Actinomycin Synthesis in Streptomyces antibioticus

PURIFICATION AND PROPERTIES OF A 3-HYDROXYANTHRANILATE 4-METHYLTRANSFERASE

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A methyltransferase, which utilizes 3-hydroxyanthranilic acid (HAA) as a substrate, has been purified to near homogeneity from 30-36-h mycelium of the bacterium Streptomyces antibioticus. The enzyme was obtained in approximately 20% yield with a purification of 130-fold. Polyacrylamide gel electrophoresis under denaturing conditions indicates that the enzyme is composed of a single subunit with Mₐ of about 36,000. On chromatography in 0.5 M NaCl, the enzyme displays a molecular weight of about 37,000. The specific activity of the enzyme in S. antibioticus mycelium is maximal between 30 and 36 h following inoculation of galactose/glutamic acid medium and, at those times post-inoculation, the specific activity is essentially the same in extracts of mycelium obtained from cultures grown on glucose rather than galactose as the carbon source. The enzyme activity is stimulated by Na₂EDTA (in crude extracts) and by 2-mercaptoethanol and the methyltransferase shows a strong preference for HAA as substrate as compared with a number of HAA analogs. Thin layer chromatography of ethyl acetate extracts of large-scale incubation mixtures confirms that the product of the reaction is 4-methyl-3-hydroxyanthranilic acid. The reaction product was also a substrate for phenoxazinone synthase and was incorporated into actinomycin by S. antibioticus mycelium. Kinetic parameters for the methyltransferase reaction was determined.

Considerable progress has been made in recent years in characterizing the enzymes which are involved in the biosynthesis of the antibiotic, actinomycin. Thus, Keller and co-workers (1) have described the isolation of an enzyme that modifies and polymerizes the amino acids in the actinomycin pentapeptide chains. There is strong evidence to suggest that it is the MHA-pentapeptides which are the substrates in the ultimate or penultimate step in the biosynthetic pathway, the synthesis of the phenoxazinone ring which comprises the actinomycin chromophore (3, 4). Phenoxazinone synthase, the enzyme which catalyzes this reaction, at least in Streptomyces antibioticus, was identified a number of years ago by Katz and Weissbach (5) and has more recently been purified to homogeneity in the author’s laboratory (6).

In a previous report from this laboratory (7), another enzyme was described which is apparently involved in actinomycin biosynthesis. That enzyme catalyzes the transfer of a methyl group from S-adenosylmethionine to 3-hydroxyanthranilic acid to form MHA (Fig. 1). The methyltransferase has been purified to near homogeneity and the details of the purification and the properties of the enzyme are described in the present report.

MATERIALS AND METHODS

Radioisotopes and Chemicals

[methyl-¹⁴C] S-adenosyl-L-methionine, 58.5 mCi/mmol, was supplied by Du Pont-New England Nuclear and HAA, 3-hydroxy-DL-kynurenine, anthranilic acid, and sodium benzoate were obtained from Sigma. Nonidet P-40 was from Shell Chemicals while a-aminophenol and 3-hydroxybenzoic acid were from Aldrich. MHA was synthesized by Dr. A. B. Mauger, Washington Hospital Center, Washington, D. C. and was generously donated by Dr. Edward Katz, Georgetown University, Washington, D. C. Actinocin and cinnabaric acid were prepared by reaction of MHA and HAA, respectively, with phenoxazinone synthase. The reaction products were obtained from reaction mixtures by ethyl acetate extraction and concentration as described below.

Growth of Organisms

S. antibioticus IMRU3720 was grown as described previously (6). After 48 h in NZ-amime medium the mycelium from four flasks was washed, transferred to four 2-liter flasks, each containing 500 ml of galactose/glutamic acid medium, and growth was continued for 30–35 h. Mycelium was harvested by suction filtration and washed as described (6).

