Formation of a Biotin Precursor, Pimelic Acid, in Yeasts from C₁₈ Fatty Acids

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Summary The biosynthesis of biotin-vitimers from C₁₈ fatty acids in the resting cell reaction system of yeasts was investigated. The formation of pimelic acid (a biotin precursor) and biotin-vitimers (a mixture of 7-keto-8-aminopelargonic acid and dethiobiotin) from linolenic, linoleic, and oleic acids was observed in certain yeasts belonging to the genera *Cryptococcus*, *Candida*, *Rhodotorula*, and *Trichosporon*. Metabolites from linolenic acid were analyzed by gas-liquid chromatography and mass spectrometry. Linoleic, oleic, stearic, heptadecanoic, palmitic, pentadecanoic, and pimelic acids were identified as metabolites of linolenic acid. The existence of a route to pimelic acid from linolenic acid in a strain of *Rhodotorula rubra* AKU 4836 was also indicated.

Key Words C₁₈ fatty acids and biotin, biotin biosynthesis in yeasts

Since Mueller *et al.* demonstrated that oleic acid (C₁₈₌₁) was able to replace biotin in a biotin-requiring microorganism (1), several subsequent investigations have failed to clarify the details of the participation of C₁₈₌₁.

Based on our investigation using a strain of *Bacillus sphaericus* AKU 0277, we previously reported the participation of linolenic acid (C₁₈₌₃), linoleic acid (C₁₈₌₂), and C₁₈₌₁ in the formation of biotin-vitimers and proposed the existence of a route to pimelic acid, which is an important precursor of biotin (2). In this organism, C₁₈₌₃, C₁₈₌₂, and C₁₈₌₁ hydrogenated to form stearic acid (C₁₈); C₁₈ and its β -oxidation product palmitic acid (C₁₆) decarboxylated to form heptadecanoic acid (C₁₇) and pentadecanoic acid (C₁₅), respectively. We investigated the occurrence of biotin biosynthesis from certain fatty acids in some yeasts, with special attention to the incorporation of C₁₈₌₁-1-¹⁴C into biotin-vitimers (3), the cultivation conditions for biotin-vitamer formation from C₁₈₌₁ (4), and the formation of pimelic acid from azelaic acid (5). Nothing is known

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concerning the participation of C_{18} fatty acids ($C_{18=3}$, $C_{18=2}$, $C_{18=1}$, and C_{18}) on biotin biosynthesis in yeasts. This paper will discuss the formation of biotin-vitamers from C_{18} fatty acids in *Rhodotorula rubra* AKU 4836.

EXPERIMENTAL

Strains and medium. Six strains of yeasts which produce pimelic acid from azelaic acid (as described previously (5)) were used in this study. These organisms were grown in a medium consisting of 1% glucose, 0.5% peptone, 0.3% malt extract, and 0.3% yeast extract in tap water. The initial pH of the medium was adjusted to 6.0.

Preparation and reaction system of resting cells. Each organism was grown at 28°C for 38 h while being constantly shaken in a 500 ml flask containing 100 ml of medium. The harvested cells were washed three times with saline solution and finally suspended in 1/200 M potassium phosphate buffer (pH 7.0). The cell suspension (20–40 mg/ml each) was incubated with or without $C_{18=3}$, $C_{18=2}$, $C_{18=1}$, or C_{18} (4 mg/ml) in 2 ml of 0.2 M Tris-HCl buffer (pH 7.5) at 28°C and constantly shaken. After a 24- or 48-h incubation, the cells were removed by centrifugation. The supernatant was used as a sample for identification of metabolites from $C_{18=3}$, $C_{18=2}$, or $C_{18=1}$.

Determination of pimelic acid and biotin-vitamers. Pimelic acid and biotin-vitamers in the supernatant were assayed with *Bacillus sphaericus* (6), and *Saccharomyces cerevisiae* (7), respectively. Separation of metabolites derived from $C_{18=3}$, $C_{18=2}$, and $C_{18=1}$ was accomplished by gas-liquid chromatography. The supernatant was acidified with 4 N HCl and the metabolites were extracted three times with 4 ml each of ethylether. The extract was concentrated, dehydrated with anhydrous Na_2SO_4 , methylated with BF_3 -methanol reagent (8), and analyzed by gas-liquid chromatography using the same procedure as described previously (2).

