4-Methyl-3-hydroxyanthranilic Acid Activating Enzyme from Actinomycin-Producing *Streptomyces chrysomallus*†

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ABSTRACT: A 4-methyl-3-hydroxyanthranilic acid (4-MHA) activating enzyme was purified 24-fold from a crude protein extract of *Streptomyces chrysomallus*. The enzyme catalyzes both 4-MHA-dependent ATP/PP_i exchange and the formation of the corresponding adenylate. No AMP was formed during the reaction, indicating that no covalent binding of 4-MHA takes place. Besides 4-MHA, the enzyme also catalyzes the formation of adenylates from 3-hydroxyanthranilic acid (3-HA), anthranilic acid (AA), benzoic acid (BA), 3-hydroxybenzoic acid (3-HB), 4-methyl-3-hydroxybenzoic acid (4-MHB), 4-methyl-3-methoxybenzoic acid (4-MMB), and 4-aminobenzoic acid (4-AB). No such adenylates were formed from 2-aminophenol (2-AP), 2-hydroxybenzoic acid (2-HB), 3-hydroxykynurenine (3-HK), and tryptophan (Trp). 3-HA, 4-MHB, and 4-AB were among the structural analogues of 4-MHA that were the most effective for adenylate synthesis. In the case of 3-HA, considerable AMP release was observed, most probably due to nonenzymatic hydrolysis of the corresponding adenylate. A molecular weight between 53 000 and 57 000 was estimated. The specific activity of the enzyme was correlated with the titer of antibiotic in the cultures, and feeding experiments with whole mycelium of *S. chrysomallus* showed that 4-MHB was a strong inhibitor of actinomycin synthesis in vivo. The data strongly suggest that the enzyme is involved in the biosynthesis of actinomycin.

The compound 4-methyl-3-hydroxyanthranilic acid (4-MHA) is the precursor of the chromophore moiety of the actinomycins (Weissbach et al., 1965). These pentapeptide lactone antibiotics are synthesized by members of the genus *Streptomyces* (Figure 1a). It is well established that in *Streptomyces antibioticus*, 4-MHA (Figure 2) is derived from tryptophan via the kynurenine pathway (Weissbach et al., 1965; Perlman et al., 1973). The methyl group in the molecule is donated by methionine (Sivak et al., 1962). Low levels of free 4-MHA could be detected in mycelium and protoplasts of *S. antibioticus* (Weissbach et al., 1965; Keller & Kleinkauf, 1977), and also, a mutant of another actinomycin-producing strain, *Streptomyces parvulus*, excreting large amounts of 4-MHA has been isolated (Troost & Katz, 1979).

These findings led us to assume that 4-MHA as a free intermediate should play an active role in the formation of 4-MHA pentapeptide lactones (Figure 1b), which have been claimed to be condensed by action of phenoxazinone synthase as in *S. antibioticus* (Katz et al., 1962; Salzman et al., 1969). The mechanism of the synthesis of the yet hypothetical 4-MHA pentapeptide is unknown due to the lack of a cell-free system of actinomycin synthesis, but in vivo data indicate that it should resemble other well-documented examples of nonribosomal peptide synthesis (Katz, 1967; Kleinkauf, 1979). Compared to the known reaction sequence in gramicidin S synthesis (Lipmann, 1971), the activation reaction of 4-MHA should be an important step that either initiates the synthesis of the 4-MHA peptide or leads to the acylation of a preformed peptide with 4-MHA.

We describe here the isolation and characterization of an enzyme fraction from *Streptomyces chrysomallus* catalyzing the formation of the 4-MHA adenylate. In addition, data from feeding experiments with a 4-MHA substrate analogue will support the idea that the enzyme is involved in the process of 4-MHA peptide and, hence, actinomycin synthesis in *S. chrysomallus*.

Materials and Methods

Radioisotopes and Chemicals. [U-^{14}C]Adenosine triphosphate (586 Ci/mol), L-[U-^{14}C]valine (285 Ci/mol), L-[U-^{14}C]threonine (232 Ci/mol), L-[*methyl*-^{14}C]methionine (56.7 Ci/mol), and L-[U-^{14}C]proline and L-[G-^{3}H]tryptophan (5.4 Ci/mmol) were obtained from the Radiochemical Centre; [α-^{32}P]adenosine triphosphate (37 Ci/mmol) and tetrasodium [^{32}P]pyrophosphate (2.9 Ci/mmol) were purchased from New England Nuclear Corp. 3-Hydroxyanthranilic acid, anthranilic acid, 4-aminobenzoic acid, 2-hydroxybenzoic acid, benzoic acid, and 2-aminophenol were from Merck, Darmstadt. 4-Methyl-3-hydroxy-2-nitrobenzoic acid, 4-methyl-3-hydroxybenzoic acid, and 4-methyl-3-methoxybenzoic acid were from Ega-Aldrich Chemical Co. 3-Hydroxybenzoic acid was obtained from Fluka and 3-hydroxykynurenine from Sigma. Adenosine monophosphoric acid (from yeast) was from Sigma. Adenosine triphosphate disodium salt and DNase I (grade II) were from Boehringer Mannheim. All other chemicals were of the highest purity commercially available.

