Purification and Characterization of Actinomycin Synthetase I, a 4-Methyl-3-hydroxyanthranilic Acid-AMP Ligase from Streptomyces chrysomallus*

(Received for publication, November 1, 1991)

Ulrich Keller† and Wilhelm Schlumbohm‡
From the Institut für Biochemie und Molekularbiologie, D-1000 Berlin 10, Federal Republic of Germany

Actinomycin synthetase I was purified to homogeneity from actinomycin-producing *Streptomyces chrysomallus*. The purified enzyme is a single polypeptide chain of Mr 45,000. It catalyzes the formation of the adenylate of 4-methyl-3-hydroxyanthranilic acid (4-MHA) from the free acid and ATP in an equilibrium reaction. 4-MHA is the precursor of the chromophoric part of actinomycin. By using the 4-MHA analogue, 4-methyl-3-hydroxybenzoic acid, as a model substrate it could be established that the equilibrium constant $K_e$ is independent on enzyme concentration, which suggests that no stoichiometric acyladenylate-enzyme complex is formed in contrast to observations made with aminoacyl adenylates formed by aminoacyl-tRNA synthetases or multifunctional peptide synthetases. Actinomycin synthetase I does not charge itself with substrate carboxylic acid via a covalent thioester bond as is usual for amino acid activation in non-ribosomal peptide synthesis. In addition, the enzyme does not act as an acyl-coenzyme A ligase as revealed by its inability to release AMP in the presence of 4-MHA or other structurally related aromatic carboxylic acids, coenzyme A and ATP. Additional analysis of the activation reaction showed that it is exothermic, whereas the free enthalpy change $\Delta G^\circ$ is positive due to a negative entropy change indicating a strong influence of restriction of random motion on the course of the reaction. Determinations of $K_m$ and $k_{cat}$ of various substrate carboxylic acids revealed the highest $k_{cat}/K_m$ ratio for the natural substrate 4-MHA. From these properties, actinomycin synthetase I represents the prototype of novel chromophore activating enzymes involved in non-ribosomal synthesis of chromopeptide lactones in streptomycetes.

Actinomycin, a bicyclic chromopeptidemycin produced by various streptomycetes, originates from the oxidative condensation of two molecules of a monocyclic precursor pentapeptide lactone which contains 4-methyl-3-hydroxyanthranilic acid (4-MHA) at the amino terminus (Fig. 1) (Keller, 1984). Despite the fact that a cell-free system of actinomycin or of 4-MHA pentapeptide lactone synthesis could not be accomplished, we were able to identify the enzymes responsible for peptide synthesis by dissecting the synthetic pathway into distinct steps that are catalyzed by partial activities of a multienzyme complex (Keller et al., 1984; Keller, 1987). By this way, it could be shown previously that partially purified protein fractions of actinomycin-producing *Streptomyces chrysomallus* contain an enzyme of Mr 52,000–55,000, called actinomycin synthetase I, that activates 4-MHA as an adenylate (Keller et al., 1984). Indirect evidence for its involvement in the attachment of 4-MHA to the peptide chain of the actinomycin half-molecule came from the finding that a number of structural analogues of 4-MHA, which are also activated by the enzyme, are efficiently incorporated in vivo into the pentapeptide lactone instead of 4-MHA (Keller, 1984).

Furthermore, two multifunctional peptide synthetases (called actinomycin synthetase II and III) of Mr 225,000 and 280,000, respectively, are responsible for the assembly of the pentapeptide lactone rings of the antibiotic (Keller, 1987). These enzymes, which have also been isolated from *S. chrysomallus*, are able to catalyze the activation of the amino acids present in the peptide ring as thioesters via the corresponding adenylates. In addition, they harbor functions for epimerization and N-methylation of covalently bound substrates and intermediates (Keller, 1987). However, neither of these two enzymes activates 4-MHA, and this implies that actinomycin synthetase I is an essential part of the enzyme system involved in actinomycin biosynthesis.

The successful isolation and purification of enzymes functionally related to actinomycin synthetase I which are involved in chromophore activation of other chromopeptide lactones from streptomycetes such as the quinoxaline antibiotics (Glund et al., 1990) and the mikamycin B antibiotics (Schlumbohm and Keller, 1990) provided strong evidence that actinomycin synthetase I is the prototype of a new class of activating enzymes. These enzymes and actinomycin synthetase I appear to share a great deal of similarities with each other as expressed by nearly the same purification protocol, comparable sizes, PI values, and, in respect of one substrate, some homology in the active site of two of the enzymes (Schlumbohm and Keller, 1990).

In the present paper we describe the purification to homogeneity and a more detailed characterization of actinomycin synthetase I than was possible in our previous report, when only a limited amount of material was available (Keller et al., 1984). It is shown that this enzyme displays properties which are unusual in non-ribosomal peptide synthesis. The enzyme

* This work was supported by Grant Sfb 9, D5 from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement." in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 0049-30-314-24168; Fax: 0049-30-31424783.

