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Purification and characterization of α(2-6)-sialyltransferase from human liver

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A Galβ1-4GlcNAc α(2-6)-sialyltransferase from human liver was purified 34 340-fold with 18% yield by dye chromatography on Cibacron Blue F3GA and cation exchange FPLC. The enzyme preparation was free of other sialyltransferases. It did not contain CMP-NeuAc hydrolase, protease, or sialidase activity, and was stable at −20°C for at least eight months. The donor substrate specificity was examined with CMP-NeuAc analogues modified at C-5 or C-9 of the *N*-acetylneuraminic acid moiety. Affinity of the human enzyme for parent CMP-NeuAc and each CMP-NeuAc analogue was substantially higher than the corresponding Galβ1-4GlcNAc α(2-6)-sialyltransferase from rat liver.

Keywords: human liver α(2-6)-sialyltransferase, CMP-NeuAc analogues, dye chromatography

Abbreviations: FPLC, fast protein liquid chromatography; NeuAc, 5-*N*-acetyl-D-neuraminic acid; 9-amino-NeuAc, 5-acetamido-9-amino-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulosonic acid; 9-acetamido-NeuAc, 5,9-diacetamido-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulosonic acid; 9-benzamido-NeuAc, 5-acetamido-9-benzamido-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulosonic acid; 9-fluoresceinyl-NeuAc, 9-fluoresceinylthioureido-NeuAc; 5-formyl-Neu, 5-formyl-β-D-neuraminic acid; 5-aminoacetyl-Neu, 5-aminoacetyl-β-D-neuraminic acid; CMP-NeuAc, cytidine-5′-monophospho-*N*-acetylneuraminic acid; G_{M1}, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-ceramide; ST, sialyltransferase; DTE, 1,4-dithioerythritol.

Enzyme: Galβ1-4GlcNAc α(2-6)-sialyltransferase, EC 2.4.99.1.

Sialyltransferases are important enzymes required in the biosynthesis of sialoglycoproteins and sialoglycolipids [1]. They catalyse the transfer of sialic acid from the respective CMP-glycosides to distinct glycan acceptor structures [2–5]. Therefore sialyltransferases are of increasing interest in various fields of biochemistry. Expression of different sialyltransferases in tissues of the same animal may be responsible for the sialoglycoconjugate pattern occurring on the respective cell surfaces [6]. In this context, sialyltransferase activity in transformed cells has been studied intensively [7]. Purified sialyltransferases are useful tools for incorporation of naturally occurring or synthetic sialic acids into cell surface glycoconjugates or into solubilized glycoproteins and glycolipids [8–14]. Sialylation with purified transferases has served to identify sialic acids as the receptor determinant for several viruses [15–17], and to elucidate the role of specific surface sialoglycoconjugates in the stimulation of lymphocytes for tumor cell recognition by immunocompetent cells [18].

Up to the present, sialyltransferases with four different acceptor specificities have been purified from mammalian tissues to near or complete homogeneity [19–27]. This report presents a purification procedure yielding a 34 340-fold enriched, stable Galβ1-4GlcNAc α(2-6)-sialyltransferase from human liver. Acceptor and donor substrate specificity of the human liver enzyme have been extensively analysed. Favourable kinetic data obtained for CMP-activated *N*-acetylneuraminic acid analogues indicates high suitability for resialylation studies with these useful sialic acid analogues.

Materials and methods

Materials

All chemicals were of analytical grade and purchased from Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Sigma (Munich, Germany) or Biomol (Ilvesheim, Germany). Cibacron Blue F3GA-Sepharose 6B was synthesized as described [27], and Affi-Gel Blue obtained from Bio-Rad (Munich, Germany). α_1-Acid glycoprotein and Zn-α_2-glycoprotein were generously donated by Dr Karl Schmid (Boston, USA). Anti-freeze glycoprotein (Fraction 3-5, from serum of *Pagothenia borchgrevinki*) was supplied by Dr Robert E. Feeney (Davis,

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USA). Antithrombin III was kindly donated by Behring Werke (Marburg, Germany). Ganglioside G_{M1} was from Dr Pallmann KG (Munich, Germany) and Fetuin from Sigma (Munich, Germany). CMP-NeuAc and its analogues were prepared as described previously [11, 12]. They contained less than 4% free CMP; in the case of CMP-9-amino-NeuAc, less than 2%. Rat liver Galβ1-4GlcNAc α(2-6)-sialyltransferase (1.7 units per mg) was purified using dye chromatography [27]. CMP-[^{3}H]NeuAc (11.5 Ci mol^{-1}) was obtained from New England Nuclear and Quickszint 212 for liquid scintillation counting from Zinsser (Frankfurt, Germany).

Sialyltransferase assay

The standard incubation assay for determination of sialyltransferase activity contained 50 µg of bovine serum albumin, enzyme (0–0.4 milliunit) and different acceptor glycoproteins in 50 mM sodium cacodylate, pH 6.5, with 0.1% Triton X-100 in a total volume of 100 µl [27]. The enzymatic reaction was initiated by addition of 10 nmol of CMP-[^{3}H]NeuAc (7000 cpm nmol^{-1}). The following acceptor substrates were used: asialofetuin containing 200 nmol of galactose sites mg^{-1}, asialo-α_1-acid glycoprotein (430 nmol), asialo-antithrombin III (110 nmol), asialo-Zn-α_2-glycoprotein (135 nmol), antifreeze glycoprotein (1400 nmol), and ganglioside G_{M1} (1022 nmol). The assay was carried out as described previously [27]. Asialofetuin was used to monitor sialyltransferase activity during the purification procedure. Asialo-α_1-acid glycoprotein served to analyse enzyme activity specific for the terminal sequence Galβ1-4GlcNAc of oligosaccharide chains; antifreeze glycoprotein and G_{M1} were the acceptor substrates for sialyltransferases specific for Galβ1-3GalNAc glycans. One unit of activity is defined as the amount of enzyme catalysing the formation of 1 µmol of product min^{-1} under standard assay conditions.

The effect of a number of different compounds on enzyme activity was examined using standard assay conditions, in the presence of $MgCl_2$, $MnCl_2$, $ZnCl_2$, EDTA, and DTE at concentrations varying from 0.4 to 15 mM; in the case of NaCl, from 0.025 M to 0.7 M. Concentrations of 10 µM and 100 µM were used to estimate the effect of $HgCl_2$ and $CuSO_4$ on sialyltransferase activity.