Determination of acetic acid. Reaction mixture contained C_{18} – C_{10} fatty acid (10 μ mol each), resting cells (32 mg), and potassium phosphate buffer (pH 7.4, 67 mM) in a total volume of 1.5 ml. For the control, fatty acid was replaced with water. Incubation was carried out for 90 min at 28°C while being constantly shaken. After incubation, the cells were removed by centrifugation. The resulting supernatant was assayed for acetic acid with acetokinase (9). The amount of acetohydroxamate formed was determined by the method of Lipmann and Tuttle (10), using Hiranuma EPO-3 type photometer at 530 nm.

Decarboxylation of [$1-^{14}C$]fatty acid by resting cells. Warburg vessel (2 ml) contained [$1-^{14}C$]fatty acid (0.17 μ mol, 0.2 μ Ci), resting cells (2.5 mg dry weight), 0.02 M (pH 7.0) potassium phosphate buffer, mixed with unlabeled fatty acid (5 μ mol). After a 30-min incubation at 37°C, an aliquot of 10 μ l drawn from 0.2 ml of 15% KOH in the center well was transferred to vials containing a scintillation mixture of the same composition as that described by Goldmark and Linn (11). Radioactivity was determined using an automatic liquid scintillation analyzer

(Beckman, LS-3155P Liquid Scintillation System).

Mass spectrometry. A Hitachi Model RMU-6M mass spectrometer was used to identify the components fractionated by the gas chromatogram. The operating parameters were as follows: interface temperature, 200°C; injection temperature, 200°C; ion acceleration voltage, 3.2 kV; chamber voltage, 20 eV.

Materials. Acetokinase (1,530 units/mg, lyophilized) from *Bacillus stearothermophilus* was purchased from Unitika Co., Ltd., Japan. [$1\text{-}^{14}\text{C}$]Fatty acids were obtained from Amersham Radiochemical Centre, England.

RESULTS

Formation of biotin-vitimers from C_{18} fatty acids

In a previous paper (5), we demonstrated that pimelic acid was formed in the resting cell reaction system of certain yeasts from azelaic acid. Here, the formation of biotin-vitimers in *R. rubra* AKU 4836 from C_{18} fatty acids was examined. Figure 1 shows the time course formation of biotin-vitimers from pimelic acid, azelaic acid, $C_{18=3}$, $C_{18=2}$, and $C_{18=1}$. Biotin-vitimers, namely, mixtures of dethiobiotin and 7-keto-8-aminopelargonic acid were identified through paper chromatography and assay with *S. cerevisiae*. The amount of biotin-vitimers formed from pimelic acid and azelaic acids increased as a function of incubation time. The formation of biotin-vitimers from $C_{18=3}$, $C_{18=2}$, and $C_{18=1}$ was found to begin after about 20, 15, and 10 h of incubation, respectively. No biotin-vitimers were leaked from resting cells.

Fig. 1. Time course of formation of biotin-vitimers from several fatty acids in resting cell reaction system of *Rhodotorula rubra* AKU 4836. Reaction mixture composition and reaction conditions were the same as those described in EXPERIMENTAL except that pimelic acid (a), azelaic acid (b), $C_{18=2}$ (c), $C_{18=1}$ (d), and $C_{18=3}$ (e) was added to the reaction mixture. For the control, abbreviated as (f) in the figure, fatty acid was replaced with water.

Fig. 2. Gas-liquid chromatogram of reaction products derived from $C_{18=3}$ in resting cell reaction system of *Rhodotorula rubra* AKU 4836. The reaction mixture composition and reaction conditions were the same as in EXPERIMENTAL. (a), incubation with addition of $C_{18=3}$; (b), incubation without addition of $C_{18=3}$.

Table 1. Summary of metabolites derived from $C_{18=3}$, $C_{18=2}$, or $C_{18=1}$ in resting cell reaction system of certain yeasts.

