Enzymatic synthesis of trisialoganglioside by particulate fractions of rat liver

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Summary. Rat liver contains a particulate (membrane-bound) glycosyl transferase, concentrated in Golgi apparatus fractions, which catalyzes the synthesis of a trisialoganglioside from the ganglioside precursor disialohematoside (G_{D3}). Sialic acid was not incorporated into exogenous G_{D1a} from CMP-N-acetylneuraminic acid suggesting that G_{D1a} is an endproduct of the monosialoganglioside pathway. Thus, the disialoganglioside pathway may be a primary source of trisialoganglioside and higher ganglioside homologs in adult rat liver.

Elucidation of the pathways of ganglioside biosynthesis has come primarily from studies of ganglioside glycosyltransferases in brain[1-7]. Studies by Kaufman et al.[2] showed that the monosialogangliosides G_{M3}, G_{M2} and G_{M1}[8], and the disialoganglioside G_{D1a} of the monosialoganglioside pathway were synthesized sequentially; the product of one glycosyl transferase acted as the substrate for the next transferase in the sequence. A 2nd pathway of ganglioside biosynthesis was indicated from experiments in which G_{M3} was shown to accept a 2nd moiety of sialic acid from CMP-NAN to form the disialoganglioside G_{D3}[3]. Further sequential synthesis resulted in the disialogangliosides G_{D2}[4] and G_{D1b}[5], and the corresponding trisialoganglioside G_{T1b}[6]. In previous studies from our laboratory, enzymes of the monosialoganglioside pathway (LacCer → G_{M3} → G_{M2} → G_{M1} → G_{D1a}) were localized in Golgi apparatus fractions of rat liver[9]. The possibility of a 2nd pathway, the disialoganglioside pathway (G_{M3} → G_{D3} → G_{D2} → G_{D1b}, etc.), was provided by demonstration of the formation of G_{D3} by a CMP-NAN: G_{M3} sialyltransferase[9]. In the present report we present evidence that G_{D3} accepts a 3rd sialic acid in a reaction catalyzed by an enzymatic activity of the Golgi apparatus.

Materials and methods. Fractions: Golgi apparatus were according to Morré[10]. Total particulate fractions were as described[11]. Protein was determined by the Lowry et al.[12] procedure.

Sialyltransferase assay: The procedure of Keenan et al.[9] was followed (table). Unreacted substrate and degradation products were removed from glycolipids by paper chromatography[13]. Reaction mixtures were applied to 3MM Whatman paper strips and developed overnight in 1% sodium tetraborate. The origins were cut out, placed in vials containing 15 ml of chloroform: methanol: water (2:1:0.2 v/v), and incubated at 37 °C for 1 h. Papers were removed, the extracts evaporated to dryness and radioactivity determined.

Product characterization: Methanol-terminated reaction mixtures were evaporated to dryness and the residue was extracted twice with chloroform: methanol (1:1, v/v). This extract was spotted on silica gel G thin layer plates which were then developed in chloroform: methanol: ammonium hydroxide: water (60:35:7:3, v/v) with appropriate standards. Bands in each lane which cochromatographed with the standards were scraped into vials and radioactivity determined. Methyl benzethonium hydroxide (1 ml) and toluene-based scintillation fluid (15 ml) were added to each vial before determination of radioactivity.

Results. Disialohematoside, G_{D3}, accepted a 3rd sialic acid moiety from CMP-N-acetylneuraminic acid via an enzyme-catalyzed reaction of relatively high specific activity. The product of this reaction cochromatographed with authentic brain trisialoganglioside. The specific activity was comparable to that of the sialyltransferase catalyzing the transfer of a 2nd sialic acid to G_{M1} with the formation of the disialoganglioside G_{D1a}. Both activities were found in Golgi apparatus. In other experiments (data not shown), conversions of G_{D3} to G_{D2} by transfer of N-acetylgalactosamine from UDP-N-acetylgalactosamine and G_{D1b} to G_{T1b} by transfer of N-acetylneuraminic acid from CMP-N-acetylneuraminic acid were observed at rates of 13 and 5 pmoles/h/mg protein, respectively with total particulate fractions. However, using the same reaction conditions and G_{D1a} as acceptor, no significant incorporation of sialic acid was observed; we found no evidence for formation of a ganglioside corresponding to G_{T1a}.

