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ARTICLE *in* BIOCHEMISTRY · APRIL 1990

Impact Factor: 3.02 · DOI: 10.1021/bi00466a015 · Source: PubMed

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Biosynthesis of Quinoxaline Antibiotics: Purification and Characterization of the Quinoxaline-2-carboxylic Acid Activating Enzyme from *Streptomyces triostinicus*†

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Received September 20, 1989; Revised Manuscript Received December 1, 1989

ABSTRACT: A quinoxaline-2-carboxylic acid activating enzyme was purified to homogeneity from triostin-producing *Streptomyces triostinicus*. It could also be purified from quinomycin-producing *Streptomyces echinatus*. Triostins and quinomycins are peptide lactones that contain quinoxaline-2-carboxylic acid as chromophoric moiety. The enzyme catalyzes the ATP–pyrophosphate exchange reaction dependent on quinoxaline-2-carboxylic acid and the formation of the corresponding adenylate. Besides quinoxaline-2-carboxylic acid, the enzyme also catalyzes the formation of adenylates from quinoline-2-carboxylic acid and thieno[3,2-*b*]pyridine-5-carboxylic acid. No adenylates were seen from quinoline-3-carboxylic acid, quinoline-4-carboxylic acid, pyridine-2-carboxylic acid, and 2-pyrazinecarboxylic acid. Previous work [Gauvreau, D., & Waring, M. J. (1984) *Can. J. Microbiol. 30*, 439–450] revealed that quinoline-2-carboxylic acid and thieno[3,2-*b*]pyridine-5-carboxylic acid became efficiently incorporated into the corresponding quinoxaline antibiotic analogues in vivo. Together with the data described here, this suggests that the enzyme is part of the quinoxaline antibiotics synthesizing enzyme system. The enzyme displays a native molecular weight of 42 000, whereas in its denatured form it is a polypeptide of M_r 52 000–53 000. It resembles in its behavior actinomycin synthetase I, the chromophore activating enzyme involved in actinomycin biosynthesis [Keller, U., Kleinkauf, H., & Zocher, R. (1984) *Biochemistry 23*, 1479–1484].

Quinoxaline antibiotics are chromodepsipeptides produced by several *Streptomyces* strains. They can be divided into two groups, namely, the triostins and the quinomycins (Okumura, 1983). Both groups of compounds possess the same peptide backbone characterized by the antiparallel arrangement of two quinoxaline-2-carboxylic acid tetrapeptides which are connected with each other via ester bonds to form octadepsipeptide rings as shown in Figure 1. A peculiar characteristic of these compounds is that they each contain a cross bridge which arises in the case of triostins by formation of a disulfide bridge between the *N*-methylcysteine residues present in the peptide chains. Correspondingly, in the case of quinomycins the cross bridge is formed by a dithioacetal linkage between a *N*-methylcysteine and a *N*,*S*-dimethylcysteine residue (Dell et al., 1975). Both types of antibiotics are active against Gram-positive bacteria and display inhibitory activity against a variety of tumors (Lee & Waring, 1978; Waring & Wakelin, 1974).

Studies on the biosynthesis of quinoxaline antibiotics revealed that the constituent amino acids are derived from the naturally occurring ones. The quinoxaline-2-carboxylic acid portion of the antibiotics is derived from tryptophan, whereas the methyl groups of the *N*-methyl amino acids are donated by methionine (Yoshida & Katagiri, 1969). The biosynthetic relationship between triostins and quinomycins has been established by showing that protoplasts of *Streptomyces echinatus* are able to convert externally added triostin A into quinomycin A (Cornish et al., 1983). It is suggested that the thioacetal cross bridge in the quinomycins arises from the methylation of the disulfide bond in triostin with *S*-adenosyl-L-methionine. A cell-free system of quinomycin A (echinomycin) biosynthesis has been described (Arif et al., 1970). However, a characterization of the enzymes involved in the various biosynthetic steps has as yet not been presented.

Studies on the controlled biosynthesis in vivo of both triostins and quinomycins have shown that new compounds could be obtained when intact mycelia of *Streptomyces triostinicus* or *S. echinatus* were fed with several structural analogues of the chromophore quinoxaline-2-carboxylic acid (Gauvreau & Waring, 1984a,b; Santikarn et al., 1983). Among others, quinoline-2-carboxylic acid or thieno[3,2-*b*]pyridine-5-carboxylic acid (Figure 2) was efficiently incorporated into the corresponding triostin or quinomycin analogue. The data indicate that quinoxaline-2-carboxylic acid and its analogues act as free intermediates in the biosynthetic process. It appears to be likely that they have to be activated by a specific enzyme belonging to the enzyme system responsible for the biosynthesis of quinoxaline antibiotics.