The reaction mixture composition and reaction conditions were the same as in EXPERIMENTAL. Each fatty acid derived from $C_{18=3}$, $C_{18=2}$, or $C_{18=1}$ was determined and identified by gas-liquid chromatography and mass spectrometry. The hyphen (—) in the table indicates negligible or undetectable amount of fatty acid. The abbreviation (DC_7) in the table means pimelic acid.

Microbes	Substrate	Relative percent (%) of fatty acid found							
		$C_{18=3}$	$C_{18=2}$	$C_{18=1}$	C_{18}	C_{17}	C_{16}	C_{15}	DC_7
<i>Cryptococcus neoformans</i> AKU 4501	$C_{18=3}$	52.3	15.4	14.3	—	—	4.8	—	0.9
	$C_{18=2}$	—	51.3	18.3	3.4	2.8	9.5	1.4	—
<i>Rhodotorula rubra</i> AKU 4817	$C_{18=3}$	51.3	9.2	16.8	2.0	3.6	6.8	—	1.3
	$C_{18=2}$	—	57.6	19.5	—	4.1	4.1	0.9	1.0
<i>Rhodotorula rubra</i> AKU 4836	$C_{18=3}$	31.6	11.9	27.6	1.4	1.1	10.6	0.5	1.1
<i>Trichosporon cutaneum</i> AKU 4864	$C_{18=2}$	—	65.8	2.5	—	4.2	6.4	10.1	—
	$C_{18=1}$	—	—	54.8	2.3	5.9	5.2	13.5	—

Identification of metabolites derived from C₁₈ fatty acids

As described above, the bioassay showed that the organism formed biotin-vitamins from each of the C₁₈₌₃, C₁₈₌₂, and C₁₈₌₁ in the resting cell reaction system. Metabolites derived from C₁₈₌₃ in the resting cell reaction system of the organism were fractionated by gas chromatography. Figure 2 shows the gas chromatogram of several fatty acids derived as metabolites from C₁₈₌₃ (peak 8) in a strain of *R. rubra* AKU 4836. Figure 3 shows the mass spectra of these fatty acids. Peaks 1, 3, 4, 6, and 7 were identified as pimelic acid, palmitic acid (C₁₆), heptadecanoic acid (C₁₇), C₁₈₌₁, and C₁₈₌₂, respectively. Peaks 2 and 5 (mass spectra not shown in Fig. 3) showed m/z of {74, 87, 143, 213(M-43)⁺, 225(M-31)⁺, 256(M)⁺} and {74, 87, 143, 255(M-43)⁺, 267(M-31)⁺, 298(M)⁺},

Fig. 3. Mass spectra of peaks 1, 3, 4, 6, and 7.

and were identified as pentadecanoic acid (C_{15}) and C_{18} , respectively. The other components separated remained unidentified. Table 1 shows the metabolites derived from $C_{18=3}$, $C_{18=2}$, and $C_{18=1}$ in *R. rubra* AKU 4836 and three other yeast strains. The metabolic components derived from $C_{18=3}$ in a strain of *R. rubra* AKU 4836 were almost identical with those derived from $C_{18=3}$ in both strains of *Cryptococcus neoformans* AKU 4501 and *R. rubra* AKU 4817. The metabolites derived from $C_{18=2}$ and $C_{18=1}$ were similar to those derived from $C_{18=3}$.

*Formation of a biotin precursor, pimelic acid, from C_{18} fatty acids in the resting cell reaction system of *R. rubra* AKU 4836*

1) *Decarboxylation (α -oxidation).* As shown in Figs. 2 and 3 and in Table 1, C_{15} and C_{17} were identified as metabolites derived from $C_{18=3}$ in the strains tested. The oxidation of several fatty acids was examined, using a radioactively labeled free acid as substrate, in accordance with the conventional Warburg manometric technique. Table 2 shows the results of the measurements of $^{14}CO_2$ produced from $[1-^{14}C]$ fatty acids during incubation with the resting cells. C_{18} and C_{16} were effective substrates, but shorter saturated fatty acids such as myristic acid (C_{14}), lauric acid (C_{12}), and octanoic acid (C_8), as well as C_{18} unsaturated fatty acids ($C_{18=3}$, $C_{18=2}$, and $C_{18=1}$) were ineffective. Metabolites derived from C_{18} and C_{16} by the organism were also investigated using GC-MS analysis. Decarboxylation of C_{18} and C_{16} occurred and caused the formation of odd-chain fatty acids of C_{17} and C_{15} (Table 3). Table 3 also shows the formation of odd-chain fatty acids from C_{18} and C_{16} in the other five strains, including the formation of C_{16} from C_{18} .