Conditions for Methyltransferase Assay

Reaction mixtures (0.1 ml) for methyltransferase assay were prepared in 1.5-ml Eppendorf centrifuge tubes and contained: sodium phosphate, pH 7, 50 mM; Na₂EDTA, 10 mM; 2-mercaptoethanol, 5 mM; HAA, 1 mM; [¹⁴C]AdoMet, 0.5 Ci/ml (8.5 μM); enzyme, 0.15 mg/ml. Assays of highly purified enzyme fractions also contained 50 μg/ml bovine serum albumin. In some experiments, other substrates, at concentrations of 1 mM, were substituted for HAA while in other experiments the HAA concentration was varied between 0 and 2 mM and the AdoMet concentration was varied between 8.5 μM and 0.21 mM. Reaction mixtures were incubated for up to 2 h at 30 °C (30-min incubations were performed for the estimation of kinetic parameters. The reaction proceeded linearly at all substrate concentrations tested for at least 30 min.) The reactions were stopped by adding 0.4 ml of 0.25 M HCl followed by 0.5 ml of ethyl acetate to reaction mixtures. After brief vortexing, phases were separated by centrifugation of the reaction tubes for 1 min in an Eppendorf centrifuge. Aliquots of the ethyl acetate layer (0.1 ml) were analyzed by liquid scintillation counting. One unit of methyltransferase activity is defined as that amount of enzyme catalyzing the transfer of HAA of 1 pmol of methyl groups per min under the assay conditions described above.
**Purification of HAA 4-Methyltransferase**

The solution was applied to a 1.2 x 95-cm column of Bio-Gel A0.5m, eluted with the same buffer. Fractions of 1 ml were collected at a flow rate of 20 ml/h and a typical column profile is depicted in Fig. 2B. Fractions 85-96 were pooled and concentrated as in Step 5.

**Step 7**—The precipitated step 6 protein was dissolved in 0.5 ml of Buffer B and applied to a 1.2 x 120-cm column of Sephadex G-50 eluted with the same buffer. Fractions of 1.1 ml were collected at a flow rate of 35 ml/h, and a typical column profile is depicted in Fig. 2C. Fractions from this column were analyzed by polyacrylamide gel electrophoresis (9) and numbers 59-68 were pooled and concentrated as above. The resulting precipitate was then dissolved in 0.5 ml of Buffer 2 (with the glycerol concentration raised to 5%), dialyzed for 1 h against the same buffer and the enzyme solution was stored in small aliquots at −70 °C.

**Analysis of the Products of the Methyltransferase Reaction**

Large-scale incubations (1.2–2 ml) were prepared by scaling up reaction mixtures containing the components indicated above in the description of the assay conditions. Those mixtures contained 1 mM HAA and 0.15 mg/ml enzyme and were incubated for 2 h at 30 °C. At the end of these incubations, the mixtures were acidified and extracted with 3 ml of ethyl acetate essentially as described above. The aqueous phase from the first extraction was re-extracted with 1.5 ml of ethyl acetate and the layers were again separated by centrifugation. The combined ethyl acetate extracts were washed twice with 2 ml of distilled water by mixing and centrifugation. The washed ethyl acetate layer was then concentrated to dryness under a stream of nitrogen.

The final samples were dissolved in 30 μl of methanol and centrifuged for 1 min in an Eppendorf centrifuge. In some experiments, an aliquot (2 x 10⁶ cpm) of the concentrated product preparation was utilized as a substrate for phenoxazinone synthase (6). The radioactive product of the phenoxazinone synthase reaction was obtained by ethyl acetate extraction and concentrated as described above. In other experiments, the radioactive product of the methyltransferase reaction was incubated with washed S. antibioticus mycelium (10). Mycelium from a 50 ml 48-h culture of S. antibioticus was washed twice with 0.9% NaCl and resuspended in 15 ml of 100 mM sodium phosphate, pH 7.0, also containing 0.1 mM L-proline, L-valine, L-threonine, and glycine (10). Radioactive product from the methyltransferase reaction (3.7 x 10⁶ cpm) was added and the mixture was incubated with shaking for 6 h at 30 °C. Actinomycin was recovered by neutral ethyl acetate extraction of the reaction mixture (medium and mycelium) and concentration as described above.

Samples of the concentrated products obtained from the three assays just described were analyzed by thin layer chromatography performed on silica gel plates using chloroform/glacial acetic acid (9:1, v/v, solvent I) or ethyl acetate/methanol/water (100:5:5, v/v/v, solvent II). In all chromatographic experiments HAA, MHA, cinna- barinic acid, and actinoin were run as standards.

