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Reduced hepatic iron uptake from rat aglycotransferrin

Wei-Li Hu, Paul A. Chindemi and Erwin Regoeczi

Department of Pathology, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

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Summary. Rat aglycotransferrin (rAgTf) was produced from the disialosyl diantennary fraction of rat transferrin (rTf) by treatment with peptide:*N*-glycosidase F. Following removal of the enzyme by gel filtration and isolation of the deglycosylated protein by lectin chromatography, rAgTf was compared to rTf both in vitro and in vivo. No significant differences were found between the two proteins with respect to affinity for iron and kinetics of Fe release from the N-lobe and C-lobe. The fluorescence emission spectrum of apo-rTf was red-shfited by approximately 3 nm relative to diferric rTf; however, no spectral difference was detected between rTf and rAgTf when the analogous forms (apo or diferric) were compared. Plasma clearance of radioactive iron administered to rats as either rTf or rAgTf was comparable. Reticulocytes took up iron from rAgTf slightly faster than from rTf. In contrast, Fe acquisition by the liver from rAgTf was significantly reduced relative to rTf. This finding contrasts sharply with earlier observations with asialotransferrin (rAsTf) and provides a basis for discounting charge loss as the mechanism of enhanced hepatic Fe uptake from rAsTf. It is suggested that the glycan complement of rTf, while unimportant for interaction of the protein with specific receptors, probably plays a role in the interaction with low-affinity hepatic binding sites.

Key words: Deglycosylation - Glycoprotein - Hepatic iron uptake - Transferrin

Introduction

In rats, the principal iron-transporting protein, transferrin, contains one complex oligosaccharide attachment that is of the diantennary type (Spik et al. 1988). Earlier studies in this laboratory showed that removal of the two sialyl residues from rat transferrin (rTf) glycan shortened the biological life time of the protein by 25-30% (Rudolph et al. 1986). Two possible mechanisms, one specific and one nonspecific, can be considered to account for the phenomenon: (1) specific interaction of rat asialotransferrin (rAsTf) with the hepatic galactose/galactosamine-specific (Gal/GalNAc-specific) lectin (Ashwell and Harford 1982); (2) a nonspecific molecular alteration resulting from the loss of neuraminic acid (NeuAc) carboxyl charges. A distinction between these possibilities was afforded by a subsequent study with rat aglycotransferrin (rAgTf), which showed that the metabolic change following sialidase treatment was overwhelmingly caused by altered charge (Regoeczi et al. 1989).

Desialylation also endows rTf with the property of preferentially directing its iron to the liver (Rudolph et al. 1986), a feature which might be of importance for the development of hepatic siderosis in alcoholism (Regoeczi et al. 1984). Based on the effect of competitive inhibitors, we suggested that synergistic action between transferrin receptors and the Gal/GalNAc-specific lectin might explain the enhanced hepatic uptake of Fe from rAsTf (Rudolph et al. 1986). However, a role of charge loss could not be excluded at that time.

In the course of the present study, which was undertaken to clarify the above point, an unexpected observation was made: namely, that the hepatic iron-donating properties of rAgTf deviated from those of both rAsTf and control rTf. This prompted further studies of the molecule in vitro as well as its function in vivo.

Materials and methods

Proteins. The main component of rTf, containing a disialosyl diantennary glycan, was used throughout. It was isolated from pooled rat plasma as described elsewhere (Regoeczi et al. 1987). Unless stated otherwise, it was saturated with Fe before use. A batch of rAgTf was prepared by incubating apo-rTf (150 mg) with peptide:*N*-glycosidase F (Boehringer, Mannheim, FRG) under conditions established previously (Regoeczi et al. 1989). After 36 h at 37° C, rTf was reconstituted as a holoprotein, followed by separation of deglycosylated molecules from those still glycosy-

Offprint requests to: E. Regoeczi

lated by chromatography on concanavalin-A-Sepharose (ConA-Sepharose) as before (Regoeczi et al. 1989). The yield in rAgTf was 30%. Residual enzyme activity was removed from the preparation by filtration through a column (1.5 cm × 1.1 m) of Sephadex G-100 (Tarentino et al. 1985). To obtain rAsTf, rTf was incubated with sialidase from *Vibrio cholerae* (Gibco). Conditions and the technique used to separate reaction products were the same as employed in an earlier study (Rudolph et al. 1986). Preparations were labeled with ^{125}I and/or ^{59}Fe (New England Nuclear) as required according to routine protocols (Regoeczi 1983; Rudolph et al. 1986).