2) *β -Oxidation.* In addition to C_{17} and C_{15} , C_{16} was identified as an oxidation product of C_{18} in the strains tested (Table 3). Figure 4 shows the formation of acetic acid, the major product of the β -oxidation of fatty acids, from various fatty acids in

Table 2. $^{14}CO_2$ production from $[1-^{14}C]$ fatty acids in resting cell reaction system of *Rhodotorula rubra* AKU 4836.

The reaction mixture composition and reaction conditions are described in EXPERIMENTAL. As the control, each fatty acid and boiled resting cells were allowed to react.

$[1-^{14}C]$ Fatty acid	$^{14}CO_2$ produced (% of counts added)	
	Boiled	Unboiled
$C_{18=3}$	0	0
$C_{18=2}$	0.1	0.1
$C_{18=1}$	0	0
C_{18}	0.9	34.3
C_{16}	1.1	13.7
C_{14}	0.2	0.3
C_{12}	0.1	0.9
C_8	1.1	1.2

Table 3. Formation of odd-chain fatty acids from C_{18} and C_{16} in resting cell reaction system of certain yeasts.

The reaction mixture composition and reaction conditions were the same as in EXPERIMENTAL. Each fatty acid derived from C_{18} and C_{16} was determined and identified by gas-liquid chromatography and mass spectrometry. The hyphen (—) in the table indicates negligible or undetectable amount of fatty acid.

Microbes	Substrate	Relative percent (%) of		
		C_{17}	C_{16}	C_{15}
<i>Cryptococcus neoformans</i> AKU 4501	C_{18}	4.3	5.7	4.6
	C_{16}	—	—	5.1
<i>Candida lipolytica</i> AKU 4582	C_{18}	1.5	2.9	1.4
	C_{16}	—	—	2.9
<i>Rhodotorula rubra</i> AKU 4817	C_{18}	5.1	6.3	5.1
	C_{16}	—	—	9.2
<i>Rhodotorula rubra</i> AKU 4836	C_{18}	5.2	3.7	6.4
	C_{16}	—	—	1.9
<i>Rhodotorula</i> sp. AKU 4853	C_{18}	9.4	11.9	17.4
	C_{16}	—	—	8.6
<i>Trichosporon cutaneum</i> AKU 4864	C_{18}	4.3	12.1	8.8
	C_{16}	—	—	7.8

Fig. 4. Formation of acetic acid from several fatty acids in resting cell reaction system of *Rhodotorula rubra* AKU 4836. The reaction mixture composition and reaction conditions were the same as in EXPERIMENTAL. None, without addition of fatty acid; C_{11} , hendecanoic acid.

the course of the resting cells reaction. The formation of acetic acid from C_{12} was most pronounced. Acetic acid formation was detectable in both odd- and even-chain fatty acids tested. Also unsaturated fatty acids such as $C_{18=3}$, $C_{18=2}$, and

Fig. 5. Formation of pimelic acid from various fatty acids in resting cell reaction system of *Rhodotorula rubra* AKU 4836. The reaction mixture composition and reaction conditions were the same as in EXPERIMENTAL. None, incubation without addition of fatty acid; DC₉, azelaic acid.

C₁₈₌₁ served as substrates for the formation of acetic acid.

3) *Pimelic acid formation.* In the previous paper (5), we reported the formation of pimelic acid as a degradation product of azelaic acid in the six strains used in this study. Figure 5 shows the formation of pimelic acid from C₉–C₁₈ fatty acids by the organism in the resting cell reaction system. However, no formation of pimelic acid from C₁₄, C₁₂ or decanoic acid (C₁₀) was observed.

