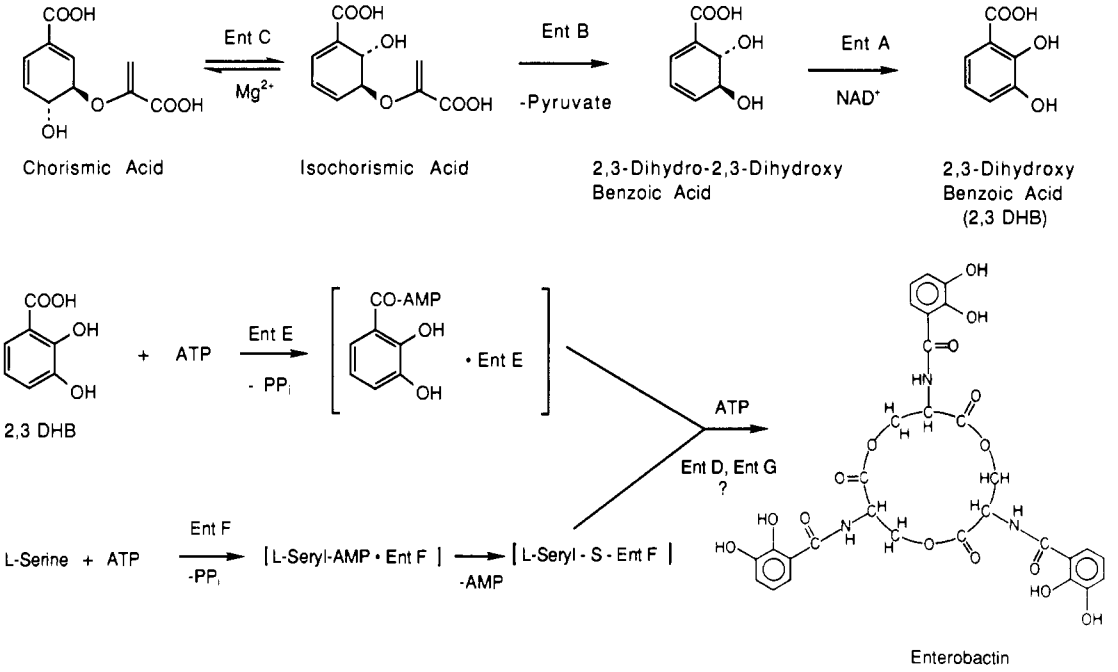
Scheme I



vided by Dr. I. G. Young of the Australian National University.

DNA Manipulations. DNA manipulations were carried out as described (Maniatis et al., 1982). All restriction enzymes were obtained from New England Biolabs and were used in accordance with the manufacturer's instructions. T4 DNA ligase and ligase 10× buffer were from IBI; X-Gal and IPTG were from Boehringer-Mannheim Biochemicals. Mutagenesis was performed with the oligonucleotide-directed in vitro mutagenesis system, Version 2, from Amersham.

Construction of a 2,3-Dihydroxybenzoate-AMP Ligase Overproducing Strain. (*a*) *Subcloning into M13mp19.* With the restriction map of pMS111 obtained (Liu et al., 1989), a 2.5-kb *Eco*RI/*Pvu*II fragment containing the *entE* gene was isolated by electrophoresis and purified by ion-exchange chromatography on an Elutip (Schleicher & Schuell) column. This 2.5-kb fragment was subsequently ligated into the *Eco*RI/*Sma*I-digested replicative form of M13mp19 with T4 DNA ligase and incubation at 16 °C for 20 h. The ligation mixture was then transformed into *E. coli* JM101, and clear plaques were isolated from H plates. Preparation of single-stranded template following the Amersham protocol led to the isolation of single-stranded DNA. Sequencing of the template showed presence of the insert in the proper orientation. The M13 phage containing the 2.5-kb *entE* insert is called mSF100 (Figure 1A).

(*b*) *Mutagenesis.* To introduce an *Eco*RI restriction site at the beginning of the *entE* gene, a two base pair change was required. This was accomplished with the Amersham oligonucleotide-directed in vitro mutagenesis protocol. A mismatched oligonucleotide primer (Figure 1B) corresponding to the proper sequence was synthesized, phosphorylated, and annealed to the single-stranded form of mSF100. Mutagenesis using the Amersham procedure yielded a 40% success rate, determined by sequencing and restriction analysis. The M13 phage containing the 2.5-kb *entE* insert with the *new Eco*RI site is designated mSF101 (Figure 1C).

(*c*) *Subcloning into pKK223-3.* The replicative form of mSF101 was incubated with *Eco*RI/*Hin*dIII, and the 2.1-kb fragment containing the *entE* gene was isolated and purified by LMP–agarose gel electrophoresis and ion-exchange chromatography (Elutip). The plasmid pKK223-3 was linearized by *Eco*RI/*Hin*dIII digest and isolated and purified in a similar fashion. Ligation of the 2.1-kb fragment containing the *entE* gene into pKK223-3 using T4 DNA ligase as described above was followed by transformation into *E. coli* JM105. After overnight incubation at 37 °C, individual colonies were picked from the plate and grown in 5 mL of LB broth containing 100 μg/mL ampicillin. The cells were grown to an OD_{595} of ≈0.6 and collected by centrifugation. A DNA miniprep and subsequent restriction mapping showed the presence of the recombinant pKK223-3 plasmid containing the 2.1-kb insert, herein described as pSF105.

Purification of 2,3DHB-AMP Ligase. (*a*) *Crude Cell Extract.* Two 1-L cultures of *E. coli* pSF105/JM105 were grown in LB broth containing ampicillin (100 μg/mL) at 37 °C until the OD_{595} reached 0.7, at which point IPTG was added to a final concentration of 1 mM. Three hours later, the cells (6 g) were harvested by centrifugation, washed in 0.10 M Tris-HCl, pH 8.0, resuspended in 14.4 mL of buffer containing 10 mM $MgCl_2$, 5.0 mM dithiothreitol, and 0.10 M Tris-HCl, pH 8.0, and lysed by three passages through a French press operating at 12000 psi at the orifice. Cell debris was removed by centrifugation (15 min, 10000*g*), and nucleic acids were precipitated by the addition of one-fifth volume of 2% protamine sulfate to yield crude extract.

(*b*) *Ammonium Sulfate Fractionation.* To the crude extract, ammonium sulfate was added slowly with stirring on ice to give a final concentration of 30% saturation. After centrifugation (15 min, 10000*g*) to remove the precipitate, 2,3DHB-AMP ligase was precipitated by bringing the ammonium sulfate concentration of the supernatant up to 50% saturation by slow addition with stirring on ice. The precipitate was collected by centrifugation (15 min, 10000*g*) and dissolved in 6.0 mL of 25 mM Tris-HCl buffer, pH 8.0, containing 5 mM dithiothreitol and 10 mM magnesium chloride (buffer A).