‡ Present address: Institut für Arzneimittel, Bundesgesundheitsamt, Seestrasse 10, D-1000 Berlin 65, Germany.

The abbreviations used are: 4-MHA, 4-methyl-3-hydroxyanthranilic acid; 4-MHB, 4-methyl-3-hydroxybenzoic acid; 3- HA, 3-hydroxybenzoic acid; 4-AB, 4-aminobenzoic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAG, polyacrylamide gel electrophoresis; DTE, dithioerythritol.

1 A. Stindl and U. Keller, manuscript in preparation.
activates its substrates as adenylates but forms neither an enzyme-adenylate complex, as has been shown for peptide synthetases and aminoacyl tRNA synthetases, nor the covalent acid-enzyme thioester complexes typically observed in enzymatic peptide synthesis. An investigation of the incorporation of 4-MHA into the peptide chains of actinomycin will be presented in a forthcoming publication.

MATERIALS AND METHODS

Radioisotopes and Chemicals

1-[(methyl-14C)]Methionine (86.7 Ci/mol) and [U-14C]adenosine triphosphate ammonium salt (563 Ci/mol) were from Amersham International. Tetradecium [35P]pyrophosphate (2.9 Ci/mmol) and [32P] adenosine triphosphate (35 Ci/mmol) were from Du Pont-New England Nuclear. The following chemicals were obtained as indicated: 4-aminobenzoic acid, 2,4-dihydroxybenzoic acid, and 3-hydroxyanthranilic acid (Merck, Darmstadt, F.R.G.); 4-methyl-3-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-methylbenzoic acid, and 2,3-dihydroxybenzoic acid (Ega-Aldrich, Steinheim, F.R.G.); 4-hydroxybenzoic acid, 3-amino benzoic acid, 3-methylbenzoic acid, adenosine monophosphoric acid, adenosine triphosphoric acid disodium salt, and DNase I (grade II) (Sigma, Deisenhofen, F.R.G.); polyethyleneimine-impregnated cellulose sheets (Macherey & Nagel, Düren, F.R.G.); silica gel sheets (Schleicher & Schuell, Dassel, F.R.G.). Polymipin P was a gift of BASF AG (Ludwigshafen, F.R.G.). 4-MHA was prepared as described previously (Keller et al., 1984). All other chemicals were of the highest purity commercially available.

Strains and Cultures

S. chrysomallus strain X2-18 was used throughout this study. It was obtained through two rounds of mutagenic treatment of spores of S. chrysomallus wild type strain ATCC 11523 with NTG as described previously (Keller et al., 1985). The strain produces 250–400 mg/liter actinomycin C and does not produce spores. It was kept on complete medium agar as described (Keller et al., 1985). For the inoculation of liquid cultures the mycelium of strain X2-18 grown for 3 days on a Petri dish containing complete medium agar was scraped into 8–10 ml of water. After vigorous agitation and filtering of the resultant suspension of mycelial fragments through cotton, 0.2 ml of the suspension served as the inoculum for one flask of liquid culture. The liquid medium was complete medium without agar as described (Keller et al., 1985).

Growth Conditions

 Cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of liquid complete medium with shaking at 220 rpm and 28 °C (New Brunswick Environmental Shaker, model G 25). Flasks were equipped with baffles (steel springs) as described (Hopwood et al., 1985). After 42–48 h of growth cultures had reached the peak level of actinomycin production rate as assessed by measuring the extinctions of ethylacetate extracts of the cultures at 440 nm. Control short term harvests were immediately dried with a stream of cold air. Plates were then developed in ice-cold solvent system II for 8–12 h in the cold room. For determinations of Km of the adenylate formation reaction, reaction conditions were varied by changing initial concentrations of substrates such as acid, ATP, or pyrophosphate at various enzyme concentrations. Determinations of Km were done by measuring initial rates obtained by varying concentrations of one substrate and keeping the concentration of the other ones at saturation. In some rare cases such measurements were done in the additional presence of inorganic pyrophosphatase.

Enzyme Purification

All operations were carried out at 2 °C.

Step 1—Some 60 g of freshly harvested mycelium of S. chrysomallus strain X2-18 were suspended in buffer A to give a total volume of 350–400 ml. The suspension was passed through a French press (Amino) at a cell pressure of 10,000 psi. 0.5 ml of 1 M MgCl2 and 0.5 mg DNase I (grade II) (Sigma) was added. After stirring for 30 min the suspension was centrifuged for 20 min at 10,000 rpm in the GSA rotor of a Sorvall RC-2B centrifuge.