The pH optimum of the purified Galβ1-4GlcNAc α(2-6)-sialyltransferase activity was determined in the standard assay with (150 mM) and without NaCl by varying the buffer pH over the range of 5.0 to 8.0.

Assays for kinetic measurements were performed in duplicate using 0.25 milliunits of enzyme preparation. CMP-NeuAc concentration varied over 6.25 to 200 µM, while asialo-α_1-acid glycoprotein was fixed at 0.7 mM in terms of galactose sites. Kinetic data for the various glycoprotein acceptors were obtained at five different concentrations of galactose sites, with CMP-NeuAc concentration at the K_M value (200 µM). In each case, consumption of the limiting substrate was kept below 12%. All assays were performed over the linear time course of reaction rate by restricting the consumption of donor and acceptor substrates to less than 15%.

For calculation of specific activity and purification level, enzyme activity determined in the standard assay was multiplied by a calculated factor of 1.8 to reflect maximal velocity obtainable at saturating concentrations of CMP-NeuAc (0.2 mM) and asialo-α_1-acid glycoprotein (1.0 mM). Kinetic parameters were calculated from Hanes plots by linear regression analysis [28].

Protein determination

Protein content was analysed by the amidoblack dye binding technique [29].

Desialylation of glycoproteins

After treatment with *Vibrio cholerae* sialidase as described [30], asialo-α_1-acid glycoprotein, asialofetuin, asialo-Zn-α_2-glycoprotein and asialo-antithrombin III contained approximately 0.2% bound *N*-acetylneuraminic acid.

Galactose acceptor sites

Sites were expressed in terms of galactose content of asialo-α_1-acid glycoprotein, asialo-Zn-α_2-glycoprotein, antifreeze glycoprotein, asialofetuin, asialo-antithrombin III, and the sialoglycolipid G_{M1}, and determined after acid hydrolysis (1 N HCl, 100°C, 2, 4 and 6 h) using the galactose dehydrogenase assay [31].

Buffers

The following buffers were used: A, 25 mM sodium cacodylate, pH 6.0, 20 mM $MnCl_2$; B, 10 mM sodium cacodylate, pH 6.0, 5 mM $MgCl_2$; C, 10 mM sodium cacodylate, pH 6.0, 50 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100; D, 10 mM sodium cacodylate, pH 6.0, 130 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100; E, 10 mM sodium cacodylate, pH 5.4, 50 mM NaCl, 5 mM $MgCl_2$, 15% glycerol, 1% Triton X-100; F, 10 mM sodium cacodylate, pH 6.0, 100 mM NaCl, 5 mM $MgCl_2$, 15% glycerol, 1% Triton X-100.

Purification of α(2-6)-sialyltransferase from human liver

Adult human liver which appeared normal on a gross pathological examination was obtained at autopsy and stored at −80°C. All operations during the purification procedure were performed at 4°C.

Triton extraction. Human liver (120 g) was homogenized in buffer A (1:5, w/v) using a Waring Blender (three 10 s bursts on "low" separated by 1 min rest intervals). Before Triton extraction of the human liver sialyltransferase, soluble proteins and blood components were removed by washing the crude liver membrane fraction [20] and collecting the crude homogenate by centrifugation at 18000 × **g** for 1 h. After removal of the supernatant the pellet was homogenized again in buffer A and recentrifuged. The washed pellet was

suspended in 400 ml of buffer B by rehomogenization in the blender (three 10 s bursts with 30 s rest intervals). The resulting suspension was brought to 1% (w/v) Triton X-100. After stirring for 1 h at 4°C the crude extract was centrifuged for 1 h at 14000 × **g**, and the resulting supernatant filtered through glass wool.

Cibacron Blue F3GA-Sepharose 6B (NaCl gradient). The Triton X-100 extract was applied to a column (13 cm × 2.5 cm) of immobilized Cibacron Blue F3GA (4.3 μmol ml^{-1}) equilibrated with buffer C. The column was washed extensively with buffer C (1 litre) to remove inert protein. Sialyltransferase was eluted using a linear NaCl gradient with 120 ml of buffer C as starting buffer and 120 ml of buffer C/2.5 M NaCl as limit buffer. Fractions containing sialyltransferase activity (>8 milliunits ml^{-1}) were pooled and dialysed against buffer C.

Affi-Gel Blue (CDP gradient). The dialysed eluate (70 ml) was applied to a column (10 cm × 1.6 cm) of Affi-Gel Blue pre-equilibrated with buffer C. The column was washed with buffer D (200 ml) and eluted by a linear CDP gradient with 40 ml of buffer D as starting buffer and 40 ml of buffer D containing 10 mM CDP as limit buffer. Active fractions (activity >4 milliunits ml^{-1}) were pooled (21 ml) and dialysed against buffer C.

FPLC on Mono S column (NaCl gradient). The dialysate was adjusted to pH 5.4 with 1 N HCl and applied to a Mono S (Pharmacia, Freiburg, Germany) FPLC column (2 cm × 0.5 cm) equilibrated with buffer E (flow rate 0.2 ml min^{-1}, 0.5 MPa). After washing with buffer F, the column was eluted with a linear NaCl gradient (5 ml of buffer F as starting buffer and 5 ml of buffer F containing 1.0 M NaCl as limit buffer (flow rate 0.1 ml min^{-1}) using a Pharmacia FPLC system. Active fractions (>15 milliunits ml^{-1}) were pooled (3 ml) and dialysed against buffer F.

Concentration of sialyltransferase activity. Active fractions from step 4 were concentrated in the dialysis tube with dry Sephadex G-100 (Pharmacia, Freiburg, Germany). After several changes of Sephadex G-100 the dialysed pool was concentrated approximately six-fold. After concentration, the enzyme preparation was brought to a level of 50% glycerol and stored at −20°C.

SDS/polyacrylamide gel electrophoresis

This was performed by the method of Laemmli [32], and the gel was stained with Coomassie Blue R-50.