(*c*) *Gel-Filtration Chromatography.* The protein fraction after ammonium sulfate precipitation was applied to a 115 × 2.5 cm column of Sephadex G-150-120 (Sigma) and eluted with buffer A at a flow rate of 20 mL/h at 4 °C; 6.0-mL fractions were taken and assayed for protein by measuring the

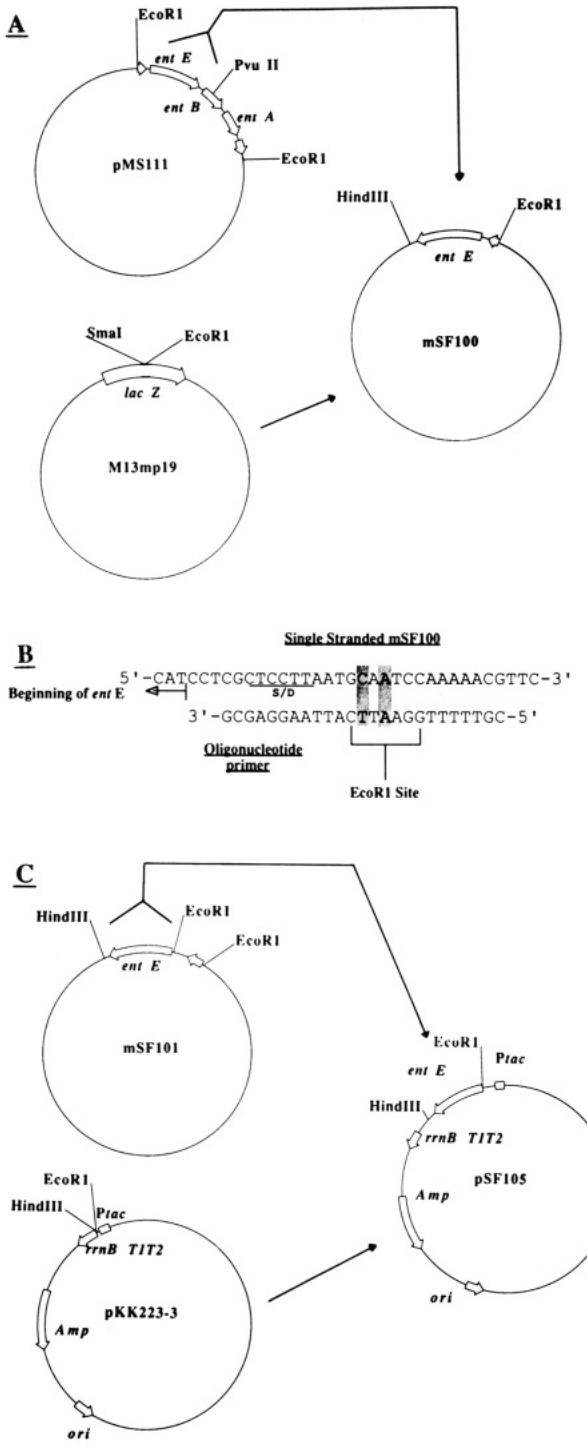


FIGURE 1: Subcloning strategy for *entE*. (A) A 2.5-kb *Eco*RI/*Pvu*II fragment from pMS111 containing the *entE* gene was ligated into M13mp19 which had been linearized with *Eco*RI/*Sma*I. The small fragment upstream from *entE* in pMS111 is the gene coding for the carboxy terminus of *entC* while the unlabeled operon after *entA* codes for a small 15-kDa polypeptide of unknown function (Liu et al., 1989; Nahlik et al., 1989). The M13 phage containing the 2.5-kb *entE* insert is called mSF100. (B) Oligonucleotide primer complementary to mSF100 used to mutagenize two base pairs upstream from the beginning of the *entE* gene to create a new *Eco*RI site. (C) Double-stranded DNA of the M13 phage with the new *Eco*RI site (mSF101) is linearized with *Eco*RI/*Hin*dIII (using the *Hin*dIII site present originally in M13mp19) and the *entE* fragment ligated into pKK223-3 to give the plasmid pSF105.

absorbance at 280 nm. Fractions of the major peaks which eluted were assayed for the exchange of radioactive pyrophosphate into ATP (see below). A single peak which eluted after the void volume was found to catalyze ATP–PP_i exchange in the presence of 2,3-dihydroxybenzoic acid. The fractions from this peak were pooled for the next purification step.

(*d*) *Anion-Exchange Chromatography.* The fractions containing 2,3DHB-AMP ligase activity were next applied to a prepacked mono-Q 16/10 column (Pharmacia) at 4 °C and washed with 250 mL of buffer A. The ligase eluted from the column with a linear 600-mL gradient of 0.01–0.3 M KCl at about 0.2 M KCl. The flow rate under these conditions was 6.0 mL/min.

(*e*) *Analysis of Proteins.* Polyacrylamide gel electrophoresis under denaturing conditions was performed on 12.5% gels as previously described (Laemmli, 1970). Molecular mass standards were from Pharmacia. Protein determinations were performed by Bradford's method (Bradford, 1976) using bovine serum albumin as a standard.

(*f*) *Storage of Purified Protein.* It was found that under dilute conditions (i.e., after anion-exchange chromatography) purified fractions of 2,3DHB-AMP ligase precipitated and lost activity. In order to stabilize the enzyme, the fractions after anion-exchange chromatography were pooled and dialyzed quickly by repetitive concentration/dilution twice with buffer A in a 50-mL Amicon filtration cell with a PM-30 membrane and further concentrated to about 20 mL. This solution was then dialyzed further in Spectra/Por 2 dialysis tubing (American Scientific Products Inc.) against a solution of 50% buffer A and 50% glycerol over 24 h with three changes of buffer. The resulting solution appeared stable over several months at −70, −20, and +2 °C.

(*g*) *Native Molecular Mass Determination.* The native molecular mass for 2,3DHB-AMP ligase was determined by chromatography on a Superose 12 (Pharmacia) gel-filtration column of the purified protein using ferritin (440 000 daltons), catalase (232 000 daltons), bovine serum albumin (67 000 daltons), hemoglobin (64 500 daltons), and carbonic annhydrase (29 000 daltons) as standards. A flow rate of 0.5 mL/min with 25 mM Tris, pH 7.0, at 4 °C as eluate was employed. A plot of $(V_e - V_0)/(V_t - V_0)$, where V_e = elution volume, V_0 = void volume, and V_t = total column volume, versus log molecular mass for the standards gave a linear curve which could be used to predict the molecular mass.

