Human acetyl-coenzyme A:α-glucosaminide *N*-acetyltransferase

Kinetic characterization and mechanistic interpretation

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Acetyl-CoA:α-glucosaminide *N*-acetyltransferase (*N*-acetyltransferase) is an integral lysosomal membrane protein which catalyses the transfer of acetyl groups from acetyl-CoA on to the terminal glucosamine in heparin and heparan sulphate chains within the lysosome. *In vitro*, the enzyme is capable of acetylating a number of mono- and oligo-saccharides derived from heparin, provided that a non-reducing terminal glucosamine is present. We have prepared highly enriched lysosomal membrane fractions from human placenta by a combination of differential centrifugation and density-gradient centrifugation in Percoll. This preparation was used to investigate the kinetics of the enzyme with three acetyl-acceptor substrates, i.e. glucosamine and a disaccharide and a tetrasaccharide derived from heparin, each containing a terminal glucosamine residue. The enzyme showed a pH optimum at 6.5, extending to 8.0 for the mono- and di-saccharide substrates but falling off sharply above pH 6.5 for the tetrasaccharide substrate. We identified two distinct K_m values for the glucosamine substrate at both pH 7.0 and pH 5.0, whereas the tetrasaccharide substrate displayed only a single K_m value at each pH. The K_m values were found to be highly pH-dependent, and at pH 5.0 the values for the acetyl-acceptor substrates showed a decreasing trend as the size of the substrate increased, suggesting that the enzyme recognizes an extended region of the non-reducing terminus of the heparin or heparan sulphate polysaccharides. Double-reciprocal analysis, isotope exchange between *N*-acetylglucosamine and glucosamine, and inhibition studies with desulpho-CoA indicate that the enzyme operates by a random-order ternary-complex mechanism. Product inhibition studies display a complex pattern of dead-end inhibition. Taken in context with what is known about lysosomal utilization and physiological levels of acetyl-CoA, these results suggest that *in vivo* the enzyme operates via a random-order ternary-complex mechanism which involves the utilization of cytosolic acetyl-CoA to transfer acetyl groups on to the terminal glucosamine residues of heparin within the lysosome.

INTRODUCTION

The integral lysosomal membrane protein acetyl-CoA (AcCoA):α-glucosaminide *N*-acetyltransferase (*N*-acetyltransferase) catalyses the transfer of acetyl groups from AcCoA on to the amino residue of the non-reducing terminal GlcN in heparin and heparan sulphate chains within the lysosome. The autosomal recessive disorder Sanfilippo syndrome type C (mucopolysaccharidosis III C) results from a deficiency in this enzyme. Clinical symptoms of the disorder show considerable variation in severity, and include behavioural problems, severe progressive mental retardation, relatively mild skeletal deformities and death, usually in the late teens. Biochemically the disease is characterized by the intra-lysosomal storage of heparin and heparan sulphate fragments in all organs, as well as excessive excretion of these fragments in the urine [1,2].

Degradation of heparin occurs within the lysosomes by the concerted action of a group of at least eight enzymes: these include four sulphatases, three exo-glycosidases and *N*-acetyltransferase [3,4]. These enzymes act sequentially at the non-reducing terminus of heparin chains to catabolize the glycosaminoglycan, producing free sulphate and monosaccharide residues which are subsequently transported out of the lysosome to be utilized by the cell. Within this process, *N*-acetyltransferase is required to *N*-acetylate the terminal GlcN residues, a reaction that must occur before their hydrolysis by α-*N*-acetylglucosaminidase. The requirement for a biosynthetic reaction in a catabolic process is unusual, and to our knowledge this is the only instance where such a process occurs within the lysosome. The mechanism by which this is achieved has been the topic of considerable investigation. Evidence presented by Rome and Crain [5] and Rome et al. [6] on the degradation of glycosaminoglycans in intact lysosomes indicate that *in vivo* AcCoA is supplied to *N*-acetyltransferase from the cytosol. Based on detergent activation studies and AcCoA/CoA uptake experiments [7], it is thought that AcCoA cannot enter the lysosome. The pH optima reported for *N*-acetyltransferase [1,7] in the range 5.5–8.0 also suggest a cytosolic binding site for AcCoA. In addition, as has been previously suggested [6], and corroborated by our own results, AcCoA is unlikely to survive in the hydrolytic environment of the lysosome. Consequently *N*-acetyltransferase is required to transfer acetyl groups from cytosolic AcCoA across the lysosomal membrane on to the terminal GlcN residues of heparin.

We have investigated this process in highly purified lysosomal membranes isolated from human placenta, using a range of oligosaccharide substrates. Contrary to earlier reports, which suggested a substituted-enzyme mechanism [7], our results indicate a random-order ternary-complex mechanism.

EXPERIMENTAL

Reagents

D-[1-^{3}H]GlcN hydrochloride (2.3 Ci/mmol) was purchased from Amersham Australia; all other reagents were from Sigma, St. Louis, MO, U.S.A.

Preparation of substrates

D-[1-^{3}H]GlcN hydrochloride (2.3 Ci/mmol) was chromatographed on Dowex AG 50W-X8 cation-exchange resin to remove

Abbreviations used: AcCoA, acetyl-CoA; *N*-acetyltransferase, acetyl-CoA:α-glucosaminide *N*-acetyltransferase (EC 2.3.1.78).

