ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 222, No. 1, April 1, pp. 145–149, 1983

Demonstration of the Enzymatic Mechanisms of α-N-Acetyl-D-Glucosamine-1-Phosphodiester N-Acetylglucosaminidase (Formerly Called α-N-Acetylglucosaminylphosphodiesterase) and Lysosomal α-N-Acetylglucosaminidase[1]

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Received October 1, 1982, and in revised form November 29, 1982

An enzyme that is capable of removing the outer N-acetylglucosamine residues from phosphodiesters present on the high-mannose-type oligosaccharides of newly synthesized lysosomal enzymes has been described. This enzyme has been called an α-N-acetylglucosaminylphosphodiesterase, based upon its substrate specificity and on inhibitor studies. In this study it is demonstrated by the ^{18}O enrichment method that the enzyme cleaves the C–O bond rather than the O–P bond, and therefore acts by a glycosidase type of mechanism. In addition, the enzyme has no significant activity toward α-N-acetylglucosamine 1-phosphate, and therefore requires an underlying phosphodiester for activity. In accordance with the IUB recommendations for enzyme nomenclature, it is therefore suggested that the enzyme be renamed α-N-acetyl-D-glucosamine-1-phosphodiester N-acetylglucosaminidase (systematic name, 2-acetamido-2-deoxy-α-D-glucose 1-phosphodiester acetamidodeoxyglucohydrolase). For convenience, the trivial name phosphodiester glycosidase is proposed. Lysosomal α-N-acetylglucosaminidase also has a glycosidase type of mechanism but it is active toward α-N-acetylglucosamine 1-phosphate as well as phosphodiesters with outer N-acetylglucosamine residues.

Newly synthesized lysosomal enzymes acquire a phosphomannosyl recognition marker that is involved in their translocation to lysosomes (see (1, 2) for recent reviews). This post-translation modification of aspargine-linked high-mannose-type oligosaccharides is generated in two steps. First, phosphorylation occurs by transfer of N-acetylglucosamine 1-phosphate to the hydroxyl at C-6 of a mannose residue (3–5). Next the outer α-linked N-acetylglucosamine residue is removed to generate the mature phosphomonoester recognition signal (6–8).

The enzymatic activity capable of removing the outer N-acetylglucosamine residue is distinct from lysosomal α-N-acetylglucosaminidase (9–12) by several criteria (6). The enzyme has been partially purified from rat liver (8) and human placenta (7) and its properties have been studied. We initially named the enzyme α-N - acetylglucosaminylphosphodiesterase based upon the following observations. First, the enzyme is capable of cleaving α-linked N-acetylglucosamine residues from an underlying phosphate residue, but has no activity against p-nitrophenol α-N-acetylglucosaminide. Second, the enzymatic activity is inhibited by both N-acetylglucosamine and phosphate ions (6). This nomenclature was subsequently followed by

[1] This work was supported in part by United States Public Health Service Grants RO1 CA08759, T32 HL007088, and RR-00954 from the National Institutes of Health.

0003-9861/83 $3.00
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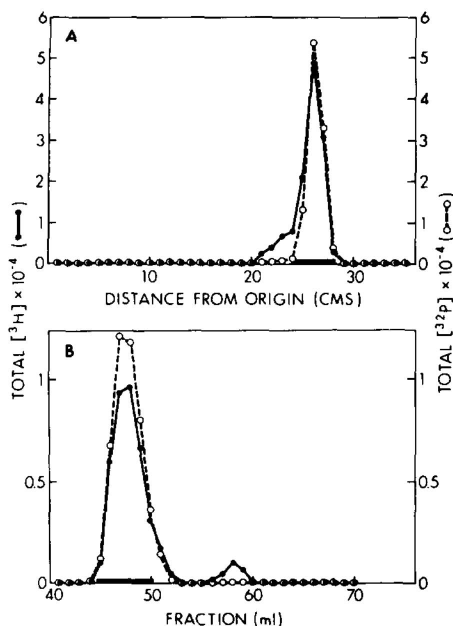

FIG. 1. Purification of GlcNAc-1-P-6-(α-methyl)-mannoside. (A) Chromatography of the enzymatically synthesized material on Whatman 3MM paper as described under Materials and Methods; (B) the further purification of the material by gel filtration on a column of Bio-Gel P2 in water. In both cases aliquots were monitored for ^{32}P and ^{3}H.