Step 2—12% Polymipin P was added to the supernatant to give 0.3% final concentration, and the resulting suspension was stirred for 30 min. The suspension was centrifuged as above, and the precipitate was discarded.

Step 3—The supernatant was passed through a DEAE-cellulose column (8 x 5.5 cm) which had been previously equilibrated with distilled water. For preparation of enzyme cells were kept frozen not longer than 2 weeks at −80°C. The yield of wet weight mycelium was about 2 g/flask.

Syntheses

Adenylates of various benzene carboxylic acids for chromatographic purposes were synthesized essentially as described previously (Keller et al., 1984).

Buffers and Solvent Systems

Buffer A consisted of 50 mM potassium phosphate, pH 6.8, 3 mM dithioerythritol (DTT), 15% (v/v) glycerol, 1 mM EDTA, 1 mM benzanilide, and 1 mM phenylmethylene sulfonate fluoride (PMSSF). Buffer B consisted of 0.1 M Tris- HCl, pH 8.0, 3 mM DTT, 15% glycerol, 1 mM EDTA, 1 mM benzanilide, and 1 mM PMSSF. Thin-layer chromatography (TLC) of acyladenylates on silica gel was done using n-butanol/acetate/water (4/1/1; by volume) as solvent systems. Polyethyleneimine-cellulose sheets were developed in 1.2 M LiCl.

Assays for direct measurement of adenylate formation were run in Eppendorf tubes at enzyme concentrations from 0.25 to 2.5 μM in the presence of 10 mM MgCl2. The buffer in these assays was 30 mM Tris- HCl, pH 8.0, 4% glycerol (w/v), 1 mM benzamidine, 1 mM PMSSF, 0.5 mM EDTA, and the reaction volume was between 15 and 40 μl. The reaction was started by the addition of the enzyme and incubation was carried at 25 °C. Aliquots (10 μl) were quickly removed in 1–min intervals and pipetted into charcoal suspensions. Determination of radioactive ATP was as above. This procedure served for indirect determinations of Km values of aromatic carboxylic acids in the adenylate formation reaction.

Enzyme Assays

The ATP-pyrophosphate exchange reaction was performed in two different versions. For routine measurements in the course of protein purification such as for localization of activity peaks and determinations of specific activities, the incubation mixture contained 1 mM 4-methyl-3-hydroxybenzoic acid (4-MHB), 5 mM ATP, 10 mM MgCl2, 0.5 mM sodium pyrophosphate, 10 μM sodium pyrophosphate, and 50 μl of enzyme fraction in a total volume of 100 μl. Incubation was for 10 min at 28 °C. Radioactive ATP was adsorbed to charcoal and measured as described (Keller et al., 1984).

In the second version, the incubation mixture contained 0.5 mM ATP, 10 mM MgCl2, 0.1 mM sodium pyrophosphate, 5 × 104 pM sodium [32P]pyrophosphate, 0.25–2.5 μM enzyme, and varying amounts of individual aromatic carboxylic acid in a total volume of 100 μl. The reaction was started by the addition of the enzyme and incubation was carried at 25 °C. Aliquots (10 μl) were quickly removed in 1–min intervals and pipetted into charcoal suspensions. Determination of radioactive ATP was as above. This procedure served for indirect determinations of Km values of aromatic carboxylic acids in the adenylate formation reaction.

For determination of Km of the adenylate formation reaction, reaction conditions were varied by changing initial concentrations of substrates such as acid, ATP, or pyrophosphate at various enzyme concentrations. Determinations of Km were done by measuring initial rates obtained by varying concentrations of one substrate and keeping the concentration of the other ones at saturation. In some rare cases such measurements were done in the additional presence of inorganic pyrophosphatase.

FIG. 1. Structure of actinomycin D (C1, left) and the corresponding actinomycin half-molecule 4-MHA pentapeptide lactone (right). Sar, sarcosine-N-methylglycine; Me-Val, N-methyl-L-valine.
Actinomycin Synthetase I from Streptomyces chrysomallus

Table I

Purification of actinomycin synthetase I from Streptomyces chrysomallus

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (nkat)</th>
<th>Specific Activity (nkat/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>280</td>
<td>2520</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymyxin P precipitation</td>
<td>286</td>
<td>1960</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. DEAE-cellulose (NH₄)₂SO₄ precipitation step</td>
<td>590</td>
<td>243</td>
<td>100.5</td>
<td>0.41</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Aca-44 gel filtration</td>
<td>96</td>
<td>76</td>
<td>52.7</td>
<td>0.58</td>
<td>1.44</td>
<td>52</td>
</tr>
<tr>
<td>2. DEAE-cellulose</td>
<td>120</td>
<td>26</td>
<td>28.1</td>
<td>1.08</td>
<td>2.63</td>
<td>28</td>
</tr>
<tr>
<td>Aminohexyl-Sepharose</td>
<td>58</td>
<td>8.1</td>
<td>18.3</td>
<td>2.26</td>
<td>5.51</td>
<td>18</td>
</tr>
<tr>
<td>Aca-54 gel filtration</td>
<td>16</td>
<td>2.8</td>
<td>11.4</td>
<td>4.07</td>
<td>9.92</td>
<td>11</td>
</tr>
<tr>
<td>Mono Q</td>
<td>5</td>
<td>1.4</td>
<td>8.8</td>
<td>6.29</td>
<td>15.34</td>
<td>9</td>
</tr>
</tbody>
</table>

60 g of wet weight mycelium was used.