During the biosynthesis of the bicyclic chromopentapeptide lactone actinomycin in *Streptomyces chrysomallus*, the chromophoric precursor of these antibiotics, 4-methyl-3-hydroxyanthranilic acid (4-MHA) has been shown to be a free intermediate that is activated as an adenylate. The activating enzyme in this case has a M_r between 52 000 and 55 000 and

† This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 9, Teilprojekt D5).
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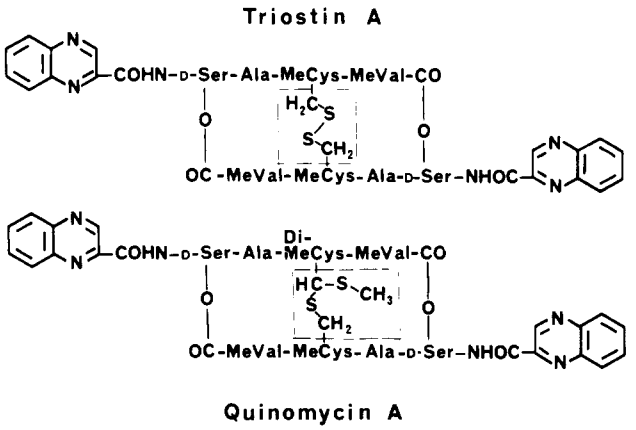

FIGURE 1: Structures of quinoxaline antibiotics. Triostin A contains a disulfide cross bridge between the two *N*-methyl-L-cysteine residues (the dashed box shows the disulfide together with the β-CH_2 groups of the amino acids). Quinomycin A (echinomycin) contains a thioacetal cross bridge between the *N*,*S*-dimethylcysteine and the *N*-methylcysteine residues (the dashed box shows this cross bridge together with the β-CH_2 groups of the two amino acids). MeCys = *N*-methylcysteine; MeVal = *N*-methylvaline; Di-MeCys = *N*,*S*-dimethylcysteine.

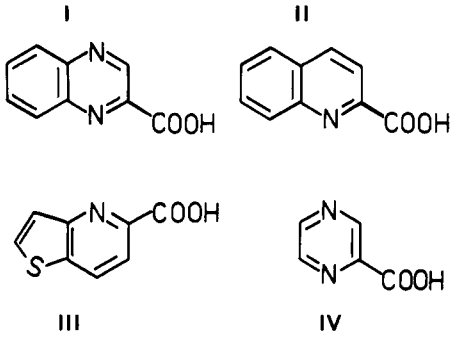

FIGURE 2: Structures of quinoxaline-2-carboxylic acid and several of its structural analogues. (I) Quinoxaline-2-carboxylic acid; (II) quinoline-2-carboxylic acid; (III) thieno[3,2-*b*]pyridine-5-carboxylic acid; (IV) 2-pyrazinecarboxylic acid.

was designated actinomycin synthetase I (Keller et al., 1984; Keller, 1987). By contrast, the enzymes responsible for the synthesis of the peptide chains of actinomycin are large multifunctional enzymes, each activating more than one amino acid (actinomycin synthetase II, M_r 225 000; actinomycin synthetase III, M_r 280 000) (Keller, 1987). Structural analogues of 4-MHA are efficiently incorporated in vivo into the corresponding pentapeptide lactones that represent half-actinomycins (Keller, 1984).

In the following we describe the isolation and characterization of an enzyme that activates quinoxaline-2-carboxylic acid and appears to play a similar role in the biosynthesis of quinoxaline antibiotics in *S. triostinicus* and *S. echinatus* as actinomycin synthetase I in actinomycin synthesis.

Materials and Methods

Radioisotopes and Chemicals. L-[*methyl*-^{14}C]Methionine (56.7 Ci/mol), [U-^{14}C]adenosine triphosphate (586 Ci/mol), and [α-^{32}P]adenosine triphosphate (410 Ci/mmol) were from Amersham International. Tetrasodium [^{32}P]Pyrophosphate (2.9 Ci/mmol) was from New England Nuclear Corp. Quinoxaline-2-carboxylic acid chloride, 2-pyrazinecarboxylic acid, and quinoline-3-carboxylic acid were obtained from Aldrich Chemicals. Quinoline-2-carboxylic acid was from Janssen (Beerse, Belgium), quinoline-4-carboxylic acid was obtained from EGA-Chemie (Steinheim, FRG), and pyridine-2-carboxylic acid was from Sigma. Thieno[3,2-*b*]-pyridine-5-carboxylic acid was donated by Dr. S. Gronowitz (University of Lund, Sweden). Echinomycin was a gift from Dr. H. Peter (Ciba Geigy AG, Basel, Switzerland). Silica gel 60 plastic sheets were from Merck (Darmstadt, FRG), and poly(ethylene imine) (PEI) impregnated cellulose sheets were from Macherey & Nagel (Düren, FRG). Polymin P was from BASF (Ludwigshafen, FRG).

