Acetylation of Sphingosine Bases and long-Chain Amines by Cell-Free Preparations of Hansenula Ciferri

Yechezkel Barenholz and Shimon Gatt

Department of Biochemistry, Hebrew University
Hadassah Medical School, Jerusalem, Israel

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Summary. A cell-free preparation of the yeast, Hansenula ciferri catalyzed the transfer of the acetyl group of acetyl coenzyme-A to the sphinosine bases at both their amino and hydroxyl groups. The enzyme also transferred acetyl groups to the hydroxyls of the N-acetylated sphingosine bases as well as to the amino groups of primary amines of ten or more carbon atoms. A mixture of acetate, ATP and coenzyme-A could be employed instead of acetyl CoA. The reaction had an optimal pH at about 7.8 and was inhibited by free coenzyme-A.

The yeast, Hansenula ciferri, produces large quantities of phytosphingosine[1] and lesser amounts of dihydrosphingosine; these are excreted into the medium as partially or fully-acetylated bases (Maister et al. 1962; Greene et al., 1965). These compounds differ from mammalian "ceramide" (N-acyl sphingosine) in which the sphingosine base is acylated, at the amino group, to a long-chain fatty acid (Marinetti & Stotz, 1957; Gatt & Berman, 1963).

When added to the growth medium, serine and palmitic acid were incorporated into the base portion of the acetylated derivatives (Greene et al., 1965); similar results were obtained by using short incubation periods of 10 minutes to 3 hours. But when we added sodium 1-^{14}C acetate to the growth medium, the radioactivity after one hour was found only in the acetyl moiety. The probability that by neutralizing the excessive quantities of base the acetylation is important for preservation of the yeast, led to an investigation of the acetylation system. This resulted in the isolation of a cell-free system of H. ciferri which catalyzed a transfer of the acetyl group of acetyl coenzyme-A to the sphingosine bases at both their amino and hydroxyl groups. The enzyme also transferred acetyl groups to the hydroxyls of the N-acetylated bases as well as to the amino groups of primary amines of ten or more carbon atoms

[1] The following are the structures of the three main sphingosine bases.
Phytosphingosine: $CH_3\ (CH_2)_{13}\ CHOH \cdot CHOH \cdot CHNH_2 \cdot CH_2OH$;
dihydrosphigosine: $CH_3(CH_2)_{14}\ CHOH \cdot CHNH_2 \cdot CH_2OH$;
sphingosine: $CH_3(CH_2)_{12}\ CH{=}CH \cdot CHOH \cdot CHNH_2 \cdot CH_2OH$

Materials and Methods

Substrates: Individual sphingosine bases were prepared according to Barenholz and Gatt (1968). Synthetic DL-erythro sphingosine and DL-erythro dihydrosphingosine were gifts of Prof. D. Shapiro. N-acetyl phytosphingosine (97% pure) was prepared by mild alkaline hydrolysis of the acetylated bases, isolated from the growth medium of H. ciferri. N-acetyl sphingosine and N-acetyl dihydrosphingosine were prepared according to Gatt (1966). N-acetyl hexadecylamine was prepared by acetylation of hexadecylamine with acetic anhydride (Carter and Gaver, 1967a, b). Acetyl CoA and butyryl CoA were synthesized using the corresponding anhydrides as acylation reagents of coenzyme-A (Smith, 1965); the acyl derivatives contained only about 2% of free coenzyme-A. Tritium-labelled acetyl CoA was similarly prepared, using tritiated acetic anhydride.

Hansenula ciferri (NRRL 1031 . mating type F-60-10) was a gift of Dr. L. J. Wickerham.

Preparation of Enzyme. The yeast was cultured in a New Brunswick fermentor and harvested according to Braun & Snell (1967). Washed cells were suspended in two volumes of 0.05 M potassium phosphate buffer, pH 7.0 containing 20% glycerol (v/v) and 1mM dithiothreitol. They were disrupted in an Aminco pressure cell at 20,000 p.s.i. Whole cells and debris were removed by centrifuging for 10 min. at 1,000xg. The supernatant was then centrifuged for 30 min at 14,000xg and the sediment was discarded. The supernatant was further centrifuged for 4 hours at 105,000xg, the sediment was suspended in the above phosphate-glycerol-dithiothreitol buffer at a concentration of 7.5 mg protein/ml. This preparation retained most of its activity for at least 10 months.