**Miscellaneous Methods**

For the determination of the native M₄ of the methyltransferase the enzyme was chromatographed on a 1.2 x 45-cm column of Sephadex G-75, eluted with Buffer 3. Molecular weight standards run simultaneously were bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c. Protein was determined by the method of Bradford (11) using the Bio-Rad assay reagent.

**RESULTS**

**Time Course of Methyltransferase Appearance**—Before attempting the purification of the HAA methyltransferase, it seemed advisable to examine the time course of its synthesis in S. antibioticus mycelium. To that end, 500 ml of galactose/glutamic acid culture were inoculated and aliquots were removed at various times thereafter. Methyltransferase was prepared from those aliquots through step 2 of the purification scheme described above. The enzyme specific activities at various times post-inoculation are shown in Fig. 3. For comparison, the relative specific activity of phenoxazinone synthase is also shown. Based on the data of Fig. 3, methyltransferase was purified from 30 to 36-h S. antibioticus mycelium. Unlike phenoxazinone synthase, synthesis of the methyltransferase did not appear to be subject to glucose repression.
Thus, comparable methyltransferase specific activities were obtained whether the organism was grown on glucose or galactose (data not shown).

Comments on the Purification—The procedure described above for the preparation of mycelial extracts differs in several respects from that reported previously (7). In the present procedure, DNase treatment of crude extracts was omitted to allow complete precipitation of protein during centrifugation. While no increase in enzyme specific activity was observed following polyethyleneimine precipitation, this treatment was effective in removing nucleic acids from the enzyme extract. Although the native Mr of the enzyme (see below) is well below the exclusion limits of one of the gel filtration resins used in the purification (Bio-Gel A0.5m), the use of this resin was more effective initially in removing impurities from the methyltransferase than resins with limits closer to the Mr.

A summary of the purification is provided in Table I. The methyltransferase was purified about 130-fold and was obtained in 20% yield as compared with the crude extract. Approximately 2 mg of protein were obtained from 12 g of starting mycelium.

**Purity and Molecular Weight of the Methyltransferase—**
The purity of the enzyme at each step of the purification was assessed by polyacrylamide gel electrophoresis as shown in Fig. 4. Step 7 enzyme was nearly homogenous and was composed of a single polypeptide with a molecular weight of about 36,000 (calculated using the size standards shown in Fig. 4). The native Mr of the enzyme was estimated by chromatography of step 7 enzyme on Sephadex G-75 as described under "Materials and Methods." As shown in Fig. 5, the mobility of the enzyme on that column indicated an Mr of about 37,000. The apparent Mr was essentially unaffected by the omission of NaCl from the elution buffer. Thus, the methyltransferase seems to be composed of a single polypeptide chain with a molecular weight between 36,000 and 37,000.

**Properties of the Methyltransferase Reaction—** As demonstrated in Table II, the methyltransferase activity was stimulated in crude extracts by Na2EDTA while the effects of this additive were diminished in highly purified preparations. This observation may reflect the presence in crude extracts of metal ions which inhibit the enzyme. Those metals are presumably removed from highly purified enzyme preparations. The activity of both crude extracts and purified preparations was

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**TABLE I**

Purification of HAA methyltransferase from *S. antibioticus*

Starting from 12 g of 33-h mycelium.

| Step          | Volume | Protein | Units* | Specific activitya | Yield | Purification |
|---------------|--------|---------|--------|-------------------|-------|--------------|
| 1. Crude extract | 91     | 1,292   | 29,979 | 23.2              | 100   | 1.0          |
| 2. 12,000 x g supernatant | 108   | 626     | 22,500 | 35.9              | 75    | 1.6          |
| 3. Polymin P supernatant | 106   | 583     | 17,372 | 29.8              | 58    | 1.3          |
| 4. 50-75% (NH4)2SO4 | 14.3  | 405     | 17,835 | 44.0              | 59    | 1.9          |
| 5. DEAE-cellulose | 1.0   | 36.5    | 8,415  | 231               | 28    | 9.9          |
| 6. A0.5m | 0.57   | 7.2     | 5,001  | 695               | 17    | 29.9         |
| 7. G-50 | 1.0    | 2.1     | 6,103  | 3,052             | 20    | 132          |

*Picomoles of methyl groups transferred per min.