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uncharged trace contaminants. The sample was applied to a column (3 ml) of resin in water, and the column was washed with 30 vol. of water. The GlcN was eluted with 0.3 M HCl, freeze-dried and finally taken up in 10% ethanol/water (50000 c.p.m./μl) and stored at −20 °C. The disaccharide *O*-(α-2-amino-2-deoxy-D-glucopyranosyl)-(1→3)-L-[6-^{3}H]idonic acid (0.10 Ci/mmol) and the tetrasaccharide *O*-(2-amino-2-deoxy-α-D-glucopyranosyl *N*-sulphate)-(1→4)-β-D-uronic acid-(1→4)-(2-amino-2-deoxy-α-D-glucopyranosyl *N*-sulphate)-(1→3)-L-[6-^{3}H]idonic acid (or -2,5-anhydro-L-[6-^{3}H]idonic acid or -L-[6-^{3}H]gulonic acid) (0.35 Ci/mmol) were prepared from heparin as described previously [8]. The N-sulphate on the non-reducing terminal GlcN residue of the tetrasaccharide was removed by incubating the tetrasaccharide (55 nmol) with a dialysed homogenate supernatant (100000 *g*, 1 h) of human skin fibroblasts (1 mg of protein) in 0.1 M sodium acetate buffer (400 μl), pH 5.6, for 60 h. The partially N-desulphated tetrasaccharide was separated from the undigested material on a column (0.6 cm × 8.0 cm) of Dowex AG1-X8 anion-exchange resin, eluted with a 0–1 M linear gradient of NaCl.

Assay of the *N*-acetyltransferase

In a typical reaction, membranes (1–5 μg of protein) were suspended in 50 mM dimethylglutaric acid/NaOH buffer (pH 4.5–8.0), 50 mM sodium phosphate/50 mM sodium tartrate buffer (pH 7.0) or 100 mM sodium phosphate/50 mM sodium citrate buffer (pH 5.0) with AcCoA to a final concentration of 1–4000 μM. The reaction was started by addition of the second ^{3}H-labelled substrate, i.e. GlcN, the disaccharide substrate or the partially N-desulphated tetrasaccharide substrate, to a final concentration of 1–500 μM. Reactions were performed in a total volume of 20 μl in 96-well microtitre plates (half-area wells). The wells were covered with Parafilm and the plates incubated at 37 °C for 1–4 h. The reaction was stopped by addition of 20% formic acid (2 μl) and the reaction mixture was analysed by high-voltage electrophoresis [8] performed on Whatman 3MM filter paper in 3% (v/v) formic acid at 45 V/cm for 30 min (GlcN) or 45 min (di- and tetra-saccharide substrates). The paper strips were dried, sectioned and counted for radioactivity in 3.3 ml of water plus 10 ml of Optiphase 3 scintillant. The ratio of radioactivity associated with the substrate to that with the product was used to determine the amount of substrate acetylated.

Preparation of lysosomal membranes

Human placentas were stored at 4 °C immediately after birth and used within 2 h; all subsequent work was performed at 4 °C. Placentas (300–500 g) were weighed, dissected into 1 cm strips, and washed in 3 × 500 ml of 0.25 M sucrose/1 mM EDTA, pH 7.0. The washed placenta was minced and then homogenized by two passes through a Mouli hand homogenizer with 750 ml of the same buffer. The homogenate was centrifuged (750 *g*, 10 min), the supernatant was kept and the pellet minced, homogenized and centrifuged again, as described above. The two supernatants were pooled, filtered through two layers of cotton gauze and then centrifuged (8000 *g*, 20 min). The pelleted granular fraction was resuspended in 40 ml of sucrose buffer and layered above two 10 ml 50%-Percoll cushions prepared in the same buffer; the cushions were centrifuged (1000 *g*, 10 min) to remove contaminating red blood cells and the granular fraction was removed from the top of the cushions. The granular fraction was made up to 320 ml in 0.25 M sucrose/1 mM EDTA, pH 7.0, containing 25% Percoll, and eight 40 ml fractions were centrifuged (31000 *g*, 60 min). The gradients were fractionated, and the lower 10 ml from each gradient was pooled and centrifuged (31000 *g*, 60 min) to form two 40 ml gradients. The gradients were fractionated (10 × 4 ml) and those fractions (8–10) containing highly enriched lysosomes were made up to 1.0 M NaCl, freeze/thawed three times, and then centrifuged (100000 *g*, 60 min). The membrane pellet was resuspended in 20 mM dimethylglutaric acid/NaOH (pH 7.0)/1.0 M NaCl, freeze/thawed three times and centrifuged (100000 *g*, 60 min). The washed lysosomal membranes were resuspended in 20 mM dimethylglutaric acid/NaOH, pH 7.0, at 1.0 mg/ml and stored at −20 °C.

