Galactosyltransferase-dependent sialylation of complex and endo-*N*-acetylglucosaminidase H-treated core *N*-glycans in vitro

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Purified β-*N*-acetylglucosaminide β(1–4)galactosyltransferase and partially purified β-galactoside α(2–6)-sialyltransferase were used to elongate and terminate glycan chains of agalacto-ovalbumin and endo-β-*N*-acetylglucosaminidase H-treated yeast invertase in vitro. In the presence of both transferases, 0.1 mol sialic acid was incorporated per mol agalacto-ovalbumin within 24 h. Evidence is presented to show that purification of the galactosylated intermediate increases the efficiency of sialylation. Incorporation of sialic acid into endo-β-*N*-acetylglucosaminidase H-treated oligomannose glycoproteins may be useful for in vivo stabilization of these glycoproteins by preventing uptake in liver or reticuloendothelial cells.

Oligomannose glycan *Ovalbumin* *Endo-β-*N*-acetylglucosaminidase H*[2] *Invertase* (Saccharomyces cerevisiae) *Glycosylation*

1. INTRODUCTION

Optimum conditions for in vitro synthesis of biopolymers are difficult to achieve. This holds for template-based biosynthetic reactions such as replication, transcription and translation (review [1]) as well as for non-template based mechanisms of glycan biosynthesis (review [2]). Although progress has been made in recent years in understanding Golgi-associated glycosylation [3,4], much remains to be learned about the molecular mechanisms of glycan biosynthesis in vivo. Current evidence favors a model of Golgi-associated glycosylation in which each step in chain elongation and termination of complex glycans takes place in different compartments. This is in accordance with the 'assembly-line' model for glycan biosynthesis as opposed to the template-based process of protein and nucleic acid biosynthesis [3].

Single glycosylation steps have been carried out in vitro using crude or purified glycosyltransferases in soluble phase [2] or solid phase [5,6] for many years. In vitro chain elongation and termination (i.e. the incorporation of terminal sialic acid), however, has not been reported; such an in vitro glycosation system may be useful for at least two potential applications. First, mammalian glycoproteins expressed in yeasts by a recombinant DNA approach ([7,8] and references cited therein) may not be suitable for in vivo application since they are likely to be substituted by mannans; second, immunotoxins containing ricin A chains (an oligomannosidic glycoprotein) injected into the circulation of laboratory animals had a very short half-life due to rapid removal by the reticuloendothelial system [9].

As a first attempt toward the objective of introducing sialic acid into yeast glycoproteins, we

Abbreviations: NeuAc, *N*-acetylneuraminic acid; gal, galactose; GlcNAc, *N*-acetylglucosamine; UDP, uridine diphosphate; CMP, cytidine monophosphate; Gal-T, galactosyltransferase; Sialyl-T, sialyltransferase; Endo H, endo-β-*N*-acetylglucosaminidase H

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investigated chain elongation and termination in vitro by use of purified and well characterized glycosyltransferases. An incubation system was devised which brought about Gal-T-dependent incorporation of sialic acid into agalacto-ovalbumin and was then applied to Endo H-treated yeast invertase by the following sequence of enzymatic modifications:

1. (Oligomannose)-invertase $\xrightarrow{\text{Endo H}}$ (GlcNAc)-invertase
2. (GlcNAc)-invertase $\xrightarrow{\text{Gal-T}}$ (Gal-GlcNAc)-invertase
3. (Gal-GlcNAc)-invertase $\xrightarrow{\text{Sialyl-T}}$ (NeuAc-Gal-GlcNAc)-invertase

2. MATERIALS AND METHODS

2.1. *Biological material*

Human milk was obtained through the help of La Leche League; bovine colostrum was obtained from farms near Berne. Ovalbumin (grade 5) was from Sigma. α-Lactalbumin was purified from human milk according to [10]. Fetuin and sialidase (*Vibrio cholerae*, 20 U/mg) were from Calbiochem. Endo H was purified from 6 l of a cell-free filtrate from a stationary culture of *Streptomyces plicatus* essentially according to [11]. Gal-T (EC 2.4.1.22) was purified from human milk by two sequential affinity chromatography steps on columns of GlcNAc-Sepharose and α-lactalbumin-Sepharose [12] and Sialyl-T (EC not specified) was partially purified from bovine colostrum on CDP-ethanolamine-Sepharose [13]. Invertase obtained from Boehringer was further purified by ion-exchange chromatography on a 20 ml DEAE-Sepharose column equilibrated in 0.01 M Na phosphate, pH 6.5, and subjected to a linear gradient of 0–0.2 M NaCl. Fractions containing activity were pooled, dialyzed against 0.01 M Na acetate, pH 5.3, and stored at −20°C. Periodate oxidation of ovalbumin was carried out according to Spiro [14] to remove terminal galactose residues [15].