Strains and Cultures. S. triostinicus 21043 was from the American Type Culture Collection. *S. echinatus* A 8331 was obtained from Drs. J. Nüesch and K. Scheibli (Ciba Geigy AG, Basel, Switzerland). For maintenance, strains were streaked on agar slants containing complete medium. Complete medium was as described (Keller et al., 1985). After incubation for 3 days at 28–30 °C, slant cultures were kept frozen at −20 °C for culture preservation. For inocula preparation for liquid culture, spores were scraped from one slant and 6 mL of water was added. After the spore suspension was vigorously vortexed and filtered through cotton, spores were sedimented by centrifugation for 10 min at 3000*g* in a bench-top centrifuge at room temperature. The spore pellet was taken up in 2 mL of water, and 0.1 mL from the suspension served as the inoculum for one flask of liquid culture. Liquid medium was complete medium without agar.

Growth of Organism. Erlenmeyer baffled flasks (250 mL) containing 100 mL of liquid medium were inoculated each with 0.1 mL of spore suspension of *S. triostinicus* or *S. echinatus* and incubated with shaking (220 rpm) at 28 °C in a New Brunswick Environmental Shaker (Model G 25) for 36 (*S. triostinicus*) or 24 h (*S. echinatus*). Baffles were steel springs as described (Hopwood et al., 1985). After this time, antibiotic production was measured in short-term labeling experiments (see below), and cells were harvested by suction filtration on a Büchner funnel. Generally after these periods, the cultures showed maximum radiolabel incorporation into the antibiotics. The yields of wet weight mycelium were 1.5 g with *S. triostinicus* and 1 g with *S. echinatus* per flask.

Short-Term Labeling Experiments. Five-milliliter portions of cultures of *S. triostinicus* or *S. echinatus* were centrifuged for 5 min at 3000*g* in a bench-top centrifuge at room temperature. The pelleted mycelia were washed twice with distilled water by repeated centrifugation and finally suspended in 5 mL of water. The suspensions were transferred to 25-mL Erlenmeyer flasks, and 0.5 μCi L-[*methyl*-^{14}C]methionine was added per flask. The flasks were incubated for 1 h in the shaker under conditions described above. After this period, radioactive antibiotic formed was extracted twice with 2-mL portions of ethyl acetate. The combined extracts were evaporated to dryness, and the residues were taken up in 100 μL of ethyl acetate. The samples were either applied to silica gel plates for subsequent chromatography or subjected to liquid scintillation counting.

Identification of Quinoxaline Antibiotics. The identity of the compounds formed by *S. triostinicus* or *S. echinatus* was checked in the case of echinomycin by thin-layer cochromatography of the radioactive compound with authentic echinomycin using solvent systems I and II. In the case of triostin, no authentic material was available. Therefore, the triostin formed by *S. triostinicus* was identified by two criteria: (1) Nonlabeled triostin was isolated from 10 flasks of cultures of *S. triostinicus* by ethyl acetate extraction and subjected to thin-layer chromatography on silica gel in solvent system I with subsequent bioautography. The main antibiotically active spot was isolated from parallel run plates and subjected to acid hydrolysis. Thin-layer chromatographic comparison in solvent system III with the acid hydrolysate of authentic echinomycin

gave the same amino acid composition and thus proved that the compound was most probably triostin A. (2) Radioactively labeled triostin A, formed by *S. triostinicus* after administration of L-[*methyl*-^{14}C]methionine, comigrated with the nonlabeled antibiotic and, after acid hydrolysis, yielded L-[*N-methyl*-^{14}C]valine.

Synthesis. Quinoxaline-2-carboxylic acid was prepared by hydrolysis of quinoxaline-2-carboxylic acid chloride in 2 N NaOH at 50 °C for 1 h. After this period, the reaction mixture was brought to pH 1.8 with HCl and extracted with ethyl acetate. The ethyl acetate phase was dried with Na_2SO_4 and evaporated to dryness. The dry residue was quinoxaline-2-carboxylic acid as checked by mass spectrometry.

Adenylates of quinoxaline-2-carboxylic acid and quinoline-2-carboxylic acid were prepared according to the method of Berg (1958). They were identified by thin-layer chromatography on silica gel at 4 °C in solvent system III. Visualization of the compounds on the plates was done by detection of their fluorescence with long-wave UV. The adenylates were characterized as follows: Treating the compounds with 1 N NaOH resulted in the release of AMP and the free acids. After reaction with hydroxylamine (1 M) for 10 min at 25 °C, addition of a solution containing 0.37 M $FeCl_3$, 0.31 M trichloroacetic acid, and 0.65 M HCl (Stulberg & Novelli, 1962) resulted in the formation of a colored Fe^{3+}–hydroxamate complex, which was not seen when a mixture of free acid and AMP was treated with hydroxylamine. The R_f values of the adenylates of quinoxaline-2-carboxylic acid and quinoline-2-carboxylic acid in solvent system III were 0.25 and 0.33, respectively. By contrast, in this solvent system AMP had a R_f value of 0.1, whereas quinoxaline-2-carboxylic acid and quinoline-2-carboxylic acid had R_f values of 0.66 and 0.78, respectively.