Protein/enzyme marker assays

Protein was assayed by the bicinchoninic acid method [9], with bovine serum albumin as a standard. Samples taken at various stages of the lysosomal membrane preparation were assayed for organelle marker enzymes to assess the purity of the lysosomes. *N*-Acetyltransferase was assayed as described above, by using 50 mM dimethylglutaric acid/NaOH (pH 7.0) containing 5 μM [^{3}H]GlcN and 2 mM AcCoA, with incubation for 2 h at 37 °C. Samples in sucrose buffer were dialysed against 20 mM sodium phosphate-buffered saline before assay. 5′-Nucleotidase activity was assayed by the method described by El-Aaser and Reid [10] and glucose-6-phosphatase by the method of Swanson [11]. In both assays 10 mM sodium (+)-tartrate was added to inhibit non-specific acid phosphatase activity [12,13]. P_i was determined by the method of Chen et al. [14]. Cytochrome *c* oxidase activity was measured by the method described by Cooperstein and Lazarow [15]. Galactosyltransferase activity was measured by a modification of the method of Brew et al. [16] as described by Rome et al. [17]. Total *N*-acetyl-β-D-glucosaminidase and acid phosphatase activities were measured fluorimetrically with 4-methylumbelliferyl substrates by the methods of Leaback and Walker [18] and Kolodny and Mumford [19] respectively. Catalase activity was measured by the method described by Bergmeyer et al. [20].

Isotope-exchange studies

Isotope-exchange studies were performed as follows: membranes (10 μg) were incubated in 50 mM sodium phosphate/50 mM sodium tartrate buffer, pH 7.0, containing 1.0 μM [^{3}H]GlcN (50000 c.p.m.), 0–100 mM GlcNAc and 0–31.6 mM CoA or 1.0 mM desulpho-CoA for 4 h at 37 °C. The reactions were performed in a total volume of 20 μl in 96-well microtitre plates (half-area wells) and stopped by addition of 2 μl of 20% formic acid. The reaction mixtures were analysed as described for the *N*-acetyltransferase assay.

RESULTS

Preparation of lysosomal membranes

The results of the enzymic analysis of the fractions from the first and second Percoll gradients are shown in Tables 1 and 2. In the first gradient, the lysosomal marker enzymes *N*-acetyl-β-D-glucosaminidase and *N*-acetyltransferase were concentrated in the lower fractions, with 65% and 49% respectively being found in the lower 25% of the gradient at a density of 1.09–1.11 g/ml; this region was subsequently used to generate the second Percoll gradient. Acid phosphatase, which is found in both lysosomes and endosomes, showed a typical biphasic distribution, indicating that the endosomes were concentrating in the upper three fractions. The enzyme markers specific for plasma membrane, Golgi, peroxisomes, mitochondria and endoplasmic reticulum were all concentrated in the upper region of the gradient, with only a small percentage of the respective activities co-migrating with the lysosomes.

Table 1 Enzyme analysis of the first Percoll gradient

Fractions from the first Percoll gradient were assayed for protein content and marker enzyme activity as described in the Experimental section. The results are expressed as mg/ml (Protein) and percentage of total activity in each fraction of *N*-acetyl-β-D-glucosaminidase (β-Hex), cytochrome *c* oxidase (Cyt *c* oxid), *N*-acetyltransferase (GNAT), 5′-nucleotidase (5′Nuc), glucose-6-phosphatase (Glc-6-P), Catalase, acid phosphatase (ACP) and galactosyltransferase (Gal trans).

Fraction	Protein	β-Hex	Cyt *c* oxid	GNAT	5′Nuc	Glc-6-P	Catalase	ACP	Gal trans
1	7.7	6	18	20	46	43	40	25	21
2	3.8	4	24	7	35	30	31	20	18
3	2.1	4	12	3	8	8	16	11	11
4	1.9	4	13	3	3	3	6	4	14
5	1.7	4	9	4	2	3	2	4	13
6	1.4	4	8	7	1	2	1	4	10
7	1.2	5	6	3	2	2	1	5	7
8	1.2	8	6	5	1	2	1	6	3
9	1.2	19	3	15	1	3	1	11	2
10	1.6	42	2	33	1	4	1	10	1

Table 2 Enzyme analysis of the second Percoll gradient

Fractions from the second Percoll gradient were assayed for protein content and marker enzyme activity as described in the Experimental section. The results are expressed as mg/ml (Protein) and percentage of total activity in each fraction of *N*-acetyl-β-D-glucosaminidase (β-Hex), cytochrome *c* oxidase (Cyt *c* oxid), *N*-acetyltransferase (GNAT), 5′-nucleotidase (5′Nuc), glucose-6-phosphatase (Glc-6-P), Catalase, acid phosphatase (ACP) and galactosyltransferase (Gal trans).

Fraction	Protein	β-Hex	Cyt *c* oxid	GNAT	5′Nuc	Glc-6-P	Catalase	ACP	Gal trans
1	1.1	2	17	5	17	14	15	9	32
2	1.3	4	23	4	23	16	18	15	23
3	1.2	5	18	5	18	14	13	24	11
4	1.0	6	12	5	11	10	10	12	8
5	1.0	6	9	5	7	8	9	9	8
6	1.0	7	6	6	7	8	8	8	6
7	1.1	10	4	10	6	8	8	8	5
8	1.4	15	4	16	5	8	7	8	4
9	1.9	29	4	25	3	7	7	5	2
10	1.8	16	3	19	3	7	5	2	1

Table 3 Specific activities of marker enzymes in purified organelles

Specific activities of the marker enzymes in the different stages of organelle preparation were determined as described in the Experimental section. The values reported for lysosomes are the average of fractions 8–10 from the second Percoll gradient. Lysosomal membranes were prepared from those fractions. The results are expressed as specific activities of *N*-acetyl-β-D-glucosaminidase (β-Hex; nmol/min per mg), cytochrome *c* oxidase (Cyt *c* oxid; nmol/min per mg), *N*-acetyltransferase (GNAT; pmol/min per mg), 5′-nucleotidase (5′Nuc; nmol/min per mg), glucose-6-phosphatase (Glc-6-P; nmol/min per mg), catalase (μmol/min per mg), acid phosphatase (ACP; nmol/min per mg) and galactosyltransferase (Gal trans; pmol/min per mg): nd, not detected.

