Reinvestigation of Compound X, a Suspected Biotin Intermediate: Identification of N-Formyl Derivatives of Biotin and Dethiobiotin

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Summary Compound X, reported as an intermediate in the biosynthesis of biotin from dethiobiotin (DTB) (Biochem. Biophys. Res. Commun., 88, 312 (1979)), was found to contain N-formyl DTB and biotin. The methyl ester of N-formyl biotin was considered to be a product from the biotin, which was biosynthesized from DTB by resting cells of E. coli C 124, through treatment with diazomethane in the presence of a trace amount of formic acid after Dowex 1X2 column chromatography. NMR analysis revealed that biotin was formylated at 1'-N. N-Formylated DTB and biotin are new biotin derivatives.

Key Words compound X, biotin intermediate, N-formyl biotin, N-formyl dethiobiotin

Salib et al. (1) reported that compound X was isolated from the reaction mixture of resting cells of Escherichia coli C124 (bio A −, His−) with [3H] and/or [14C]DTB and that the compound was possibly an intermediate in the biosynthetic pathway between DTB and biotin. However, the structure of compound X could not be established because it was not yet completely pure and only available in minute quantities. In the course of our study on compound X, we found that compound X consisted of at least two components. The two components were found to be new compounds which are artificially synthesized N-formyl derivatives of DTB and biotin. This paper describes the reinvestigation of compound X as a biotin intermediate and the determination of the structure of these two components.

MATERIALS AND METHODS

Materials. [3,4-3H]DTB (200 mCi/mmol) were prepared as described previously (2). Other chemicals were commercial products.

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Analytical techniques. Radioactive biotin vitamers were identified using silica gel thin layer chromatoplates (TLC plate) (aluminium sheet, Merck, 60 F$_{254}$).

The developing solvent was ethyl acetate–acetone (4:1) for methyl esters of biotin vitamers. With this solvent, compound X could be more distinctly separated from DTB and biotin methyl esters than with the previous solvent, ethyl acetate–methanol (4:1). Spots with radioactivities were detected as follows: After the solvent front moved 17 cm, the silica gel plate was cut into 1 x 1 cm pieces. Each piece was put into Bray's liquid scintillator (3). Radioactivity of each piece was detected with a Beckman LS-100 liquid scintillation counter. Biotin vitamers containing a ureido ring were also detected on developed chromatograms by spraying with p-dimethylaminocinnamaldehyde (p-DACA)(4).

Bacterial strains and cultivation. E. coli C 124 (bio A–, His–) was cultivated as described previously (1) or cultivated in a 70-liter jar fermenter at 37°C for 24 h.

Incubation experiments and isolation of compound X. The incubation experiments and isolation of compound X were carried out as described previously (1), except that Dowex 1X2 (formate 100–200 mesh) was used instead of A-G 1X2.

Preparation of methyl esters of N-formyl DTB and biotin. DTB or biotin (10 mg) was dissolved in 20 ml of formic acid and evaporated to dryness under reduced pressure. After being dissolved in 5 ml of methanol, the solution was treated twice with diazomethane. p-DACA-positive compounds were isolated from a TLC plate developed with the solvent, ethyl acetate–acetone (4:1). N-Formyl biotin methyl ester was also synthesized by the method of Sheehan and Yang (5). Cold biotin methyl ester (5 mg) was incubated overnight with 3 ml of formic acid and 1 ml of acetic anhydride at room temperature. N-Formyl biotin methyl ester was isolated by silica gel column chromatography using the solvent system of ethyl acetate–acetone (4:1).

Assay of E. coli C 162 activity. Aliquots of fractions in silica gel column chromatography were saponified using NaOH (1 N) for 3 h at room temperature and neutralized with HCl. In the case of TLC, each 1 x 1 cm piece was subjected to extraction with methanol for about 1 h, and the extracts were saponified in the same way. Microbiological activities (E. coli C 162 activity) of saponified samples were bioassayed using E. coli C162 (bio B+, His−), a mutant blocked between DTB and biotin, with biotin as the standard by the turbidimetric method as described elsewhere (6).

Instrumental analysis. Mass spectra analyses were carried out on a Hitachi M-80 instrument in the conditions of In-Beam EI (20 eV). NMR spectra were measured on a JEOL-JNM GX-400 spectrometer at 400 MHz in CD$_3$OD and chemical shifts were given in ppm with tetramethylsilane as 0.00 ppm.

RESULTS AND DISCUSSION

Reinvestigation of homogeneity of compound X

Compound X methyl ester was obtained from the reaction mixture of resting

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cells of *E. coli* C 124 with [3H]DTB, through ethanol treatment, Dowex 1X2 column chromatography, esterification by diazomethane and three silica gel column chromatographies, in the same manner as described previously (1). In a previous paper (1), compound X was referred to as the substance showing a radioactive single peak in the third silica gel column chromatography (the peak of Fractions No. 42 to No. 62 in Fig. 1A). Since we established the microbiological assay method of biotin with *E. coli* C 162, the *E. coli* C 162 activity of each fraction in the third silica gel column chromatography was measured as well as radioactivity. As a result, the peaks of the two activities were separated slightly from each other (Fig. 1A). Furthermore, the fraction samples of each peak (Fractions No. 47 and No. 50) were chromatographed on the TLC plate. As shown in Fig. 1B, Fraction No. 47 showed radioactivity, but no *E. coli* C 162 activity, at a Rf value of 0.43, while Fraction No. 50 showed *E. coli* C 162 activity at a Rf value of 0.38 and radioactivity in the Rf value range of 0.38–0.50. These results suggest that compound X consists of at least two components, one having *E. coli* C 162 activity (referred to as C 162-active X) and the other having no such activity (referred to as C 162-inactive X).