Buffer and Solvent Systems. Buffer A contained 50 mM potassium phosphate, pH 6.8, 4 mM dithioerythritol (DTE),[1] 15% (w/v) glycerol, 1 mM EDTA, 1 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride (PMSF). Buffer B consisted of 0.1 M Tris-HCl, pH 8.0, 4 mM DTE, 15% (w/v) glycerol, 1 mM EDTA, 1 mM benzamidine, and 1 mM PMSF.

For thin-layer chromatography on silica gel the following solvent systems were used: (I) ethyl acetate/methanol/water (100/5/5); (II) ethyl methyl ketone; and (III) *n*-butanol/acetic acid/water (4/1/1). PEI-cellulose plates for chromatography of ATP, AMP, and adenylates were developed in 1.2 M LiCl (IV).

Enzyme Assay. Assay conditions for enzymatic adenylate synthesis and for enzymatic synthesis of [^{32}P]ATP from [^{32}P]pyrophosphate and chemically synthesized adenylate were the same as described previously (Keller et al., 1984). In several experiments reaction mixtures for adenylate synthesis contained in addition 0.08 μg of inorganic pyrophosphatase (Boehringer Mannheim, FRG). The ATP–pyrophosphate exchange reaction mixture contained besides [^{32}P]pyrophosphate (5–10 × 10^4 cpm) 10 nmol of nonlabeled pyrophosphate (Keller et al., 1984).

Enzyme Purification. All operations were carried out at 0–4 °C. Some 80 g of freshly harvested mycelium of *S. triostinicus*, which had been washed first with 0.3 M NaCl and then with distilled water, was suspended in buffer A containing 10 mM $MgCl_2$ to give a total volume of about 450 mL. The suspension was passed through a French press (Aminco) at a cell pressure of 5000 psi. Five milligrams of DNase I (grade II) (Boehringer Mannheim, FRG) was added, and the suspension was stirred on ice for 30 min. The homogenate was centrifuged for 20 min at 10000 rpm in a Sorvall RC-2B centrifuge (GSA rotor) (step 1). A solution of 14% (w/v) Polymin P was added to the supernatant to give a final concentration of 0.3%. After 30 min of stirring, the solution was centrifuged as above (step 2). The supernatant was then passed through a DEAE-cellulose column (8 × 5.5 cm) that had been previously equilibrated with buffer A. The protein peak appearing in the eluate was collected (step 3). Solid ammonium sulfate was added to this fraction until a saturation of 65% was reached. The mixture was left on ice overnight. The precipitate was collected by centrifugation for 30 min as described above. The pellet was dissolved in a minute volume of buffer B (step 4). The protein was then applied onto an Ultrogel AcA 44 column (40 × 3.2 cm) that had been equilibrated with buffer B. Fractions of 6.3 mL were collected (Figure 3). Fractions catalyzing quinoxaline-2-carboxylic acid dependent ATP–pyrophosphate exchange were pooled (step 5). After dilution of this pool with 1 volume of water [containing 15% (w/v) glycerol, 4 mM DTE, and the protease inhibitors indicated above], enzyme was applied onto a DEAE-cellulose column (13 × 2.1 cm), which had been equilibrated with buffer B. Under these conditions, enzyme adsorbs to the anion exchanger. After the column was washed with 30 mL of buffer B, protein was eluted with 400 mL of a linear gradient from 0 to 0.1 M NaCl in buffer B (fraction size 6.3 mL). Enzyme activity appeared at a NaCl concentration of 15–25 mM (step 6). The combined enzyme activity containing fractions were diluted with 1 volume of water (containing the same ingredients as in step 6) and applied onto an aminohexyl-Sepharose column (6 × 1 cm) previously equilibrated with buffer B. The column was washed with 20 mL of buffer B and then developed with a 400-mL gradient from 0 to 0.2 M NaCl in buffer B. Enzyme elutes at about 50 mM NaCl (step 7). Enzyme-containing fractions were pooled and concentrated to a final volume of 2.5 mL by using Centricon 30 microconcentrators (Amicon). The concentrated enzyme was loaded onto an Ultrogel AcA 54 column (48 × 2.1 cm), which was equilibrated with buffer B. Fractions of 2.8 mL were collected (step 8). Enzyme-containing fractions were combined, and the enzyme was finally purified by ion-exchange chromatography on Mono Q HR 5/5 (5 × 0.5 cm) performed on a Pharmacia FPLC system. The buffer used contained 20 mM Tris-HCl, pH 8.0, 10% (w/v) glycerol, 1 mM EDTA, and 4 mM DTE. A gradient from 0 to 0.2 M NaCl was used, and 1-mL fractions were collected (step 9).