DISCUSSION

It is well known that pimelic acid is an important precursor of biotin biosynthesis, and that dethiobiotin is normally formed in the intermediate stage of biotin biosynthesis. However, the details of the biosynthetic route to pimelic acid still remain obscure (we discussed the relationship between fatty acid metabolism and biotin biosynthesis in microorganisms previously (2–5, 12–18). In one paper (2), we proposed the existence of the following route from C₁₈₌₃ to pimelic acid in a strain of *B. sphaericus* AKU 0227: 1) hydrogenation of C₁₈₌₃ to form C₁₈ via C₁₈₌₂ and C₁₈₌₁; 2) decarboxylation of C₁₈ and C₁₆ to form C₁₇ and C₁₅, respectively; 3) repeated β -oxidation of C₁₇ and C₁₅ to form pelargonic acid (C₉); 4) ω -oxidation of C₉ to form azelaic acid; and finally, 5) β -oxidation of azelaic acid to form pimelic acid.

Here, we have described the biosynthesis of biotin-vitimers from the substrates of C₁₈₌₃, C₁₈₌₂, and C₁₈₌₁ in the resting cells of yeasts belonging to the genera *Cryptococcus*, *Candida*, *Rhodotorula*, and *Trichosporon*. As reported previously (4), these strains formed biotin-vitimers (mainly dethiobiotin) from C₁₈₌₁ during cultivation. The cultivation of *C. neoformans* AKU 4501 showed that C₁₈₌₁-1-¹⁴C is

Fig. 6. Possible route from C_{18} unsaturated fatty acids to pimelic acid in biotin biosynthesis in *Rhodotorula rubra* AKU 4836. Dotted line in the figure means repeating β -oxidation. DC_9 , azelaic acid; DC_7 , pimelic acid.

incorporated into biotin-vitamers such as 7-keto-8-aminopelargonic acid, de-thiobiotin, and biotin (3). In the present paper, we have shown that biotin-vitamers are formed from $C_{18=3}$, $C_{18=2}$, and $C_{18=1}$ in the resting cell reaction system of these strains (Fig. 1). The metabolic intermediates derived from $C_{18=3}$ in a strain of *R. rubra* AKU 4836 were similar to those intermediates found in strains of *C. neoformans* AKU 4501 and *R. rubra* AKU 4817 (Table 1). These findings suggest that the route of biosynthesis of biotin from $C_{18=3}$ is identical in these yeast strains. The results of our manometric experiment and GC-MS analysis showed that C_{17} and C_{15} were formed from C_{18} and C_{16} , respectively (Tables 2 and 3). These odd-chain fatty acids degraded to form acetic acid by β -oxidation just as did the even-chain fatty acids (Fig. 4). We previously showed that, in *Pseudomonas* sp. strain 393, azelaic acid must be derived from C_9 by ω -oxidation (18). In our present investigation, several experiments on the decomposition of C_9 were undertaken by incubating C_9 together with resting cells of *R. rubra* AKU 4836 until azelaic acid was formed. Azelaic acid was identified by gas-liquid chromatography. During a 5-h incubation (data not shown), both azelaic and pimelic acids were formed.

Based upon the above results, we propose the scheme shown in Fig. 6 for the route from C_{18} unsaturated fatty acids to pimelic acid in *R. rubra* AKU 4836. The biotin precursor (pimelic acid) can be seen to be formed from $C_{18=3}$ as follows: 1) saturation (or hydrogenation) of $C_{18=3}$ to form C_{18} via $C_{18=2}$ and $C_{18=1}$ as intermediates; 2) the conversion of C_{18} into the odd-chain fatty acid C_{17} by decarboxylation (or α -oxidation); 3) the formation of C_9 by repeated β -oxidation of C_{17} ; 4) ω -oxidation of C_9 to form the dicarboxylic acid azelaic acid; and 5) the formation of pimelic acid from azelaic acid by β -oxidation. Therefore, the metabolic route from C_{18} unsaturated fatty acids to pimelic acid is identical in both bacteria and yeasts.

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