Assay of enzyme activity. The enzymatic activity could be determined by measuring either the free coenzyme-A released, or, when using ^{3}H-acetyl CoA, the degree of incorporation of tritium-labelled acetate into acetylated base. When free coenzyme-A was measured, the enzyme preparations were previously dialyzed to remove the dithiothreitol. The dialysis media were phosphate-glycerol buffer containing 10^{-5}M DTNB (5, 5-dithiobis-(2-nitrobenzoic acid) followed by phosphate-glyerol; the dialyzed preparations retained their activity for at least 6 months. Incubation mixtures, in volumes of 0.2ml, contained 20 μmoles of potassium phosphate buffer, pH 7.8, 0.1-0.2 μmoles of either substrate, enzyme and other additions as required. After 30-90 min. at 37°, 1.6ml of a mixture of chloroform-methanol 1:1 was added, followed by 0.6ml of water. The phases were separated and the coenzyme-A content in the upper phase was determined with DTNB according to Ellman (1958), using 0.5M phosphate, pH 7.8, instead of water.

When total acetylation of the bases was measured, using ^{3}H-acetyl CoA, the reaction was terminated as above. The lower, chloroform phase was washed

three times with a mixture of chloroform-methanol-water, 6:94:96 and a solution containing 1mg of lecithin was added. The solvents were evaporated, 10 ml of scintillation fluid were added and the radioactivity was determined in a scintillation spectrometer. If measurement of the acetylation of only the amino group was required ("N-acetylation"), the reaction mixtures were terminated by adding 0.6 N KOH in 60% methanol. After hydrolyzing the ester bonds for 10 min at 60°, the N-acetylated derivatives were extracted twice with 1.5ml each of ether. The ether was washed twice with 1 ml of water and counted as above.

Identification of the Reaction Products: The tritium-labeled acetylated bases were identified by chromatography on thin layer silica gel plates, using ether-chloroform-methanol, 7:3:1, $^{v}/v$ (Greene et al, 1965). For identification of N-acetyl hexadecylamine a mixture of chloroform methanol -2N NH_4OH, 90:10:1, was used. The silica gel corresponding to the respective spots was scraped off the plate and transferred to counting vials. 2ml of a mixture of Triton X 100-absolute ethanol, 1:1 ($^{v}/v$) was added, followed by 10ml of scintillation fluid. The vials were then counted in a liquid scintillation spectrometer.

The N-acetylated bases were also identified by gas-liquid chromatography of their trimethylsilyl ethers. The effluent was directed into a fraction collector and condensed onto glass wool in small glass vials; these were then transferred to counting vials containing 20ml of scintillation fluid and were counted in a liquid scintillation spectrometer.

Results

Table I shows some of the results obtained. The enzyme catalyzed the acetylation of sphingosine, dihydrosphingosine and phytosphingosine, both at the amino group ("N-acetylation") and at the hydroxyls ("O-acetylation"). After incubating for 15-30 minutes about 60% and after 2 hours less than 50% of the acetyls were bound to the amino group, the rest were present as acetate esters linked to the hydroxyls. The enzyme also further acetylated the N-acetyl derivatives of the above three bases, yielding N and O-acetylated bases. It did not act on the N-octanoyl and N-palmitoyl derivatives of sphingosine or dihydrosphingosine nor on psychosine (1-O-galactosyl-sphingosine). Acetylation was obtained using primary amines with 10-16 carbon atoms, but not lower primary amines, nitroaniline, glucosamine or dodecyl alcohol. Butyryl CoA could not replace acetyl CoA as substrate for acetylation of either the free or N-acetylated bases. 1 ^{14}C-acetate was utilized for acetylation of the free or N-acetylated bases, as well as of hexadecylamine, only in the presence of both ATP and coenzyme-A. (Since the enzyme contained appreciable thiokinase activity, Y. Bartana personal communication, addition of this enzyme was not necessary).

