A tryptophan C-methyltransferase involved in streptonigrin biosynthesis in *Streptomyces flocculus*

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A C-methyltransferase that catalyses the transfer of a methyl group from S-adenosylmethionine to C-3 of tryptophan, resulting in β -methyltryptophan, has been identified in cell-free extracts of streptonigrin-producing *Streptomyces flocculus*. The absolute configuration of the product was shown to be (2S,3R)- β -methyltryptophan by high-pressure liquid chromatography and reactivity with D- and L-amino acid oxidases. In shake culture, maximum specific activity occurs after *S. flocculus* enters stationary phase, but before significant streptonigrin accumulates.

Streptonigrin is an anti-tumour antibiotic produced by *Streptomyces flocculus* (A.T.C.C. 13257) (Rao & Cullen, 1959–1960). Although the complete biosynthetic pathway to streptonigrin is not known, it has been reported that the quinoline quinone portion is derived, in part, from glucose (Gould & Cane, 1982), and that tryptophan provides the 4-phenylpicolinic acid moiety through an unique cleavage of the indole ring (Gould & Chang, 1978). The biosynthetic pathway described by Gould & Chang (1977) proposes that the first step that commits tryptophan to antibiotic biosynthesis is a methylation at the β -carbon atom of the side chain of tryptophan. In support of this proposal, Gould & Darling (1978) isolated β -methyltryptophan from culture filtrates of the producing organism and found that labelled β -methyltryptophan was efficiently incorporated into streptonigrin by cultures of *S. flocculus* to which it was fed.

In the present paper we report the presence of enzyme activity in dialysed cell-free extracts of *S. flocculus* that can catalyse the transfer of a methyl group from S-adenosylmethionine to tryptophan to form β -methyltryptophan, in the absence of added cofactors.

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

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Experimental

Materials

Isomers A and B of β -methyltryptophan and streptonigrin were gifts from Dr. S. J. Gould (Corvallis, OR, U.S.A.), who synthesized the β -methyltryptophan isomers by the procedure of Snyder & Matteson (1957) and separated and identified them as previously reported (Gould & Darling, 1978). Yeast extract and beef extract were purchased from Difco Laboratories (Detroit, MI, U.S.A.). Trypticase Soy Broth, dextrose and Gelysate Peptone were from Baltimore Biological Laboratories (Baltimore, MD, U.S.A.). S-Adenosyl-L-[methyl- 14 C]methionine (55 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Its radiochemical purity was examined periodically by paper chromatography and by liquid-scintillation counting. DL-[methylene- 14 C]-Tryptophan (57 mCi/mmol) was from Amersham-Searle (Arlington Heights, IL, U.S.A.). Acetonitrile was purchased from Burdick and Jackson Laboratories (Muskegon, MI, U.S.A.). All other chemicals were reagent grade or better and available commercially.

Culture conditions

S. flocculus (A.T.C.C. 13257) was maintained on Emerson agar slants. Inoculated slants were incubated at 26–27°C for 5–7 days and then stored at 4°C until needed. Inoculum for shake cultures was prepared by washing the surface of the slant

with 5 ml of sterile water and gently scraping the surface with a sterile pipette. The liquid was suction-filtered through sterile Whatman no. 1 filter paper. The resulting spore suspension (3×10^6 – 6×10^6 spores/ml) was added to 100 ml of Emerson medium (beef extract, 4.0 g; Gelysate Peptone, 4.0 g; yeast extract, 1.0 g; NaCl, 2.5 g; dextrose, 10.0 g; distilled water to 1 litre) in a 1-litre Erlenmeyer flask and incubated at 26–27°C on a New Brunswick Scientific rotary shaker at 150 rev./min for 48 h. A 25 ml portion of this inoculum was added to 500 ml of Emerson medium in a 4-litre Erlenmeyer flask and incubated as above for the appropriate periods of time.

Determination of streptonigrin

Cultures were harvested after various periods of incubation by suction filtration through Whatman no. 1 filter paper. Then 10 ml of each filtrate was extracted and assayed for streptonigrin content by the procedure of Ponomarenko (1971). A standard curve was constructed with the use of pure streptonigrin.