(*h*) *N-Terminal Sequence Determination.* Twelve residues of the N-terminal sequence of 2,3DHB-AMP ligase were determined according to the Edman degradation procedure by William Lane at Harvard Microchemistry Facility, Harvard University, Cambridge, MA.

Assays for 2,3DHB-AMP Ligase. (*a*) *ATP–[^{32}P]PP_i Exchange.* ATP–pyrophosphate exchange was assayed as previously described (Bryce & Brot, 1972; Greenwood & Luke, (1976) with minor modifications. The assay mixture contained 10 mM magnesium chloride, 5.0 mM ATP, 1.0 mM 2,3-dihydroxybenzoic acid, 2.0 mM disodium hydrogen [^{32}P]pyrophosphate, 10 mM Tris-HCl, pH 9.0, and enough enzyme fraction to give detectable counts in a final volume of 0.1 mL. After incubation at 37 °C for 15 min, the reaction was quenched by the addition of 0.4 mL of 0.5 M perchloric acid; 0.4 mL of 0.10 M tetrasodium pyrophosphate was added and ATP adsorbed by adding 0.2 mL of a 4% charcoal suspension (w/v in water). After being washed four times with water, the charcoal was resuspended in 1.0 mL of water and transferred to a scintillation vial. The Eppendorf tube was washed with an additional 1.0 mL of water and transferred to the scintillation vial. To the combined 2.0-mL charcoal suspension was added 5.0 mL of scintillation fluid prior to counting. The principal differences between the present assay and those reported previously (Bryce & Brot, 1972; Greenwood & Luke,

Table I: Purification of 2,3DHB-AMP Ligase Catalyzed ATP–Pyrophosphate Exchange Activity from JM105/pSF105

	vol (mL)	act.[a]	protein (mg/mL)	sp act.[b]	purification (x-fold)	yield (%)
crude extract	19.0	25.0	14.4	1.74	1	100
$(NH_4)_2SO_4$, 0–30%	19.2	37.4	16.6	2.26	1.3	151[c]
$(NH_4)_2SO_4$, 30–50%	6.0	66.2	24.0	2.76	1.6	84
G-150 Sephadex	38.0	9.49	1.73	5.48	3.2	76
mono-Q anion exchange	78.0	2.25	0.27	8.33	4.8	37

[a] Activity is defined as the numbver of μmol of [^{32}P]ATP synthesized min^{-1} ml^{-1}. [b] Specific activity is defined as the number of μmol [^{32}P]ATP synthesized min^{-1} (mg of protein)$^{-1}$. [c] The increase of activity observed after the first ammonium sulfate fractionation is most likely a result of errors involved in performing the exchange assay.

1976) are a higher ATP concentration (from 2.0 to 5.0 mM) and a higher pH (from pH 8.0 to 9.0). The rate measured under the present conditions is closer to V_{max} and the pH optimum for the reaction. K_m and k_{cat} values were usually calculated from eight different substrate concentrations while the second substrate was kept at saturating concentrations (e.g., vary 2,3-dihydroxybenzoate while [ATP] kept at 5 mM). The values for these kinetic constants were obtained by the fitting routine HYPER (Cleland, 1979).

(*b*) *Pyrophosphate Release.* To monitor the release of pyrophosphate from 2,3DHB-AMP ligase, the enzyme (90–270 μg, 1.5–4.5 nmol of subunit) was incubated in an Eppendorf tube containing 10 mM magnesium chloride, 5.0 mM ATP, 1.0 mM 2,3-dihydroxybenzoic acid or salicylic acid, and 10.0 mM Tris-HCl, pH 9.0, in a final volume of 0.10 mL. At specified time intervals, the reaction mixture was quenched by boiling for 1 min. After cooling and centrifugation to pellet the precipitated protein, 1 unit of inorganic pyrophosphatase was added, and the reaction was allowed to proceed for 20 min at 37 °C. An appropriate aliquot was removed and assayed for phosphate by the ammonium molybdate–malachite green method (such that 1 nmol ≤ total P_i ≤ 20 nmol) (Lanzetta et al., 1979). Values were corrected for the amount of free phosphate present in ATP.

Detection of Enzyme–Substrate Complexes. (*a*) *Separation by Gel-Filtration Chromatography.* Enzyme (0.2 mg) was incubated with $MgCl_2$, ATP, and salicylic acid as described above (pyrophosphate release assay) in which the corresponding radiolabeled substrates were used. This reaction was allowed to proceed for 1 min at 0 °C and quickly applied to a 5.5 × 1.0 cm column containing G-25 (40 mesh) Sephadex (Sigma) at 4 °C and eluted with ice-cold buffer containing 10 mM magnesium chloride and 25 mM Tris-HCl, pH 7.5, at a flow rate of 1.0 mL/min. Fractions (0.4 mL) were collected and counted with 5.0 mL of scintillation fluid. The absorbance at 280 nm was determined continuously by a detector after emergence from the column. The final concentration and specific activity of the radiolabeled substrates used in these incubations were 0.010 M and 5.82 μCi/μmol for [7-^{14}C]salicylate, 0.020 M and 2.58 μCi/μmol for [U-^{14}C]-ATP, and 0.050 M and 50 μCi/μmol for [γ-^{32}P]ATP. The counts detected in the enzyme peak were corrected for losses due to injection by dividing by the yield of total counts per run. To correct for backround and nonspecific binding of radiolabeled substrate to enzyme, the counts from the enzyme peak run in the absence of the counter substrate (e.g., ATP in the case of [7-^{14}C]salicylic acid) were subtracted from the total counts detected in the enzyme peak in the presence of all substrates.

(*b*) *Acid Precipitation of Reaction Mixtures.* This was performed in two ways. In the first case, the radiolabeled enzyme peak after incubation with [7-^{14}C]salicylate and ATP was collected after gel-filtration chromatography as described above (approximately 2.5 mL), and a 0.5-mL aliquot of this was removed for scintillation counting. The remainder was acidified with ice-cold trichloroacetic acid to a final concentration of 7% (w/v) after addition of bovine serum albumin (1 mg). The precipitated protein was pelleted quckly by centrifugation and immediately washed once with 1.0 mL of ice-cold 7% trichloroacetic acid. After further centrifugation, the pellet was resuspended in 1.0 mL of distilled water and transferred to a scintillation vial for counting. Alternatively, the total reaction mixture (containing [^{14}C]salicylate with and without ATP) was allowed to proceed for 1 min at 0 °C after which ice-cold trichloroacetic acid was added to a final concentration of 7%. The precipitated protein was quickly pelleted by centrifugation and washed four times with ice-cold trichloroacetic acid (1.0 mL of 7% each time). This process typically required about 4–5 min to complete. After the final wash step, the pellet was resuspended in 1.0 mL of water and transfered to a scintillation vial for counting.