Colorimetric transfer assay

Assay for transfer of NeuAc analogues was performed as described previously [13]. The reaction mixture (160 μl) contained 10 μmol sodium cacodylate buffer pH 6.5, 160 μg BSA, 0.16 mg Triton X-100, 0.25 mg asialo-α_1-acid glycoprotein (104 nmol galactose acceptor sites), and different concentrations of CMP-glycosides (20 μM–1 mM). The concentration of asialo-α_1-acid glycoprotein in terms of galactose acceptor sites was two-fold higher than the apparent K_M value of rat and human liver ST (see Results). Transfer reaction was started by addition of human or rat liver Galβ1-4GlcNAc α(2-6)-sialyltransferase, and the assays were processed further as described earlier [11, 13]. Transferred *N*-acetylneuraminic acid or its analogues were released from the glycoprotein by acid hydrolysis [11, 13] and quantified by the thiobarbituric acid procedure [33]. The extent of degradation during acid hydrolysis was as follows: NeuAc, 10%; 9-amino-NeuAc, 10%; 9-benzamido-NeuAc, 15%; 9-acetamido-NeuAc, 20%; 5-formyl-Neu, 50%; and 5-aminoacetyl-Neu, 10%.

For kinetic measurements, initial rates were determined in duplicate at five different concentrations of the respective CMP-glycoside near the K_M value. To ensure linearity of transfer rates with incubation time, consumption of the donor substrates was restricted to below 20%, and in the case of CMP-9-amino-NeuAc, below 7%. Furthermore, the consumption of the acceptor substrate was always below 10%. Kinetic data were obtained from Hanes plots [28].

Fluorometric transfer assay

Kinetic data of rat liver and human liver Galβ1-4GlcNAc α(2-6)-sialyltransferase for CMP-9-fluoresceinyl-NeuAc were determined by the fluorometric assay system as described [14], with asialo-α_1-acid glycoprotein as acceptor substrate.

Results

Purification of α(2-6)-sialyltransferase from human liver was 34340-fold with good yield (18%) as summarized in Table 1.

Triton extraction

Extraction of sialyltransferase required 1% (w/v) Triton X-100, but higher concentrations of detergent (up to 2%) did not improve the yield. Solubilization was virtually complete after one Triton extraction.

Cibacron Blue F3GA-Sepharose 6B (NaCl gradient)

Sialyltransferase was partially purified and concentrated from 425 ml of Triton extract to 70 ml by adsorption on Cibacron Blue F3GA-Sepharose 6B (4.3 μmol ml^{-1}) and subsequent elution with a linear NaCl gradient (Fig. 1). The overall yield in this step was 82% with an 8.3-fold purification. Neither protease nor sialidase activity could be detected in the pooled fractions.

Affi-Gel Blue (CDP gradient)

After rechromatography on Affi-Gel Blue, purification was considerably enhanced by specific elution with CDP; to improve yields, ionic strength of the buffer system was increased (130 mM NaCl). Sialyltransferase (82%) was eluted

Table 1. Purification of α(2-6)-sialyltransferase from human liver.
Results are shown for an enzyme preparation obtained from 120 g of human liver. Details are described in the Materials and methods section. Activities have been multiplied by a factor of 1.8 to reflect maximal velocities under saturating conditions.

	Volume (ml)	*Protein* (mg)	*Activity* (munits)	*Yield* (%)	*Specific activity* (munits mg^{-1})	*Purification*
Triton extract	425	8214	1124	100	0.137	1
Cibacron Blue F3GA-Sepharose (NaCl gradient)	70	799	899	82	1.13	8.3
Affi-Gel Blue (CDP gradient)	21	17.8	226[a]	20[a]	12.7[a]	92.7[a]
Mono S FPLC column (NaCl gradient)	0.6	0.044	207	18	4705	34 340

[a] Activity measured in presence of contaminating CDP.

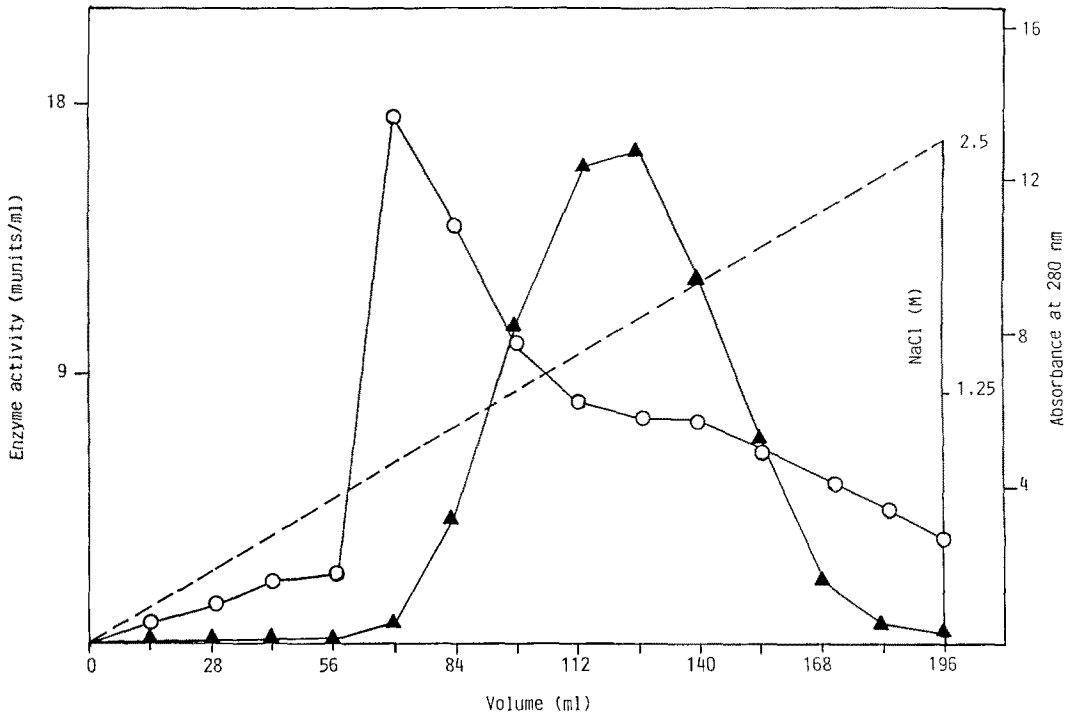

Figure 1. NaCl gradient elution of α(2-6)-sialyltransferase from Cibacron Blue F3GA-Sepharose 6B. Chromatography of the Triton extract was performed as described in the Materials and methods section. (– – –), NaCl gradient; (▲), ST activity; (○), protein concentration.

ahead of the inert protein peak (Fig. 2). The remainder of the enzyme activity (18%) eluted with a 2.5 M NaCl pulse together with the bulk of protein. Apparently human liver sialyltransferase binds to Affi-Gel Blue with higher affinity than rat liver ST [27], since the former ST required a higher concentration of NaCl (130 mM) in buffer D to promote elution with CDP.