Discussion. A sialyltransferase activity was identified which catalyzed the incorporation of a 3rd moiety of sialic acid from CMP-N-acetylneuraminic acid with G_{D3} as acceptor; this branch of the disialoganglioside pathway may be an important source of trisialoganglioside in liver. G_{M1} served as an acceptor for the synthesis of G_{D1a} but not of G_{D1b} (see, however, Arce et al.[14]) in agreement with findings of Yip[15]. In other experiments in which incorporation of sialic

Ganglioside sialyltransferase activities of total particulate (TP) and Golgi apparatus (GA) fractions and chromatographic properties of the products of the reaction

Enzymatic activity acceptor → product	Specific activity, pmoles/h/mg protein				
	G_{M1}/G_{D3} TP	G_{D1a} TP	G_{D1a} GA	G_{D1b} TP	G_T GA
A $G_{M1} \rightarrow G_{D1a}$					
plus G_{M1} acceptor	127±7	67±3	618±7	364±11	951±11
minus acceptor (endogenous)	189±4	31±2	26±1	782±54	951± 5
net	-	36±1	592±8	-	0
B $G_{D3} \rightarrow G_{T3}$					
plus G_{D3} acceptor			54±1		1,366±3
minus acceptor (endogenous)			24±1		946±3
net			30±0		420±1

Sialyltransferase assays contained in a total volume of 0.1 ml: 600 µg Tween 80-Triton CF-54 (1:2, w/w), 50 nmoles glycolipid acceptor (G_{M1} or G_{D3}), 50 nmoles CMP-N-acetylneuraminic acid (5×10^6 cpm/µmole), 15 µmoles sodium cacodylate, 0.1 µmoles $MgCl_2$ and 50 µl of membrane suspension (125-300 µg protein), final pH 6.6. Radioactive products were scraped from TLC plates at regions corresponding to authentic G_{M1}, G_{D1a}, G_{D1b} or G_T from bovine brain and radioactivity determined (see text). Values are from 2 experiments ± mean average deviations.

acid was localized in endogenous ganglioside products by thin layer chromatography, incorporation into G_{D1b} was at least 30 times that of incorporation into G_{D1a}. With total particulate fractions, the branch point enzyme of the disialoganglioside pathway, CMP-N-acetylneuraminic acid: G_{M3} sialyltransferase, was 36 times more active than the UDP-N-acetylgalactosamine: G_{M3} N-acetylgalactosaminyltransferase, the entry enzyme into the monosialoganglioside pathway[9]. Thus, the synthesis of higher homologues of disialogangliosides in rat liver apparently occurs predominantly if not exclusively via the disialoganglioside pathway; CMP-N-acetylneuraminic acid was not incorporated into G_{D1a} by either total particulate or Golgi apparatus fractions of rat liver suggesting that G_{D1a} is an endproduct of the monosialoganglioside pathway in this tissue.

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8 Abbreviations used: Cer, ceramide; Gal, galactose; GalNAc, N-acetyl galactosamine, Glc, glucose; G_{M3}, NAN-Gal-Glc-Cer; G_{M2}, GalNAc-(NAN)-Gal-Glc-Cer; G_{M1}, Gal-GalNAc-(NAN)-Gal-Glc-Cer, G_{D1a}, NAN-Gal-GalNAc-(NAN)-Gal-Glc-Ceramide; G_{D3}, $(NAN)_2$-Gal-Glc-Cer; G_{D2}, GalNAc-$(NAN)_2$-Gal-Glc-Cer; G_{D1b}, Gal-GalNAc-$(NAN)_2$-Gal-Glc-Cer; G_{T1a}, $(NAN)_2$-Gal-GalNAc-(NAN)-Gal-Glc-Cer; G_{T1b}, NAN-Gal-GalNAc-$(NAN)_2$-Gal-Glc-Cer; G_{T3}, $(NAN)_3$-Gal-Glc-Cer; NAN, N-acetylneuraminic acid (sialic acid).
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Mise en évidence d'une activité α-amylasique dans les lysosomes d'*Aspergillus oryzae*

