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ON THE MECHANISM OF CONVERSION OF DETHIOBIOTIN TO BIOTIN IN *Escherichia coli*. III ISOLATION OF AN INTERMEDIATE IN THE BIOSYNTHESIS OF BIOTIN FROM DETHIOBIOTIN (1,2)

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SUMMARY : An intermediate in the biosynthetic pathway between dethiobiotin and biotin has been isolated for the first time, from the incubation medium of resting cells of *E. Coli C124* (bio A⁻, His⁻) with |^{3}H| or/and |^{14}C| dethiotiotin. This compound contains sulfur. It promotes the growth of *E. Coli 124* as well as other strains which are strictly biotin requiring, i.e. blocked between dethiobiotin and biotin. The conversion of the labeled intermediate into biotin by growing cells of *E. Coli C124* has been established.

The biosynthesis of biotin 1 has already been intensively studied (3). All the intermediates in the biosynthetic pathway from pimelic acid to dethiobiotin 2 are presently known and formed by classical biochemical reactions. But the mechanism of the conversion of dethiobiotin 2 into biotin, a very unusual transformation, is still completely unknown.

The mechanism of the formation of C - S bonds in sulfur containing natural products raises a general problem which is not solved, either for other biologically important molecules such as thiamin(4), lipoic acid (5) or penicillin(6).

In our first investigation (1) of biotin biogenesis, we have shown that the formerly proposed hypothesis of an unsaturated intermediate of type *i* was highly improbable (scheme 1).

Another obvious hypothesis for the functionalization of the saturated carbons was an hydroxylation process. But we have recently shown (2) that the dethiobiotin derivatives hydroxylated at position 2 or 5 or 2 and 5, *ii*, are very likely not intermediates, either.

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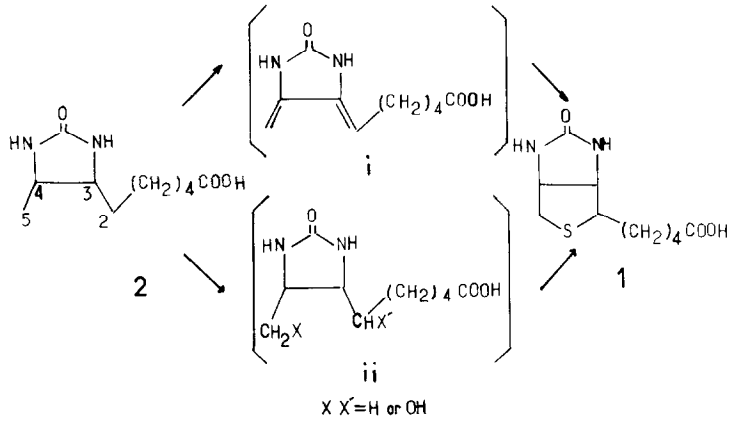

Scheme 1

It is clear that isolation of an active intermediate from the culture medium would provide the most direct help to solve this difficult problem. But till now, in spite of many efforts, such an approach has always failed.

We report here that we have now succeeded to trap a new biotin vitamer, which is an intermediate in the biosynthetic pathway between dethiobiotin and biotin.

MATERIALS AND METHODS

Materials

All chemicals were of the highest purity available. The $|^{35}S|$ as sulfate, in aqueous solution (carrier-free) and d$|2' - ^{14}C|$ biotin (58 mCi/mmol) were purchased from Radioactive Centre Ltd, Amersham. $|3,4 - ^{3}H|$ dethiobiotin (200 mCi/mmol) and $|10 - ^{14}C|$ dethiobiotin (34 mCi/mmol) were prepared in our laboratory (7).

Analytical Technics

Radioactive biotin or biotin vitamers were identified using silica gel thin layer chromatoplates (Merck, 60 F_{254}). The developing solvent was n BuOH, H_2O, C_6H_6, MeOH ; 2:1:1:1 +ε for biotin vitamers and ethyl acetate, methanol 4:1 for their methyl esters. Scanning was carried out by using Berthold NIM-2300, series Scanner. Biotin vitamers containing ureido ring were detected on developed chromatograms by p-dimethylaminocinnamaldehyde (P-DACA) (8).

Scintillation Counting

Scintillation Counting was carried out with an Intertechnique SL_{30} spectrometer in Bray's liquor (9). Simultaneous $|^{3}H|$, $|^{14}C|$ or $|^{35}S|$ counting was achieved through dual channel discriminator. The quench curves were established with $|^{3}H|$ dethiobiotin, $|2' - ^{14}C|$ biotin and $|^{35}S|$ sulfate as standards. All the results were corrected for quenching by external standard method.