Units/mg of protein.

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**FIG. 4.** Polyacrylamide gel electrophoresis of fractions from the methyltransferase purification. Lane 1, size standards (phosphorylase b, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,699; carboxic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400); lane 2, crude extract (110 µg); lane 3, 12,000 x g supernatant (152 µg); lane 4, polyethyleneimine supernatant (107 µg); lane 5, 50-75% ammonium sulfate fraction (119 µg); lane 6, DEAE fraction (63 µg); lane 7, Bio-Gel A0.5 peak (6.2 µg); lane 8, Sephadex G-50 (5 µg).

**FIG. 5.** Estimation of the native Mr of the methyltransferase. Chromatography on Sephadex G-75 was performed as described under "Materials and Methods." Size standards other than the two listed in the legend to Fig. 4 were chymotrypsinogen (25,000) and cytochrome c (12,500).
TABLE II
Properties of the methyltransferase reaction

| System                  | Activity | units |
|-------------------------|----------|-------|
| Complete                | 54.3     |       |
| Crude extract* - Na₂EDTA| 41.6     |       |
| Crude extract - mercaptoethanol | 7.1  |       |
| Step 7 enzyme*          | 67.1     |       |
| Step 7 enzyme - Na₂EDTA | 70.3     |       |
| Step 7 enzyme - mercaptoethanol | 9.6  |       |
| Step 7 enzyme - HAA + anthranilic acid | 0.9 |       |
| Step 7 enzyme - HAA + MHA | 0.3    |       |
| Step 7 enzyme - HAA + 3-hydroxy-DL-kynurenine | 1.4 |       |
| Step 7 enzyme - HAA + o-aminophenol | 0      |       |
| Step 7 enzyme - HAA + benzoic acid | 0.5    |       |
| Step 7 enzyme - HAA + 3-hydroxybenzoic acid | 0.4  |       |

* 140 µg of extract protein.
+ 7.5 µg of enzyme.
* Reaction mixtures were incubated for 2 h.

**Fig. 6. Thin layer chromatography of the products of the methyltransferase reaction.** Large-scale incubations were prepared and extracted as described under "Materials and Methods." Panel A depicts an autoradiogram of the reaction products chromatographed in solvent II. The experiment whose results are shown in panel B, the product of the methyltransferase reaction was treated with phenoxazinone synthase and the products of the last reaction were extracted and chromatographed in solvent I. Panel C shows the products obtained from incubation of the methyltransferase reaction product with S. antibioticus mycelium. The arrow indicates the origin in each panel.

stimulated by 2-mercaptoethanol. At 30 °C and under the other assay conditions specified under "Materials and Methods," the reaction was linear for up to 2 h. With purified enzyme no lag period was observed before the onset of linear product formation. The enzyme was also active at 37 °C, indeed, initial velocities were higher at that temperature than at 30 °C. However, the reaction was linear for only about 1 h at 37 °C.

Some information on the substrate specificity of the methyltransferase reaction is also shown in Table II. It can be seen that the enzyme is quite specific for HAA and is essentially inactive with a number of analogs of that substrate. It must be noted that the ability to detect radioactive products in the assay used here depends on the ability of those products to partition preferentially into ethyl acetate upon extraction of acidified reaction mixtures. It was necessary, therefore to determine whether the substrates used here, and where possible, whether the methylated derivatives of those substrates, were extractable from the acidified reaction mixtures with ethyl acetate. In such experiments, it was observed that about 75% of HAA or MHA, in reaction mixtures containing those compounds at 2 mM, could be extracted into the ethyl acetate phase following HCl treatment. While methylated derivatives of the other putative substrates tested were not available, it was observed that 51, 98, 100, 75, and 32%, respectively, of added 3-hydroxy-DL-kynurenine, benzoic acid, 3-hydroxybenzoic acid, anthranilic acid, and o-aminophenol (each at 2 mM) could be extracted from reaction mixtures following acidification. Assuming the methylated derivatives of these compounds partitioned to the same extent as the unmethylated form (as was the case for HAA and MHA, see above) and given the estimated detection limits of the radioactive assay for methyl transfer, it can be calculated that the assay would allow the facile detection of the methylated product even if that product partitioned into ethyl acetate 20 times less effectively than MHA or if the substrate in question were utilized 20 times less efficiently than HAA.