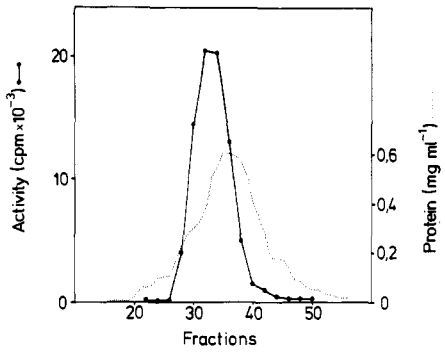

FIGURE 3: Gel filtration of an ammonium sulfate fractionated protein extract of *S. triostinicus* on AcA 44 Ultrogel. 11 mL of the protein concentrate obtained after ammonium sulfate precipitation (Table I, step 4) was chromatographed on Ultrogel AcA 44. 6.3 mL fractions were collected. (···) Protein concentration; (●—●) ATP–pyrophosphate exchange dependent on quinoxaline-2-carboxylic acid.

[1] Abbreviations: FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; DTE, dithioerythritol; PMSF, phenylmethanesulfonyl fluoride.

Table I: Purification of the Quinoxaline-2-carboxylic Acid Activating Enzyme[a]

step	volume (mL)	protein (mg)	units[b] (nkat)	sp act. (nkat/mg)	recovery (%)	purification (x-fold)
(1) crude extract	425	2550				
(2) Polymin P precipitation	420	2100				
(3) DEAE-cellulose (50 mM potassium phosphate, pH 6.8)	480	134.4	8.45	0.063	100	1
(4) $(NH_4)_2SO_4$ precipitation (65%)	11	72.6	7.36	0.102	87	1.62
(5) Ultrogel AcA 44 gel filtration	82	51.7	5.90	0.114	70	1.81
(6) DEAE-cellulose (0.1 M Tris-HCl, pH 8.0)	108	24.8	5.22	0.21	62	3.33
(7) aminohexyl-Sepharose	69.5	8.34	3.96	0.47	47	7.4
(8) Ultrogel AcA 54 gel filtration	11	1.38	1.21	0.88	14.3	14.0
(9) Mono Q HR 5/5	4	0.40	0.444	1.11	5.2	17.6

[a] Eighty grams of cells (wet weight) of *S. triostinicus* was used. [b] Enzymatic activity was determined by measuring the ATP–PP_i exchange reaction dependent on quinoxaline-2-carboxylic acid. One nanokatal (1 nkat) is the amount of enzyme catalyzing the incorporation of 1 nmol of pyrophosphate into ATP per second.

Radioactivity Measurements. Radioactivity was determined with a Tricarb liquid scintillation counter (Packard Instruments). Charcoal filters were counted by using toluene-based scintillation fluid. ^{14}C-Labeled compounds on thin-layer sheets were eluted with suitable solvents from scraped silica gel and then counted. For visualization of radioactive bands on the plates, autoradiography was performed with Kodak X-ray film X-omat S.

Methods of Analysis. Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done according to the method of Laemmli (1970). Silver staining of slab gels was performed according to the method of Blum et al. (1987).

Results

Purification of the Quinoxaline-2-carboxylic Acid Activating Enzyme. The quinoxaline-2-carboxylic acid activating enzyme could be easily detected in protein extracts from *S. triostinicus* or *S. echinatus* by means of the ATP–pyrophosphate exchange reaction. The purification of the enzyme is given in Table I. Polymin P precipitation of the crude protein extract and following passage through DEAE-cellulose resulted in the complete removal of exchange activity independent of quinoxaline-2-carboxylic acid. Concomitantly, more than 90% of total protein was removed. After $(NH_4)_2SO_4$ precipitation, enzyme was subjected to gel filtration on AcA 44 Ultrogel (Figure 3). Enzyme activity eluted as one single peak of activity. Further steps of purification involved chromatography on DEAE-cellulose at pH 8.0 and on aminohexyl-Sepharose and gel filtration on Ultrogel AcA 54. In these separations enzyme was also detected as one single peak of activity. Finally, enzyme was subjected to FPLC on Mono Q HR 5/5 (not shown). The progress of purification is demonstrated in Table I and Figure 4. SDS–PAGE shows one single band in the last purification step.

Substrate Specificity of the Quinoxaline-2-carboxylic Acid Activating Enzyme. Purified enzyme was tested for its ability to catalyze the ATP–pyrophosphate exchange dependent on structural analogues of quinoxaline-2-carboxylic acid such as quinoline-2-carboxylic acid, quinoline-3-carboxylic acid, quinoline-4-carboxylic acid, 2-pyrazinecarboxylic acid, pyridine-2-carboxylic acid, and thieno[3,2-*b*]pyridine-5-carboxylic acid. Table II indicates a strong response of the enzyme when incubated with quinoline-2-carboxylic acid and thieno[3,2-*b*]pyridine-5-carboxylic acid, whereas the others gave only weak or no response.