	β-Hex	Cyt *c* oxid	GNAT	5′Nuc	Glc-6-P	Catalase	ACP	Gal trans
Cell homogenate	36	17	2	57	9	254	49	96
Cell contents	15	13	1	111	16	207	49	128
Cell content supernatant	11	3	0.7	101	19	203	36	89
Granular fraction	85	149	6	386	22	60	210	176
Lysosomes	1164	11	53	21	11	9	672	5
Lysosomal membranes	57	nd	673	1	nd	nd	703	nd

After the lower fractions of the first gradient had been mixed and re-formed into a new gradient, the lysosomes still concentrated in the lower fractions 7–10, at a density of 1.10–1.15 g/ml. The other organelles all concentrated in the upper fractions of this second gradient, further decreasing the level of these contaminants in the lysosome-containing fractions (Table 2). Table 3 shows the specific activities of the marker enzymes in the various fractions. The lysosomal fraction showed an enrichment of *N*-acetyl-β-D-glucosaminidase and *N*-acetyltransferase of 32- and 27-fold respectively over the total cell homogenate. The other marker enzymes all showed less than 1.2-fold enrichment over the cell homogenate. The washed lysosomal membrane fraction showed a 336-fold enrichment of the lysosomal-membrane marker enzyme *N*-acetyltransferase over

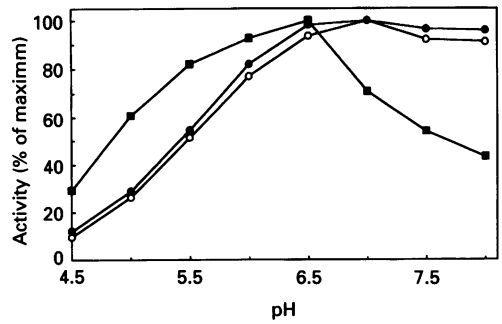

Figure 1 pH-dependence of the *N*-acetyltransferase reaction

Lysosomal membranes were assayed for *N*-acetyltransferase activity in 50 mM dimethylglutaric acid buffer at pH 4.5–8.0, by using a 1 h incubation at 37 °C as described in the Experimental section. Substrate concentrations used were 4 mM AcCoA with 10 μM GlcN (●), 4 μM disaccharide (○) or 4 μM tetrasaccharide (■).

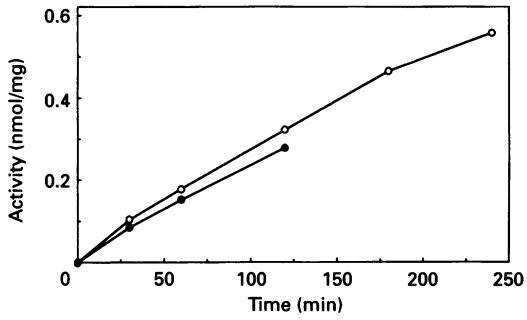

Figure 2 Time-dependence of the *N*-acetyltransferase reaction

Lysosomal membranes were assayed for *N*-acetyltransferase activity in 50 mM sodium phosphate/50 mM sodium tartrate buffer at pH 7.0 with 1 μM AcCoA/1 μM GlcN (●) and in 100 mM sodium phosphate/50 mM sodium citrate buffer at pH 5.0 with 1.0 μM AcCoA/1 μM GlcN (○) as described in the Experimental section.

the cell homogenate, a 12-fold enrichment over the whole lysosomes.

pH optima and time course of the *N*-acetyltransferase reaction

The pH optima of *N*-acetyltransferase was investigated by using three different acetyl-acceptor substrates, the monosaccharide GlcN and the di- and tetra-saccharides derived from heparin (Figure 1). The mono- and di-saccharides gave identical profiles, with the pH optima at 6.5 and extending up as far as pH 8.0. The tetrasaccharide had the same pH optimum of 6.5; however, the profile declined sharply above pH 6.5, with higher relative activities in the lower pH range. Ionic strength of the assay buffer was shown to have no effect on the enzyme activity over the range 0–500 mM NaCl (results not shown). The activity time-course of the *N*-acetyltransferase reaction (Figure 2) was shown to be almost linear for up to 2 h at pH 7.0 and up to 4 h at pH 5.0, with 1 μM GlcN and with 1 and 10 μM AcCoA respectively. These conditions represented the minimum substrate concentrations and maximum incubation times used.

Kinetic studies of the *N*-acetyltransferase reaction

Double-reciprocal analysis was carried out on the *N*-acetyltransferase reaction at pH 7.0, using substrate concentrations in the range 1–8 μM (Figure 3). The Lineweaver–Burk plots clearly show converging sets of lines, indicating that the reaction proceeds via a ternary-complex mechanism. Non-linear regression analysis of the primary data with the Enzfitter program (Biosoft, Cambridge, U.K.) was used to determine K_m and $V_{max.}$ values. These values were subsequently used to calculate the gradients and intercepts of the primary plots (Figure 3). Secondary plots of the data in Figure 3(a), i.e. 1/[AcCoA] versus gradient and 1/[AcCoA] versus ordinate intercept, were used to calculate the K_m values for AcCoA and GlcN; at pH 7.0 these were 2.5 μM and 3.0 μM respectively (Table 4). Double-reciprocal analysis was performed at pH 5.0, with substrate concentrations in the range 50–400 μM (results not shown). This analysis also produced sets of converging lines, and from the secondary plots the K_m values were calculated to be 54 μM and 370 μM for AcCoA and GlcN respectively (Table 4). The relative $V_{max.}$ value at pH 5.0 was 9 times the corresponding value at pH 7.0.