Syntheses. 4-Methyl-3-hydroxyanthranilic acid was prepared by catalytic hydrogenation of 4-methyl-3-hydroxy-2-nitrobenzoic acid with Pd/H_2 (courtesy of Dr. Z. Palacz from this institute). The reaction product was isolated as the hydrochloride and was chromatographically pure in solvent systems I, IV, and V; mp 225–227 °C dec. A mass spectrometric analysis of the compound revealed its identity (courtesy of Dr. G. Bojesen, Institut für Organische Chemie, Technische Universität Berlin).

The adenylates of 4-methyl-3-hydroxyanthranilic acid (4-MHA), 3-hydroxyanthranilic acid (3-HA), and 4-methyl-3-hydroxybenzoic acid (4-MHB) were prepared by a combination of the procedures developed by Berg (1958) and Moldave et al. (1959): 0.2 mmol of acid hydrochloride or free acid (4-MHB) each was mixed with 0.2 mmol of AMP, 0.32 mL of cold H_2O, and 1.04 mL of pyridine in a 10-mL glass-stoppered flask. After addition of 5 mmol of dicyclohexylcarbodiimide (DCCI) dissolved in 1.2 mL of pyridine, each flask was shaken vigorously in the cold (2 °C) for 6 h. The reaction mixtures were then filtered in the cold through glass-fiber filters, and the filter cakes of dicyclohexylurea were washed with 2 mL of cold 75% pyridine. On standing on ice,

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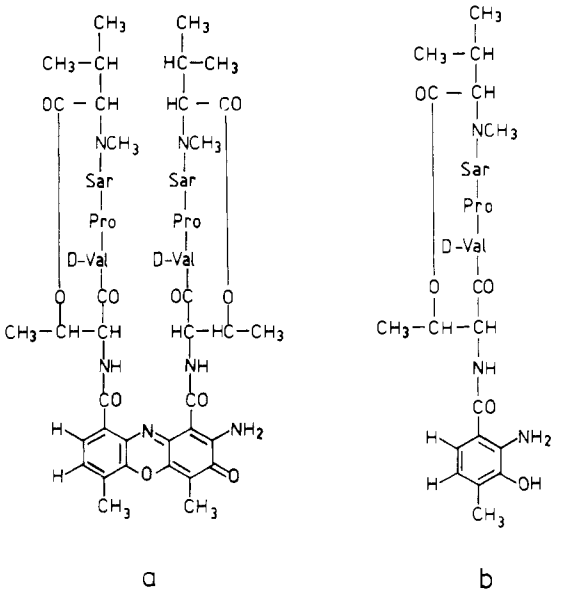

FIGURE 1: (a) Structural formula of actinomycin C_1: D-Val (D-valine), Pro (L-proline), Sar (sarcosine = *N*-methylglycine). (b) Structural formula of the corresponding 4-methyl-3-hydroxyanthraniloyl pentapeptide lactone.

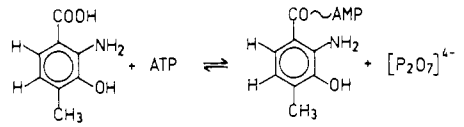

FIGURE 2: Proposed reaction scheme of activation of 4-methyl-3-hydroxyanthranilic acid (4-MHA).

the combined filtrate and washing separated into two layers. The lower layer was added to 20 mL of acetone at −20 °C. The precipitate was washed by centrifugation with two portions of 20 mL of acetone (−20 °C). After being allowed to drain for a few minutes, the precipates were leached with 5-mL portions of ethylene glycol monomethyl ether. In the case of the 4-MHB adenylate preparation, nearly all of the precipitate dissolved, whereas the 4-MHA and 3-HA adenylates were much less soluble. Therefore, the leaching procedure had to be repeated (6 times; in the case of 4-MHB twice).

The combined extracts of the adenylates were kept at −20 °C until prior to use. Usually, 1–2 mL of the adenylate solution was removed, and a 5-fold volume of diethyl ether (−20 °C) was added. The precipitate was washed with the same volume of ether by centrifugation, and the pellet was taken up in 50–100 μL of cold buffer A. The concentration of the adenylate solution was estimated by measuring the OD_{260}, taking a 1:1 mixture of acid and AMP as standard. The aqueous solutions of adenylates had to be used up quickly because of instability of the compounds. TLC[1] on silica gel plates of the freshly prepared aqueous solutions of the adenylate preparations in solvent system I at 4 °C revealed the following R_f values: AMP, 0.05–0.1; 4-MHA adenylate, 0.30–0.32; 3-HA adenylate, 0.28–0.30; 4-MHB adenylate, 0.35–0.38; 3-HA and 4-MHA, 0.8–0.9; MHB, 0.9. Both the 4-MHA and 3-HA adenylate preparations contained a trace band with slightly higher R_f value, presumably representing the 2',3'-hydroxyl esters of the two acids having the same blue fluorescence in the UV like these, whereas the adenylates show a green-blueish fluorescence. Besides by their R_f values, which differ from those of AMP and the acids, the adenylates were identified by several further criteria established by Berg (1958) and Moldave et al. (1959): On addition of 1 N NaOH to the adenylates, release of AMP and free acids occurred. Addition of 5 M salt-free hydroxylamine, pH 6.8, yielded AMP and yet uncharacterized compounds, presumably the hydroxamic acids. At pH 3.1, the 4-MHA and 3-HA adenylates were slightly cationic and moved to the cathode in the electric field. 4-MHA and 3-HA moved much faster.