Step 9—Finally, enzyme was separated to homogeneity by ion exchange chromatography on a Mono Q HR 5/5 column using a fast protein liquid chromatography system (Pharmacia). The buffer used was 20 mM Tris-HCl, pH 8, 10% (v/v) glycerol, 1 mM EDTA, and 4 mM DTE. A 90-min gradient from 0 to 0.2 M NaCl was applied, and 1 ml/min was collected (step 9).

Methods of Analyses

Protein was determined according to Bradford (1972) or to Warburg and Christian (1941). SDS-polyacrylamide gel electrophoresis was done according to Laemmli (1970). Silver staining of slab gels was performed according to Blum et al. (1987). The pl of the enzyme was determined by isoelectric focusing on Servalyt Precotes sheets (pH range of 4–7). Radioactive bands (adenylates) on thin-layer plates were localized by autoradiography with Konica x-ray films. Corresponding zones (0.5–2 cm²) were then scraped off the plates and placed into scintillation vials. Portions of 1 ml of 0.5 M ammonia and 8 ml of scintillation mixture for aqueous samples (Zinsser, Frankfurt, F.R.G.) were added. After thorough mixing samples were counted in a liquid scintillation counter. Calibration curves were established by mixing known amounts of radioactivity between 0.5 × 10⁻³ and 15 × 10⁻³ μCi with scapped silica gel (2 cm²). These standards were treated as the samples above. Charcoal filters with radioactive phosphorus were measured either by Cerenkov counting in the H-channel or by liquid scintillation counting as described.

RESULTS

Purification of 4-Methyl-3-hydroxyanthranilic Acid-AMP Ligase—4-Methyl-3-hydroxyanthranilic acid-AMP ligase was purified from S. chrysomallus strain X2-18. Table I shows the various steps of purification beginning with a Polymyxin P precipitation for removal of nucleic acids. The enzyme activity was monitored by the 4-MHA-dependent ATP-pyrophosphate exchange chromatography, and fast performance liquid chromatography on Mono Q (Pharmacia LKB Biotechnology Inc.) afforded the separation of the pure enzyme. Gel electrophoretic analysis of the various fractions under denaturing conditions are shown in Fig. 2. It can be seen that in the last purification step only one band is present which represents actinomycin synthetase I with an Mᵣ of 45,000. The yield of enzyme was 9%. Assuming that no loss of enzyme in the first two steps of the purification takes place, the culture of S. chrysomallus X2-18 contains 4-5 mg of enzyme/liter of culture, which is much higher than the 0.5-1 mg produced by the wild type S. chrysomallus strain. This high yield enabled us to purify enough enzyme for the study of its kinetic and thermodynamic properties.

Analysis of the Reaction—During this study and also when investigating other related chromophore-activating enzymes...
(Keller et al., 1984; Schlumbohm and Keller, 1990; Glund et al., 1990), we found that a series of chemically as well as enzymatically synthesized aromatic carboxylic acid adenylates are highly stable in aqueous solutions when compared with aminoacyl adenylates. In particular, 4-methyl-3-hydroxybenzoic acid adenylate (4-MHB-AMP) is a compound which decomposes very slowly under the assay conditions (less than 10% in 3 h at 28 °C) and is absolutely unaffected by the chromatographic procedures as illustrated by the thin-layer chromatograms in Fig. 5 which show the apparent stability of the 4-MHB-AMP in the incubation mixtures at various temperatures and time periods. There was no significant decomposition as revealed by the faint band represented by AMP. Also, 4-MHB has the highest $k_{cat}$ value of all substrates tested with actinomycin synthetase I and the highest $K_{eq}$ value in the adenylate formation reaction (Equation 1, a and b) (Table III). In vivo incorporation studies showed that it is the strongest competitor of the natural substrate 4-MHA (Keller, 1984). 4-MHA is oxidized by air to the corresponding phenoxazinone with a high rate, and this applies also to 4-MHA-AMP, which is not as stable as 4-MHB-AMP. We therefore chose 4-MHB as substrate for characterization of the enzyme.