Fluorescence spectroscopy. A Perkin-Elmer spectrofluorometer (model LS 50), interfaced with an IBM computer, was used. A 5-nm bandpass was set for both excitation and emission. A binomial filter was used for smoothing. Proteins (5–30 μg protein/ml) were dissolved in 10 mM Tris/HCl pH 7.4 containing 0.15 M NaCl. The solvent for apoproteins also included 0.001% (mass/vol.) desferrioxamine mesylate (Ciba). Samples were excited at 275 nm or 295 nm and emission was recorded between 300–400 nm. Spectra were corrected for solvent scatter and then normalized.

Kinetics of iron release from rTf and rAgTf. An isotopic modification of the spectrophotometric method of Morgan et al. (1978) was used which distinguishes between the two iron-binding sites of Tf with respect to release kinetics. In brief, [^{59}Fe]rTf or [^{59}Fe]rAgTf (2 mg/ml of either) was equilibrated with 0.1 M Hepes pH 7.4 containing 0.15 M NaCl. Release was initiated by adding an equal volume of a solution containing 10 mM 2,3-bisphosphoglycerate (Sigma) and 10 mM desferrioxamine in the above Hepes/NaCl buffer. Samples (100 μl) were removed at intervals for the determination of protein-bound and chelated ^{59}Fe by rapid gel filtration on PD-10 columns (Pharmacia). The whole procedure was carried out at room temperature.

Iron exchange between rTf and rAgTf. An adaptation of the method of Aisen and Leibman (1968) was used. A mixture of both apoproteins (50 μg of each) was labeled with ^{59}Fe. In addition, rTf was also labeled with ^{125}I. The latter label served as an internal standard to monitor changes in ConA affinity of rTf that might arise during prolonged incubation (see below). After removing unbound radioactivity (Regoeczi et al. 1989) and reducing the volume in a Centricon 30 microconcentrator (Amicon), the preparation was added to 0.5 ml Hepes/NaCl pH 7.4 (see above) containing 450 μg each of apo-rTf and apo-rAgTf, 1 mM sodium citrate and 0.05% (mass/vol.) NaN_3, and then incubated at 37° C. Samples (50 μl) were taken at intervals up to 168 h for the determination by ConA chromatography of the partition of ^{59}Fe between rTf and rAgTf. A similar study was also carried out using a combination of rTf molecules possessing a disialosyl diantennary and a trisialosyl diantennary glycan, respectively.

Induction of reticulocytosis. Rats were given a series of intraperitoneal injections of phenylhydrazine (Sigma), typically 1.5 ml of a 1% solution (mass/vol.) on days 1 and 2, and 0.8 ml on days 4 and 6. Two days later, they were exsanguinated using acid/citrate/glucose (ACD; Dacie and Lewis 1975) as an anticoagulant. The cells were first washed twice in ACD and then twice in RPMI 1640 medium (Flow Lab, Costa Mesa, CA) containing 5 μM adenine (Sigma). Enrichment of the preparations with reticulocytes, as established by new methylene blue staining (Dacie and Lewis 1975), was better than 95%.

Uptake of ^{59}Fe by reticulocytes. Cells ($8–9 \times 10^7$/ml) were suspended in cold (4° C) RPMI 1640 medium containing 5 μM adenine and bovine serum albumin (5 mg/ml; Sigma). (Traces of transferrin detected in the albumin were removed beforehand by cation-exchange chromatography.) Double-labeled (^{59}Fe and ^{125}I), rTf, rAsTf, or rAgTf was added at a final concentration of 20 μg/ml before warming the suspension to 37° C in an atmosphere of 95% O_2 and 5% CO_2. Samples (150 μl) were obtained at intervals for the separation of cell-associated and free radioactivities in an Eppendorf microcentrifuge as described elsewhere for hepatocytes (Rudolph et al. 1986). The $^{125}I/^{59}Fe$ ratio in the cell-free medium was used in conjunction with the ^{125}I activity in the cell pellet to calculate 'net' ^{59}Fe uptake by the reticulocytes.