The data were obtained by TLC of the compounds and their degradation products. Adenylates could also be separated by HPLC, where the 4-MHA and 3-HA adenylates could be eluted from a Lichrosorb RP-8 column with 16–18% acetonitrile in 0.1 M potassium phosphate, pH 2.1. The ethylene glycol monomethyl ether solutions of adenylates could be kept at −20 °C for 2 months without significant degradation of the compounds.

Enzyme Assay. The buffer in all assays was buffer A. The ATP/PP_i exchange reaction was measured by a modification of the method of Lee & Lipmann (1975): In a total volume of 210 μL, 100 μL of enzyme, 3×10^5 cpm tetrasodium [^{32}P]pyprophosphate, 1 μmol of $MgCl_2$, 0.5 μmol of ATP, 0.01 μmol of tetrasodium phosphate, and 0.1 μmol of 4-MHA or structural analogue were incubated for 10 min at 26 °C. The reaction was stopped by the addition of 0.5% norit charcoal suspension, and radioactive ATP was determined as described.

The assay for formation of adenylate was performed by incubating 30–70 μL of enzyme in total volumes of 50–100 μL in the presence of either 10^6 dpm [^{32}P]ATP or 3×10^5 dpm [^{14}C]ATP, up to 100 nmol of unlabeled ATP, 0.5 μmol of $MgCl_2$, and 10–50 nmol of 4-MHA or structural analogues for 10 min at 26 °C. Aliquots of 10–50 μL from the reaction mixtures were applied to silica gel plates (Merck), with use of warm air to accellerate drying. Development of the plates was done as described below. Alternatively, reaction mixtures could also be applied to PEI-impregnated cellulose sheets (Macherey-Nagel, Düren, FRG), and these were developed as described below.

Formation of radioactive ATP from [^{32}P]pyrophosphate and synthetic adenylate was measured in a total volume of 200 μL containing 8×10^5 cpm [^{32}P]pyrophosphate, 50–150 nmol of adenylate, and 70–130 μL of enzyme. After 10 min at 26 °C, portions from the reaction mixture were given into 1 mL of charcoal suspension. After repeated washing with distilled water, radioactive ATP was eluted from the charcoal with 10% pyridine. After evaporation to dryness, the residue was taken up in 20 μL of water and applied to a PEI–cellulose sheet, and this was developed with solvent system II. The additional formation of ADP in this assay could strongly be reduced, when unlabeled pyrophosphate was present in 0.2–1 mM concentrations.

Enzyme Purification. All operations were carried out at 0–4 °C and should not exceed a total length of 5 h. (Step 1) Buffer A contains 50 mM potassium phosphate, pH 6.8, 5 mM DTE, and 1 mM PMSF. Some 48-h-old mycelium of *S. chrysomallus* from 200 mL of medium S (20–30 mg of actinomycin/L of culture) was harvested by suction on a Büchner funnel and washed with distilled water. Generally, 6 g (wet wt) of mycelia was suspended with 6 mL of buffer A. The paste was passed through a French press (Aminco) with 10 000 psi, and to the homogenate was added 0.4 mL of 1 M $MgCl_2$. After this was mixed, a spatula tip of DNase I was added, and the suspension was left on ice for 1 h. During this period, occasional gentle shaking supported the decrease in viscosity. The suspension was then centrifuged for 30 min at 36000*g* in

[1] Abbreviations: DTE, dithioerythritol; PMSF, phenylmethanesulfonyl fluoride; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; PEI, poly(ethylenimine).

a Sorvall RC-2B centrifuge (rotor SS-34). (Step 2) The supernatant (9–11 mL) was applied to a DEAE-cellulose column (Whatman DE-52, bed dimensions 1 × 6.5 cm) previously equilibrated with Buffer A, and the protein peak appearing was collected (10–12 mL). For detection of activity, 2 mL of this fraction was desalted on an Ultrogel AcA-202 column (bed dimension 1 × 11 cm) and then tested. (Step 3) A total of 2.8 mL from the DEAE fraction was applied onto an Ultrogel AcA-44 (LKB) column (bed dimension 1.8 × 47 cm) previously equilibrated with buffer A. Fractions of 2.9 mL were collected and assayed for the presence of 4-MHA activating enzyme. The peak fractions were pooled and used for characterization of enzyme.

Specific Activity. Specific activity is defined in picokatals (pkat). One picokatal is the amount of enzyme catalyzing the incorporation of 1 pmol of pyrophosphate into ATP/s under conditions described above.