Attempts were undertaken to isolate an actinomycin synthetase I 4-MHB-AMP complex as has been shown for ami-noacyl adenylates and their corresponding aminoacyl-tRNA synthetases. These form stoichiometric enzyme-ami-noacyl adenylate complexes (Eigner and Loftfield, 1972), which can be measured by binding of the charged enzymes to nitrocel-lulose or DEAE-paper filters (Yarus and Berg, 1970; Bartmann et al., 1976), by isolation through gel filtration (Allende and Allende, 1971), or by active site titrations with [γ-32P] ATP (Fersht et al., 1975; Mulvey and Fersht, 1977). Since the formation of enzyme-adenylate complexes has also been described for amino acid activating enzymes of non-ribosomal systems (Kleinkauf and Koischwitz, 1979; Billich et al., 1987), we tried to isolate a complex of 4-MHB·AMP and the ligase. However, no acyladenylate binding was detected either in filter binding or gel filtration experiments using Sephadex G 25 (fine) or Ultrogel Aca-202. By contrast, detailed analyses revealed the presence of 4-MHB adenylate in the low molecular weight range of the columns (not shown).

Therefore, formation of acyladenylate was measured by a thin-layer chromatographic assay that permits the estimation of total acyladenylate in the presence of ATP, carboxylic acid, and enzyme under incubation conditions (Jakubowski et al., 1977, Keller et al. 1984). Fig. 3 shows the time course of adenylate formation from 4-MHB at various enzyme concentrations and fixed substrate concentrations. It can be seen that, at each enzyme concentration, the amount of acyladenylate steadily increases with time until it reaches a plateau level which is the same in all three cases. Obviously, only the rate with which the plateau value is reached is dependent on enzyme concentration as is to be expected from an enzyme-catalyzed reaction. The result of this experiment precludes the existence of a stoichiometric ligase-acyl adenylate complex. Otherwise, for each enzyme concentration one should have observed an individual plateau value. Correspondingly, the plateau level is independent of the enzyme concentration but determined by the chemical equilibrium constant. Also from the shape of the curves an initial burst of adenylate formation as observed with, e.g., aminoacyl-tRNA synthetases cannot be seen. Such an initial burst would indicate the synthesis of a proportional amount of acyladenylate and simultaneous binding to the enzyme (Fersht et al., 1975).

In order to prove that the plateau value in Fig. 3 represented the adenylate concentration in the assay determined by the equilibrium constant $K_{eq}$ of the reaction and was not due to substrate exhaustion, $K_{eq}$ was determined according to Equation 1 in the presence of constant enzyme concentration at various initial substrate concentrations (Table II).

\[
4\text{-MHB} + \text{ATP} \xrightleftharpoons[k_1^-]{k_1^+} 4\text{-MHB-AMP} + \text{PP}_i \quad (1a)
\]

\[
\frac{[4\text{-MHB-AMP}][\text{PP}_i]}{[4\text{-MHB}][\text{ATP}]} = \frac{k_1}{k_1^-} = K_{eq} \quad (1b)
\]

In every case, the formation of 4-MHB-AMP had reached equilibrium at the latest after 45 min and did not change for at least 160 min. The constancy of $K_{eq}$ over large changes in initial concentrations of substrates such as 4-MHB, pyrophosphate, and ATP clearly indicates that the reaction obeys the law of chemical equilibrium. In addition, the fact that at sufficiently high initial substrate concentrations the amount of acyladenylate formed exceeds that of the enzyme present in the incubation confirms the independence of $K_{eq}$ on enzyme concentration. Data in Table II also show that with increasing 4-MHB concentration there is a constant decrease in $K_{eq}$ from 4.6–4.8 to 2.8–3.0 × 10⁻⁴. By contrast, changes in ATP concentration had a much less pronounced influence on $K_{eq}$. The reasons for these effects are not known. A possible explanation could be an increase of the activity coefficient of 4-MHB when increasing its concentration, which would result in a lower $K_{eq}$ due to the presence of [4-MHB] in the denominator of Equation 1b. In fact, the effect of 4-MHB becomes more severe at concentrations >100 μM where an additional decrease of $K_{eq}$ by 1 order of magnitude is observed (not shown).

**Thermodynamic Parameters of the Reaction**—The time course of 4-MHB-AMP formation was followed at temperatures ranging from 6 to 35 °C, and Arrhenius plots were constructed from initial rates at the individual temperatures. Fig. 4 shows that there is a straight line in the plot which revealed an activation energy $E_a$ of 10.6 kcal/mol. From the data given in Table II a standard free enthalpy change of the reaction $\Delta G^\circ$ (28 °C, 50 μM 4-MHB and 114.8 μM ATP) was
**Actinomycin Synthetase I from Streptomyces chrysomallus**