Results

Subcloning and Overproduction. The subcloning of the *entE* gene from pMS111 into M13mp19 as described under Materials and Methods is illustrated in Figure 1A, resulting in mSF100. In order to construct an overproducer by use of the *tac* promoter in pKK223-3, an *Eco*RI restriction site was produced by mutagenizing two base pairs prior to the beginning of the *entE* gene (at positions corresponding to −16 and −18 base pairs upstream from the ATG start codon of *entE*, Figure 1B). Subcloning of the fragment containing the new *Eco*RI restriction site (from mSF101) into the multicopy plasmid pKK223-3 led to construction of the plasmid pSF105, which was transformed into *E. coli* JM105. After induction by IPTG, a measurement of 2,3DHB-AMP ligase activity in crude protein extract indicates that greater than ≈8% of the soluble protein in the crude extract is present as ligase, qualitatively consistent with the size of the protein band on the sodium dodecyl sulfate (SDS)–polyacrylamide gel of crude extract (Figure 2, lane 3).

Purification and Characterization of 2,3DHB-AMP Ligase. The purification of 2,3DHB-AMP ligase was accomplished by a five-step procedure (Table I), yielding 20 mg of essentially homogeneous enzyme from 2 L of growth (Figure 2, lanes 7–9). The 5-fold increase in the specific activity of the pure enzyme over the crude extract is likely a lower limit due to the lability of the enzyme in the later stages of purification. Gel-filtration chromatography removed most of the minor contaminants and a few, more significant proteins (Figure 2, lane 6). However, the most significant purification step in the process was chromatography on the mono-Q 16/10 anion-exchange column, which gave complete separation between the ligase and the contaminants remaining after gel filtration. Although a large fraction (≈50%) of the remaining activity was lost at this stage, the high flow rates attainable with the mono-Q 16/10 column relative to those of conventional anion-exchange resins most likely kept these losses to a minimum.

SDS–polyacrylamide gel electrophoresis of the protein fraction after anion exchange indicated an essentially pure

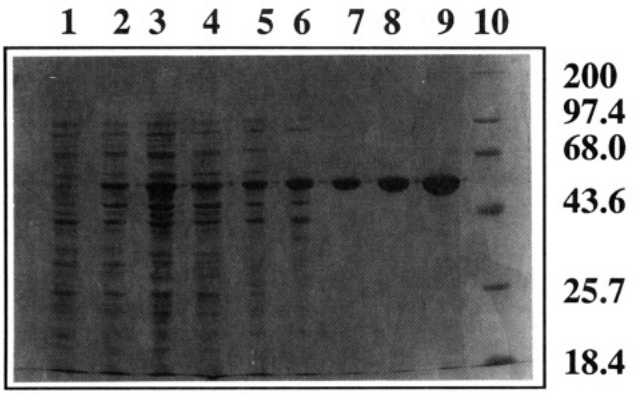


FIGURE 2: Purification of 2,3DHB-AMP ligase as followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (Lane 1) Whole cells of uninduced pSF105/JM105, 50 μg of protein; (lane 2) whole cells of pSF105/JM105 3 h after adding the inducer IPTG, 50 μg of protein; (lane 3) crude extract, 50 μg of protein; (lane 4) 0–30% ammonium sulfate fractionation (supernatant), 40 μg of protein; (lane 5) 30–50% ammonium sulfate fractionation (precipitate dissolved in buffer A), 30 μg of protein; (lane 6) pooled 2,3DHB-AMP ligase fractions after Sephadex G-150 gel-filtration chromatography, 15 μg of protein; (lanes 7–9) pooled 2,3DHB-AMP ligase fractions after mono-Q anion-exchange chromatography [the amount of protein loaded was 10 (lane 7), 20 (lane 8), and 50 μg (lane 9)]; (lane 10) molecular mass standards. The corresponding molecular masses (kDa) are listed to the right of the gel.

protein (Figure 1, lanes 7–9; overloading of the protein in lane 9 indicates <1% contamination remaining). The subunit molecular mass was calculated to be 59 000 daltons, which compares quite favorably with the calculated molecular mass obtained from the DNA sequence of 59 299 daltons (Staab et al., 1989). Gel filtration on a Superose 12 gel filtration column against molecular mass standards yielded a native molecular mass for 2,3DHB-AMP ligase of 115 000 daltons, indicating an α_2 quaternary structure. It is unknown whether 2,3DHB-AMP ligase acts as a dimer in the putative enterobactin synthase complex with Ent D, Ent F, and Ent G.

Amino-terminal sequencing of the first 12 residues of 2,3DHB-AMP ligase gave the sequence H_2N-S-I-P-F-T-"X"-W-P-E-E-F-A-...-COOH, where residue X could not be determined by the procedure employed. This sequence is identical with that predicted from the DNA sequence (Staab et al., 1989) if X = R and the starting methionine has been processed after translation. These are likely since arginine is destroyed under the conditions employed in protein sequencing and the N-terminal methionine is often removed after formylation in bacteria.

The specific activity for the purified protein after anion-exchange chromatography in the ATP–PP_i exchange assay was 8.3 μmol of [^{32}P]ATP synthesized min^{-1} (mg of protein)$^{-1}$. The specific activity was independent of whether extraneous thiols (e.g., dithiothreitol) were present in the assay (data not shown). Upon dialysis of the protein into a solution containing 50% glycerol to stabilize the activity, the specific activity had decreased to 5.8 μmol of [^{32}P]ATP synthesized min^{-1} mg^{-1} but remained stable for several months at 2 °C. Preparations could also be stored at −70 and −20 °C with no observable loss of activity over several months. It is interesting to note that partially purified preparations of 2,3DHB-AMP ligase have been reported to be stable over several days when stored at 4 °C (Greenwood & Luke, 1976), in contrast to the results obtained in the present report. However, these previous preparations containing ligase may have been contaminated by additional enzymes involved in enterobactin biosynthesis, for example, components of the proposed enterobactin synthase. The effect of these on the stability of the ligase is unknown. On the basis of the subunit molecular mass of 59.3 kDa, the turnover number for the 50% glycerol solution of 2,3DHB-AMP ligase is 340 min^{-1} at pH 9.0 and 37 °C. The K_m values calculated for ATP and 2,3-dihydroxybenzoic acid are 1.1 mM and 2.7 μM, respectively (Table II).