Molecular Weight Determination. The molecular weight of the active enzyme was determined by gel filtration on Ultrogel AcA-44 (bed dimension 1 × 62 cm) in buffer A. The column was calibrated with bovine serum albumin (66 000), ovalbumin (45 000), chymotrypsinogen (25 000), and lysozyme (14 300).

Protein Concentrations. These were determined according to Bradford (1976).

Thin-Layer Chromatographic Analyses. Enzymatically formed and synthetic adenylates were routinely chromatographed on silica gel plates (Merck) with BuOH/HOAc/H_2O (4:1:1) (solvent system I) at 4 °C (Jakubowsky et al., 1977). Visualization of synthetic adenylate was done by detection of their fluorescence with long- (4-MHA and 3-HA) and short- (4-MHB) wave UV. Radioactive adenylates or other metabolites were detected by autoradiography with Kodak X-ray film X-Omat. Alternatively, PEI-impregnated cellulose sheets were used for chromatography of adenylates and developed with either 1.2 M LiCl (solvent system II) or 0.2 M NH_4HCO_3 (solvent system III) at 4 °C. 4-MHA and 3-HA were chromatographed on silica gel in either toluene/AcOH (3:1, solvent system IV) (Keller & Kleinkauf, 1977) or $CHCl_3$/AcOH (9:1, solvent system V) (Ochi, 1982). Actinomycins were chromatographed on silica gel in EtOAc/MeOH/H_2O (100:5:5, solvent system VI) (Keller & Kleinkauf, 1977). High-voltage electrophoresis of adenylates on cellulose plates (Macherey-Nagel, Cel 300) was done at 25 V/cm in 0.02 M sodium citrate, pH 3.1 (Berg, 1958).

High-Pressure Liquid Chromatography. HPLC of adenylates was performed on a Knauer HPLC system with a Lichrosorb RP-8 (Pharmacia) column, which was run with 0.1 M potassium phosphate, pH 2.1. Adenylates were eluted with a linear gradient of acetonitrile in the same buffer from 0 to 40% (U. Keller, unpublished data).

Radioactivity Measurements. Radioactivity was determined with a Tricarb liquid scintillation counter (Packard Instruments). In the case of ^{32}P-labeled compounds on TLC plates, radioactive zones were scraped off the plates and, after addition of 1–2 mL of water to the silica gel, were directly counted with Bray's solution (Bray, 1960). Counts were corrected for counting efficiency with freshly prepared standards of ^{32}P-labeled compounds on TLC plates with known radioactivity. Charcoal filters were counted under a toluene-based scintillation fluid. In the case of ^{14}C-labeled compounds on TLC, these were eluted with suitable solvents from scraped silica gel and then counted.

Methods of Cultivation. S. chrysomallus 11523 was obtained from the American Type Culture Collection. It was maintained on agar slants and kept as spore suspensions as described previously (Keller et al., 1982). Submerged culture was done in 250-mL flasks with steel springs containing 50 mL of medium S (Okanishi et al., 1974) at 30 °C and 250 rpm in a controlled environmental shaker (New Brunswick Instruments, Model G-25).

Short-Term Labeling Experiments. Portions of a 48-h-old culture of *S. chrysomallus* were centrifuged and washed with physiological saline. After resuspension in the same volume of 50 mM potassium phosphate, pH 6.8, 5-mL portions were transferred into 25-mL Erlenmeyer flasks. After addition of 2 μCi of labeled amino acids, the flasks were incubated with shaking for indicated periods. After filtration through cotton, neutral and acidic EtOAc extracts were prepared as described (Ochi, 1982) and subjected to chromatography.

Table I: Purification of 4-MHA Activating Enzyme[a]

step	vol (mL)	total protein (mg)	total act. (pkat)	sp act. (pkat/mg)	yield (%)
(1) crude extract[b]	10	270	*c*	*c*	*c*
(2) DEAE-cellulose	10.8	73.5	83.2	1.13	100
(3) AcA-44	40.5 (10.5)[d]	13.9	103.7	7.4	124

[a] Based on 3-HA-dependent ATP/PP_i exchange. [b] A total of 6 g of mycelium (wet wt) was used. [c] Activity of the enzyme could not be estimated in the crude extract due to nonspecific ATP cleaving activity. On the basis of the assumption that the crude extract contains the same amount of enzyme as the DEAE fraction, the entire purification is 24-fold. [d] Correction was made for 10.8 mL of step 2 since only 2.8 mL from this step was applied to the AcA-44 column.

Results

Protein Purification. Adenylate formation in ribosomal and nonribosomal systems is commonly measured by the ATP/pyrophosphate exchange reaction (Eigner & Loftfield, 1974). According to the postulated reaction scheme for MHA activation (Figure 2), we first used this reaction for detection of the MHA activating enzyme.

As can be seen (Table I), the 4-MHA activating enzyme (monitored by 3-HA-dependent ATP/PP_i exchange) could be purified about 24-fold in two steps from the supernatant of a cell homogenate. During the DEAE-cellulose step, 73% of total protein was removed, including all nonspecific ATP/PP_i exchange activity, whereas in the AcA-44 step a 7-fold increase of specific activity was obtained. The latter separation is shown in Figure 3. The activity resided in fractions 22–26, and the nonspecific activity was less than 4% of the total. Normally, fractions 23–25 were pooled and served for studies of adenylate formation, since further purification was not possible (see below).