**Table II**

**Determination of 4-MHB-AMP and $K_{eq}$ at different initial concentrations of 4-MHB, ATP, and pyrophosphate**

Substrates at the indicated initial concentrations were reacted in the presence of 2.5 μM enzyme, 10 mM MgCl₂ at 28 °C for 1 h. The total volumes were 15 μl in every case and were applied at the end of the incubation to silica gel plates as described under “Materials and Methods.” For calculations of $K_{eq}$, [ATP] and [4-MHB] were estimated by subtracting [4-MHB-AMP] from initial [ATP] or [4-MHB] in millimolar. [PPi] was set equal to the sum of externally added [PPi] plus enzymically formed [PPi] = [4-MHB-AMP]. In those incubations with different initial concentrations of PPi, [PPi] was the sum of externally added [PPi] plus enzymically formed [PPi] = [4-MHB-AMP].

<table>
<thead>
<tr>
<th>Initial concentrations of ATP (μM)</th>
<th>Initial concentrations of 4-MHB (μM)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>31.5</td>
<td>1.27 μM</td>
<td>$K_{eq} = 4.6 \times 10^{-3}$</td>
<td>$K_{eq} = 3.7 \times 10^{-3}$</td>
<td>$K_{eq} = 2.06 \mu M$</td>
</tr>
<tr>
<td>64.8</td>
<td>1.76 μM</td>
<td>$K_{eq} = 4.6 \times 10^{-3}$</td>
<td>$K_{eq} = 2.24 \mu M$</td>
<td>$K_{eq} = 3.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>114.8</td>
<td>2.29 μM</td>
<td>$K_{eq} = 4.6 \times 10^{-3}$</td>
<td>$K_{eq} = 2.92 \mu M$</td>
<td>$K_{eq} = 3.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>181.5</td>
<td>2.87 μM</td>
<td>$K_{eq} = 4.8 \times 10^{-3}$</td>
<td>$K_{eq} = 3.59 \mu M$</td>
<td>$K_{eq} = 4.7 \times 10^{-3}$</td>
</tr>
<tr>
<td>514.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 4.** Time course of 4-MHB-AMP formation at different temperatures and determination of the activation energy $E_a$. △, 6 °C; □, 16 °C; ●, 28 °C; ○, 35 °C. Incubation mixtures (30 μl final volume) contained 50 μM MHB, 114.8 μM ATP, 10 mM MgCl₂, and 2.5 μM enzyme. At the indicated times, 5.5-μl aliquots from the reaction mixtures were applied to silica gel sheets for chromatography and determination of adenylate. The Arrhenius plot in the inset was constructed by plotting ln $k$ (Equation 1) versus 1/T. The slope is $-E_a/R$. $E_a$ is +10.6 kcal/mol.

![Graph showing time course of 4-MHB-AMP formation](image)

**FIG. 5.** Temperature dependence of $K_{eq}$. Incubation mixtures (15 μl total volume) contained 50 μM 4-MHB, 114.8 μM ATP, 10 mM MgCl₂, and 3.4 μM enzyme. After incubation at the indicated temperatures for 1–2.5 h, the mixtures were applied to silica gel sheets and chromatographed as described. Autoradiographs of the various chromatograms are shown on the right. Time of exposure to x-ray film was 1 week. $\Delta H^o$ was calculated by plotting $-\log K_{eq}$ versus 1/T and determining the slope which is $\Delta H^o/2.3 R$. $\Delta H^o$ is −3.7 kcal/mol.

![Graph showing temperature dependence of $K_{eq}$](image)

Calculated according to $\Delta G^o = -RT \ln K_{eq}$ to be +3.4 kcal/mol. This would indicate that the reaction is endergonic.

By contrast, measurements of the temperature dependence of acyladenylate formation revealed a decrease of the equilibrium constant with increasing temperature (Fig. 5). This would indicate an exothermic reaction. From the van’t Hoff plot a standard enthalpy change for the reaction, $\Delta H^o = -3.7$ kcal/mol was calculated, a value with the enthalpy change for the hydrolysis of ATP with −4 kcal/mol. Apparently, the opposite signs of $\Delta G^o$ and $\Delta H^o$ indicate a strong contribution by entropic factors.

The reaction itself was strictly enzyme-dependent, as was shown by incubating 4-MHB, ATP, chemically synthesized 4-MHB-AMP, and pyrophosphate in various ratios (using [14C]ATP or [32P]pyrophosphate) in the absence of enzyme. No spontaneous formation of adenylyl-ate or ATP was observed. The same result was obtained when other aromatic carboxylic acids and their adenylylates were tested (not shown).

From the Gibbs-Helmholtz equation $\Delta G^o = \Delta H^o - T \Delta S^o$ the standard entropy change of the reaction at 28 °C was found to be be negative with a value of −23.7 cal mol⁻¹ K⁻¹. A large part of the negative $\Delta S^o$ may result from a strong restriction in random motion of the substrates when forming the enzyme-substrate complex.