Enzyme preparation

The 54–62 h cultures of *S. flocculus* were harvested by suction filtration. The mycelial pad was washed once with distilled water and resuspended in 0.01 M-sodium phosphate buffer, pH 8.0, containing 1 mM-dithiothreitol (buffer I) to form a thick slurry (1 g of cells/3 ml of buffer). All subsequent steps were carried out at 4°C. A cell-free extract was prepared by ultrasonic disruption of the cell slurry for 5 min at 23 W (Branson Sonifier, model W-140, 1 cm probe) followed by centrifugation at 30000 g for 20 min at 2°C (Damon/IEC Division IEC B-20A centrifuge). The pellet formed during centrifugation was re-dissolved in 2 ml of buffer I, dialysed overnight against two changes of 100 ml of buffer I without dithiothreitol (buffer II), and assayed for tryptophan C-methyltransferase activity. The supernatant from the centrifugation was dialysed overnight against two changes of 2 litres of buffer II and purified 1.2-fold by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Solid $(\text{NH}_4)_2\text{SO}_4$ was first added to 35% saturation. After being stirred for 20 min, the preparation was centrifuged at 30000 g for 20 min at 2°C. The resulting supernatant was adjusted to 60% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ as described above. The precipitate was collected by centrifugation as before and either frozen at –20°C for storage or resuspended in buffer I to a final protein concentration of 6–8 mg/ml and dialysed against two changes of 2 litres of buffer II.

Protein was determined by the biuret method (Layne, 1957), with bovine serum albumin (fraction V) as the standard.

Assay of activity enzyme

Tryptophan C-methyltransferase (S-adenosyl-methionine:L-tryptophan 3-methyltransferase) activity was assayed by incubating 1.0 ml of enzyme preparation with 5 mM-L-tryptophan, 5 mM-S-adenosylmethionine, 0.03 mM-pyridoxal 5'-phosphate and 0.1 μCi of S-adenosyl-L-[methyl- ^{14}C]-methionine in a total of 1.4 ml. The control consisted of 1 ml of enzyme that had been boiled for 5 min and the same concentration of all other reagents. After incubation at 37°C for 90 min–3 h the reaction was stopped by the addition of 2 ml of water-saturated butan-1-ol. The assay tubes were agitated on a vortex mixer for 15–30 s and centrifuged at low speed to separate the two phases. The butanol was removed and the extraction was repeated twice more with 1.5 ml of butanol. This procedure removed 70–80% of the β -methyltryptophan from the aqueous phase. The butanol extracts were pooled and dried under N_2 . The dried residue was redissolved in methanol and chromatographed on silica-gel plates (EM Laboratories; 0.25 mm thickness, aluminium-backed, with fluorescent indicator) in butan-2-one/pyridine/water/acetic acid (70:15:15:2, by vol.). Alternatively, the extract was spotted on cellulose plates (EM Laboratories; 0.25 mm thickness, aluminium-backed, with fluorescent indicator) and developed in butan-1-ol/acetic acid/water (12:3:5, by vol.). The developed plates were sprayed with a solution of 0.25% ninhydrin in propan-2-ol. The zone corresponding to β -methyltryptophan was scraped into a mini scintillation vial (Research Products, Mount Prospect, IL, U.S.A.) containing 0.40 ml of methanol. After agitation, 6 ml of scintillation cocktail (Beckman Ready-Solv EP) was added and radioactivities of samples were counted in a liquid-scintillation counter (Searle Mark III, model 6880) to a least 2% standard error. One unit of enzyme activity is defined as that quantity of enzyme that will catalyse the formation of 1 μmol of product/min under the assay conditions described.

H.p.l.c.

The h.p.l.c. system consisted of a Waters Associates model M-6000A solvent-delivery system, a Waters Associates model U-6K injector with a 2 ml loop, a Waters Associates $\mu\text{Bondapak-C}_{18}$ column (either analytical, 3.9 mm $\times$ 30 cm, or semi-preparative, 7.8 mm $\times$ 30 cm), a Varian model 635 UV-Visible spectrophotometer set at 260 nm and a Varian model A-25 chart recorder. The mobile phase was 5% (v/v) acetonitrile in 0.01 M-sodium acetate, adjusted to pH 4.0 with acetic acid (Anderson & Purdy, 1977). The recoveries of β -methyltryptophan and tryptophan from the semi-

preparative column were 82.3% and 79.4% respectively.

Kuhn–Roth oxidation

The β -[^{14}C]methyltryptophan that had been isolated from the enzyme reaction mixture and purified by h.p.l.c. followed by chromatography on Dowex 50X to remove acetate was subjected to Kuhn–Roth oxidation, and the acetic acid was collected by fractional distillation as described by Wissenberger (1948).