Enzymatic Synthesis of Quinoxaline-2-carboxylic Acid Adenylate and Adenylates of Structural Analogues. Purified enzyme was incubated with quinoxaline-2-carboxylic acid and

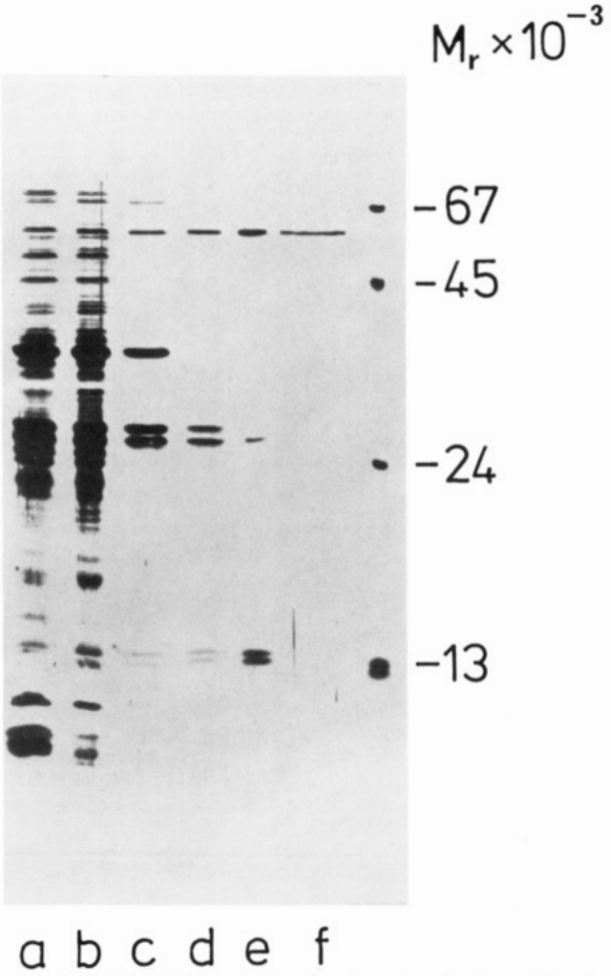

FIGURE 4: SDS–PAGE analysis of various steps in the purification of the quinoxaline-2-carboxylic acid activating enzyme. 20–50-μL portions of the steps from Table I were subjected to SDS–PAGE. (a) Ammonium sulfate precipitation (65%) (step 4); (b) Ultrogel AcA 44 gelfiltration (step 5); (c) DEAE-cellulose (step 6); (d) aminohexyl-Sepharose (step 7); (e) Ultrogel AcA 54 gelfiltration (step 8); (f) FPLC on Mono Q HR 5/5 (step 9). A 15% slab gel was used. A silver staining method was used as described under Materials and Methods. Marker proteins were BSA (67 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), and lysozyme (13 kDa).

[^{14}C]ATP or [^{32}P]ATP, and the reaction mixture was subjected to thin-layer chromatography. Figure 5 (lane B) shows that in the reaction mixture with quinoxaline-2-carboxylic acid a radioactive band appeared that was missing in a parallel experiment without this compound (lane A). Cochromatography of the new radioactive compound (R_f = 0.25) and chemically synthesized quinoxaline-2-carboxylic acid adenylate showed comigration.

Table II: Rates of Adenylate Formation and ATP–Pyrophosphate Exchange Reaction Dependent on Quinoxaline-2-carboxylic Acid and Several Structural Analogues

substrate	adenylate formed[a] (pmol)	pyrophosphate exchanged[b] (nmol)
quinoxaline-2-carboxylic acid	12	11.9
quinoline-2-carboxylic acid	39	5.6
quinoline-3-carboxylic acid	0	0.36
quinoline-4-carboxylic acid	0	0
2-pyrazinecarboxylic acid	0	0.24
pyridine-2-carboxylic acid	0	0
thieno[3,2-*b*]pyridine-5-carboxylic acid	35	8.4

[a] Enzyme (0.27 nkat) was reacted with the respective carboxylic acids (0.5 mM) in the presence of 50 μM ATP and 0.1 mM $MgCl_2$ in buffer B without glycerol in a total volume of 50 μL for 15 min at 26 °C. [b] Enzyme (0.013 nkat) was reacted with the respective carboxylic acids (0.5 mM) in a total volume of 200 μL of buffer B for 15 min at 26 °C as described under Materials and Methods. The ATP–pyrophosphate exchange reaction mixture contained 50 nmol of unlabeled pyrophosphate.