Adenylate Synthesis. The purified enzyme was incubated with 4-MHA and [^{14}C]ATP or [^{32}P]ATP as described under Materials and Methods. Aliquots from the reaction mixtures were applied to thin-layer plates and chromatographed as described. Figure 4 shows that on such chromatograms (solvent system I) a radioactive band appeared due to enzymatic action, which was missing in the parallel experiment without 4-MHA. The R_f value of this band was 0.30–0.32. In other chromatographic systems (PEI–cellulose, solvent systems II and III), the same band could also distinctly be determined. Cochromatography of this compound and chemically synthesized 4-MHA adenylate always showed comigration. Also, treatment of the radioactive compound with NaOH or hydroxylamine resulted in the liberation of radio-

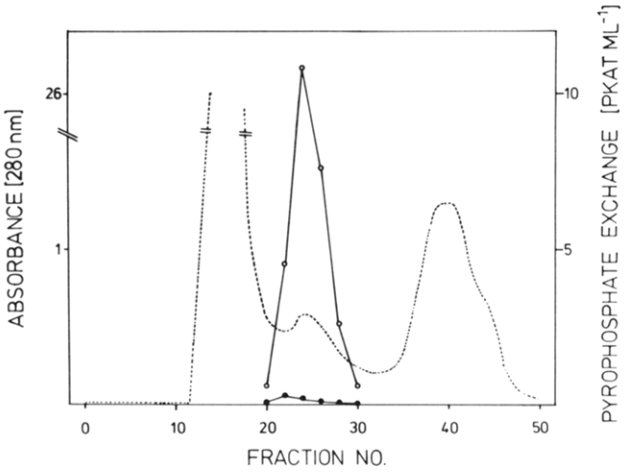

FIGURE 3: Gel filtration of 2.8 mL of the DEAE-cellulose enzyme fraction on Ultrogel AcA-44 (1.8 × 47 cm). The flow rate was 0.6 mL/min; 2.9-mL fractions were collected. (---) Absorbance at 280 nm; (○) 3-HA-dependent ATP/PP_i exchange; (●) nonspecific ATP/PP_i exchange.

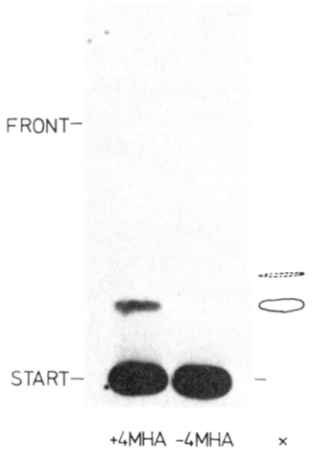

FIGURE 4: Formation of the 4-MHA [^{32}P]adenylate catalyzed by the 4-MHA activating enzyme. A total of 75 μL (0.2 pkat) was incubated together with 1 nmol of [α-^{32}P]ATP, 50 nmol of MHA, and 500 nmol of $MgCl_2$ in a total volume of 100 μL for 10 min at 26 °C. A 30-μL aliquot from this mixture was applied to silica gel and chromatographed as described under Materials and Methods. Exposure of the chromatogram to X-ray film was for 24 h. The right lane (×) shows cochromatography of the chemically synthesized 4-MHA adenylate.

active AMP. Further evidence for the identity of the radioactive and chemically synthesized compound came from experiments where the enzyme was incubated together with the chemically synthesized 4-MHA adenylate and [^{32}P]pyrophosphate (Figure 5). The formation of ATP can be clearly seen when the 4-MHA adenylate is present. In the control with AMP and 4-MHA instead of the 4-MHA adenylate, no ATP was formed. The same experiments with the 3-HA or 4-methyl-3-hydroxybenzoic acid adenylates (4-MHB, see below) gave the same result (not shown).

Substrate Specificity. The enzyme was tested against a variety of compounds structurally related to 4-MHA. The results are shown in Figure 6. Besides 3-HA (as mentioned before), the enzyme catalyzes the formation of adenylates from anthranilic acid (AA), benzoic acid (BA), 3-hydroxybenzoic acid (3-HB), 4-methyl-3-hydroxybenzoic acid (4-MHB), 4-methyl-3-methoxybenzoic acid (4-MMB), and 4-aminobenzoic acid (4-AB). No such adenylates were detectable in the presence of 2-hydroxybenzoic acid (2-HB), 3-hydroxykynurenine (3-HK), tryptophan (Trp), and, of course, 2-aminophenol (2-AP). The relative amounts of the different