Bacterial Strains and Media

E. Coli C124 (bio A^-, His^-) and *C 162* (bio B^-, His^-) are grown on their basic medium as described by Rickenberg *et al.* (10). *E. Coli B 105* (bio^-), *B 107* (bio^-) and *B 112* (bio^-) are grown on their basic medium as described by Rolfe and Eisenberg (11). Before autoclaving, the media were treated with Norit to remove traces of biotin.

Incubation Experiments

E. Coli C 124 was grown on medium containing biotin (0.3 ng/ml) at 37°C for 24 h with shaking (10^2 rpm). The cells were harvested and washed twice with the incubation medium at pH 6.6 (KH_2PO_4, 6.8g ; glucose, 3.6 g ; $MgSO_4$, 0.246 g per litre redistilled water).

The reaction mixture containing 230 mg resting cells as dry matter and 10 µg of labelled dethiobiotin |21 x 10^6 dpm of |^3H| or 5.2 x 10^6 dpm of |^{14}C| dethiobiotin or a mixture of |^3H| and |^{14}C| dethiobiotin (2 : 1 W/W)| in a total volume of 10 ml of incubation medium (pH 6.6) was shaked (10^2 rpm) for 5h at 37°C.

The experiment with |^{35}S| was carried out by growing *E. Coli C 124* on a synthetic medium containing per litre NH_4Cl, 1.62 g and $(NH_4)_2SO_4$, 0.1 g supplemented by |^{35}S| as sulfate, 20 mCi (26.4 mCi/mmol). The incubation mixture of resting cells contained 0.4 mCi |^{35}S| as sulfate per 10 ml (40 mCi/mmol |^{35}S| as $MgSO_4$).

Isolation of the Intermediate

The reaction mixture was centrifuged (10^4 rpm for 20 min) and the supernatants were treated several times with ethanol 99 % until precipitation stops (12). The supernatant solution was evaporated under reduced pressure to a small volume and poured over a column of anion exchange resin (A-G.1-X2, formate, 100-200 mesh, 17 x 600 mm). After washing out with 200 ml of distilled water, the product was eluted using a linear gradient from 500 ml of distilled water to 500 ml of 0.1 N formic acid. The eluted radioactive fractions (10 ml each) were collected, evaporated to dryness under reduced pressure and converted to methyl esters using freshly prepared diazomethane. The methyl esters were chromatographed on a silica gel-60 column (70-200 mesh, 17 x 600 mm) using ethyl acetate : methanol (4 : 1) as eluent. Two radioactive peaks were observed, one corresponding to dethiobiotin methyl ester and the other one to a new compound, X. The latter fractions were rechromatographed several times, 4 / 5 times, for purification. The purified compound did not contain more than 0.5 % of dethiobiotin methyl ester.

Growth Promoting Activity of the Intermediate

Methyl ester of the isolated intermediate was saponified using NaOH(1N) for 3 h at room temperature. The acid was purified on AG 1-X2, as before, and sterilized by filtration (Sartorius membrane, pore size 0.2µ.). It has been checked by reesterification that this treatment leaves X unchanged.

Each strain of the above-mentioned bacteria was grown, separately, on preculture containing biotin (1 ng/ml culture). Bacteria were harvested by centrifugation (10^4 rpm for 10 min.), washed twice with NaCl 9‰. The cells were suspended in 5 ml of NaCl 9‰ and diluted 100 times with NaCl 9‰. The culture medium (10 ml) containing the isolated intermediate (1 x 10^3 dpm/ml) were inoculated with one drop of the bacterial suspension. Absorbance was measured at 570 nm during the growth phase and generation time was calculated. Blank experiments were carried out without addition of biotin vitamers.

Conversion of the Isolated Intermediate to Biotin

E. Coli C124 was grown on the isolated intermediate (8.2×10^3 dpm/ml). The crude protein-bound biotin was isolated (1) and treated several times with ethanol (99%) followed by methanol : ether (4:1) until precipitation stops. The isolated bound biotin was purified according to Ogata (13).

RESULTS AND DISCUSSION

I. Isolation of the Intermediate

Resting cells of *E. Coli C124*, an auxotrophic strain whose biosynthetic pathways to biotin is blocked before dethiobiotin, were incubated with $|^3H|$ dethiobiotin. In the supernatant, a new biotin vitamer has been detected and isolated.