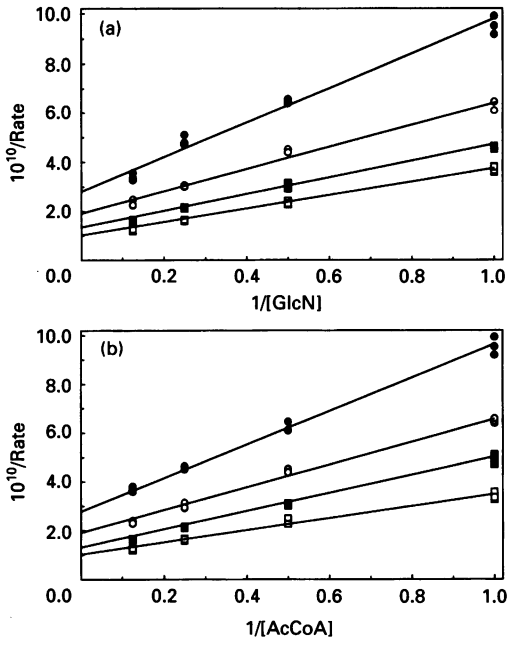

Figure 3 Double-reciprocal analysis of the *N*-acetyltransferase reaction

Lysosomal membranes were assayed for *N*-acetyltransferase activity in triplicate, at pH 7.0, in 50 mM sodium phosphate/50 mM sodium tartrate buffer as described in the Experimental section. (**a**) The concentration of GlcN was varied between 1 and 8 μM, while the concentration of AcCoA was held at 1.0 μM (●), 2.0 μM (○), 4.0 μM (■) or 8.0 μM (□). (**b**) The concentration of AcCoA was varied between 1 and 8 μM while the concentration of GlcN was held at 1.0 μM (●), 2.0 μM (○), 4.0 μM (■) or 8.0 μM (□). Line fitting was based on the K_m and $V_{max.}$ values determined from the corresponding Michaelis–Menten plots using non-linear regression analysis. 1/Rate is expressed as $mol^{-1} \cdot min \cdot mg$.

The K_m and relative $V_{max.}$ values for AcCoA and GlcN as well as the disaccharide and tetrasaccharide substrates were also determined at both pH 7.0 and 5.0 from plots of rate versus substrate, using saturating concentrations of GlcN and AcCoA respectively. The data were fitted to the standard equation for two-enzyme systems, $v = V_1[S]/(K_1+[S]) + V_2[S]/(K_2+[S])$, using the Enzfitter program. Under these conditions two K_m values were determined for GlcN at pH 7.0 and 5.0, and for the disaccharide and AcCoA at pH 7.0; however, only single K_m values were determined for the tetrasaccharide at pH 7.0 and 5.0, and for the disaccharide and AcCoA at pH 5.0 (Table 4).

Table 4 K_m and relative $V_{max.}$ values of the *N*-acetyltransferase reaction

K_m (low)* values of AcCoA and GlcN and relative $V_{max.}$ (low)* values at pH 7.0 and 5.0 were calculated from the Dalziel parameters obtained from the secondary plots of the double-reciprocal analyses of the *N*-acetyltransferase reaction. K_m values of AcCoA and GlcN and of the disaccharide and tetrasaccharide substrates, and the relative $V_{max.}$ values, were determined by fitting the rate-versus-concentration plots to the standard equation for a two-enzyme system, $v = V_1[S]/(K_1 + [S]) + V_2[S]/(K_2 + [S])$, by using the Enzfitter program. Substrate concentrations used were 200 μM GlcN with 25–400 μM. AcCoA or 4.0 mM AcCoA with 1–200 μM GlcN at pH 7.0, 5 mM GlcN with 0.5–5 mM AcCoA or 10 mM AcCoA with 0.25–4.0 mM GlcN at pH 5.0, and for the di- and tetra-saccharide substrates 4.0 mM AcCoA with 0.5–70 μM disaccharide at pH 7.0, 25–850 μM disaccharide at pH 5.0 or 0.2–20 μM tetrasaccharide at pH 5.0 and 7.0. Relative $V_{max.}$ values are given in brackets. Values at pH 5.8 are taken from [7]. Abbreviation: nd, not detected by the equation for a two-enzyme system.