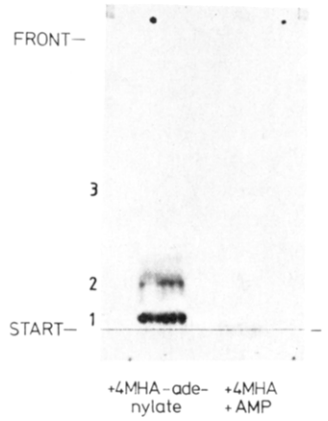

FIGURE 5: Enzymatic synthesis of ATP from the 4-MHA adenylate and pyrophosphate. A total of 120 μL of enzyme (1.6 pkat) was incubated with 750000 cpm [^{32}P]pyrophosphate, 0.1 μmol of unlabeled pyrophosphate, and 125 nmol of the MHA adenylate in a total volume of 200 μL. The control contained 125 nmol of MHA and 125 nmol of AMP instead of adenylate and the same amount of enzyme. After 10 min of incubation, 1 mL of charcoal suspension was added, and the radioactive ATP was recovered as described. Chromatography on PEI–cellulose was done in solvent system II. Exposure of the chromatogram to X-ray film was for 72 h. Numbers represent positions of cochromatographed ATP (1), ADP (2), and AMP (3).

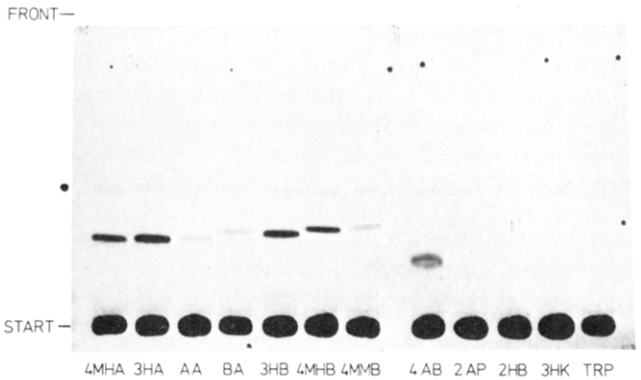

FIGURE 6: Autoradiogram of a thin-layer chromatogram of various reaction mixtures containing different structural analogues of 4-MHA. A total of 65 μL of enzyme (0.96 pkat) was reacted with 2 nmol of [^{14}C]ATP, 500 nmol of $MgCl_2$, and 50 or 100 nmol of structural analogue in a total volume of 100 μL. After incubation for 10 min, 50 μL from each reaction mixture was applied to silica gel plates and chromatographed. The fastest moving band in each track is the corresponding adenylate. Time of exposure to X-ray film was 48 h. Abbreviations and substrate concentrations: 4-MHA, 4-methyl-3-hydroxyanthranilic acid, 0.5 mM; 3-HA, 3-hydroxyanthranilic acid, 0.5 mM; AA, anthranilic acid, 1 mM; BA, benzoic acid, 1 mM; 3-HB, 3-hydroxybenzoic acid, 1 mM; 4-MHB, 4-methyl-3-hydroxybenzoic acid, 0.5 mM; 4-MMB, 4-methyl-3-methoxybenzoic acid, 1 mM; 4-AB, 4-aminobenzoic acid, 0.5 mM; 2-AP, 2-aminophenol, 1 mM; 2-HB, 2-hydroxybenzoic acid, 1 mM; 3-HK, 3-hydroxykynurenine, 1 mM; TRP, tryptophan, 1 mM. The band indicated by a dot represents a radiochemical impurity usually present in commercial preparations of [^{14}C]ATP.

adenylates formed under slightly varied conditions (see legend Figure 6) and the corresponding ATP/PP_i exchange values are given in Table II. The data clearly reveal that with respect to adenylate formation 3-HA gives the highest value, followed by 3-MHB and 4-MHA. In the exchange reaction, 3-MHB was more effective than the other two substrates. Interestingly, 4-aminobenzoic acid yields a considerable amount of adenylate, although being rather weak in the ATP/PP_i exchange reaction. Measurements of the time course of adenylate formation from 4-MHA and 3-HA revealed that at 3-HA concentrations of 0.1–0.5 mM, adenylate formation reached a plateau after 4–6 min (0.5 mM ATP). In the case of 4-MHA under the same

Table II: Formation of Adenylates from Structurally Related Analogues of 4-MHA and the Corresponding Rates of ATP/PP_i Exchange[a]

substrate	adenylate formed[b] (pmol)	pyrophosphate exchanged (pmol)
4-MHA	22.3	648
3-HA	33.7	887
AA	1.4	3.3
BA	1.6	13.3
3-HB	1.8	270
4-MHB	21	1352
4-MMB	2.1	194
4-AB	18.2	49

[a] A total of 35.2 μL (17.8 μg, 26.8 pkat/mg of protein) was reacted together with 50 nmol of substrate, 0.3 nmol of [^{14}C]ATP, and 250 nmol of $MgCl_2$ in a total volume of 50 μL as described under Materials and Methods. [b] During a 10-min incubation.