**Kinetic Parameters of the Reaction**—Determinations of $K_{eq}$ of 4-MHB and ATP by measuring initial rates in the acyladenylate formation reaction at various substrate concentrations revealed values of 6 and 110 μM, respectively. Several control experiments using inorganic pyrophosphatase (50...
units ml⁻¹) in identical assays indicated, that product formation in these experiments was affected by mass action by less than 5% provided that the amount of product formed in a given period of time had not reached more than 80% of the product level in the equilibrium. The $k_\text{m}$ value of 4-MHB as obtained from time course measurements of 4-MHB-AMP formation in V₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅₅¢
that no enzyme-acyladenylate complex could be isolated by a variety of techniques. Thus, this enzyme differs from well-known examples of single amino acids activating peptide synthetases such as gramicidin S-synthetase I (Vater and Kleinkauf, 1976) which activates the starter amino acid L-phenylalanine in the synthesis of the peptide antibiotic gramicidin S as an enzyme-thioester intermediate via the corresponding adenylate. Gramicidin S-synthetase I has a $M_r$ of 120,000 and thus is bigger than actinomycin synthetase I (45,000). Sequence analyses of the corresponding gene grsA (Krätschmar et al., 1989), the related tycA gene which codes for tyrocidin synthetase I (Weckermann et al., 1990), and the gene of the multifunctional $\alpha$-aminoacipyl-cysteinyl-valine synthetase (Diez et al., 1990; Smith et al., 1990), which codes for a single polypeptide chain activating and condensing 3 different amino acids indicate that a domain responsible for the activation of 1 amino acid within a multifunctional peptide synthetase has a size of approximately 1000 amino acid residues. This size of a domain fits well with the $M_r$ values determined in the case of the single amino acid activating synthetases gramicidin S-synthetase I and tyrocidin synthetase I. Whether the smaller size of actinomycin synthetase I, as compared with those of the latter two enzymes, reflects its inability to form an acyladenylate-enzyme complex and to charge itself with 4-MHA via thioester linkage remains to be seen when its gene sequence is available and can be compared with the other ones.

The quinoxaline-2-carboxylic acid activating enzyme and the 3-hydroxypropionic acid activating enzyme from chromopeptide lactone-producing streptomycetes are strongly related to actinomycin synthetase I in respect of their sizes and a number of physical and catalytic properties (Glund et al., 1990; Schlumbohm and Keller, 1990). It could also be demonstrated by equilibrium dialysis experiments that also these two enzymes did not bind their corresponding acyladenylates in a complex, which indicates that their role in the biosynthesis of the different peptide lactones might be the same.

Other aromatic carboxylic acid activating enzymes involve 4-coumarate-CoA ligase, the key enzyme in flavonoid and isoflavonoid biosynthesis in higher plants. It catalyzes the formation of 4-coumarate-coenzyme A from 4-coumaric acid coenzyme A with the consumption of ATP and has a $M_r$ of 60,000 (pansy) (Knobloch and Habibrock, 1975; Lozoya et al., 1988). ATP is cleaved to AMP and PP$\Pi$; however, it is not known whether the enzyme forms an enzyme-acyladenylate complex. Firefly luciferase, a 57-kDa enzyme from Photinus pyralis, catalyzes the adenylation of the aromatic carboxylic acid luciferin but forms a stable enzyme-acyladenylate complex, acyladenylate binding being essential for the subsequent oxidation of luciferin in the formation of light (DeLuca and Elroy, 1978; Wood et al., 1985; de Wet et al. 1988). These data clearly show that these enzymes differ from actinomycin synthetase I in respect of reaction mechanism and product binding, respectively.

The entE gene product from Escherichia coli is a 2,3-dihydroxybenzoic acid-AMP ligase (Rusnak et al., 1989) involved in the biosynthesis of enterobactin, an iron chelating cyclic chromopeptide of $E. coli$. Enterobactin consists of three residues of serine which form a trilactone. To the free amino groups of the ring are attached 2,3-dihydroxybenzoic acid residues in an amide-like fashion. This constellation is very similar to the positions of 4-MHA in the actinomycin half-molecule (Fig. 1) or of heteroaromatic carboxylic acid residues in the quinoxaline antibiotics and etamycin (Schlumbohm and Keller, 1990). In these molecules, like in enterobactin, the aromatic carboxylic acid is always attached to the amino group of a hydroxyamino acid such as serine or threonine. 2,3-Dihydroxybenzoic acid-AMP ligase ($M_r$ 57,000) may play a similar role in enterobactin synthesis as does actinomycin synthetase I in actinomycin biosynthesis. However, Rusnak et al. (1989) reported that the isolation of an enzyme-adenylate complex of 2,3-dihydroxybenzoic acid-AMP ligase and also the evaluation of stoichiometric ratios revealed the formation of a 2,3-dihydroxybenzoic acid-enzyme thioester. Therefore, the mechanism of aromatic carboxylic acid activation and peptide formation in the case of enterobactin synthesis in $E. coli$ would differ from the corresponding steps in chromopeptide lactone biosynthesis in the streptomycetes. It is noteworthy to point to the fact that actinomycin synthetase I activates 2,3-dihydroxybenzoic acid although only to a much less extent and also that the $K_m$ values of actinomycin synthetase I and the entE enzyme in respect of their most efficient substrates are very similar (0.44 compared with 0.41). We believe that the two enzymes should carry homologous sequences in, at least, the regions comprising their active sites.