Waheed *et al.* (7), who pointed out, however, that it was not clear whether the enzymatic reaction proceeds by a phosphodiesterase or a glycosidase type of mechanism.

The generalization proposed by Koshland and Stein states that if an enzyme shows a high specificity for the group R of an R-O-Q sequence, then cleavage of the R-O bond probably occurs (13). In this instance, the bond being attacked is C-O-P, between the 1 carbon of the *N*-acetylglucosamine, and the underlying phosphomannose. The studies of the substrate specificity and the inhibitors of the enzyme indicated that both the *N*-acetylglucosamine and the phosphate group are recognized (6, 8). We therefore undertook a study to directly define the mechanism of this enzyme. Reactions were carried out in ^{18}O-enriched water and the products were analyzed for ^{18}O enrichment, thus allowing a differentiation between attack on the C-O and the O-P bond. We simultaneously studied the enzymatic mechanism of pig liver lysosomal α-*N*-acetylglucosaminidase, an acid hydrolase which is known to cleave the same bond (14-16).

MATERIALS AND METHODS

Chemicals and reagents. $H_2{}^{18}O$ (99.12 atom% enriched) was purchased from Bio-Rad. Regisil (*N,O*-bis(trimethylsilyl)triflouroacetamide containing 10% trimethylsilylchloride) was from Regis, Inc., Morton Grove, Illinois. [β-^{32}P]UDP-*N*-acetylglucosamine was prepared as previously described (3). UDP-[6-^{3}H]*N*-acetylglucosamine (6.6 Ci/mmol) was from New England Nuclear. UDP-*N*-acetylglucosamine, α-*N*-acetylglucosamine 1-phosphate, *N*-acetylglucosamine, QAE-Sephadex (Q-25-120), α-methylmannoside, and ATP were from Sigma. All other chemicals were of reagent grade and were purchased from commercial sources.

Enzymes. α-*N*-Acetylglucosamine-1-phosphodiester *N*-acetylglucosaminidase (formerly called α-*N*-acetylglucosaminylphosphodiesterase) was purified 1800-fold from rat liver as previously described, and concentrated 5-fold on DEAE-Sephadex before use to a final protein concentration of 1.3 mg/ml (8). Lysosomal α-*N*-acetylglucosaminidase was purified from pig liver as previously described (17). Neither preparation was homogenous, but each was free of the other activity (8).

Preparation of α-N-acetylglucosamine-1-phosphodiester-6-(α-methyl)mannoside. This compound can be made enzymatically by transfer of *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to the 6 position of α-methylmannoside (5, 18). It closely resembles the natural substrate for the rat liver enzyme (14-16). Crude smooth membranes containing UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosaminylphosphotransferase activity was prepared from rat liver as previously described (5). The residue left behind after the initial detergent extraction steps still contained substantial amounts of the activity in a relatively concentrated form. This pellet (107 mg) was resuspended in 4 ml of 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1% Triton X-100, 0.25 mM dithiothreitol, 5 mM ATP, 200 mM α-methylmannoside, 50 mM GlcNAc,[2] and 0.5 mM UDP-GlcNAc. The mixture was incubated at 37°C in a shaking water bath, and 20 μl of 100 mM UDP-GlcNAc was added every 2 h. At the end of 6 h, the reaction mixture was boiled for 5 min and centrifuged at 10,000*g* for 20 min. To follow the subsequent purifi-

[2] Abbreviations used: GlcNAc, *N*-acetylglucosamine; GC-MS, gas chromatography-mass spectrometry; TMS, trimethylsilyl.

