EVIDENCE FOR THE INCORPORATION OF L-THIAZOLIDINE-4-CARBOXYLATE INTO ACTINOMYCINS BY STREPTOMYCES ANTIBIOTICUS

Jonathan S. Nishimura and William F. Bowers

Department of Biochemistry, Tufts University
School of Medicine, Boston, Massachusetts 02111

Received July 13, 1967

During the past few years several new actinomycins have been discovered through studies in which certain amino acids and amino acid analogs were added to cultures of the producing organisms, resulting in amino acid replacements in the two pentapeptide chains of these chromopeptide antibiotics. The sites most susceptible to replacement appear to be those occupied by proline.Thus, a number of imino acids, such as sarcosine, hydroxyproline, azetidine-2-carboxylic acid, 4-methyl proline and 5-methyl proline have been shown to be incorporated in the place of proline into actinomycins (Katz and Weissbach, 1965; Yoshida et al., 1966). The observation that L-thiazolidine-4-carboxylate (T-4-C) interfered with the incorporation of ^{14}C proline into rat liver ribosomes, presumably acting as a proline analog (Bekhor et al., 1965) suggested to us the possibility that this sulfur-containing compound might also be incorporated into actinomycins. Evidence is presented here that T-4-C not only affects the composition of the actinomycin mixture produced by Streptomyces antibioticus, but is also incorporated by the organism into at least two new actinomycins.

MATERIALS AND METHODS

Streptomyces antibioticus (ATCC 14888; Rutgers Institute of Microbiology Culture Collection No. 3720) was cultured in the galactose-glutamic acid minimal medium described by Sivak, et al. (1962). The actinomycins were extracted from culture filtrates and chromatographed on silica gel G plates (Katz, et al., 1965). Sulfur-35-labeled

L-cystine (34.8 mcuries/mmole) was obtained from Schwarz BioResearch and was converted to L-cysteine by reduction with 2,3-dithiothreitol (Cleland, 1964) at pH 8.5 under nitrogen. Thiazolidine-4-carboxylic acid, unlabeled and labeled, was synthesized from L-cysteine and formaldehyde according to the procedure of Ratner and Clarke (1937). "Highly polymerized" calf thymus DNA was a product of General Biochemicals. Polydeoxy (alternating adenylic-thymidylic) acid (dAT co-polymer) was kindly provided by Dr. Saul A. Slapikoff. Sephadex G-100 (medium) was purchased from Pharmacia. Small columns of Sephadex were prepared using 1 ml Corning glass disposable serological pipets which were plugged at the tips with glass wool. The column bed volumes were approximately 0.75 ml. Single-drop fractions (.045-.050 ml) were collected on aluminum planchets, dried and counted in a gas-flow planchet counter. Thin layer plates were counted in a Nuclear Chicago Actigraph III scanner. The scan rate used was 30 cm/hr with a slit width of 6 mm.

RESULTS

Initial experiments were directed toward studying the effect of varied concentrations (1 to 100 ug/ml) of T-4-C on the production of actinomycins I (containing 1 proline and 1 4-hydroxyproline residue), IV (containing 2 proline residues) and V (containing 1 proline and 1 4-ketoproline residue) by cultures of S. antibioticus. Actinomycin synthesis, as indicated by the periodic measurement of antibiotic titer over several days, was inhibited 50%at a T-4-C concentration of 100 ug/ml. It is of interest that the decreased synthesis of antibiotic was accounted for principally by the almost complete cessation of actinomycin V formation and, to a much lesser degree, by a decreased rate of synthesis of actinomycins I and IV. These results were corroborated by studies in which 72-hour cultures to which T-4-C (100 ug/ml) had been added at 48 hours were incubated for short periods (15 to 60 minutes) with ^{14}C-valine or ^{14}C-proline. In these experiments the amount of label found in actinomycin V dropped from 31-33%(based on radioactivity incorporated into total actinomycins) in the controls to 6%in the analog-treated cultures. Correspondingly, the

amount of radioactivity found in actinomycin IV rose from the control value of 41%to 76%. It would appear that T-4-C interferes with 4-ketoproline synthesis from proline or with 4-ketoproline utilization in antibiotic polypeptide chain synthesis. Aside from actinomycin I no other labeled actinomycins were evident. The likelihood remained that actinomycins containing T-4-C might be synthesized at a relatively slow rate and would not, as a consequence, be readily detectable. In view of this possibility, it appeared that the employment of radioactive T-4-C in such experiments would be desirable. The ^{35}S-labeled compound was therefore prepared.

Cultures of S. antibioticus were incubated for various periods of time in the presence of ^{35}S-T-4-C. Significant quantities of radioactivity were found in the actinomycin fraction with maximal incorporation occurring between 8-12 hours after addition of the labeled compound. The actinomycins were extracted from the cultures and subjected to thin layer chromatography. A representative chromatogram is described in Figure 1.