Mono S FPLC column (NaCl gradient)

Enzyme activity obtained was completely adsorbed to the Mono S column at pH 5.4. Developing the column with buffer F removed inert protein and, during the subsequent NaCl gradient, sialyltransferase activity eluted as a sharp peak in front of the protein (Fig. 3).

Final enzyme preparation

Dry Sephadex G-100 served to concentrate the final enzyme preparation from 2 ml to 0.3 ml in only 1 h without loss of activity. The enzyme was stored at −20°C in 50% glycerol, maintaining full activity for at least eight months. Furthermore the purified sialyltransferase was highly stable and maintained more than 90% activity in the standard assay at 37°C for at least 4 h.

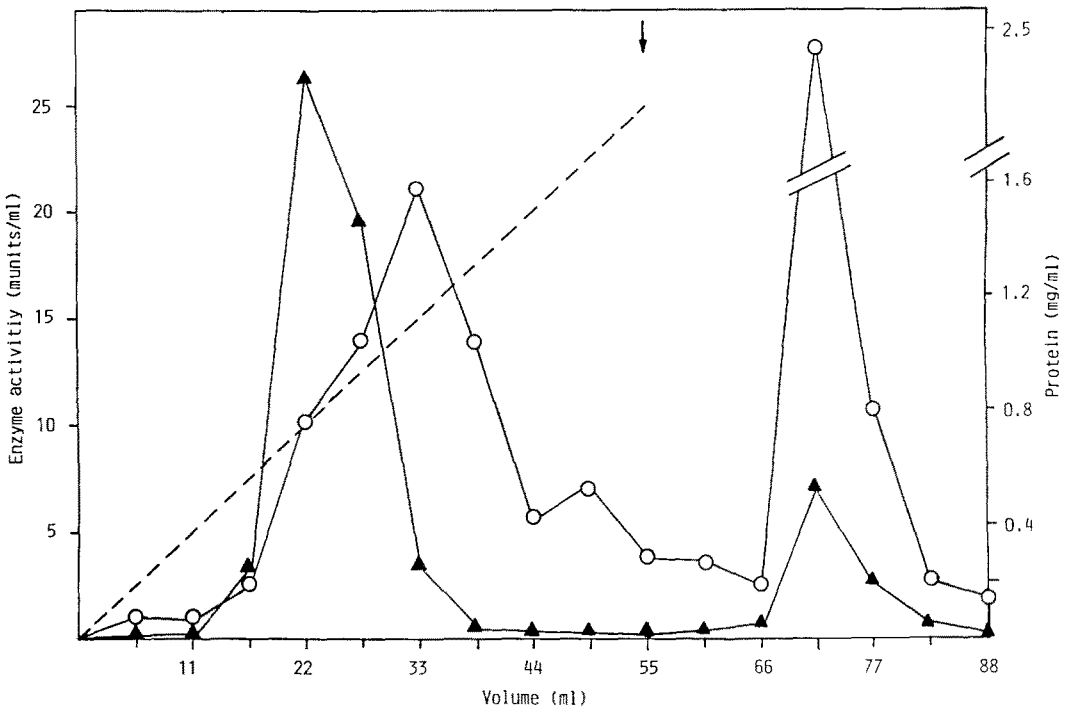

Figure 2. CDP gradient elution of α(2-6)-sialyltransferase from Affi-Gel Blue. Dialysed enzyme from Cibacron Blue chromatography was rechromatographed as described in the Materials and methods section. Elution was achieved with a linear CDP gradient (– – –) up to 10 mM, followed by elution with a 2.5 M NaCl pulse. The end of the CDP gradient (10 mM) and the start of the NaCl pulse is indicated by an arrow. Fractions were assayed for sialyltransferase activity (▲) and protein (○). Protein content in the fractions was determined using the Bio-Rad protein assay (Bio-Rad, Munich).

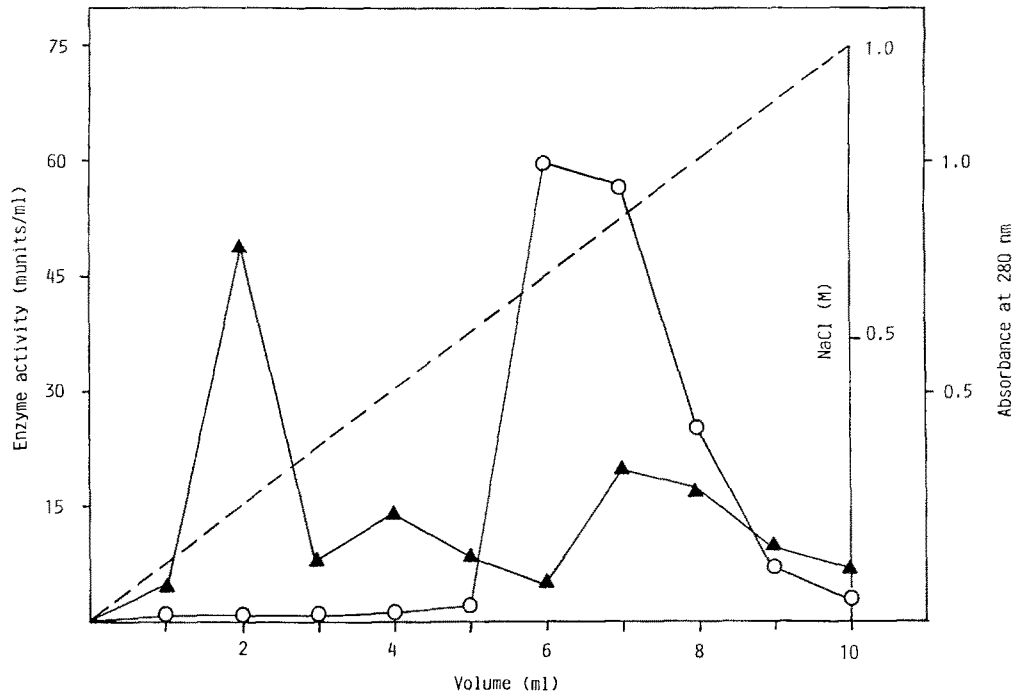

Figure 3. NaCl gradient elution of α(2-6)-sialyltransferase from Mono S. The enzyme from step 3 was applied as described in the Materials and methods section. Following adsorption the column was eluted with a linear NaCl gradient up to 1.0 M (– – –). (▲), sialyltransferase activity; (○), protein concentration..