2.2. *Standard assay for Gal-T*

10 μmol Na cacodylate, 1 μmol $MnCl_2$, 1 mg native ovalbumin, 20 nmol UDP-[^{3}H]galactose (5 mCi/mmol) and 75 μU Gal-T were incubated in a final volume of 100 μl at pH 7.35 for 15 min at 37°C. The reaction was stopped by the addition of approx. 1 ml phosphotungstic acid in 2 N HCl, the precipitate filtered over glass fiber filters (Whatman GFA), washed with cold ethanol, dried and counted in a toluene-based liquid scintillation counting system at an efficiency of 45%.

2.3. *Standard assay of Sialyl-T*

10 μmol Na cacodylate, 0.2 mg asialofetuin prepared by mild acid hydrolysis [16], 0.2 mg bovine serum albumin, 0.1 μmol CMP-[^{3}H]NeuAc (0.9 mCi/mmol) and 24 mU Sialyl-T were incubated in a final volume of 55 μl at pH 6.8 for 120 min at 37°C. The incubation was stopped by addition of approx. 1 ml phosphotungstic acid (5%, w/v) and trichloroacetic acid (15%, w/v) in H_2O; the precipitate was treated and assessed for radioactivity as indicated above.

2.4. *Incubation conditions for Gal-T-dependent sialylation of agalacto-ovalbumin*

The incubation contained in a final volume of 2 ml: 10 μmol Na cacodylate, 2 μmol $MnCl_2$, 40 nmol UDPGal, 1 mg agalacto-ovalbumin, 2.5 nmol CMP-[^{3}H]NeuAc (7.15 mCi/mmol), 0.13 mU purified Gal-T and 70 mU partially purified Sialyl-T.

2.5. *Deglycosylation of invertase*

Purified invertase (22 mg) was incubated with 10 mU Endo H in 8 ml of 0.1 M Na citrate, pH 5.3, containing 4% (v/v) thiophene, tetrahydro-1,1-dioxide (sulfolane, Fluka) and a few drops of toluene at 37°C for 48 h. The incubation mixture was brought to 1% Triton X-100, boiled for 10 min and dialyzed against 0.005 M Tris-HCl, pH 7.0, containing 1% Triton X-100. The extent of deglycosylation was assessed by the increase in electrophoretic mobility of invertase according to [17]. Electrophoresis and fluorography were carried out according to Maniatis et al. [18].

3. RESULTS

3.1. *Sialylation of agalacto-ovalbumin*

Native ovalbumin is known to contain hybrid-type glycans terminated by Galβ(1-4)GlcNAcβ(1-2/4)R and GlcNAcβ(1-2/4)R branches. Thus,

ovalbumin exposes acceptor sites for both Sialyl-T and Gal-T, respectively [15]. Preliminary assays using Sialyl-T and Gal-T with native ovalbumin as acceptor substrate confirmed this assumption (not shown). Since we aimed at finding incubation conditions which would allow the demonstration of Gal-T-dependent sialylation of terminal *N*-acetylglucosamine residues only, we subjected native ovalbumin to periodate oxidation to produce agalacto-ovalbumin [15]. By comparison with native ovalbumin, periodate-treated ovalbumin preserved 62% of its original acceptor activity for Gal-T, whereas acceptor activity for Sialyl-T was completely abolished (not shown). Next, we studied glycosylation of agalacto-ovalbumin in a system in which both Gal-T and Sialyl-T and their respective donor substrates were present. Up to 2.09 nmol sialic acid per mg agalacto-ovalbumin were incorporated within 24 h during which sialylation was linear; this corresponded to an efficiency of 0.1 mol sialic acid per mol ovalbumin. In control incubations in which Gal-T or agalacto-ovalbumin was omitted, small but similar amounts of sialic acid were incorporated into acid-precipitable material which probably represented endogenous acceptor present in the partially purified Sialyl-T preparation. Treatment of the sialylated ovalbumin with *V. cholerae* sialidase for 3 h at 37°C completely removed the radiolabel. From these data we drew the conclusion that glycosylation in soluble phase by combining sequentially acting glycosyltransferases is possible but relatively slow since maximum incorporation was achieved only after 24 h. We then tested a system in which galactosylated ovalbumin was first purified by separating unreacted UDP-galactose from UDP generated by the reaction on a Bio-Gel P2 column, removing Gal-T by affinity chromatography on α-lactalbumin-Sepharose [19] and desalting on a PD 10^R column with subsequent concentration of the purified galactosylated product in a Minicon^R device. After this stepwise procedure, incorporation of sialic acid into galactosylated ovalbumin was approx. 2.5-times higher than in the combined assay with the same overall incubation time (table 1).