	K_m (μM)		
Substrate	pH 5.0	pH 5.8	pH 7.0
AcCoA (low)*	54 ± 8 [9]		2.5 ± 0.5 [1]
GlcN (low)*	370 ± 30 [46]		3.0 ± 0.4 [11]
AcCoA (low)	nd		21 ± 14 [1]
AcCoA (high)	580 ± 30 [46]	550	304 ± 153 [6]
GlcN (low)	600 ± 258 [20]		6.3 ± 1.1 [6]
GlcN (high)	3660 ± 1870 [45]	300	289 ± 113 [18]
Disaccharide (low)	210 ± 11 [4]		7.8 ± 4.4 [1]
Disaccharide (high)	nd		79 ± 27 [5]
Tetrasaccharide	6.9 ± 0.6 [1]		5.7 ± 0.4 [1]

* Values determined from double-reciprocal analysis

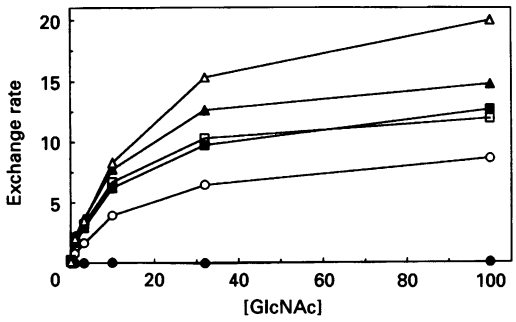

Figure 4 Isotope exchange in the *N*-acetyltransferase reaction

Lysosomal membranes were assayed for the rate of acetate exchange from GlcNAc to GlcN as described in the Experimental section. The concentration of GlcNAc was varied from 0 to 100 mM while the concentration of [^{3}H]GlcN was kept constant at 1.0 μM; CoA was included at 0 μM (●), 10 μM (○), 100 μM (■), 1.0 mM (□), 10 mM (▲) or 31.6 mM (△). The exchange rate was calculated from the final concentration of GlcN obtained at equilibrium as determined from the K_{ia} value of GlcN and the K_{is} value of GlcNAc with respect to GlcN (3.0 μM and 5.6 mM respectively) and is expressed as pmol/min per mg.

Inhibition of the *N*-acetyltransferase reaction

Product-inhibition studies were performed at pH 7.0, with low substrate concentrations, in order to investigate the relationship between the substrate and product binding sites and thereby to gain more insight into the reaction mechanism. Inspection of the Lineweaver–Burk plots (results not shown) revealed that, within the concentration range used, GlcNAc is a competitive inhibitor of GlcN and a non-competitive inhibitor of AcCoA, whereas CoA is a non-competitive inhibitor of both AcCoA and GlcN. Secondary plots of gradient or ordinate (determined from non-linear regression analysis of Michaelis–Menten plots of the primary data) versus inhibitor concentration were used to determine the K_{is} and K_{ii} values respectively (Table 5).

Using the same conditions the CoA analogue desulpho-CoA was determined to be a competitive inhibitor of AcCoA and a non-competitive inhibitor of GlcN (Table 5).

Isotope exchange in the *N*-acetyltransferase reaction

Isotope-exchange studies were performed to examine the exchange of acetate groups from GlcNAc to GlcN at pH 7.0. The rate of exchange was found to be dependent on the concentrations of both GlcNAc and CoA (Figure 4); in the absence of CoA there was no exchange even at very high concentrations of GlcNAc. Desulpho-CoA, an analogue of CoA lacking the free thiol group and therefore unable to accept acetyl groups, was found to be unable to participate in the acetate exchange between GlcNAc and GlcN (results not shown).

Table 5 K_i values of inhibitors of the *N*-acetyltransferase reaction

Lysosomal membranes were assayed for *N*-acetyltransferase activity in duplicate, at pH 7.0, by using the following sets of substrate/inhibitor concentrations to determine the different product/substrate inhibition types. (1) CoA inhibition of AcCoA: the concentration of AcCoA was varied between 1.0 and 8.0 μM while the concentration of GlcN was held at 2.0 μM; CoA was included at 0, 50, 100 and 200 μM. (2) CoA inhibition of GlcN: the concentration of GlcN was varied between 1.0 and 8.0 μM, while the concentration of AcCoA was held at 4.0 μM; CoA was included at 0, 100, 200 and 400 μM. (3) GlcNAc inhibition of AcCoA: the concentration of AcCoA was varied between 1.0 and 8.0 μM, while the concentration of GlcN was held at 2.0 μM; GlcNAc was included at 0, 2.0, 4.0 and 8.0 mM. (4) GlcNAc inhibition of GlcN: the concentration of GlcN was varied between 1.0 and 8.0 μM, while the concentration of AcCoA was held at 4 μM; GlcNAc was included at 0, 4.0, 8.0 and 12.0 mM. To determine the inhibition type and inhibition constants associated with the dead-end inhibitor desulpho-CoA, the following conditions were used. (5) Desulpho-CoA inhibition of AcCoA: the concentration of AcCoA was varied between 1.0 and 8.0 μM, while the concentration of GlcN was held at 2.0 μM; desulpho-CoA was included at 0, 250, 500 and 1000 μM. (6) Desulpho-CoA inhibition of GlcN: the concentration of GlcN was varied between 1.0 and 8.0 μM, while the concentration of AcCoA was held at 4.0 μM; desulpho-CoA was included at 0, 250, 500 and 1000 μM. In all cases line fitting was based on the K_m and $V_{max.}$ values determined from non-linear regression analysis of the corresponding Michaelis–Menton plots.