Column chromatography on AG I-X2 gave a broad radioactive peak, the R_F of which on thin layer chromatoplates was close to that of dethiobiotin as shown by autoradiography (Fig.1-A).

Chromatography of the methyl esters of these fractions on silica gel revealed two peaks, one corresponding to dethiobiotin, the other one to a new compound X (fig. 1-B). X was rechromatographed several times. The absence of significant amounts of dethiobiotin after that treatment was ensured by cochromatography of a mixture of methyl esters of $|^3H|$X and $|^{14}C|$ dethiobiotin (Fig. 2). Nearly 10 % of the radioactivity of the incubated dethiobiotin was recovered in X compound.

II. Growth Promoting Activity of X and Conversion into Biotin

We checked the growth promoting activity of this intermediate for several biotin or dethiobiotin requiring mutants of *E. Coli*. It is active

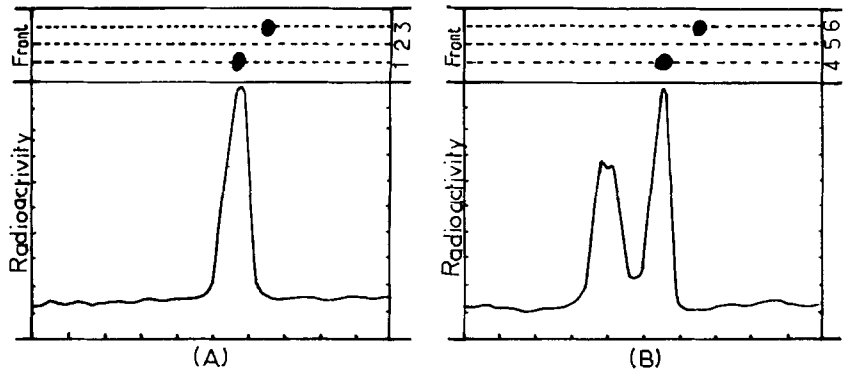

Fig. 1. Autoradiography of the isolated biotin vitamers from *E. Coli C124*

(A) before-esterification, (B) after esterification.
(1) dethiobiotin, (2) biotin vitamer, (3) biotin,
(4) dethiobiotin methyl ester, (5) biotin vitamer methyl ester, (6) biotin methyl ester.

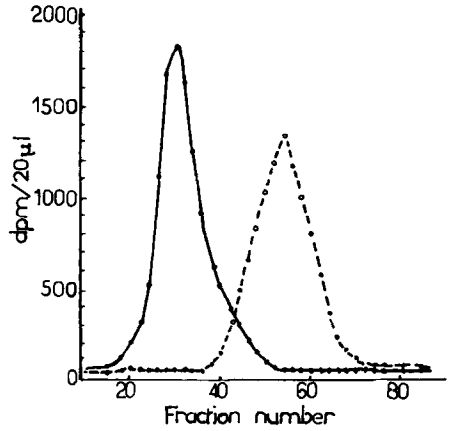

Fig. 2. Column chromatography of a mixture of methyl esters of $|^{3}H|$ X compound (-•-) and $|^{14}C|$ dethiobiotin (-∘-).
Column : silica gel-60 (70-200 mesh, 17 X 600 mm)
Eluant : Ethyl acetate : methanol 4 : 1
Fraction volume : 1 ml

for all the strains tested, not only for *C124*, from which it was produced, but also for other ones (Table I), which are biotin requiring and cannot convert dethiobiotin into biotin.

This shows, that X is, very likely, on the biosynthetic pathway going from dethiobiotin to biotin and that the biotin requiring mutants are blocked before the formation of X.

The generation times are given in Table I. As we know (*vide infra*.) that X sample is not completely pure, a quantitative comparison is not possible. However it is clearly seen from Table I that the generation times are of the same order of magnitude as those observed with biotin or dethiobiotin.

Table I

Generation time of different *E.Coli* strains grown on dethiobiotin, biotin or $|^{3}H|$X-intermediate.