Table II: Measured Values of K_m, k_{cat}, and the Ratio k_{cat}/K_m for ATP, 2,3DHB, and Some Alternate Substrates of 2,3DHB-AMP Ligase in the ATP–PP_i Exchange Reaction[a]

substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM min^{-1})
ATP	1120 (160)	350 (19)	
2,3-dihydroxybenzoic acid	2.7 (0.4)	330 (19)	122
benzoic acid		<1	
salicylic acid (*o*-hydroxybenzoic acid)	91 (4)	150 (2)	1.6
m-hydroxybenzoic acid		<1	
p-hydroxybenzoic acid		<1	
2,4-dihydroxybenzoic acid	242 (36)	194 (14)	1.3
gentisic acid (2,5-dihydroxybenzoic acid)	552 (93)	189 (10)	2.9
2,6-dihydroxybenzoic acid		<1	
2,3,4-trihydroxybenzoic acid	93 (30)	27 (2)	0.29
2,4,6-trihydroxybenzoic acid		<1	
anthranilic acid		<1	
thiosalicylic acid		<1	

[a] The numbers in parentheses after each value are the standard errors of that value.

Substrate Specificity of 2,3-Dihydroxybenzoate-AMP Ligase. A number of analogues of 2,3-dihydroxybenzoate had been tested previously with partially purified enzyme to determine whether they would support the ATP–PP_i exchange reaction (Bryce & Brot, 1972). We have expanded on these studies to gain further insight into the specificity of the aromatic substrate; the results are summarized in Table II. We found that 2,3-dihydroxybenzoic acid (the natural substrate), salicylic acid (*o*-hydroxybenzoic acid), 2,4-dihydroxybenzoic acid, gentisic acid (2,5-dihydroxybenzoic acid), and 2,3,4-trihydroxybenzoic acid all supported the exchange while benzoic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, anthranilic acid (*o*-aminobenzoic acid), and thiosalicylic acid (*o*-thiobenzoic acid) did not support exchange. The natural substrate 2,3-dihydroxybenzoate is by far the best substrate in the exchange reaction with k_{cat}/K_m at least 30 times larger than that of any of the other substrates which promoted exchange. The results with benzoate, the three isomers of the monohydroxybenzoates, and the amino and thio analogues of salicylic acid suggest that the minimum requirement for the aromatic substrate is a hydroxyl function ortho to the carboxylate group. It is interesting to note that 2,6-dihydroxybenzoate and 2,4,6-trihydroxybenzoate do not support exchange whereas salicylate and 2,4-dihydroxybenzoate do. Thus, there may be significant steric interference between the enzyme and the 6-position of the aromatic ring. It has been reported that 2,3,4-trihydroxybenzoate does not promote ATP–PP_i exchange (Bryce & Brot, 1972); although the rate is low, we were able to measure significant exchange. These results are surprising since 2,3- and 2,4-dihydroxybenzoate are both fairly good substrates. The low value for k_{cat}/K_m observed for the trihydroxybenzoate may be a result of either impurities present from oxidation and/or polymerization which could inhibit the reaction or possibly an improper chelation of the Mg^{2+} ion required for the reaction with ATP.

Pyrophosphate Release from 2,3DHB-AMP Ligase. The observation that 2,3-dihydroxybenzoic acid supports the ATP–PP_i exchange reaction indicates that the function of

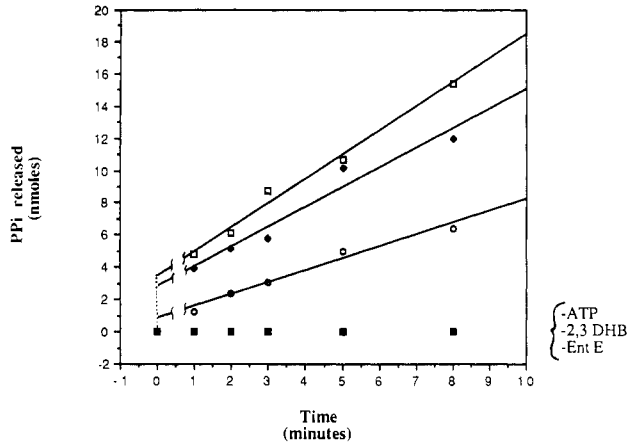


FIGURE 3: Pyrophosphate release as a function of time. Each reaction contained 1 mM 2,3-dihydroxybenzoic acid, 5 mM ATP, and 2,3DHB-AMP ligase [1.5 (○), 3.0 (◆), and 4.5 nmol (□), based on the subunit molecular mass], in addition to buffer as described under Materials and Methods. Control experiments in which either 2,3-dihydroxybenzoate, ATP, or ligase was omitted from the reaction are grouped together (■). The lines through the data for each different enzyme concentration were obtained by linear regression. The initial burst of pyrophosphate released (see text) was calculated by extrapolating back to zero time.

2,3DHB-AMP ligase is to activate the aromatic substrate for subsequent amide bond formation with serine in the biosynthesis of enterobactin (Scheme I). When 2,3DHB-AMP ligase was assayed for the release of pyrophosphate, an initial burst was followed by a slower release (Figure 3). The initial burst probably corresponds to the stoichiometric formation of the (2,3-dihydroxybenzoyl)adenylate complex since, under the conditions of the assay, the formation of this complex would be rapid ($t_{1/2}$ < 1 s) and should release an amount of pyrophosphate proportional to the amount of enzyme present. As seen in Figure 3, when 1.5, 3.0, and 4.5 nmol of ligase is present in the assay (molecular mass = 59.3 kDa), the corresponding amounts of pyrophosphate released immediately are 0.80, 2.8, and 3.5 nmol. This corresponds to an average of 0.7 ± 0.3 nmol of PP_i released per nanomole of ligase subunit.

Measurement of the rate of pyrophosphate release after the initial burst indicates that this release is due to turnover by the enzyme (Figure 3). Thus, as the amount of enzyme is increased, the rate of pyrophosphate release increases accordingly. A calculation from these data yielded a turnover number for pyrophosphate release of 0.41 ± 0.08 min^{-1} at 37 °C. Measurement of AMP formation by HPLC analysis was in agreement with both the rate of pyrophosphate release and the total amount released at any given time (data not shown). Furthermore, when any of the constituents of the assay were omitted, neither the burst nor the slow release of pyrophosphate was evident (Figure 3). These results are consistent with the proposal that pyrophosphate (and AMP) release is due to the reaction of 2,3DHB-AMP ligase with ATP and 2,3-dihydroxybenzoate.