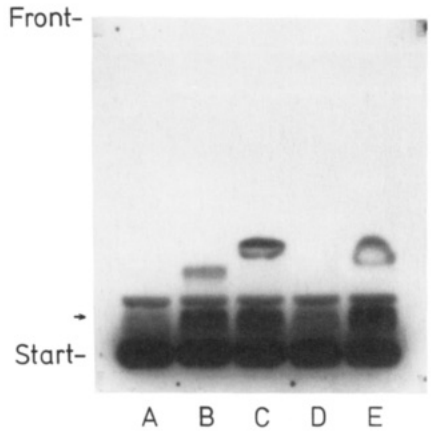

FIGURE 5: Enzymatic formation of adenylates of quinoxaline-2-carboxylic acid and its analogues. Autoradiogram of a thin-layer chromatogram of various reaction mixtures containing different structural analogues of quinoxaline-2-carboxylic acid. A total of 0.27 nkat enzyme was reacted with the substrate carboxylic acid (0.5 mM) in the presence of 50 μM [^{32}P]ATP (2.2×10^6 dpm) and 0.1 mM $MgCl_2$ in a total volume of 50 μL for 15 min at 26 °C. 30 μL from each reaction mixture was applied to a silica gel plate and chromatographed in solvent system III. The fastest moving band in lanes B, C, and E is the corresponding adenylate. Time of exposure to X-ray film was 48 h. (A) No carboxylic acid; (B) quinoxaline-2-carboxylic acid; (C) quinoline-2-carboxylic acid; (D) 2-pyrazinecarboxylic acid; (E) thieno[3,2-*b*]pyridine-5-carboxylic acid. The arrow denotes the position of AMP.

Likewise, in the case of the chemically synthesized adenylate, treatment of the radioactive compound with either 1 N NaOH or 1 M hydroxylamine resulted in the formation of AMP. Furthermore, if the enzyme was incubated with quinoline-2-carboxylic acid or thieno[3,2-*b*]pyridine-5-carboxylic acid in the presence of radioactive ATP, new bands with higher R_f values (Figure 5, lanes C and E, respectively) could be observed. Chromatographic comparison of the band formed in the presence of quinoline-2-carboxylic acid with chemically synthesized quinoline-2-carboxylic acid adenylate revealed its identity as enzymatically formed adenylate. A similar analysis of the compound formed in the presence of thieno[3,2-*b*]pyridine-5-carboxylic acid was not carried out. However, the coordinately increased R_f value of the radioactive compound indicates its identity with the corresponding adenylate since free thieno[3,2-*b*]pyridine-5-carboxylic acid has a R_f value of about 0.8 compared to 0.66 for quinoxaline-2-carboxylic acid. In addition, each of the radioactive bands when treated with NaOH yielded radioactive AMP. These data suggest strong evidence that the newly formed compounds shown in Figure 5 are the adenylates of the various carboxylic acids. It is noteworthy that 2-pyrazinecarboxylic acid (Figure 5, lane D) did not react to give an adenylate under the same conditions, which is not surprising because this compound gave a low response in the ATP–pyrophosphate exchange reaction. By contrast, when 2-pyrazinecarboxylic acid was incubated with enzyme and ATP in the additional presence of inorganic pyrophosphatase, a low but significant amount of adenylate was formed (not shown).

Further Properties of the Quinoxaline-2-carboxylic Acid Activating Enzyme. The amount of adenylate formed from quinoxaline-2-carboxylic acid was proportional to the amount of enzyme present in reaction mixtures. This was also the case for the ATP–pyrophosphate exchange, where a linear dependence vs time (up to 30 min) was also observed. A broad optimum of enzymatic activity was detected between pH 7 and 9. Enzyme could be kept frozen in buffer B at −80 °C for at least 6 weeks without loss of activity. The native molecular weight was estimated to be 42000 by gel filtration on Ultrogel AcA 44 using bovine serum albumin (67000), ovalbumin (45000), trypsinogen (24000), and cytochrome *c* (12000) as standards. SDS gel electrophoretic analysis of the various purification steps in Figure 4 shows that the enzyme is homogeneous and is a single polypeptide chain of M_r between 52000 and 53000. The enzyme was found in both *S. triostinicus* and *S. echinatus*. The amount of enzyme in *S. triostinicus* was 3-fold higher than in *S. echinatus* on the basis of the activity in step 3 of the purification protocol (Table I). Enzyme from *S. echinatus* behaves absolutely identically with that from *S. triostinicus* up to step 7 (further steps not compared), and in the ATP–pyrophosphate exchange reaction it shows the same behavior against structural analogues as described for the *S. triostinicus* enzyme in Table II.

The apparent analogy between chromophore activation in quinoxaline antibiotic biosynthesis and the activation of 4-methyl-3-hydroxyanthranilic acid (4-MHA) in actinomycin biosynthesis (Keller et al., 1984) led us to compare the two enzymes in their specificity against their substrates. Neither enzyme activated the substrates of the other one.