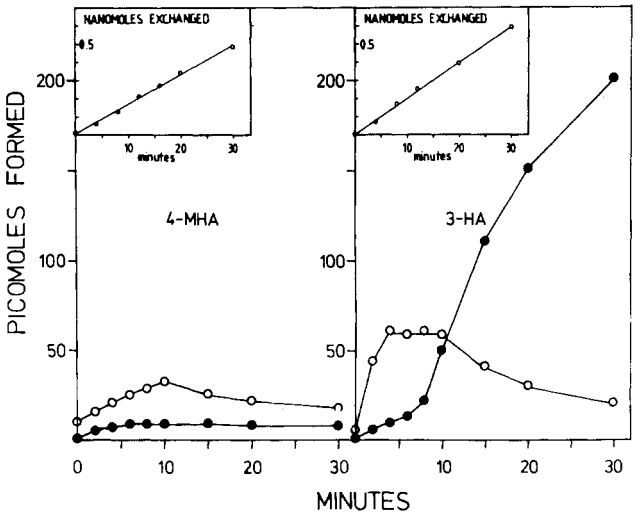

FIGURE 7: Time course of adenylate formation with respect to 4-MHA and 3-HA: (○) adenylate; (●) AMP; (inset) ATP/PP_i exchange. A total of 60 μL of enzyme (0.5 pkat) was mixed with 50 nmol of [^{32}P]ATP, 500 nmol of $MgCl_2$, and 400 nmol of 4-MHA or 300 nmol of 3-HA in a total volume of 100 μL. At the indicated times, 10-μL aliquots were removed and applied to silica gel plates. When the last aliquot had been applied, the plate was developed as described. Zones of [^{32}P]adenylates and [^{32}P]AMP were scraped off the plates and counted.

conditions, still after 20 min a net increase in adenylate synthesis was measured. Only when 4-MHA concentrations >1 mM were present, a maximum level of adenylate could be attained after 10 min as is depicted in Figure 7. Also under these conditions, the 3-HA adenylate concentration reached the plateau after 4–6 min (right side of Figure 7), and it is clear that 3-HA yields nearly twice as much adenylate than 4-MHA. AMP release started after 6–8 min and showed a dramatic increase up to the 30th minute. By contrast, the amount of AMP formed in the presence of 4-MHA did not significantly exceed the normal background level of AMP, which is a consequence of the chromatographical procedures. From the data, it appears that due to the lower rate of synthesis the 4-MHA adenylate is tighter bound to the enzyme than the 3-HA adenylate, which seems to dissociate from the enzyme with considerable subsequent hydrolysis. Most of the dissociated 3-HA adenylate seems constantly to be replenished through enzymatic action, as indicated by the enormous increase in the AMP concentration.

The decrease of adenylate concentration that is detectable after 15 min for both acids was found to be a consequence of

Table III: Inhibition of Actinomycin Synthesis in *S. chrysomallus* by 4-Methyl-3-hydroxybenzoic Acid (4-MHB) and D-Valine[a]

radiolabel	[^{14}C]actinomycin formed: control (cpm)	4-MHB (cpm): 0.5 mM	1 mM	2 mM	2 mM D-Val (cpm)
L-[*methyl*-^{14}C]methionine	29790	5226	2499	1071	8523
L-[U-^{14}C]threonine	6210	1808	624		
L-[U-^{14}C]valine	4424	827	390		

[a] Mycelial suspensions were prepared as described under Materials and Methods. After reincubation with inhibitor at the indicated concentration for 1 h, radiolabel was added, and incubation was continued for a further 90 min. After this period, mycelia were removed by filtration through cotton, and the filtrate was extracted twice with 5 mL of EtOAc. Evaporated extracts were subjected to TLC; bands corresponding to radioactive actinomycins were scraped off the plates and after elution with EtOAc/MeOH (1:1) were counted.

the high concentrations of 4-MHA and 3-HA (3 and 4 mM, respectively) used in this experiment because both acids, at such high concentrations, have a deleterious effect on the enzyme. This effect was not observed at low concentrations as was also confirmed by the finding that the ATP/PP_i exchange reaction dependent on both substrates (0.5 mM) proceeds linearly with time up to 30 min (upper part of Figure 7). Obviously, the enzyme is still functional after this period.

Further Properties of Enzyme. The amount of adenylate formed from 4-MHA or 3-HA was proportional to the amount of enzyme used. The same held true in respect of the ATP/PP_i exchange reaction. Adenylate formation was strictly dependent on the presence of divalent metal ions such as Mg^{2+}, Mn^{2+}, or Co^{2+}. Also, the enzyme did not catalyze the ATP/PP_i exchange dependent on any of the constituent amino acids of actinomycin with or without 4-MHA. By the use of a calibrated Ultrogel AcA-44 column, the molecular weight of the native MHA activating enzyme was found to lie between 53 000 and 57 000. The synthetic activity of the enzyme was rather unstable at 0–4 °C with a half-time of 4–8 h depending on the quality of the preparation. This denaturation could not be reduced by higher DTE or PMSF concentrations and was accelerated when enzyme was brought to a pH higher than 7.5. Freezing the purified enzyme in 15% glycerol (−20 °C) for 1 week did reduce the activity to about 50%, as judged by the ATP/PP_i exchange reaction.

Further purification of the enzyme was not yet possible. Adsorption of it to DEAE-cellulose, CM-cellulose, or hydroxylapatite at various pH values completely abolished synthetic and ATP/PP_i exchange activity, as well. Dialysis of the enzyme against various buffers led to heavy losses of units.