A comparison of the rate of ATP–PP_i exchange with the rate of pyrophosphate release indicates that exchange is occurring about 1000-fold faster. This observation strongly suggests that intermediates are accumulating on the enzyme surface (see Discussion). In comparison with other amide bond forming enzyme systems which catalyze such an ATP–PP_i exchange reaction (Geevers et al., 1968, 1969; Roskoski et al., 1970a,b), these intermediates should correspond to either the initially formed (2,3-dihydroxybenzoyl)adenylate or the covalent enzyme thioester of 2,3-dihydroxybenzoate formed by subsequent enzymatic nucleophilic displacement of AMP. The continuous release of pyrophosphate indicates that, if formed, these complexes are unstable, with a half-life of about 1.7 min. We have observed that the release of pyrophosphate is dependent upon the presence of extraneous thiols. For example, when pyrophosphate release was monitored in the presence of 5.0 mM dithiothreitol, the turnover number doubled to ≈1 min^{-1}. Thus, the presence of thiols in the assay increased the rate of pyrophosphate release presumably by rendering the product complex of 2,3DHB-AMP ligase less stable under these conditions.

Observation of Enzyme–Substrate Complexes. In order to detect any enzyme intermediates which might be accumulating, a series of experiments were undertaken with radiolabeled substrates and gel-filtration chromatography. [7-^{14}C]Salicylic acid, [U-^{14}C]ATP, and [γ-^{32}P]ATP were used in an effort to determine whether any portions of these compounds remained bound to the enzyme under turnover conditions. [7-^{14}C]Salicyclic acid was chosen since it is more readily available than radiolabeled 2,3-dihydroxybenzoate and it also supported the ATP–PP_i exchange reaction. However, the turnover numbers at 37 °C for ATP–PP_i exchange and pyrophosphate release (in the absence of thiols) with salicylate were 150 and ≈10 min^{-1}, respectively. Thus, the enzyme–substrate complex of 2,3DHB-AMP ligase with salicylate is about 20-fold less stable than the corresponding complex with 2,3-dihydroxybenzoate. In order to stabilize the salicylyl–enzyme complex, the reactions and chromatography were carried out at low temperatures (0–4 °C), and in the absence of dithiothreitol. In this case, the turnover numbers with salicylate in the ATP–PP_2 exchange and pyrophosphate release reactions at 0.4 °C in the absence of DTT are ≈11 and ≤1 min^{-1}, respectively.

Incubation of 2,3DHB-AMP ligase with either [7-^{14}C]-salicylic acid or [U-^{14}C]ATP followed by separation of the enzyme from starting material by gel-filtration chromatography allowed for the isolation of an enzyme fraction containing radioactive label. For instance, when the ligase was incubated for 1 min at 0–4 °C with ATP and [7-^{14}C]salicylic acid and then quickly chromatographed, the fractions which contained ligase also contained [7-^{14}C]salicylate (Figure 4A). To determine whether this represented nonspecific binding of salicylic acid to 2,3DHB-AMP ligase, [7-^{14}C]salicylic acid and ligase were incubated in the absence of ATP. As seen in Figure 4A, very little radioactivity coelutes in the fractions containing ligase when ATP is absent. A quantitation of salicylate bound per enzyme (see Materials and Methods) indicates that approximately 0.15 mol of salicylate is bound per mole of ligase subunit for the experiment shown in Figure 4A. When this experiment was repeated two additional times, the number of molecules of ligase containing radiolabel was 7% and 11%.

When a parallel experiment using [U-^{14}C]ATP in the presence of salicylate was performed, it was found that roughly 0.08 mol of the adenosyl moiety is bound per mole of 2,3DHB-AMP ligase (Figure 4B). As described above, running the reaction in the absence of salicylate resulted in no significant binding of ATP to the enzyme. Two additional incubations with [U-^{14}C]ATP yielded 10% and 4% of enzyme molecules labeled. Finally, 2,3DHB-AMP ligase was incubated with [γ-^{32}P]ATP in the presence and absence of salicylate. In these experiments, there was no significant difference between the amount of radioactivity associated with the enzyme fractions in the presence or absence of salicylate (Figure 4C). These results strongly suggest that the (β-