Discussion

The quinoxaline antibiotics are cyclodepsipeptides produced by a number of streptomycetes which are active against several experimental tumors (Katagiri et al., 1975; Waring, 1981). Their activity as cytostatics is greatly influenced by interaction between its chromophore and the DNA base pairs (Waring & Wakelin, 1974). Thus, studies on chromophore incorporation into echinomycin or triostin are of great importance for the design of new intercalating drugs with better DNA-binding efficiency and/or less toxicity. Previous data in the case of the DNA-intercalating chromopeptide lactone actinomycin had shown that replacement of the natural precursor of the chromophoric part of actinomycin, 4-methyl-3-hydroxyanthranilic acid (4-MHA), by certain structural analogues resulted in new compounds, actinomycin half-molecules (Keller, 1984). Testing of one of such compounds, 4-methyl-3-hydroxybenzoic acid pentapeptide lactone, displayed moderate antibiotic activity against a variety of Gram-positive microorganisms without any cytotoxic activity (Keller and Schlumbohm, unpublished results). The rationale for these experiments came from the finding that a specific enzyme in *S. chrysomallus*, which we now call actinomycin synthetase I, is able to activate 4-MHA and a series of structural ana-

logues as adenylates (Keller et al., 1984). From the structural similarity of the peptide chain of actinomycin and the tetrapeptide chain of the quinoxaline antibiotics, we argued that a similar enzyme like actinomycin synthetase I may operate in the case of triostins and quinomycins. In fact, the study described here clearly reveals the presence of such an enzyme in both *S. triostinicus* and *S. echinatus*. It activates quinoxaline-2-carboxylic acid as an adenylate and catalyzes the quinoxaline-2-carboxylic acid dependent ATP–pyrophosphate exchange. Gauvreau and Waring (1984a,b) described the in vivo incorporation of various analogues of quinoxaline-2-carboxylic acid into quinomycin analogues, and this had also been found in the case of triostins (Santikarn et al., 1983). We tested several structural analogues, mentioned by these authors to be effective analogues in feeding experiments for their ability to become activated by the quinoxaline-2-carboxylic acid activating enzyme. Among others, quinoline-2-carboxylic acid and thieno[3,2-*b*]pyridine-5-carboxylic acid were efficiently incorporated into the corresponding quinomycin analogues, and these two compounds were also efficient substrates for the quinoxaline-2-carboxylic acid activating enzyme (Table II). On the other hand, quinoline-3-carboxylic acid, which was a weak substrate, decreased the yield of total quinomycins elaborated by *S. echinatus* significantly, indicating a correlation between the substrate specificity of our enzyme and the behavior of these compounds when fed to the *Streptomyces* culture. It was interesting to see that quinoline-4-carboxylic acid was not activated by the enzyme at all, and this indicates together with the finding obtained with quinoline-3-carboxylic acid that for optimal substrate recognition a heteroaromatic nitrogen and its proximity to a carboxyl group are required. In addition, the lack of significant activity of the enzyme against 2-pyrazinecarboxylate acid and pyridine-2-carboxylic acid indicates the necessity of a two-ring system for recognition by the enzyme. The same conclusions were reached by Gauvreau and Waring from their in vivo experiments. In the light of all the data it appears most likely that the quinoxaline-2-carboxylic acid activating enzyme is a part of the enzyme system that produces quinoxaline antibiotics.

From its physical properties the quinoxaline-2-carboxylic acid activating enzyme displays a considerable similarity with the actinomycin synthetase I. The purification protocol given in Table I is the same for both enzymes (Keller, unpublished results). From their sizes the enzymes do not differ significantly from each other, and they catalyze the same type of reaction. Actinomycin synthetase I appears to be involved in the initiation of chromopeptide synthesis catalyzing the acylation with 4-MHA of peptide intermediates covalently bound to actinomycin synthetase II (Stindl & Keller, 1989). It appears plausible to assume a similar role for the quinoxaline-2-carboxylic acid activating enzyme in the case of quinoxaline antibiotic biosynthesis. Other work in this laboratory dealing with the activation of D-lysergic acid, the chromophoric moiety of the ergopeptide alkaloids, has revealed the presence of a D-lysergic acid activating enzyme in the fungus *Claviceps purpurea* (Keller et al., 1988). This enzyme shows behavior similar to that of the two *Streptomyces* enzymes, and its purification protocol is very similar to that described here. Also, its molecular weight is in the expected range (62 000).

We believe that chromophore activation in chromopeptide synthesis is accomplished by a characteristic class of enzymes that may be widespread among microbial organisms capable of synthesizing these secondary metabolites. Therefore, it should be of interest to isolate more chromophore activating enzymes from both *Streptomyces* and filamentous fungi to support this assumption. Such attempts may be successful in those streptomycetes that produce chromopeptide lactones such as virginiamycin S_1, etamycin, and pyridomycin containing 3-hydroxypicolinic acid as chromophore (Okumura, 1983). These chromopeptide lactones show considerable structural similarity with actinomycin and its half-molecules. The investigation of such an enzyme is currently being performed in this laboratory.

ACKNOWLEDGMENTS

M.B. gratefully acknowledges the receipt of a DAAD fellowship.

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