Role of the Enzyme in Actinomycin Synthesis. In most preparations the specific activity of the enzyme laid between 6 and 12 pkat/mg of protein. This corresponds to an actinomycin titer in the cultures of 20–30 mg/L after 48 h of growth. In some instances, higher actinomycin concentrations in such cultures were obtained after the same period (>50 mg), which resulted in enzyme preparations containing 20–30 pkat/mg of protein.

As 4-MHB was the most potent structural analogue of 4-MHA with respect to enzymatic adenylate formation, this compound was tested as a competitor in vivo. Mycelium of *S. chrysomallus* actively synthesizing actinomycin was incubated with radioactive precursor of actinomycin in the presence of 4-MHB. The results are shown in Table III. The data

clearly show that 4-MHB is a potent inhibitor of antibiotic synthesis in vivo already at a concentration of 0.5 mM. Control experiments revealed that the inhibitor did not affect the uptake of radiolabel used in the experiments. By contrast, D-valine, which is known to inhibit actinomycin synthesis in *S. antibioticus* (Beaven et al., 1967), exerts a rather weak influence, still allowing considerable synthesis of actinomycin at 2 mM. 4-MHA, which is known to accumulate when D-valine is added to actinomycin synthesizing cells, accumulates in *S. chrysomallus* to a much lower degree than that described for *S. antibioticus* (Ochi, 1982) and was detectable in acidic extracts of the incubation mixtures only as a weak band after D-valine and 4-MHB inhibition with L-[*methyl*-^{14}C]methionine or L-[G-^{3}H]tryptophan as the radiolabels.

Discussion

By following the proposed mechanism of the 4-MHA adenylate synthesis in Figure 2, it was possible to obtain a partially purified enzyme fraction from *S. chrysomallus* capable of catalyzing a 4-MHA-dependent ATP/PP_i exchange and formation of the respective adenylate. The protein purification data presented illustrate the necessity for a rapid isolation of the enzyme, which is realized by the procedure described. The enzyme is rather unstable by reasons yet unknown, and this instability resembles other *Streptomyces* enzymes involved in secondary metabolism (Hori et al., 1978; Hunaiti & Kolattukudy, 1982). Although purification of the enzyme was possible to an only limited extent, all nonspecific activity (i.e., other ATP-consuming activities) could be purified off, allowing detailed studies on its nature.

The enzyme reacts with a series of structural analogues of 4-MHA. Among these, 3-hydroxyanthranilic acid (3-HA), 4-aminobenzoic acid (4-AB), and 4-methyl-3-hydroxybenzoic acid (4-MHB) were most potent as substrates for adenylate synthesis despite apparent differences in the ATP/PP_i exchange reaction (Figure 6, Table II). By comparison of the structure of different analogues and their reactivity, it turns out that for optimum activity besides the COOH groups either a bulky substituent in the 4-position of the aromatic nucleus or the presence of the 3-OH together with a 2-amino or 4-methyl group is favorable. The finding that 4-MHB is a strong inhibitor of actinomycin synthesis in vivo (even stronger than D-valine) fits well with its in vitro activity and supplies further evidence for the involvement of the enzyme in the biosynthesis of the antibiotic. Whether other compounds listed in Table II have a similar effect due to blocking the first step of 4-MHA peptide synthesis remains to be seen.

The fact that during adenylate formation from 4-MHA nearly no AMP is formed indicates that no covalent bond is formed between the acid and the enzyme. The appearance of AMP in the case of 3-HA (and 4-MHB and 3-HB, not shown) apparently seems to be a consequence of the instability of the corresponding enzyme–adenylate complex. Thus, the enzyme differs from the small subunit of gramicidin S synthetase, which is involved in the initiation of gramicidin S synthesis by adenylation and subsequent thioesterification of phenylalanine (Lipmann, 1971). Apparently, the smaller molecular weight (about 55 000 compared to 100 000) does not allow more than one catalytic function in the 4-MHA activating enzyme, and this raises the question what molecule serves as the acceptor for activated 4-MHA. Neither coenzyme A nor acetyl coenzyme A stimulated AMP release from ATP in the presence of 4-MHA (results not shown), and in view of the gramicidin S synthetase system, it seems plausible to suppose that another unknown protein subunit may serve as acceptor for 4-MHA.

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Registry No. 4-MHA, 552-14-7; 4-MHA adenylate, 88609-70-5; 3-HA, 548-93-6; 3-HA adenylate, 88609-71-6; AA, 118-92-3; AA adenylate, 88609-72-7; BA, 65-85-0; BA adenylate, 56164-09-1; 3-HB, 99-06-9; 3-HB adenylate, 88609-73-8; 4-MHB, 586-30-1; 4-MHB adenylate, 88609-74-9; 4-MMB, 7151-68-0; 4-MMB adenylate, 88609-75-0; 4-AB, 150-13-0; 4-AB adenylate, 88609-76-1; D-Val, 640-68-6; actinomycin, 1402-38-6; 4-methyl-3-hydroxy-2-nitrobenzoic acid, 6946-15-2.

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