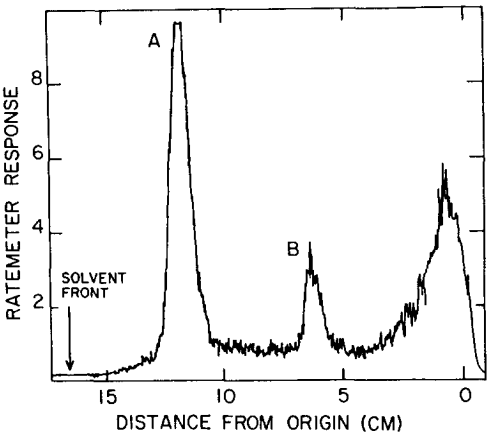

Fig. 1. Actigraph III scan of thin layer chromatogram of ^{35}S-actinomycin fraction. T-4-C (0.75 umole, 20 x 10^6 cpm) was added to a 72-hour culture (100 ml) of S. antibioticus and shaking was continued for 12 hours at 28°. Actinomycins were extracted from the culture filtrate with ethyl acetate. After evaporation of the ethyl acetate the residue was dissolved in a minimal volume of methanol. One-third of the resulting solution was streaked on a 2" x 8" silica gel G plate and chromatographed. After development the plate was scanned as described under Materials and Methods. Full scale ratemeter deflection represents 5,000 counts/min.

Two major components migrated from the origin. Component A moved between the actinomycin IV and V bands, while component B moved just ahead of actinomycin I. Radioautography of the same plate revealed that components A and B were single bands and that the radioactivity at, or near, the origin consisted of several minor bands. It was not possible to determine if components A and B had associated with them the color characteristic of the actinomycins. However, a much more sensitive test was devised to identify these compounds as actinomycins.

Component A was eluted with methanol from scrapings of the plate described above. The methanol was evaporated and the residue dissolved in Tris buffer (.01 M, pH 7.4; .01 M NaCl). The solution was diluted appropriately with the same buffer and used in the experiment described in Figure 2. This experiment was designed to test the ability of component A to bind to double-stranded DNA. The system used was in principal that

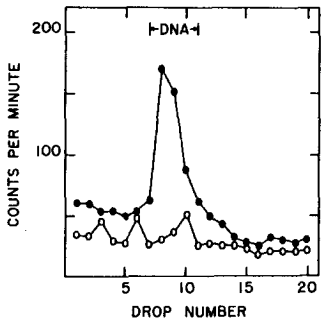

Fig. 2. Sephadex G-100 filtration of component A-DNA reaction. Columns were washed with approximately 1.8 ml of solution containing Tris buffer (.01 M, pH 7.4; .01 M NaCl) and component A. A solution (0.18 ml) of Tris buffer and component A at the same concentrations, but also containing polydeoxyribonucleotide, was passed through the column and eluted with Tris buffer. See Materials and Methods for other details. Closed circles: calf thymus DNA (0.23 umole DNA-P), component A (2200 cpm/ml); open circles: dAT co-polymer (.046 umole DNA-P), component A (1540 cpm/ml).

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described by Hummel and Dreyer (1962) for the measurement of binding of substrate or inhibitor molecules to proteins under conditions in which equilibrium dialysis is performed

during gel filtration. Since component A was contaminated by some actinomycin IV and V, no attempt was made to establish conditions whereby equilibrium could be achieved. However, the results indicate binding of componentA to calf thymus DNA. On the other hand, component A did not bind significantly to synthetic dAT co-polymer. The latter result was anticipated, as the presence of deoxyguanylic acid residues in a double-stranded polydeoxyribonucleotide is required for binding of actinomycins (Gellert, et al., 1965).

Evidence that the ^{35}S in components A and B is actually present in T-4-C residues has come from experiments in which the ^{14}C-compound (labeled with formaldehyde) was synthesized and incubated with S. antibioticus. Both components A and B were labeled with ^{14}C.

T-4-C is known to retain reactivity to certain sulfhydryl reagents and other properties displayed by cysteine (Ratner and Clarke, 1937). Although these properties would be expected to change when this compound is peptide-linked, as is the case with N-acetyl T-4-C (Ratner and Clarke, 1937), it may be possible to chemically alter the T-4-C residue in such a way that it no longer resembles proline. For example, conversion of this residue to cysteine would abolish the imino acid structure at that particular site. It would be of great interest to determine the effect of such a change on the chemical, physical and biological properties of the resulting actinomycins. The answer to this question and the determination of the location of T-4-C in the peptide chains await isolation of larger quantities of the sulfur-containing actinomycins.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Steven H. Weinograd for excellent technical assistance. This work was supported by a grant, AI-06366, U.S.P.H.S.

REFERENCES

Bekhor, I.J., Mohseni, Z., and Bavetta, L.A. (1965), Proc. Soc. Exp. Biol. and Med., 119, 765.

Cleland, W.W. (1964), Biochemistry, 3, 480.

Gellert, M., Smith, C.E., Neville, D., and Felsenfeld, G. (1965), J. Mol. Biol., 11, 445.

Hummel, J.P., and Dreyer, W.J. (1962), Biochim. Biophys. Acta, 63, 530.

Katz, E., and Weissbach, H. (1963), J. Biol. Chem., 238, 666.

Katz, E., and Weissbach, H. (1965), in "Biogenesis of Antibiotic Substances" (Z. Vanek and Z. Hostalek, eds.), p. 195. Academic Press, New York.

Katz. E., Mauger, A., and Weissbach, H. (1965), Mol. Pharmacol., 1, 107.

Ratner, S., and Clarke, H.T. (1937), J. Am. Chem. Soc., 59, 200.

Sivak, A., Meloni, M., Nobili, F., and Katz, E. (1962), Biochim. Biophys. Acta, 57, 283.

Yoshida, T., Mauger, A., Witkop, B., and Katz, E. (1966), Biochem. Biophys. Res. Commun., 25, 66.