**Product of the Methyltransferase Reaction**—The product of the methyl transfer reaction was extracted from large-scale reaction mixtures as described under "Materials and Methods" and was analyzed by thin layer chromatography. A typical chromatogram, using solvent II is shown in Fig. 6A. The radioactive product of the reaction had the mobility of MHA in this solvent and in solvent I (data not shown). Furthermore, Fig. 6B shows that reaction of the product of the methyltransferase reaction with phenoxazinone synthase leads to the formation of a compound which has the chromatographic mobility of actinocin. Phenoxazinone synthase would be expected to form actinocin from MHA. Fig. 6C shows that the radioactive product of the methyltransferase reaction can also be converted to actinomycin by S. antibioticus mycelium. Analysis of the products of the last experiment also reveals the presence of a component with the chromatographic mobility of actinocin (Fig. 6C). That product may have been formed as a result of the nonenzymatic oxidation of the added MHA rather than from the reaction of MHA with phenoxazinone synthase in the mycelium. Taken together, these data argue strongly for the identity of MHA as the product of the methyltransferase reaction.

**Kinetics of the Reaction**—To estimate kinetic parameters for the methyltransferase reaction, incubation mixtures were prepared as described under "Materials and Methods." To examine the kinetics of HAA utilization, those mixtures contained 0.109 mM AdoMet, while the kinetics of AdoMet usage were examined in the presence of 1.5 mM HAA. Eadie-Hofstee
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plots and the values of $K_m$ and $V_{max}$ estimated therefrom are provided in Fig. 7.

DISCUSSION

With the addition of the methyltransferase described in this report, a total of five enzymes have been characterized which appear to be involved in the biosynthesis of actinomycin. These include the MHA-activating enzyme and the actinomycin synthetases described by Keller and co-workers (1, 2) and phenoxazinone synthase, which catalyzes the formation of the chromophore of the antibiotic (5, 6). If, as seems likely, the formation of the lactone ring of the antibiotic also requires enzyme catalysis, the lactonizing enzyme is the only one directly involved in the biosynthetic pathway which remains to be isolated. It must be mentioned here that the enzymes just referenced have been isolated from different members of the genus *Streptomyces*. Nevertheless, it seems likely that there will be significant similarities in the enzymology of the actinomycin biosynthetic pathway among the producing organisms. It should be further noted that Katz and co-workers (12, 13) have shown that the phenoxazinone chromophore of the antibiotic is derived from the catabolism of tryptophan and those workers have isolated and characterized several of the streptomycete enzymes involved in tryptophan catabolism.

The data provided above indicate that HAA is the preferred substrate for the methyltransferase and that 3-hydroxy-DL-kynurenine is not an active substrate. This observation would seem to eliminate one of the alternative pathways which might be imagined for the formation of MHA. The result obtained is especially significant in view of the report of Perlman et al., (14), that chemically synthesized 4-methyl-3-hydroxykynurenine inhibited the incorporation by *S. antibioticus* of labeled tryptophan into actinomycin and stimulated the incorporation of other precursors into the antibiotic. These workers did find, however, that MHA and HAA exhibited the same effects. It is likely, therefore, that 3-hydroxy-DL-kynurenine is not the in vivo substrate in the methyl transfer reaction and that the results of Perlman et al. reflect the in vivo conversion of 4-methyl-3-hydroxykynurenine to MHA. It would, thus, be interesting to know if 4-methyl-3-hydroxykynurenine is a substrate for kynureninase.

To date, only one of the genes for enzymes implicated in actinomycin biosynthesis has been cloned, the gene for phenoxazinone synthase (15, 16). The development of reasonably facile assays for the enzymes which have been characterized more recently, including the methyltransferase described in this report, may well allow the isolation of the genes for those enzymes from gene libraries using a sib selection procedure similar to that employed in the cloning of the phenoxazinone synthase gene.

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