Experiment	Product/inhibitor	Substrate	Inhibition type	K_{is} (μM)	K_{ii} (μM)
(1)	CoA	AcCoA	Non-competitive	32 ± 6	114 ± 12
(2)	CoA	GlcN	Non-competitive	82 ± 25	247 ± 63
(3)	GlcNAc	AcCoA	Non-competitive	3800 ± 660	3900 ± 1000
(4)	GlcNAc	GlcN	Competitive5600 ± 640		
(5)	Desulpho-CoA	AcCoA	Competitive	220 ± 80	
(6)	Desulpho-CoA	GlcN	Non-competitive	206 ± 3	550 ± 140

DISCUSSION

Purification of lysosomal membranes from human placenta by the two-Percoll-gradient method, as outlined, gave an enrichment of *N*-acetyltransferase activity of 336-fold over the crude cell homogenate. Other reports of lysosome and lysosomal-membrane preparations from rat liver describe enrichments ranging from 30- to 260-fold, based on a number of lysosomal and lysosomal-membrane marker enzymes [21–24]. The low enrichment levels of other organelle markers indicate that the lysosomal preparations contain only a few per cent of plasma membrane, mitochondria and endoplasmic reticulum, and even lower levels of Golgi and peroxisomes; on the basis of these low levels of contamination, the preparation is estimated to contain between 80 and 90% lysosomes. These preparations show no other enzyme activity towards the GlcN-containing substrates; however, in the course of this study we noted that AcCoA was unstable in lysosomal homogenates and that purified lysosomal membranes could also degrade AcCoA. Further investigation of the product revealed it to be 3′-dephospho-AcCoA, presumably produced as a result of the action of non-specific acid phosphatases present in the lysosome. In our assays, acid phosphatase degradation of AcCoA by lysosomal enzymes affected *N*-acetyltransferase activity only when the concentration of AcCoA was in the micromolar range, and the level of phosphatase was sufficient to deplete significantly the level of AcCoA. This effect was more pronounced at pH 5.0 than at pH 7.0, and could be effectively overcome by the use of suitable phosphate- and tartrate-containing buffers (see Figure 2); these buffers were used in all situations where low AcCoA concentrations were required.

Double-reciprocal analysis of the *N*-acetyltransferase reaction at pH 7.0 with low substrate concentrations (Figure 3) clearly shows two sets of converging lines, indicating a ternary-complex mechanism. From the Dalziel parameters obtained from the secondary plots of these data, the K_m values for AcCoA and GlcN were calculated to be 2.5 and 3.0 μM respectively. A similar analysis at pH 5.0 also gave sets of converging lines, and the K_m values were calculated to be 54 and 370 μM for AcCoA and GlcN respectively. It has previously been reported [7] that the K_m values for GlcN and AcCoA at pH 5.8 for this enzyme were 300 and 550 μM respectively. We further investigated for second, higher, K_m values in our system by fitting plots of rate versus substrate concentration to the standard equation for two-enzyme systems, $v = V_1[S]/(K_1+[S]) + V_2[S]/(K_2+[S])$. This investigation revealed two K_m values for both AcCoA and GlcN at pH 7.0; however, only GlcN displayed two K_m values at pH 5.0 (Table 4). For AcCoA, at pH 5.0 the high concentrations of both GlcN (5 mM) and AcCoA (0.5–5 mM) used would result in the lower-K_m system contributing very little to the total activity and effectively being undetectable. Interestingly, when the data from the disaccharide substrate at pH 5.0 and the tetrasaccharide substrate were fitted to the two-enzyme system equation, we detected only single, low, K_m values, suggesting that the dual K_m values seen with the smaller monosaccharide substrate and the disaccharide substrate at pH 7.0 may be related to the accessibility of these substrates to different enzyme forms. It would appear unlikely that there is more than one transferase present in the human lysosomal membrane, as all of the other enzymes involved in the degradation of heparin result from single gene products [25]. However, it is possible that the different K_m values result from differential processing of the enzyme. There are a number of reports of multiple forms of lysosomal enzymes, usually corresponding to the mature and precursor forms [26,27]. A second possibility may involve different conformational forms of the enzyme within the membrane, as has been proposed for the microsomal UDP-glucuronyltransferases [28]. The smaller size of GlcN substrate in relation to the tetrasaccharide substrate, and its ability to pass through lysosomal membranes [7], could potentially enable access to a conformationally distinct population of enzyme in which the binding site is restricted. This concept is supported by the observation that only a single, low, K_m was detected for the larger tetrasaccharide substrate. The relative $V_{max.}$ values associated with the high K_m values were 2–6 times higher than the corresponding values associated with the low K_m values. However, at the low substrate concentrations used for the double-reciprocal analysis and the product-inhibition studies (1–8 μM), the contribution of the high-K_m mechanism would have been only a few per cent of the total activity.

An examination of the low K_m values of the three acetyl-acceptor substrates at pH 5.0 reveals that, as the size of the substrate increases from mono- through di- to tetra-saccharide, the K_m values decrease (600, 210 and 6.9 μM respectively). This suggests that the enzyme recognizes an extended region of the heparin molecular consisting of at least two or three sugar residues at the non-reducing terminus. In addition, the low K_m value for AcCoA at pH 5.0 (54 μM) is 22-fold higher than the corresponding K_m values at pH 7.0 (2.5 μM), indicating that the binding site for AcCoA is on the cytosolic side of the membrane at a neutral pH.