Hepatic uptake of ^{59}Fe from rTf and rAgTf. A total of 33 adult female Sprague-Dawley rats (200–240 g body mass) were used for this purpose. They were given free access to drinking water and standard Purina rat chow. Each received an intravenous dose (200 μg/100 g body mass in 0.2–0.3 ml) of either [^{59}Fe]rTf or [^{59}Fe]rAgTf. Groups of animals were exsanguinated under pentobarbital anesthesia 15, 30, 60, 90, or 120 min later for the measurement of net hepatic iron uptake by a technique described elsewhere (Regoeczi et al. 1984). (The term 'net' refers to the fact that values obtained were corrected for ^{59}Fe activity trapped post mortem in the hepatic residual blood volume by determining this volume using rat ^{125}I-albumin.)

Other techniques. To measure plasma iron clearance, rats were injected with ^{59}Fe-labeled rTf or rAgTf (80–90 μg/100 g body mass of either). Small venous blood samples were collected at 15-min intervals up to 2 h, and ^{59}Fe activity was assayed in 50-μl plasma samples in duplicate. A Packard model 5550 counter was used for radioactivity measurements with appropriate corrections for Compton radiation in multiisotope experiments. Cells were counted visually in a hemocytometer. Protein concentrations were determined by absorbance measurements at 280 nm in a Beckman Du-50 spectrophotometer.

Results

Iron delivery by rAgTf to cells and organs

Reticulocytes were incubated with ^{59}Fe-labelled rTf, rAsTf or rAgTf at a ligand/receptor ratio of 18:1, assuming 10^5 transferrin receptors (Tf-R) per cell (Iacopetta et al. 1981). The resultant ^{59}Fe uptake curves closely paralleled each other (Fig. 1). Slight inequalities

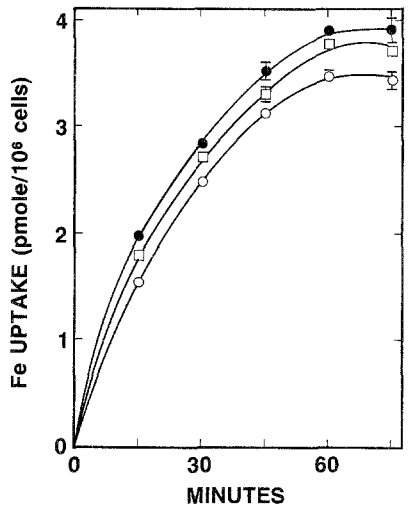

Fig. 1. Uptake of iron by reticulocytes (8.4×10^7/ml) from 20 μg/ml of each of rTf (○), rAsTf (□) and rAgTf (●). Experimental conditions are given under Materials and methods. Points are the means of measurements in quadruplicate, bars denote SE (only shown when larger than symbol). For further explanations see the text

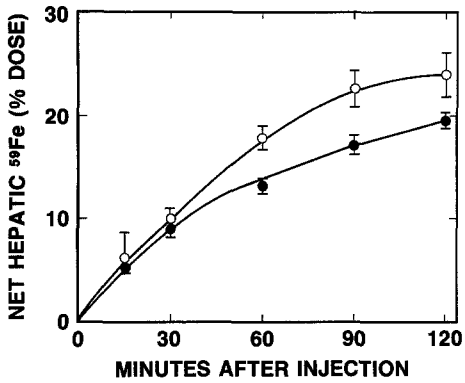

Fig. 2. Uptake of ^{59}Fe from rTf (○) and rAgTf (●) by the rat liver in vivo. Experimental conditions are given under Materials and methods. Each point is the mean of three or four experiments. Vertical bars denote SE