TABLE I

ENZYMATIC REACTIONS AND ANALYSIS OF ISOTOPE ENRICHMENT

Substrate	Treatment[a]	Product studied	Starting % $H_2{}^{18}O$	Mean[b] % ^{18}O enrichment in product
GlcNAc	None	GlcNAc	0	0[c]
	None	GlcNAc	80	14.4
α-GlcNAc-1-P	αGP	GlcNAc	80	19.7
	αLG	GlcNAc	80	78.9
UDP-GlcNAc	αGP	GlcNAc	0	−0.28
	αGP	GlcNAc	80	77.3
	αLG	GlcNAc	0	0.89
	αLG	GlcNAc	80	80.9
GlcNAc-P-α-methylmannoside	αGP	GlcNAc	80	77.1
	αLG	GlcNAc	80	77.3
	H^+	α-Methyl mannoside 6-P	0	0[c]
	αGP	α-Methyl mannoside 6-P	80	−0.76
	αLG	α-Methyl mannoside 6-P	80	0.1

[a] αGP = *N*-Acetylglucosamine 1-phosphodiester α-*N*-acetylglucosaminidase; αLG = lysosomal α-*N*-acetylglucosaminidase; H^+ = mild acid treatment. The details of each treatment are described under Materials and Methods. The % ^{18}O enrichment in the products was calculated by comparison with a standard that was kept under identical conditions in $H_2{}^{16}O$.

[b] All values are the means of 2 to 4 determinations.

[c] The % ^{18}O enrichment in the products was calculated by comparing with a standard that was kept under identical conditions in $H_2{}^{16}O$. The value with the [c] was used as the standard for % enrichment.

cation steps, a double-labeled radioactive tracer (α-[6-^{3}H]GlcNAc-1-[^{32}P]P-6-(α-methyl)mannoside) was prepared in a similar (50 μl) reaction, utilizing [β-^{32}P]UDP-GlcNAc and [6-^{3}H]UDP-GlcNAc as substrates (5, 17). The combined supernatant fluids from both reaction mixtures were diluted to 120 ml with 2 mM Tris base and applied to a column of QAE-Sephadex (4 ml) equilibrated in 2 mM Tris base. The column was washed with 30 ml of 2 mM Tris base and then eluted with 20 ml of 30 mM NaCl in 2 mM Tris base. The eluate was collected, taken to dryness, and streaked on Whatman 3MM paper. After chromatography in ethanol:1 M ammonium acetate, pH 7.5 (7.5:3), for 13 h, the paper was cut into 1-cm strips. Each strip was soaked in 2 ml of water, and an aliquot was assayed for radioactivity. As shown in Fig. 1A, most of the radioactivity migrated as a single peak. The peak fractions were pooled and chromatographed on a Bio-Gel P2 column (100 × 2 cm) in water. One-milliliter fractions were collected and monitored for radioactivity. As shown in Fig. 1B, most of the ^{32}P and ^{3}H eluted near the void volume of the column. This region was pooled and taken to dryness. The yield of GlcNAc-1-P-6-(α-methyl)mannoside was about 50 nmol, as determined by the amount of free *N*-acetylglucosamine released by mild acid hydrolysis (pH 2, 100°C, 30 min) using the amino sugar color test (19).

Enzyme reactions. The spontaneous exchange of ^{18}O into the C-1 position of free sugars is markedly diminished between pH 3 and 5.5 (20). All reactions were therefore carried out in 0.1 M acetate buffer, pH 5.0, containing 0.5% Triton X-100. The two enzymes under study retained greater than 70% of their maximal activity under these conditions. Reactions were performed in duplicate using either O or 80% $H_2{}^{18}O$ for comparison. The reactions were allowed to proceed for 15 min and then quenched by flash-freezing in a dry ice-acetone bath and lyophilized. The lyophilized samples were derivatized for GC-MS analysis by addition of 100 μl of 1:1 Regisil:pyridine and incubation at room temperature for 24 h.

Gas chromatography-mass spectrometry. Isotope analyses were carried out on the completely trimethylsilylated samples using capillary gas chromatography and chemical ionization. A Finnigan Model 3300 chemical ionization GC-MS, which had been modified to accept a Varian Model 3700 capillary injected port with an splitter injector system, was used. The injection port was used in split mode with a removable glass liner. The liner contained a glass wool plug to prevent injection residues from entering the column.