K_m values reported by Bame and Rome [7] were determined at pH 5.8, and so are not directly comparable with our results; however, the value of 550 μM for AcCoA compares favourably with our values of 304 and 580 μM at pH 7.0 and 5.0 respectively, and would appear to correspond to the high K_m value for this substrate. In contrast, an earlier study by Rome et al. [6] determined that the concentration of AcCoA required for half-maximal incorporation of acetate into heparan sulphate and GlcN in intact lysosomes at pH 7.0 was 1 μM. This is very similar to the low K_m value of 2.5 μM for AcCoA determined from double-reciprocal analysis in this study. The K_m value of GlcN at pH 5.8 reported by Bame and Rome [7] was 300 μM; this value also fits well within the high K_m values of GlcN, determined in the present study to be 289 μM and 3.6 mM at pH 7.0 and 5.0 respectively. The results from those earlier studies appear to have been derived from the high-K_m system using GlcN as the acetyl-acceptor substrate; consequently, the low-K_m mechanism was not detected, and its effect was not considered in the interpretation of the results.

Further evidence for the ternary-complex mechanism comes from the isotope-exchange studies. These studies were performed in the absence of externally added AcCoA, to ensure that any exchange observed could not be interpreted as forward reaction due to an unbalanced equilibrium. The exchange of acetyl groups from GlcNAc to GlcN was shown to be dependent not only on the concentration of GlcNAc, but also on the concentration of CoA (Figure 4), confirming the requirement for a ternary complex in the reaction mechanism. Both products were required in order for exchange to occur. Increasing the concentration of either GlcNAc or CoA resulted initially in an increased rate of exchange, up to approx. 30 mM GlcNAc and 0.1 mM CoA; however, further increases (up to 100 mM for GlcNAc and 32 mM for CoA) resulted in only slightly higher rates of exchange. These results are consistent with a random order of substrate binding. Desulpho-CoA, an analogue of CoA which does not have the thiol group and so cannot be acetylated, was unable to promote isotope exchange between GlcNAc and GlcN, indicating that, in the exchange reaction, the acetyl group is first being transferred to CoA to form AcCoA, which then feeds forward through the reaction to acetylate the labelled GlcN; this precludes any

acetylated enzyme intermediate in the reaction mechanism. The concept of an acetylated enzyme intermediate in this reaction was proposed by Bame and Rome [7], based on isotope-exchange and half-reaction studies. In our studies we have been unable to demonstrate any specific acetylation of lysosomal membranes.

Inhibition studies performed with the dead-end desulpho-CoA also indicate a random order of substrate binding. This inhibitor displays competitive inhibition with respect to AcCoA, and non-competitive inhibition with respect to GlcN (Table 5). This pattern of inhibition is consistent with either a random-order mechanism or a sequential-order mechanism with AcCoA binding before GlcN. However, product-inhibition studies show GlcNAc to be a competitive inhibitor of GlcN, thereby precluding a sequential-order mechanism with AcCoA binding before GlcN. The inhibition observed with desulpho-CoA, then, results from the ability of this inhibitor to bind to both the free enzyme and the enzyme–GlcN complex, indicating a random-order mechanism.

Results from the product-inhibition studies showed GlcNAc to be a non-competitive inhibitor of AcCoA, and CoA to be a non-competitive inhibitor of both GlcN and AcCoA. This is not the inhibition profile characteristic of a random-order ternary-complex mechanism. By themselves, these results would suggest a sequential-order mechanism, with GlcN binding before AcCoA. However, the evidence from both the isotope-exchange and the dead-end-inhibition study with desulpho-CoA indicate a random-order mechanism. The product-inhibition profile for a random-order mechanism involves both products as competitive inhibitors of both substrates; however, the formation of dead-end complexes can result in non-competitive inhibition. The dead-end complexes E–GlcN–CoA and E–AcCoA–GlcNAc result in CoA and GlcNAc being non-competitive inhibitors of GlcN and AcCoA respectively. The non-competitive inhibition of AcCoA with CoA can also be explained by the formation of a dead-end complex, E–AcCoA–CoA. This type of inhibition has been reported for phosphofructokinase [29], where the enzyme can form the dead-end complex E–(fructose 6-phosphate)–(fructose 1,6-bisphosphate).

In our investigations into the reaction mechanism of *N*-acetyltransferase we have use salt-washed membranes as a source of enzyme to enable us to study the enzyme in its membrane environment. We have seen that the acetyl-acceptor substrate used can have a significant effect on the kinetics of the enzyme. However, in such a system no allowance is made for the effects of the lysosomal pH gradient, or of other lysosomal components which may interact with the *N*-acetyltransferase and affect the kinetics of the system. It has been established that a number of lysosomal transport systems are secondarily active, in that they utilize the lysosomal pH gradient to facilitate transport [30,31]; it is also possible that protein conformation may be altered as a result of changes in pH. The degradation of heparan sulphate has been proposed to proceed by a highly organized and coupled process, such that the heparan sulphate-degradative enzymes function as a multienzyme complex built up on the lysosomal membrane [3,4]. Such a complex would involve a range of sulphatases, exo-glycosidases, transporters and the *N*-acetyltransferase. The interaction of the *N*-acetyltransferase with the other lysosomal proteins might be expected to affect both the protein conformation of the enzymes involved and the kinetics of the process. The *N*-acetyltransferase offers a potential regulatory point for this process by providing a dependent link to the cytosolic environment. Regulation of the AcCoA supply to this system has the potential to control the degradation of heparan sulphate within the lysosome, as has been demonstrated with intact lysosomes [5,6]. A more complete understanding of this system will require the purification and cloning of the *N*-acetyltransferase; this will enable the expression of larger amounts of enzyme for more detailed studies and provide the amino acid sequence for modelling of the protein. Work is proceeding in this direction.

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