α-Amylase activity in lysosomes of *Aspergillus oryzae*

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Summary. The α-amylase of mycelial cells of *Aspergillus oryzae* exists in a particular form in 8000 g pellet. The lysosomal localization of acid phosphatase is confirmed by electron microscopy. The purification of lysosomes by discontinuous gradient of sucrose in D_2O shows that α-amylase activity is bound to these particles.

De nombreux travaux ont été entrepris sur la forme soluble de l'α-amylase d'*Aspergillus oryzae* libérée en quantité importante lorsque la lyse cellulaire débute[2-4]. Un site de fixation de l'enzyme a été décelé sur la paroi cellulaire du mycélium[5]. La présence d'α-amylase a également été démontrée dans la mebrane cellulaire de *Bacillus amyloliquefaciens*[6]. Dans les cellules d'aleurone d'orge, on retrouve, à côté d'une forte proportion d'α-amylase soluble, 5-25% de cette enzyme sous forme particulaire[7]. Par ailleurs l'hydrolyse intralysosomique du glycogène sous l'action de l'α-glucosidase ou maltase acide a été démontrée dans les hépatocytes humains[8]. L'α-amylase est capable d'initier une telle hydrolyse.

Dans le présent travail après la mise en évidence de lysosomes dans les cellules mycéliales d'*Aspergillus oryzae* nous avons recherché l'existence d'une activité α-amylasique lysosomique.

Matériel et méthodes. Aspergillus oryzae (CBS 129.49) est cultivé en milieu liquide ajusté à pH 6,5, sous agitation et avec circulation d'air. La culture est effectuée à 30 °C et dure 40 h. Le terme de la culture correspond au milieu de la phase exponentielle de croissance. Les cellules mycéliales sont isolées du milieu de culture, lavées et remises en suspension, puis soumises à une digestion enzymatique de la paroi cellulaire sous l'action du suc digestif d'*Helix pomatia* suivant la méthode de Greenawalt et al.[9]. Le broyage est ensuite effectué à l'aide de l'homogénéiseur Potter-Elvhejehm type C. La fraction mitochondriale recueillie entre 1500 et 8000 g est lavée 3 fois et déposée sur un gradient discontinu constitué de 14 couches de solutions de saccharose dans un tampon tris-HCl 50 mM EDTA 1 mM, pH 7, réalisé dans l'eau lourde. Les concentrations en saccharose de ces solutions varient de 49% à 7% (densités 1,26-1,13). Les protéines sont dosées par la méthode de Lowry et al.[10]. L'activité α-amylasique est mesurée en tampon acétate-acide acétique 50 mM, NaCl 20 mM, pH 5,5 par diminution de l'intensité de la coloration du complexe bleu formé entre l'amylose et l'iode[11]. Le dosage de la phosphatase acide s'effectue par la mesure du paranitrophénol libéré à partir de paranitrophényl phosphate à pH 3[12]. La cytochrome-oxydase est dosée par disparation du cytochrome C réduit à 550 nm et à pH 7,14[13] tandis que la NADH-cytochrome C oxydoréductase se caractérise par l'apparition de cette forme réduite, à pH 7 en tampon phosphate 80 mM, cyanure de potassium 0,3 mM, roténone $0{,}15 \cdot 10^{-3}$ mM[14]. La mise en évidence de la phosphatase acide au microscope électronique se fait par une modification de la méthode de Gomori[15] après inclusion du mycélium dans la gélose et fixation par le glutaraldéhyde 3,5% pendant 4 h[16]. On prépare également un témoin sans substrat. L'inclusion est réalisée dans une résine époxy.

Résultats et discussion. La figure 1 confirme la localisation lysosomique de la phosphatase acide dans les cellules mycéliales d'*Aspergillus oryzae.* Dans des spores fongiques,