The high stability of benzene carboxylic acid adenylates made it possible to study the thermodynamic parameters of the activation reaction in detail. From Table III it is evident that $K_m$ for all substrates tested is below 1 and thus $\Delta G^\circ$ is positive. From a thermodynamic point of view, $\Delta G^\circ$ is a measure for substrate reactivity (Hinz et al., 1975). 4-MHB in this respect is the most reactive substrate with the lowest positive $\Delta G^\circ$. This behavior can be explained by the fact that the enzyme from its structure greatly favors 4-MHB as an analogue of the natural substrate 4-MHA due to the presence of the 4-methyl-group which, for example, is missing in 3-hydroxyanthranilic acid (3-HA) or 3-hydroxybenzoic acid (3-HB). The different contributions of substituents in the 3- or 4-position to enzyme reactivity become clear when one compares the $K_m$ values of 4-methylbenzoic acid, 4-hydroxybenzoic, 3-methylbenzoic acid, and 3-hydroxybenzoic acid (Table III). Such contributions may account for the negative entropy change of the reaction due to strong steric interactions during the formation of the enzyme-substrate complex. The $K_m$ of the activation reaction of 4-MHA could not be determined by technical reasons; however, we believe that it is not greater than that of 4-MHB or even 3-HA. One argument is that the steric and ionic effect of the 2-amino group at the benzene nucleus does not contribute strongly to an increase in the equilibrium constant (Table III); a second is that the $K_m$ values of 4-MHA is less than 4-MHB. $k_{cat}$ is proportional to $k_1$ (Equation 1) and hence to $K_m$. It may also be noted that the $k_{cat}$ values presented here are low if one compares them with rate constants for adenylate synthesis by aminoaoyl-tRNA synthetases. As far as possible, we roughly estimated the rates to be 10 times lower in the case of isoleucyl-tRNA synthetase from $E. coli$ (Jakuwoba et al., 1976) or valyl-tRNA synthetase from Bacillus stearothermophilus (Fersht, 1975). In the case of tyrosyl-tRNA synthetase from B. stearothermophilus, Fersht (1975) reported a $K_m$ of 15 times higher than actinomycin synthetase I in the acyladenylate formation reaction. The relatively low rate constants of actinomycin synthetase I may be related to its low substrate specificity and also to the absence of an enzyme-adenylate complex, whereas tRNA synthetases possess a high substrate specificity and affinity for the aminoaoyl adenylate, because this serves as a substrate in the process of tRNA charging.

In kinetic terms 4-MHA is the best substrate with the lowest $K_m$ more than 1 order of magnitude lower than that of 4-MHB, which may indicate that the rate of enzyme-substrate...
complex formation for 4-MHA is the highest of all substrates tested. Of course, for $K_{n}$ itself and, hence, the amount of adenylate formed in the equilibrium this does not play a role; however, it is most important for the substrate specificity of the enzyme as expressed by $k_{cat}/K_{n}$ (Table III) in the overall biosynthetic process of peptide lactone formation in the cell in which the equilibrium will never be established due to the continuous consumption of the adenylate and the presence of inorganic pyrophosphatase.

This would also explain why 4-MHA is the precursor of the actinomycin chromophore and not 3-HA. 3-HA is the direct precursor of 4-MHA (Fawaz and Jones, 1988) and could erroneously be activated by the enzyme, hence giving rise to the formation of actinomycin with missing methyl groups in the chromophore. The methylation of 3-HA converts it to a compound (4-MHA) with a $K_{n}$ of 0.4 $\mu$M compared with 18 $\mu$M for 3-HA. Since both 3-HA and 4-MHA are toxic, the methylation of 3-HA will afford a down regulation of intracellular level of 3-HA to 4-MHA in the submicromolar level. Finally 4-MHA is consumed in the biosynthesis of actinomycin. Eventually, this process may represent a detoxifying mechanism in the producing organism.

Acknowledgments—We thank Andreas Pahl and Rainer Zocher for valuable discussions and Sandor Biro for reading the manuscript.

REFERENCES


Fersht, A. R. (1975) Biochemistry 14, 5–12