TABLE I

Acetylation of Free and N-acetylated Sphingosine Bases and of Long-Chain Amines

Substrate	N-acetylation (μmoles)	O-acetylation (μmoles)
Sphingosine	2.5	2.7
Dihydrosphingosine	1.8	2.5
Phytosphingosine	2.2	2.3
Hexadecylamine	1.4	-
Decylamine	0.3	-
N-acetyl sphingosine	-	8.5
N-acetyl dihydrosphingosine	-	5.1
N-acetyl phytosphingosine	-	6.5
N-octanoyl sphingosine	-	-
N-palmitoyl sphingosine	-	-
1-O-galactosylsphingosine	-	-

Incubation mixtures, in volumes of 0.2 ml, contained 20 μmoles of potassium phosphate, pH 7.8, 0.1 μmole of substrate, 0.06 μmole of tritium-labelled acetyl coenzyme-A, 1.25 mg of sodium taurocholate and 300 μg of enzyme. After 90 min at 37°, the radioactivity in the acetylated products was determined. The values are expressed as μmoles of ^{3}H-acetate incorporated.

The N-acetylation was linear up to at least 300μg of enzyme and 30-90 min, depending on the substrate employed. The optimal pH values of the N or O-acetylations were 7.8 and 7-7.8, respectively using potassium phosphate buffer. Detergents such as Triton X100, Cutscum, sodium cholate, deoxycholate and taurocholate and Cetavlon did not increase and in many cases even decreased the reaction rates. The enzyme had only little N-acetamidase or thiol ester hydrolase activities. The reaction was inhibited by coenzyme-A, one of the two products of the reaction (85% and 70% inhibition, of the N and O-acetylations respectively, at 1mM concentrations each, of coenzyme-A, acetyl coenzyme-A and base); it was not inhibited by the second product (i.e. the N-acetyl derivatives of the sphingosine bases or of hexadecylamine), or by 1mM each, of mercaptoethanol,

p-hydroxymercuribenzoate or DTNB.

The above enzyme system describes new acetylation systems which transfer the acetyl portion of acetyl coenzyme-A to the amino and hydroxyl groups of the sphingosine bases and to the amino group of primary amines having more than 10-12 carbon atoms. Further experiments are required to determine whether the enzymatic preparation contains two separate enzymes, one for the N-acetylation and one for O-acetylation or whether one enzyme is responsible for both these activities. The acetylation system of H. ciferri differs from the acylation of sphingosine bases in animal tissues. In the latter, these bases are acylated by a long-chain fatty acid at the amino group but not at the hydroxyls (Gatt, 1966; Yavin and Gatt, 1969; Sribney, 1966).

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REFERENCES

Barenholz, Y., and Gatt, S., Biochim. Biophys. Acta, 152, 790 (1968).

Braun, P. E. &Snell, E. E., Proc. Nat. Acad. Sci., 58, 298 (1967).

Carter, H. E., and Gaver, R. C., Biochem, Biophys. Res. Comm., 29, 886 (1967a).

Carter, H. E., and Gaver, R. C., J. Lipid Res., 8, 391 (1967b).

Ellman, G. L., Arch. Biochem. Biophys. 74, 443 (1958).

Gatt, S., J. Biol. Chem., 241, 3724 (1966).

Gatt, S. and Berman, E. R. J. Neurochem., 10, 73 (1963).

Greene, M. L., Kaneshiro, T., and Law, J. H., Biochim. Biophys. Acta, 98, 582 (1965).

Maister, H. G., Rogovin, S. P., Stodola, F. H. and Wickerham, L. F., Appl. Microbiol. 10, 401 (1962).

Marinetti, E., and Stotz, E., J. Am. Chem. Soc., 79, 145 (1957).

Smith, S., Ph. D. Thesis, University of Birmingham, (1965).

Sribney, M., Biochim. Biophys. Acta, 125, 542 (1966).

Yavin, E. and Gatt, S., Biochemistry, (1969) in Press.