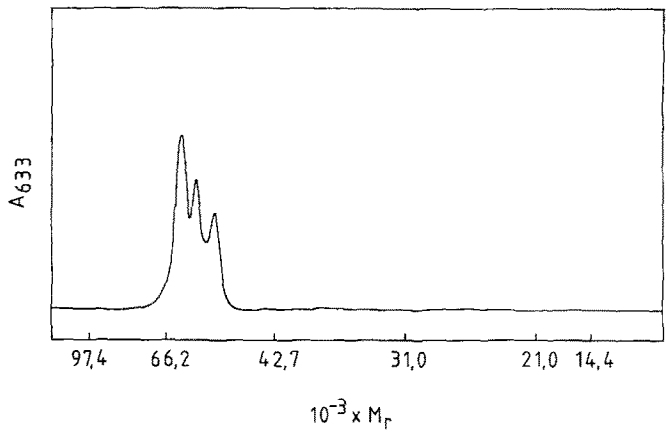

Figure 4. Scan of the purified α(2-6)-sialyltransferase from human liver. SDS/polyacrylamide-gel electrophoresis was performed under denaturing conditions. After staining the gel with Coomassie Blue R-50, it was scanned at 633 nm. The M_r values of standard proteins, electrophoresed under the same conditions, are as follows: phosphorylase b, 97 400; bovine serum albumin, 66 200; ovalbumin, 42 700; carbonic anhydrase, 21 000; lysozyme, 14 400.

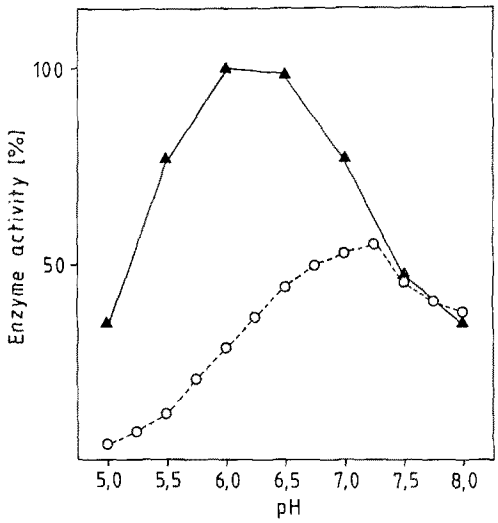

Figure 5. pH Optimum curve for the purified Galβ1-4GlcNAc α(2-6)-sialyltransferase from human liver in presence (150 mM) and in absence of NaCl. The standard assay was performed with asialo-α_1-acid glycoprotein as described in the Materials and methods section. (○), with 150 mM NaCl; (▲), without NaCl.

Electrophoresis of the purified enzyme

Polyacrylamide gel electrophoresis of the purified transferase under denaturing conditions showed three bands (Fig. 4) with approximate molecular weights of 53 000, 57 000, and 61 500 as determined from their relative migrations. Whether this is due to some proteolytic degradation during the extraction procedure is not yet known.

Absence of contaminating enzyme activities

The purified Galβ1-4GlcNAc α(2-6)-sialyltransferase from human liver was free of contaminating sialidase, protease and CMP-NeuAc hydrolase activity which was determined as described previously [27]. No contamination of sialyltransferases acting on O-linked glycans could be detected, as incorporation of [^{3}H]NeuAc into antifreeze glycoprotein or G_{M1}, both of which contain the acceptor sequence Galβ1-3GalNAc [34, 35], was below 1% relative to the value obtained with asialo-α_1-acid glycoprotein.

Specificity of the purified enzyme

Sialyltransferase assay with lactose as substrate, described previously [27], yielded only the α(2-6)-sialyllactose isomer [α(2-3)-sialyllactose isomer was below 0.5%]. This result allows identification of the purified human liver enzyme as an α(2-6)-sialyltransferase. As demonstrated above, this enzyme acts on the glycoprotein terminal acceptor sequence Galβ1-4GlcNAc.

pH Dependent activity of α(2-6)-sialyltransferase at physiological ionic strength

Figure 5 shows the dependence of activity of the purified human Galβ1-4GlcNAc α(2-6)-sialyltransferase on the buffer pH; the optimum enzyme activity was between pH 6.0 and 6.5 using standard assay conditions at low ionic strength without NaCl. This was different from the pH optimum (pH 7.0) determined for a human liver α(2-6)-ST in crude homogenate [36].

With increased ionic strength (150 mM NaCl), the pH optimum of human liver ST was significantly shifted to the more physiological pH value of 7.25, and the reaction rate was only 42% of that determined at the pH optimum of 6.5 at low ionic strength. This result differed from the data obtained with the rat liver ST which displayed a shift in the pH optimum in the presence of 150 mM NaCl only from 6.0 to 6.5; but similarly to the human enzyme the latter ST was about 50% less active at physiological ionic strength (data not shown).

Effects of different compounds on sialyltransferase activity

Presence of $MgCl_2$ stimulated sialyltransferase by approximately 20% over a concentration range of 3 to 10 mM, which was in accordance with the value measured for a crude transferase preparation [37]. Only a slight activation (about 10%) was obtained by addition of $MnCl_2$ in the range of 0.4 to 5 mM, while $CaCl_2$ had no effect on sialyltransferase activity up to a concentration of 10 mM.

$ZnCl_2$, DTE and EDTA had no effect on enzyme activity at a 1 mM concentration, but higher amounts (5 mM) caused sialyltransferase inhibition (20%). A strong inhibitory effect on enzyme activity was measured at a 100 μM concentration of $HgCl_2$ (82% inhibition) and $CuSO_4$ (40% inhibition). High ionic strength by addition of NaCl resulted in substantial lowering of activity. Activity of Galβ1-4GlcNAc α(2-6)-sialyltransferase was inhibited 35% at a concentration of 0.1 M, 81% at 0.5 M, and 89% at 0.7 M NaCl.