Determination of the stereochemistry of the reaction product

The absolute configuration of the reaction product, β -methyltryptophan, was determined by h.p.l.c. combined with oxidative deamination by stereospecific amino acid oxidases. The retention time of the reaction product in the h.p.l.c. system was compared with the retention times of authentic isomer A and isomer B of β -methyltryptophan. D- and L-Amino acid oxidases were first assayed with L- and D-tryptophan as substrates to ensure their complete stereospecificity. Subsequently, purified β -[^{14}C]methyltryptophan was added to 1 ml of buffer I, pH 7.5, containing excess catalase, 2 drops of octanol and either L-amino acid oxidase, D-amino acid oxidase or no oxidase. After incubation at 30°C for 2 h with O_2 bubbling through the assay mixture, the reaction was stopped by acidification and extracted twice with 2 vol. of ethyl acetate. The extracts were pooled, concentrated to dryness *in vacuo*, redissolved in ethyl acetate and chromatographed on silica-gel plates with fluorescent indicator in ethyl acetate/propan-2-ol/acetic acid (5:4:1, by vol.). Indolic compounds were detected visually either as quenching areas under u.v. light (254 nm) or by spraying with van Urk's reagent (Dawson *et al.*, 1969). In addition, the radioactive spots were located by radiochromatogram scanning.

Results

Confirmation of product

Incubation of the dialysed cell-free extract of *S. flocculus* with L-tryptophan and S-adenosylmethionine resulted in the formation of β -methyltryptophan. The radioactive product was extracted from the enzyme mixture with butan-1-ol and identified by co-chromatography with authentic β -methyltryptophan in two t.l.c. systems. Further confirmation of its identity was obtained by h.p.l.c. The product of the enzyme-catalysed reaction was clearly separated from L-tryptophan and co-chromatographed with β -methyltryptophan isomer A [the enantiomeric pair with (2*R*,3*S*) and (2*S*,3*R*) configurations] but not with β -methyltryp-

tophan isomer B [a mixture of (2*R*,3*R*) and (2*S*,3*S*) enantiomers].

Confirmation of the location of the labelled carbon atom in β -[^{14}C]methyltryptophan was obtained from Kuhn–Roth oxidation. It had previously been shown that treatment of streptonigrin with $\text{CrO}_3/\text{H}_2\text{SO}_4$ yielded acetic acid in stoichiometric amounts (Gould & Chang, 1980). The origin of the acetic acid was traced to the C-3 carbon atom and its attached methyl group. Similar treatment of β -[^{14}C]methyltryptophan obtained from the enzyme-catalysed reaction could be expected to yield labelled acetic acid from C-3 and the attached methyl group. A yield of 85% of the theoretical amount of acetic acid was obtained, which accounted for 92% of the radioactivity added to the oxidation reaction, indicating that the radioactivity associated with β -methyltryptophan resided specifically in the methyl group on C-3 of the side chain.

Stereochemistry of the reaction product

Since the β -methyltryptophan formed in the tryptophan C-methyltransferase assay co-chromatographed only with isomer A, the stereochemistry had to be either (2*S*,3*R*) or (2*R*,3*S*). Therefore determination of the configuration at C-2 would distinguish between the enantiomers and reveal the absolute configuration of the product. To determine the configuration at C-2, β -[^{14}C]methyltryptophan isolated from the enzyme reaction was incubated with D- or L-amino acid oxidase, or buffer alone as control. Chromatography of the butyl acetate extract from the L-amino acid oxidase reaction yielded a zone that was (1) radioactive (1221 d.p.m. versus 25 d.p.m. for the control), (2) indolic (positive reaction with van Urk's reagent), (3) quenching under u.v. light (254 nm), and (4) located at the same R_F as standard β -methylindol-3-ylpyruvate. No similar zone was observed in the butyl acetate extracts from the reactions with D-amino acid oxidase or buffer alone. Since only L-amino acid oxidase, which oxidatively deaminates amino acids with the *S* configuration at C-2, was active with the purified reaction product, the product must be a single enantiomer with the absolute configuration (2*S*,3*R*)- β -methyltryptophan.

Time course of growth, C-methylating activity and streptonigrin production

Streptonigrin was detected in culture broths after 40 h of incubation (Fig. 1), and increased in concentration over the next 32 h. This corresponded to the idiophase of the growth cycle of *S. flocculus*, since exponential growth ceased at about the same time that antibiotic production began.