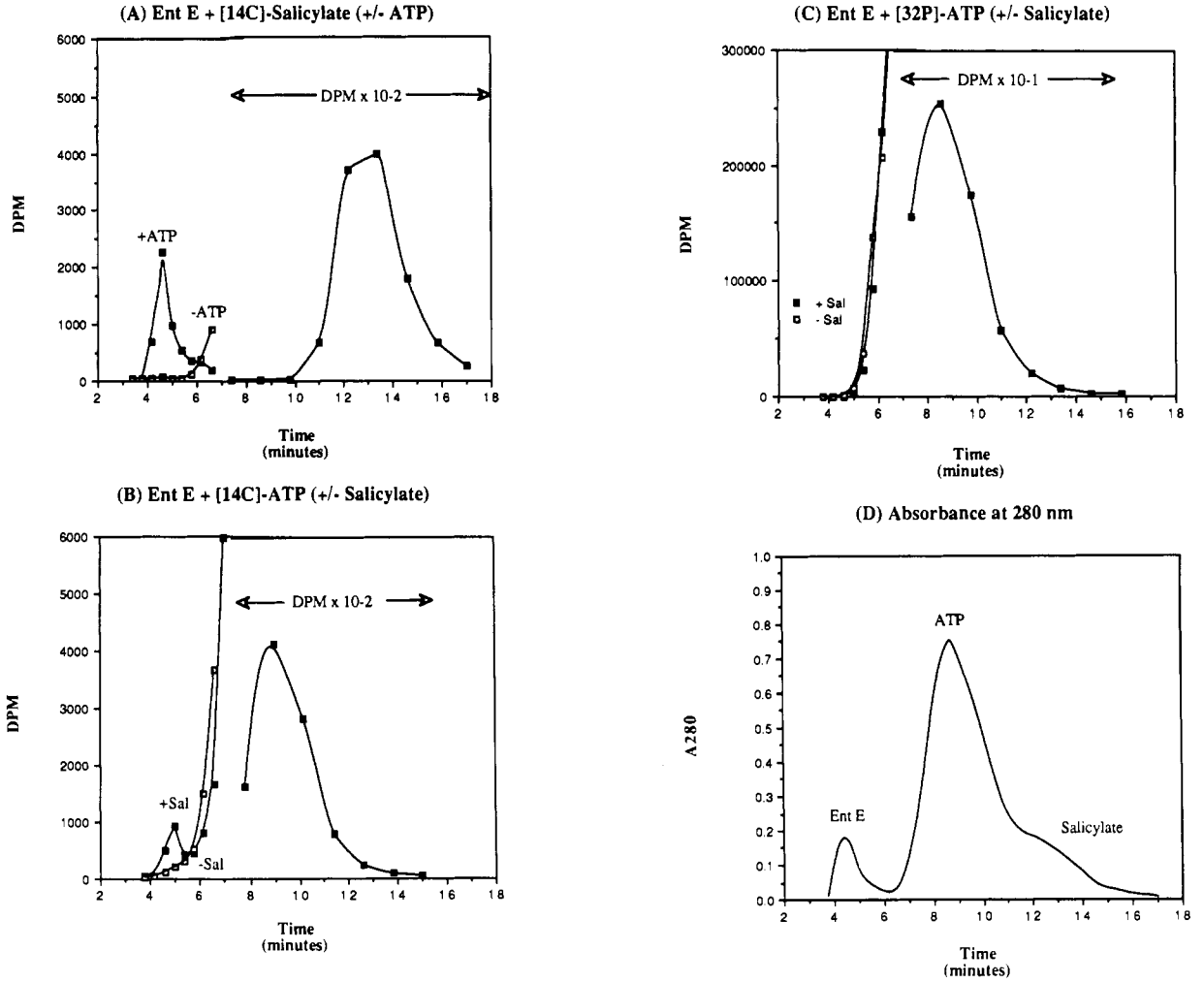


FIGURE 4: Results of labeling 2,3DHB-AMP ligase with radioactive substrates. For details, see Materials and Methods. (A) Enzyme incubated with [7-^{14}C]salicylic acid in the presence (■) and absence (□) of ATP. (B) Enzyme incubated with [U-^{14}C]ATP in the presence (■) and absence (□) of salicylic acid. (C) Enzyme incubated with [γ-^{32}P]ATP in the presence (■) and absence (□) of salicylic acid. For comparison, the positions of each of the components as they elute from the gel filtration column are shown in (D). In (A–C), the radioactive fractions corresponding to free substrate are plotted only for the experiment in which all substrates are present [(■) symbols].

phosphate and) γ-phosphates of ATP are released from the enzyme upon formation of the enzyme–substrate complex. Thus, the portion of ATP which remains bound to the ligase is mostly likely AMP. These results are totally consistent with the proposed mechanism and are strongly buttressed by the ATP–PP_i exchange data.

Although the stoichiometry of these reactions varied considerably, typically more salicylate is bound to 2,3DHB-AMP ligase than AMP. These results suggest that two complexes might be present, e.g., a mixture of [salicylyl-AMP–ligase] and [salicylyl-S-ligase]. Such a mixture has been observed in the biosynthesis of gramicidin where covalent thioester complexes with a protein have been implicated (Gevers et al., 1968, 1969; Roskoski et al., 1970a,b). Typically, thioester complexes are relatively stable in dilute acid while the acyladenylate complex rapidly decomposes. Our attempts at isolating any acid-stable complexes of salicylate with 2,3DHB-AMP ligase were unsuccessful, confirming an earlier report (Bryce & Brot, 1972). For example, after isolation of [7-^{14}C]salicylic acid labeled ligase by gel-filtration chromatography (as described under Materials and Methods), no radiolabel remained associated with the enzyme after ice-cold trichloroacetic acid precipitation. However, the enzyme complex with salicylate is relatively unstable over time and may have decayed significantly over the time course of this experiment. If the total reaction mixture was instead subjected to acid precipitation (i.e., no gel-filtration chromatography) and excess label removed by repetitive washing, again, no significant label was observed associated with the precipitated protein as judged against a control in which ATP was excluded. Since this experiment typically required about 4–5 min to complete, i.e., about the same amount of time required to isolate radiolabeled 2,3DHB-AMP ligase by gel-filtration chromatography (see Figure 4), it would be expected that detectable levels of covalently attached labeled salicylate would have remained associated with the enzyme.

Interestingly, when [U-^{14}C]ATP was incubated with 2,3-dihydroxybenzoic acid rather than salicylate, the number of molecules of [U-^{14}C]AMP bound increased to 0.34 mol/mol of 2,3DHB-AMP ligase. This is close to the number expected for stoichiometric binding after chromatography given the turnover number for pyrophosphate release (0.41 min^{-1} with 2,3-dihydroxybenzoate) and assuming an exponential decay of the enzyme–acyladenylate complex. Thus, it seems likely that the complex with 2,3DHB-AMP ligase is the (2,3-dihydroxybenzoyl)adenylate.

Discussion

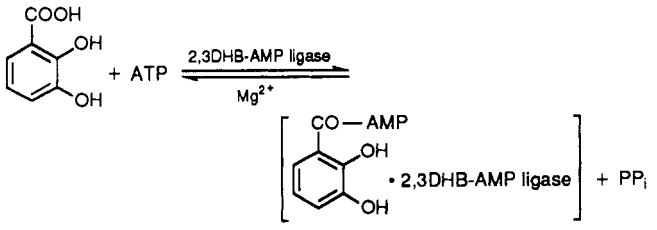
The *entE* gene, coding for the enzyme responsible for activating the carboxyl group of 2,3-dihydroxybenzoic acid in the biosynthesis of enterobactin, has been subcloned into the multicopy plasmid pKK223-3 under the control of the *tac*

promoter. The introduction of this recombinant plasmid into *E. coli* has resulted in an overproducing strain and has allowed for the first preparation of 2,3DHB-AMP ligase uncontaminated with other enzymes involved in enterobactin biosynthesis (especially components of the enterobactin synthase complex) and in suitable quantities (≈20 mg) for enzymological studies.

Reassuringly, 2,3DHB-AMP ligase purified from the overproducing *E. coli* strain is active; it catalyzes the ATP-PP_i exchange reaction dependent upon the presence of aromatic substrate. On the basis of the subunit molecular mass of 59 kDa, the enzyme has a turnover number at pH 9.0 in this assay of 340 min^{-1}. K_m values for 2,3-dihydroxybenzoic acid and ATP are 2.7 μM and 1.1 mM, respectively. Additional aromatic substrates which support the exchange reaction have a requirement for a hydroxyl group ortho to the carboxyl group, and it appears that the enzyme can tolerate substituents at all the remaining positions of the aromatic ring except the sixth position.