Table 2. Apparent kinetic parameters of human liver and rat liver Galβ1-4GlcNAc α(2-6)-sialyltransferase for different glycoprotein acceptors with CMP-[^{3}H]NeuAc as donor substrate. Radiometric assay was performed as described in the Materials and methods section with 0.05 milliunits human and 0.09 milliunits rat liver enzyme, respectively. The K_M values are given in terms of the concentration of terminal galactose sites.

Acceptor glycoprotein	*Sialyl-transferase*	K_M (μM)	V_{max} (*rel. 3%*)	V_{max}/K_M (1/mM)
Asialo-α_1-acid	Rat liver	340	100	2.95
glycoprotein	Human liver	380	100	2.65
Asialofetuin	Rat liver	235[a]	115	4.9
	Human liver	113[a]	103	8.85
Asialo-	Rat liver	160	115	7.2
antithrombin III	Human liver	63	80	12.7
Asialo-Zn-α_2	Rat liver	140	80	5.7
glycoprotein	Human liver	230	122	5.3

[a] Given in terms of the concentration of terminal galactose on N-linked glycans.

Kinetic properties of the purified sialyltransferase

The K_M value determined for CMP-NeuAc with asialo-α_1-acid glycoprotein as acceptor was 15 μM, which was significantly lower than that obtained for Galβ1-4GlcNAc α(2-6)-sialyltransferase from rat liver (K_M = 50 μM [13, 27]). The following glycoproteins with different antennae structures in their N-linked glycans were employed to measure kinetic data of the human enzyme for the acceptor substrate (Table 2): asialo-α_1-acid glycoprotein (mixture of bi-, tri- and tetra- antennary structures) [38], asialofetuin (only tri-antennary glycans) [39], asialo-antithrombin III and asialo-Zn-α_2-glycoprotein (only bi-antennary complex type structures [40, 41]. The highest affinity was obtained for the bi-antennary glycan of antithrombin III. For comparison, Table 2 shows the kinetic acceptor data of the corresponding rat liver ST, which were significantly different, except in the case of asialo-α_1-acid glycoprotein.

Enzymatic transfer of N-acetylneuraminic acid

In order to study the donor substrate specificity of α(2-6)-ST isolated from human liver, kinetic properties for several CMP-NeuAc analogues modified at position C-9 or C-5 of the sialic acid moiety were determined (structures shown in Fig. 6). Initial rates were measured at 0.2 mM and 0.5 mM donor concentration to approximate saturating conditions (Table 3). Transfer rates measured with each CMP-NeuAc analogue at 0.2 mM ranged from 0.5-fold to 1.5-fold the value obtained with parent *N*-acetylneuraminic acid. Increasing the concentration of donor to 0.5 mM did not significantly enhance transfer except in the case of CMP-9-amino-NeuAc (Table 3); the latter analogue required higher concentration (1 mM) to obtain the maximal incorporation value, a result which was in accordance with its high K_M value (see below).

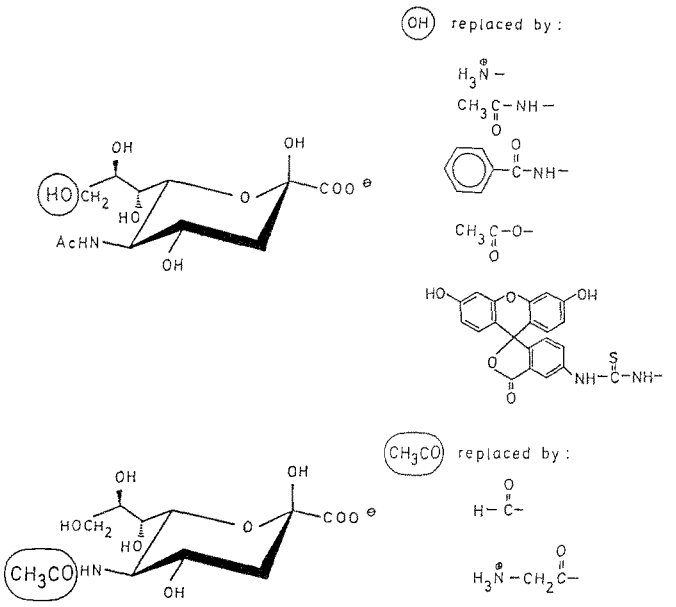

Figure 6. Structures of *N*-acetylneuraminic acid analogues modified at C-9 or C-5. The hydroxy group at C-9 was replaced by an amino-, acetamido-, benzamido-, *O*-acetyl-, or fluoresceinylthio-ureido-group; the acetyl group at C-5, by a formyl- or aminoacetyl-group.

The transfer values obtained at 0.2 mM CMP-glycoside were up to 2.4-fold higher than the values determined for the sialyltransferase from rat liver (Table 3). For optimum transfer rate the latter enzyme required a concentration above 0.5 mM CMP-NeuAc analogue: in the case of 9-amino-NeuAc, as high as 5 mM donor [13]. The initial rates determined with rat liver ST in presence of 0.1% Triton X-100 at pH 6.5 (Table 3) were in accordance with those estimated previously at pH 6.0 using 0.5% Triton CF-54 [13].

The kinetic parameters of the human liver ST obtained for CMP-9-amino-NeuAc, CMP-9-acetamido-NeuAc, CMP-9-fluoresceinyl-NeuAc and CMP-9-benzamido-NeuAc are shown in Table 4. For comparison, the corresponding data for the rat liver enzyme are included. The K_M values of the human enzyme were lower in each case; the relative V_{max} values are almost identical for both enzymes.