Table 1

Stepwise vs combined galactosylation and sialylation of agalacto-ovalbumin

Incubation	cpm[^{3}H]NeuAc	pmol[a] [^{3}H]NeuAc
Stepwise incubation[b]	837	116
Control without Gal-T	32	4
Combined incubation[c]	358	50

[a] Conversion of cpm to pmol was based on 45% counting efficiency and a specific activity of 7.15 mCi/mmol
[b] Galactosylation for 60 min at 37°C followed by purification of galactosylated ovalbumin, then sialylation for 60 min at 37°C. Incorporation of [^{3}H]NeuAc into acid-precipitable material is measured
[c] Transfer of galactose and sialic acid took place in a combined incubation for 120 min at 37°C

3.2. *Sialylation of Endo H-treated invertase*

To investigate whether an analogous glycosylation procedure could be used to incorporate sialic acid to the core *N*-acetylglucosamine of Asn-linked glycans, yeast invertase was treated with Endo H and subsequently with Gal-T and Sialyl-T. First, Endo H treatment of invertase produced an enzyme form of approx. 65 kDa as judged by electrophoretic mobility in SDS-PAGE (not shown) corresponding to the unraveling of 9 core *N*-acetylglucosamine acceptor sites per mol deglycosylated invertase [17]; this enzyme form proved to be an excellent acceptor for Gal-T. Table 2 shows the comparative incorporation of radiolabeled galactose into native and Endo H-treated invertase. Incorporation of 2.3 nmol galactose into Endo H-treated invertase corresponds to a galactosylation of 26% of available acceptor sites. A combined incubation containing both Gal-

Table 2

Glycosylation of Endo H-treated invertase

	Invertase	
	Native	Treated
nmol[^{3}H]Gal incorporated in 45 min at 37°C per 100 μg acceptor protein	0.01	2.300
Net incorporation of [^{3}H]NeuAc, nmol in 19 h per 100 μg acceptor protein (after subtraction of label incorporated in endogenous acceptors)	0	0.385

T and Sialyl-T led to the incorporation of sialic acid into Endo H-treated invertase (table 2), amounting to approx. 17% of the galactosylated acceptor sites. Control incubations in the absence of Gal-T, and Sialyl-T, UDPGal or invertase all produced similar but low amounts of labeled material due to endogenous acceptor in the Sialyl-T preparations. Subsequent treatment of sialylated invertase with *V. cholerae* sialidase removed all the incorporated label by overnight incubation at 37°C (not shown).

4. DISCUSSION

Our data demonstrate in vitro chain elongation and termination of ovalbumin in an assay system in which two sequentially acting glycosyltransferases with defined specificities were combined: Gal-T is known to catalyze the formation of a galactose β(1-4)GlcNAc-R linkage, a specificity which has been confirmed for the enzyme preparation used in this study [20]. Sialyl-T as purified according to our procedure was shown to catalyze the formation a NeuAcα(2-6)Gal linkage [13]. The specificities of purified glycosyltransferases in forming defined glycosidic bonds is considered to be rigorous and have been used to tag specifically terminal glycosyl residues on glycoproteins [21]. Therefore, we assume that the product formed by the action of the two glycosyltransferases represents authentic NeuAcα(2-6)Galβ(1-4)GlcNAc-R.

The conditions for combined Gal-T and Sialyl-T incubation as presented here to elongate and terminate glycan chains are far from being optimized. Several problems need further research such as: do the increasing concentrations of UDP or CMP generated by the action of the respective glycosyltransferase inhibit the reactions? Sialyl-T is believed to require additional binding sites for optimum activity [22] which are not present on truncated *N*-glycans; is it possible to circumvent this potential drawback by increasing the enzyme concentration or by stabilizing the enzymes on solid supports? To which extent is the biological activity of the treated glycoprotein affected by these enzymatic modifications?

In summary, a combined incubation system involving Gal-T and Sialyl-T has been shown to elongate and terminate peripheral glycan chains in the case of agalacto-ovalbumin and, albeit at low efficiency, of core *N*-glycans in the case of Endo H-treated yeast invertase. This approach represents the only possibility reported so far to incorporate sialic acid into glycoproteins of the oligomannose type. It may thus pave a way to a successful in vivo application of immunotoxins or of mammalian glycoproteins expressed in yeasts by recombinant DNA by increasing their half-life times in circulation.

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