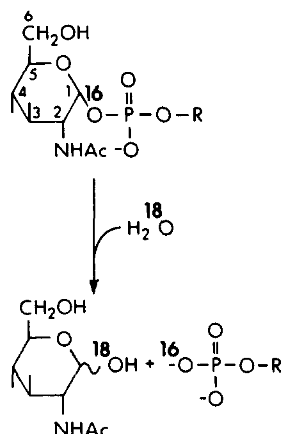

FIG. 2. Demonstration of the enzymatic mechanism. Cleavage of the glycosidic bond occurs, with consequent enrichment of the released *N*-acetylglucosamine in ^{18}O to the levels present in the starting medium. The R group used in this study was either UMP or α-methylmannoside.

A 30-DB-1 fused silica column (J. and W., Inc., Rancho Cordova, Calif.) was introduced into the injector to serve as its own split tip. It also led directly into the ionization source of the mass spectrometer, extending, by measurement, to the entrance of the ion volume. Helium carrier gas was used (10 psi). Ammonia was used as reagent gas at a total source pressure of 0.5 Torr. Retention times for the TMS-GlcNAc and TMS-(α-methyl)mannoside 6-phosphate were 2.8 min (at 170°C) and 2.5 min (at 200°C), respectively. The extent of ^{18}O incorporation into the enzyme incubation products was determined by calculating the ^{18}O content in excess of that naturally present. In the case of TMS-GlcNAc the molecular ions *m/z* 510 and 512 ($^{16}O_1$ and $^{18}O_1$) were measured. For TMS-(α-methyl)mannoside 6-phosphate the protonated molecular ions for the $^{16}O_1$ and $^{18}O_1$ species were *m/z* 635 and 637, respectively.

RESULTS AND DISCUSSION

As shown in Table I, the amount of spontaneous exchange of the $H_2{}^{18}O$ into free GlcNAc was relatively small, reaching a level of only 14.4% during a 15-min incubation, in a medium of 80% enrichment. When UDP-GlcNAc or GlcNAc-P-(α-methyl)mannoside was used as substrate for the two enzymes, the released GlcNAc was enriched with ^{18}O to an extent equal to that of the starting medium. On the other hand, there was no enrichment of ^{18}O in the other product (α-methylmannoside 6-phosphate) of the second substrate (see Table I). These data demonstrate that both enzymes cleave the C-O bond between the sugar and the phosphodiester rather than the O-P bond (see Fig. 2). Thus, the two enzymes proceed by a glycosidase rather than a phosphodiesterase type of mechanism.

The lysosomal α-*N*-acetylglucosaminidase also cleaves α-GlcNAc 1-phosphate by a glycosidase mechanism (Table I). However, the rat liver enzyme preparation had very low activity toward this substrate (8), and the small amount of GlcNAc that was formed showed little enrichment with ^{18}O. Therefore, this reaction must be proceeding primarily by a phosphomonesterase type of mechanism with cleavage of the P-O bond. This is similar to the findings of Cohn for the hydrolysis of glucose 1-phosphate by acid phosphatase or alkaline phosphatase (21). This observation indicates that the low level of activity of the rat liver enzyme preparation toward α-GlcNAc 1-P (8) is mostly, if not exclusively, due to a low level of a contaminating phosphatase rather than due to the α-*N*-acetylglucosamine-1-phosphodiester *N*-acetylglucosaminidase.

In our previous studies, we showed that the rat liver enzyme only cleaves GlcNAc residues that are α-linked to an underlying phosphate residue (8). The present data indicate that the enzyme also requires the underlying phosphate group to be in a phosphodiester linkage.

The recommendations of the Enzyme Nomenclature Committee of the IUB (22) suggest that while it is not essential that the name of an enzyme be based on its precise mechanism, "where alternative names are possible, the mechanism may be taken into account in choosing bejtween them." We therefore suggest that the enzyme be renamed α-*N*-acetyl-D-glucosamine-1-phosphodiester *N*-acetylglucosaminidase (systematic name, 2-acetamido-2-deoxy-α-D-glucose 1-phosphodiester acetamidodeoxyglucohydrolase). An appropriate trivial name would be phosphodiester glycosidase.

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