α-Glycosidically linked 9-amino-NeuAc is resistant to sialidases [30]. It was, therefore, of interest to compare the time course of incorporation of 9-amino-NeuAc and parent *N*-acetylneuraminic acid at 100 μM concentration of the respective CMP-glycosides. Transfer of *N*-acetylneuraminic acid to asialo-α_1-acid glycoprotein was linear with time for 60 min, representing 19% donor substrate consumption; transfer of 9-amino-NeuAc was linear with time for 50 min, representing only 5% donor substrate consumption. Thus, incorporation of 9-amino-NeuAc by the human ST proceeds linearly with time only over low donor substrate consumption. The incorporation rate at 100 μM CMP-NeuAc or CMP-9-amino-NeuAc was determined in the presence and

Table 3. Relative initial transfer rates of human liver and rat liver Galβ1-4GlcNAc α(2-6)-sialyltransferase for several CMP-NeuAc analogues. Colorimetric assay was performed with asialo-α_1-acid glycoprotein in duplicate at 0.2, 0.5 or 1.0 mM concentrations of the respective CMP-glycosides for 30 min with 0.35 milliunits human liver or 0.3 milliunits rat liver ST. Initial transfer rates are expressed as an average percentage with respect to the value obtained at 1.0 mM CMP-NeuAc (100% = 0.12 nmol min^{-1} for rat liver ST and 0.14 nmol min^{-1} for human liver ST; standard deviation was within a range of $\pm$ 10% of the values listed).

Donor substrate	*Initial rates (%)*					
	Human liver ST			*Rat liver ST*		
	0.2	*0.5*	*1.0*	*0.2*	*0.5*	*1.0*
	(mM *donor concentration*)					
CMP-NeuAc	99	100	100	82	92	100
CMP-9-amino-NeuAc	50	63	73	21	35	49
CMP-9-acetamido-NeuAc	103	110	–	73	96	97
CMP-9-benzamido-NeuAc	138	143	–	112	125	–
CMP-5-formyl-Neu	101	113	–	81	114	115
CMP-5-aminoacetyl-Neu	71	65	–	40	48	51
CMP-9-*O*-acetyl-NeuAc	102	115	–	95	112	–

in the absence of 10 μM CMP: Whereas *N*-acetylneuraminic acid transfer was not markedly influenced (8% reduction), incorporation of 9-amino-NeuAc was inhibited by 30%. Thus the limited linearity of product formation with time was obviously due to a marked inhibition of 9-amino-NeuAc incorporation by CMP, which is a known sialyltransferase inhibitor [42]. A similar result was described for the transfer of this analogue by the rat liver α(2-6)-ST [13].

Discussion

In vitro transfer of naturally occurring sialic acids with highly purified sialyltransferases has been a useful approach to study important biological functions of sialic acids. In this laboratory, purified sialyltransferases were applied to replace natural sialic acids either on the surface of cells or on soluble glycoproteins by synthetic analogues endowed with special properties [12, 13, 30]. This substitution may serve to influence certain biological functions of sialoglycoconjugates, or to introduce a radio-, photoaffinity- or fluorescence label.

In this study we describe an easy dye affinity purification procedure for human liver sialyltransferase avoiding the laborious synthesis of CDP-hexanolamine-Sepharose which was previously used to purify this enzyme [22]. In contrast with the very labile enzyme preparation obtained employing the affinity ligand CDP-hexanolamine, the sialyltransferase purified by dye chromatography was highly stable at both 4°C and 37°C. The final enzyme preparation was completely free of other sialyltransferases and of contaminating enzymes such as glycohydrolases which might interfere during re-sialylation studies.

The specificity of the human liver sialyltransferase for the acceptor sequence Galβ1-4GlcNAc on complex glycans was similar to the rat liver Galβ1-4GlcNAc α(2-6)-sialyltransferase. The pH optimum dependency of the human liver ST on ionic strength offers an advantage for application of sialyltransferases to physiological systems (cells, cell homogenates). Whereas activity of the rat liver enzyme at pH 7.0–7.5 in presence of 150 mM NaCl drops to about 26–32% of the activity obtained at the pH optimum of pH 6.0 at low ionic strength without added NaCl, the human liver enzyme maintains 50% activity at pH 7.0–7.5/150 mM NaCl due to a shift of the pH optimum.

Kinetic data for the human and the rat liver enzyme for several acceptor substrates differed depending on the glycoprotein without a correlation to the glycan antennae types (Table 2); the high affinity of the human enzyme for antithrombin III is noteworthy.

Synthetic sialic acid analogues were employed previously

Table 4. Apparent kinetic data of human liver Galβ1-4GlcNAc α(2-6)-sialyltransferase for several CMP-NeuAc analogues. Kinetic measurements were performed at five concentrations of CMP-glycoside using the colorimetric assay or the fluorometric assay as described in the Materials and methods section. V_{max} values are expressed relative to that determined for CMP-NeuAc (= 1.0). The kinetic data determined previously [13, 14] for rat liver α(2-6)-ST (assay performed at optimum pH of 6.0) are given for comparison.

Donor substrate	*Human liver ST*			*Rat liver ST*		
	K_M (μM)	V_{max} (*rel.*)	V_{max}/K_M (1/mM)	K_M (μM)	V_{max} (*rel.*)	V_{max}/K_M (1/mM)
CMP-NeuAc	20	1.0	50	50	1.0	20
CMP-9-amino-NeuAc	150	0.7	5	720	0.9	1.3
CMP-9-acetamido-NeuAc	84	1.1	13	120	1.1	9.2
CMP-9-benzamido-NeuAc	10	1.3	100	30	1.1	36.7
CMP-9-fluoresceinyl-NeuAc	2	0.8	400	7	1.0	142

in this laboratory to study the donor substrate specificity of purified sialyltransferases. Data on the kinetic properties of each enzyme for the transfer of differently substituted *N*-acetylneuraminic acid analogues allow a choice of suitable analogues as well as the optimal sialyltransferase for *in vitro* resialylation. To achieve transfer rates comparable to the rat liver α(2-6)-sialyltransferase, the corresponding human enzyme required lower concentrations of each CMP-NeuAc analogue. The higher donor substrate affinity of the human sialyltransferase in general, as considered from the initial rates (Table 3) or the kinetic parameters (Table 4), implies that this enzyme is superior to the rat liver α(2-6)-sialyltransferase commonly used for *in vitro* sialylation.