Fig. 1. Time course of growth, streptonigrin production and tryptophan C-methyltransferase activity in cultures of *S. flocculus*. Parallel cultures were harvested after various time periods of incubation and filtered. The mycelium from one set was dried and weighed to determine growth (●). The mycelium from a second set was used to prepare the crude cell-free extract, as described in the text, for the C-methyltransferase assay (○). Streptonigrin concentration (▲) was measured spectrophotometrically after extraction of the filtered broths.

The C-methylating activity was first detected at 30 h, when the organism was in a late-exponential growth phase. Enzyme activity peaked at 54 h, and fell to 67% of the peak level by 73 h.

Enzyme properties

The results of the initial assays with the crude cell-free extract that had been dialysed overnight indicated that the reaction proceeded in the absence of added cofactors. However, the addition of 0.03 mM-pyridoxal 5'-phosphate stimulated the reaction slightly (5–10%). D-Tryptophan could not replace L-tryptophan as a substrate, and no product was obtained in the absence of S-adenosylmethionine or enzyme. Centrifugation of the enzyme preparation and subsequent assay of the pellet established that the tryptophan-methylating activity was located exclusively in the soluble fraction of sonicated cells. The reaction was linear for 90 min under optimum conditions (37°C, pH 8.0, 5 mM substrate concentrations) for the $(\text{NH}_4)_2\text{SO}_4$ -fractionated enzyme.

The C-methyltransferase activity was quite stable at both 4°C and –20°C. No loss of enzyme activity occurred on storage of the enzyme preparation in phosphate buffer at 4°C for 60 h. Only an 8% loss of activity resulted from freezing the $(\text{NH}_4)_2\text{SO}_4$ -fractionation precipitate at –20°C for up to 3 weeks.

Discussion

We have established the presence of enzyme activity in *S. flocculus* that catalyses the transfer of a methyl group from S-adenosylmethionine to C-3 of the side chain of tryptophan, resulting in β -methyltryptophan. The time course of growth and streptonigrin production is typical of other antibiotic-producing organisms in which the product(s) of secondary metabolism do not begin to accumulate until the organism is well into stationary phase (termed 'idiophase' by Drew & Demain, 1977). Likewise, the onset of C-methyltransferase activity parallels that of secondary metabolic enzymes of other organisms, such as 6-methylsalicylate synthetase of patulin biosynthesis (Gaucher *et al.*, 1980) and L-lysine aminotransferase in the cephamycin C-biosynthetic pathway (Kern *et al.*, 1980).

Gould & Darling (1978) have previously shown that cultures of *S. flocculus* produce β -methyltryptophan isomer A, and that this compound was incorporated into streptonigrin. The β -methyltryptophan formed by the C-methyltransferase was also exclusively isomer A [specifically the (2*S*,3*R*) enantiomer]. The identity of the product, coupled with the time course of appearance of the enzyme, provides strong evidence that the enzyme assayed is involved in the streptonigrin-biosynthetic pathway.

Methylation of the side chain of tryptophan may occur in other biosynthetic pathways. β -Methyltryptophan isomer A has been reported in the hydrolysis products of telomycin, a peptide antibiotic produced by a streptomycete (Sheehan *et al.*, 1968). Also, isomer A of β -methyltryptophan was incorporated into indol-3-ylisopropionic acid by *Claviceps* at a rate 10 times greater than isomer B (Hornemann *et al.*, 1969). However, the present paper is the first reported isolation of the enzyme responsible for the tryptophan-methylation reaction, to the best of our knowledge. One other C-methyltransferase involved in antibiotic biosynthesis has been reported (Speedie *et al.*, 1975). The enzyme, S-adenosylmethionine:indol-3-ylpyruvate 3-methyltransferase, from indolmycin-producing *Streptomyces griseus*, methylates indol-3-ylpyruvate at the analogous position on the side chain. However, L-tryptophan cannot serve as a substrate for this enzyme.

The isolation of the tryptophan C-methyltransferase from *S. flocculus* raises several regulatory and mechanistic questions. The enzyme commits a primary metabolite, tryptophan, to the biosynthesis of a secondary metabolite, streptonigrin, and therefore is a likely site for regulation of the pathway. Furthermore, the β -position of the side chain of tryptophan must be activated before nucleophilic attack on the methyl group of S-adenosylmethionine can occur. The mechanism by which this activation occurs is not as yet readily apparent.

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