*Preparation and structure determination of C 162-inactive X*

During the examination of optimal culture conditions of *E. coli* C 124 and optimal reaction conditions for large-scale preparation of compound X, we found that C 162-inactive X could be detected even in the reaction mixture without *E. coli* C 124 cells or with boiled cells (Fig. 2A). Furthermore, there was no significant...
difference in the radioactivity of C 162-inactive X between the zero-time and 5-h reaction mixtures. These observations suggested that C 162-inactive X was produced artificially from DTB during the purification procedures of the reaction mixture. As we expected, this compound was produced when DTB was treated with diazomethane in the presence of formic acid as shown in Fig. 2B, while not in the absence of the acid. Therefore, to identify its structure, we synthesized a large amount of this compound by methylation of cold DTB in the presence of formic acid. Then, we found that two p-DACA-positive compounds were synthesized: one with a $R_f$ of 0.45 and the other with a $R_f$ of 0.38 on the TLC plate. The two compounds were isolated as described in the MATERIALS AND METHODS and identified from mass spectra as 1'- or 3'-N-formyl DTB methyl ester, as shown in Fig. 3. Neither E. coli C 162-inactive compound was stable and was partially transformed into DTB methyl ester after one or two months at 4°C.

Preparation and structure determination of C 162-active X

We confirmed that the C 162-active X was synthesized from [3H]DTB in the
reaction mixture with resting cells of *E. coli* C 124. However, the amount of the compound was too small to isolate it from the reaction mixture and determine its structure.

On the other hand, the question arose to us whether the C 162-active X is a biotin intermediate, because the possibility was considered that *E. coli* C 124 synthesizes biotin from DTB, which can be derivatized to 1'- and/or 3'-N-formyl biotin methyl ester during the isolation process in the same manner as observed with DTB. In fact, detectable biotin activity in the reaction mixture could be assayed with *Lactobacillus plantarum*, a biotin assay microorganism; the activity was especially high when the reaction was performed at pH 8. In addition, saponification of compound X, a mixture of C 162-active X and C 162-inactive X, with 1 N NaOH followed by methyl-esterification yielded both biotin and DTB.

Therefore, cold biotin was treated with diazomethane in the presence of formic
acid as described in the MATERIALS AND METHODS. We could see one p-DACA-positive spot besides the spot of biotin on a TLC plate, and isolated it by silica gel column chromatography.

This product was compared with C 162-active X. The $R_f$ values of both compounds in TLC were the same. The product showed microbiological activity toward E. coli C 162 when saponified with 1N NaOH. As observed with Fraction No. 50, the product was partially transformed into biotin methyl ester after one- or two-month preservation at 4°C.

From these observations, this compound was considered to be identical with C 162-active X. As shown in Fig. 4, the mass spectrum analysis indicates that the product was N-formyl biotin methyl ester.

Furthermore, according to the method of Sheehan and Yang (6), we could also synthesize more efficiently large amounts of N-formyl biotin methyl ester by treatment of biotin methyl ester with formic acid and acetic anhydride, as described in the MATERIALS AND METHODS. However, in this case, we obtained two p-DACA-positive compounds as the products, both of which showed the same mass spectrum and E. coli C 162 activity as the above N-formyl biotin methyl ester. One had a $R_f$ value of 0.39 in TLC, and the other 0.33. In the following, the former is referred to as [A] and the latter as [B]. Therefore, C 162-active X was considered to be identical to [A].

In order to determine whether the formyl group of C 162-active X is at 1'-N or 3'-N, NMR analysis of [A] and [B] was performed. From the NMR spectrum data,
these two compounds were also identified as N-formyl biotin methyl ester. The proton of formyl group appeared as a doublet at 8.8 ppm in both spectra. These spectra were characterized by their signal patterns at 4.0–4.9 ppm.

The signal of H-5 in [A] shifted in a field lower than H-4 ($\delta = 4.84$ ppm for H-5, $\delta = 4.29$ ppm for H-4), while in case of [B], that of H-4 was lower ($\delta = 4.48$ ppm for H-5, $\delta = 4.78$ ppm for H-4). It was considered that this low-field chemical shift was due to an adjacent formyl group. The H-5 signal in [A] was decoupled by irradiation of the formyl group proton, which also supports the above deduction.

The H-6a signal of [A] appeared as a doublet, while that of [B] as a double doublet. This could be explained if R' of [B] is the formyl group and steric hindrance between the formyl group and the side chain group of biotin occurs. This explanation was supported by the following observation: The H-6a signal of authentic 3'-N-methyl biotin appeared as a double doublet because of steric hindrance between the methyl and side chain groups.

All the data suggest that C 162-active X was 1'-N-formyl biotin methyl ester ($R = \text{CHO}, R' = \text{H}, \ 1$). Thus compound X previously reported to be a biotin intermediate was considered to be a mixture containing N-formyl DTB and biotin methyl esters which were synthesized through the treatment of the DTB and biotin contained in the reaction mixture with diazomethane after Dowex 1X2 column chromatography. At the moment, the possibility that compound X contains a true biotin intermediate between DTB and biotin cannot be completely excluded since the group of Marquet has observed a different radioactive spot from those of N-formyl biotin and DTB on TLC plates. Their detailed study on it is in progress.

It should be emphasized that this paper is the first demonstration of N-formyl DTB and biotin.

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