E. Coli strain	Generation time of *E. Coli* grown on: dethiobiotin (0.5ng/ml) h	min	biotin (0.5ng/ml) h	min	$|^{3}H|$X-intermediate ($1X10^{3}$dpm/ml)[a] h	min	$|^{3}H|$Y-intermediate ($1X10^{3}$dpm/ml)[a] h	min
C 124[b]	1	55	1	30	1	30	1	40
C 162[c]			1	25	1	25	1	55
B 105[d]			2	35	2	35	2	55
B 107[d]			1	30	1	40	1	55
B 112[d]			1	45	1	55	1	55

(a) corresponding approximately to 0.5 ng/ml assuming that its *Mr* equal to dethiobiotin.
(b) Able to grow on dethiobiotin (P. Cleary-personal communication).
(c) Not able to grow on dethiobiotin (P. Cleary-personal communication).
(d) Not able to grow on dethiobiotin (11).

Table II

Purification and crystallization of biotin produced form X-intermediate by *E. Coli C 124*

|3H| Biotin	total dpm |3H|	|^{14}C|	|^{3}H/14|Ratio
Crude extract	30.9×10^4		
After precipitation with ethanol	22.6×10^4		
After purification	19.3×10^4		
After addition |^{14}C| biotin	16.8×10^4	7.40×10^4	2.27
After cochromatography	$13.4 \times 10^{3*}$	$5.93 \times 10^{3*}$	2.26
After first crystallization	$11.4 \times 10^{3*}$	$4.90 \times 10^{3*}$	2.33
After second crystallization	$36.7 \times 10^{3*}$	$1.66 \times 10^{4*}$	2.21

* In the counted sample.

To prove definitely that X is a precursor of biotin, its conversion into biotin has been demonstrated (Table II).

E. Coli C124 was grown in the presence of \|^{3}H\| X (8.2×10^3dpm/ml). Bound biotin was isolated as previously described (1) and purified. It was identified by autoradiography under its acid and methyl ester form, as well as by column cochromatography with \|^{14}C\| biotin. The \|^{3}H/^{14}C\| ratio was the same after and before chromatography (Table II). This doubly labeled biotin, was then diluted with cold biotin and recrystallized twice. The constancy of the \|^{3}H/^{14}C\| ratio proves that the isolated radioactive material is pure biotin (Table II). Nearly 1.26 % of the total radioactivity of the incubated X compound was recovered in the isolated biotin. This percentage was 2.5 when the dethiobiotin was transformed to biotin under the same conditions (1).

Thus *E. Coli C 124* accumulates X, although it is able to convert dethiobiotin into biotin. The explanation to this apparent contradiction is probably to be found in the difference of experimental conditions : X is produced by resting cells and is converted into biotin by growing cells. It has been checked that no labeled biotin is produced during the incubation of \|^{3}H\| dethiobiotin with resting cells.

Some Data on the Structure and Stability of the Intermediate

The incubation experiment was repeated under the same conditions, with doubly labeled \|^{3}H\| and \|^{14}C\| dethiobiotin (^{3}H/^{14}C = 6.70). X was isolated with the same yield and the same \|^{3}H/^{14}C\| ratio (6.68). This shows that as expected (1) there is no degradation of the side chain and no loss of the 3 and 4 hydrogens during the formation of X.

An important question was to know if X contains or does not contain sulfur. To check this point, another experiment was carried out with \|^{3}H\|

Table III

Isolation of doubly labeled $|^{3}H/^{35}S|$ X compound from the filtrate of *E. Coli C 124* resting cells incubated with $|^{3}H|$ dethiobiotin and $|^{35}S|$sulfate

dpm	$\|^{3}H\|$	$\|^{35}S\|$
Total dpm added :		
$\|^{3}H\|$ dethiobiotin	10.5×10^{6}	
$\|^{35}S\|$ sulfate		88×10^{7}
Total dpm recovered in X compound	11.8×10^{5}	14.3×10^{4}

dethiobiotin and $|^{35}S|$ sulfate (using bacteria grown on a $|^{35}S|$ supplemented medium). The results reported in Table III show that X contains sulfur.

The X intermediate is not very stable. After two months at 4°C, its methyl ester is partially transformed into another compound Y, with a lower R_f value (X:0.60, Y:0.45). The product is stable after 6 months, if kept at -20°C. This transformation occurs rapidly when an aqueous solution of X is heated at 120°C for sterilization. This can be prevented by cold sterilization. Y was purified, as a methyl ester, by column chromatography on silica gel and converted back into the acid form. Its growth promoting activity for the different strains of *E. Coli* given in Table I is about the same as that of X.

The structure of X has now to be established. The amounts of product available are presently in the microgram scale and the best approach to solve the problem is Mass Spectrometry. The first spectra have revealed that the X sample is not yet completely pure and complete purification has to be achieved before the structure can be established.

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