Previous experiments on the transfer of zwitterionic 9-amino-NeuAc, which, up to now, is the only sialic acid analogue with an unaltered carbon chain that resists cleavage by either bacterial, viral or mammalian sialidases, showed that of four different sialyltransferases only the rat liver Galβ1-4GlcNAc α(2-6)-sialyltransferase accepted this *N*-acetylneuraminic acid analogue [13]. The human liver sialyltransferase described here represents an additional enzyme which allows resialylation studies using CMP-9-amino-NeuAc. Moreover, based on the higher affinity (five-fold lower K_M) the human enzyme is more convenient than the rat liver sialyltransferase as it allows lower concentrations of CMP-9-amino-NeuAc in the resialylation assay.

A low specificity for position C-9 or C-5 of the *N*-acetylneuraminic acid moiety of the donor CMP-glycoside is a common feature of both the human and the rat liver α(2-6)-sialyltransferase. With high probability, sialyltransferases with identical acceptor and, especially, identical linkage specificity conserve a similar binding site structure for the donor CMP-glycoside.

The purification procedure elaborated in this study involves only three chromatographic steps, which can easily be carried out within five days, affording a highly purified α(2-6)-sialyltransferase in good yield. The simple and economical synthesis of Cibacron Blue F3GA-Sepharose conveniently allows scaling up of the purification procedure for multiple biologically important resialylation studies.

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References

1. Basu M, De T, Das KK, Kyle JW, Chon HC, Schaeper RJ, Basu S (1987) *Methods Enzymol* **138**:575–607.
2. Corfield AP, Schauer R (1982) In *Sialic Acids* (Schauer R, ed.), Cell Biology Monographs, Vol 10, pp 195–261. Vienna: Springer-Verlag.
3. Schachter H, Narasimhan S, Gleeson P, Vella G (1983) *Methods Enzymol* **98**:98–134.
4. Durrie R, Saito M, Rosenberg A (1988) *Biochemistry* **27**:3759–64.
5. Durrie R, Rosenberg A (1989) *J Lipid Res* **30**:1259–66.
6. Paulson JC, Weinstein J, Schauer A (1989) *J Biol Chem* **264**:10931–4.
7. Yogeeswaran G, Salk PL (1981) *Science* **212**:1514–16.
8. Higa HH, Paulson JC (1985) *J Biol Chem* **260**:8838–49.
9. Kelm S, Shukla AK, Paulson JC, Schauer R (1986) *Carbohydr Res* **149**:59–64.
10. Gross HJ, Brossmer R (1987) *Glycoconjugate J* **4**:145–56.
11. Gross HJ, Bünsch A, Paulson JC, Brossmer R (1987) *Eur J Biochem* **168**:595–602.
12. Gross HJ, Brossmer R (1988) *Eur J Biochem* **177**:583–9.
13. Gross HJ, Rose U, Krause JM, Paulson JC, Schmid K, Feeney RE, Brossmer R (1989) *Biochemistry* **28**:7386–92.
14. Gross HJ, Sticher U, Brossmer R (1990) *Anal Biochem* **186**:127–34.
15. Markwell MAK, Paulson JC (1980) *Proc Natl Acad Sci USA* **77**:5693–7.
16. Paulson JC, Rogers GN, Caroll SM, Higa HH, Pritchet T, Milks G, Sabesan S (1984) *Pure Appl Chem* **56**:797–805.
17. Rogers GN, Herrler G, Paulson JC, Klenk HD (1986) *J Biol Chem* **261**:5947–51.
18. Powell LD, Whiteheart SW, Hart GW (1987) *J Immunol* **139**:262–70.
19. Paulson JC, Beranek WE, Hill RL (1977) *J Biol Chem* **252**:2356–62.
20. Sadler JE, Rearick JI, Paulson JC, Hill RL (1979) *J Biol Chem* **254**:4434–44.
21. Sadler JE, Rearick JI, Paulson JC, Hill RL (1979) *J Biol Chem* **254**:5934–41.
22. Alhadeff JA, Holzinger RT (1982) *J Biochem Biophys Meth* **6**:229–33.
23. Miyagi T, Tsuiki S (1982) *Eur J Biochem* **126**:253–61.
24. Weinstein J, de Souza-e-Silva U, Paulson JC (1982) *J Biol Chem* **257**:13835–44.
25. Hesford FJ, Berger EG, Van Halbeek H (1984) *Glycoconjugate J* **1**:141–53.
26. Joziasse DH, Bergh MLE, ter Hart HGJ, Koppen PL, Hooghwinkel GJM, Van den Eijnden, DH (1985) *J Biol Chem* **260**:4941–51.
27. Sticher U, Gross HJ, Brossmer R (1988) *Biochem J* **253**:577–80.
28. Dixon M, Webb EC (1964) In *Enzymes*, 3rd edition, pp 55–138. London: Longmans Green and Co.
29. Schaffner W, Weissmann C (1973) *Anal Biochem* **56**:502–14.
30. Gross HJ, Brossmer R (1988) *Glycoconjugate J* **5**:411–17.
31. Wallenfels K, Kurz G (1966) *Methods Enzymol* **9**:112–16.
32. Laemmli UK (1970) *Nature* **227**:680–5.
33. Warren L (1959) *J Biol Chem* **234**:1971–5.
34. Feeney RE, Burcham TS, Yeh Y (1986) *Ann Rev Biophys Biophys Chem* **15**:59–78.
35. Ledeen RW, Yu RK (1982) *Methods Enzymol* **83**:139–91.
36. Alhadeff JA, Holzinger RT (1979) *Biochim Biophys Acta* **570**:56–64.

37. Alhadeff JA, Cimino G, Janowsky A, O'Brien JS (1977) *Biochim Biophys Acta* **484**:307–21.
38. Fournet B, Montreuil J, Strecker G, Dorland L, Haverkamp J, Vliegenthart JFG, Binette JP, Schmid K (1978) *Biochemistry* **17**:5206–14.
39. Takasaki S, Kobata A (1986) *Biochemistry* **25**:5709–15.
40. Franzen LE, Svensson S (1980) *J Biol Chem* **255**:5090–3.
41. Araki T, Geiyo F, Takagaki K, Haupt H, Schwick HG, Bürgi W, Marti T, Schaller J, Rickli E, Brossmer R, Atkinson PH, Putnam FW, Schmid K (1988) *Proc Natl Acad Sci USA* **85**:679–83.
42. Beyer TA, Sadler JE, Rearick JI, Paulson JC, Hill RL (1981) *Adv Enzymol Relat Areas Mol Biol* **52**:23–175.