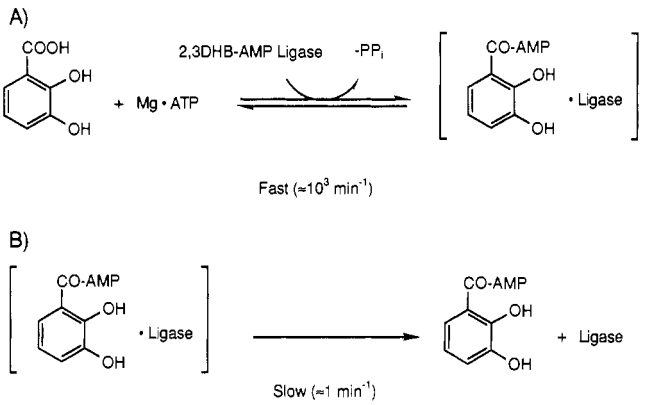
Early investigations in the biosynthesis of enterobactin showed that both impure 2,3DHB-AMP ligase and serine-AMP ligase (product of the *entF* gene) catalyzed the exchange of pyrophosphate with the β- and γ-phosphates of ATP, dependent upon 2,3-dihydroxybenzoate and serine, respectively (Bryce & Brot, 1972; Greenwood & Luke, 1976; Woodrow et al., 1979). These observations led to the proposal that the functions of 2,3DHB-AMP ligase and serine-AMP ligase were to activate 2,3-dihydroxybenzoate and serine to form (initially) acyladenylate complexes for the subsequent formation of amide and ester bonds in enterobactin (Scheme I). Furthermore, serine-AMP ligase was shown to form an acid stable complex with serine in the presence of ATP. From this and other evidence it was concluded that serine forms a covalent bond with serine-AMP ligase, presumably through a thioester linkage analogous to the complexes found in gramicidin and tyrocidin biosynthesis (Scheme I) (Gevers et al., 1968, 1969; Roskoski et al., 1970a,b). A similar acid-stable complex with partially purified 2,3DHB-AMP ligase and 2,3-dihydroxybenzoate was not isolable, and it was proposed that a thioester is not formed and 2,3-dihydroxybenzoate may remain bound to the ligase noncovalently as (2,3-dihydroxybenzoyl)adenylate.

We have set out to test this hypothesis and found that, upon incubation of pure 2,3DHB-AMP ligase with ATP and aromatic substrate, pyrophosphate is released initially, corresponding approximately to the stoichiometric amount of enzyme monomer present, and then continuously, albeit at a rate much slower (by 830-fold) than the ATP–PP_i exchange rate. The initial release of pyrophosphate most likely corresponds to the formation of stoichioimetric enzyme-bound acyladenylate:



The fact that pyrophosphate is then slowly released beyond the ≈0.7 equiv released initially might be an indication that the enzyme is turning over in the sense that a product [e.g., (2,3-dihydroxybenzoyl)adenylate] is released into solution for further enzymatic reactions. However, since free acyladenylates are notoriously unstable in aqueous solution, this is likely to be a counterproductive and adventitious release of kinetically labile, thermodynamically activated product. In a more useful sense, it is thought that such compounds remain bound to the enzyme for further reactions (Moldave et al., 1959). Alternatively, the continuous release observed might be due to an unstable enzyme complex with hydrolysis occurring on the enzyme surface. Since it has been proposed that the polypeptides coded for by the *entD–entG* genes associate into a multienzyme enterobactin synthase complex (Greenwood & Luke, 1976, 1980; Woodrow et al., 1979), it remains to be determined whether additional enzymes could influence the stability of the enzyme–acyladenylate complex of 2,3DHB-AMP ligase in vivo. Additional studies on this point and further investigations into the subsequent steps in enterobactin biosynthesis require further purification of the remaining enzymes of the enterobactin synthase complex.

Scheme II



Since the rate of 2,3-dihydroxybenzoate-dependent ATP–PP_i exchange occurs almost 3 orders of magnitude faster than pyrophosphate release, it is likely that intermediates such as an enzyme–acyladenylate complex or a covalent acyl thioester are forming and their release is dramatically rate limiting. This is because the ATP–PP_i exchange assay is a measure of the forward and reverse reactions for intermediate *formation* (Scheme IIA). Pyrophosphate release, however, is a measure of *breakdown* or *release* of acyladenylate (or the analogous thiol complex) (Scheme IIB), since dissociation of acyladenylate would be followed by subsequent formation of another acyladenylate–enzyme complex with the release of a molecule of pyrophosphate. If intermediates are formed, it might be possible to ascertain their composition through radiolabeling of substrates. In fact, such intermediates have been detected in the present study. Thus, the enzyme forms a detectable complex with [7-^{14}C]salicylate, an analogue of 2,3-dihydroxybenzoate. In addition, the adenosyl portion of ATP is also part of this complex. The fact that the terminal phosphate of ATP is not a part of this complex rules out the possiblity that unreacted ATP is bound to the enzyme.

It is uncertain whether the difference in stoichiometry between enzyme-bound [^{14}C]ATP and enzyme-bound [^{14}C]-salicylate is significant. If it is, it may represent an unusually unstable thioester bond between the enzyme and the aromatic substrate. Certainly, as is evident from the chromatogram shown in Figure 4D, the resolution of the enzyme from ATP is much less than the resolution of the enzyme with either salicylate or 2,3-dihydroxybenzoate. (These aromatic compounds appear to interact with the gel medium and elute much later than ATP.) The implication of this is that there is more error involved in computing the stoichiometry of enzyme-bound [^{14}C]ATP. For instance, the peak of [^{14}C]AMP which elutes with 2,3DHB-AMP ligase appears only as a prominent shoulder on the peak of free [^{14}C]ATP (Figure 4B) while the peak of enzyme-bound [^{14}C]salicylate is considerably more

resolved from that of free [^{14}C]salicylate (Figure 4A). Thus, the present method for assessing the stoichiometry, coupled to the inherent instability of such a complex, makes it difficult to determine whether an acyladenylate complex is formed exclusively or whether this subsequently is replaced by an intermediate in which the aromatic acid is covalently bound to the enzyme through a thioester linkage. However, the results obtained when ligase is incubated with [U-^{14}C]ATP and 2,3-dihydroxybenzoate argue more favorably that a thioester is not formed since almost 40% of the ligase subunits are radiolabeled, an amount predictable after chromatography from the rate of pyrophosphate release. This contention is strengthened by the fact that a thioester was not detected under conditions where these complexes are relatively stable.

In either case, it has been determined that the product of the reaction of 2,3DHB-AMP ligase in the presence of 2,3-dihydroxybenzoate and ATP is an *enzyme-bound activated aromatic substrate*. This complex is presumably the substrate in the subsequent reactions in the biosynthesis of enterobactin. Interestingly, an acid-insoluble complex of *N*-(2,3-dihydroxybenzoyl)serine was formed when impure preparations of 2,3DHB-AMP ligase, serine-AMP ligase, ATP, serine, and 2,3-dihydroxybenzoate were incubated together (Bryce & Brot, 1972). The authors proposed that the (2,3-dihydroxybenzoyl)adenylate complex of 2,3DHB-AMP ligase reacts with the thioester-linked serine complex of serine-AMP ligase to form an amide bond with the product, *N*-(2,3-dihydroxybenzoyl)serine, remaining bound to serine-AMP ligase. If this indeed occurs, the cyclic trimer enterobactin could be assembled by sequential assembly of three units of product-bound serine-AMP ligase. Further support for this proposal warrants the future purification of serine-AMP ligase and other enzymes of the enterobactin synthase complex.

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