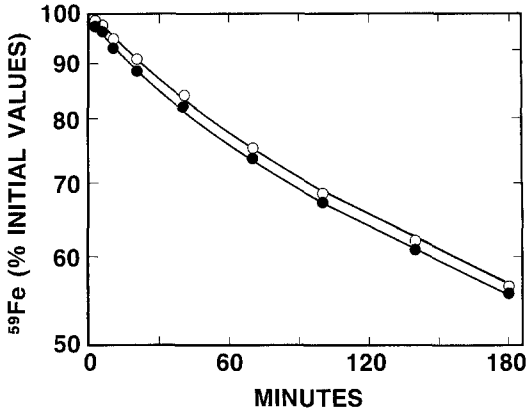

Fig. 3. Release of ^{59}Fe from rTf (○) and rAgTf (●) in the presence of 2,3-bisphosphoglycerate. Reaction conditions are given under Materials and methods. Curves, plotted semilogarithmically, denote protein-bound ^{59}Fe activity at intervals following the addition of 10 mM 2,3-bisphosphoglycerate

in ligand concentration probably account for the fact that the curves reached a plateau at different levels after about 60 min. For the interval 15–45 min, the reciprocal plots of intracellular ^{59}Fe values conformed to linear regression lines with the coefficients −0.011, −0.012 and −0.016 for rAgTf, rAsTf and rTf, respectively. The values suggest that rAgTf is marginally more efficient as an iron donor than rAsTf, which in turn is slightly more efficient than rTf.

Plasma iron clearance was measured in six rats, three of which received [^{59}Fe]rTf and the other three [^{59}Fe]rAgTf. The mean plasma half-life of ^{59}Fe was 45.9 (±0.6 SE) min in the former group compared with 47.6 (±3.4 SE) min in the latter. This difference is not significant ($t=0.45$, $P=0.70$).

Data pertaining to the acquisition of Fe by the liver in vivo from rTf and rAgTf are summarized in Fig. 2. It is seen that the uptake curves representing both proteins diverged with time in such a way that the fully glycosylated form out-performed the deglycosylated one; by 60 min, the difference became significant ($P=0.019$).

Iron release, iron exchange and fluorescence spectrum

Kinetics of iron release from rTf and rAgTf are compared in Fig. 3. The release curves of both proteins closely paralleled each other. The plots were nonlinear over the first 90 min, which is attributed to the fact that the Fe-binding site in the N-lobe is more vulnerable to proton attack than that in the C-lobe (Evans and Williams 1978). To compute half-lives ($t_{1/2}$) of iron release from the respective sites, two-term exponential functions were fitted to the data in Fig. 3. The resultant slopes, with coefficients of determination from 97.8% to 99.6%, gave a $t_{1/2}$ of 24.7 min for Fe in the N-lobe, and 293.5 min for Fe in the C-lobe of rTf, the corresponding values for rAgTf being 25.0 min and 287.7 min. These differences are too small to be of biological significance.

In the presence of iron-complexing anions (e.g. citrate), transfer of Fe is known to occur between transferrin molecules (Aisen and Leibman 1986). We took advantage of this feature to explore whether, in a mixture of rTf and rAgTf, equilibrium of Fe favored either protein: 10% of the binding sites were saturated with ^{59}Fe in the 1:1 mixture. ConA chromatography of daily subsamples showed minimal subsequent changes in the initial ^{59}Fe distribution between glycosylated and aglyco forms. Thus, after 168 h, 3.25% of the iron that had been originally associated with rTf was present in rAgTf. During the same interval, the proportion of ConA-reactive ^{125}I-rTf decreased by 3.6%. (This decrease has been taken into account when calculating ^{59}Fe distributions.) The same experimental design was used to investigate if the presence of a trisialosyl glycan in rTf (Regoeczi et al. 1987) affected the equilibrium of iron exchange. Serial measurements on a mixture of rTf molecules, containing di- and trisialosyl glycans in equal proportions, revealed redistribution of ^{59}Fe at a very low rate in favor of the trisialosyl component. By 168 h, 5.1% of iron that had been originally present in the disialosyl fraction was associated with the trisialosyl component. Clearly, the above changes are not great enough to postulate a role for the glycan, or the type of glycan, in Fe binding by rTf.

On excitation at 295 nm, both the holo and apo forms of rTf yielded a broad fluorescence peak, the former with a maximum at 337 nm and the latter at 340 nm (Fig. 4). Since tryptophans buried in proteins emit with a maximum close to 320 nm and those fully exposed close to 350 nm (Lakowicz 1983), it seems reasonable to conclude that the majority of Trp indoles in rTf are localized in a partially polar micro-environment. The slightly red-shifted spectrum of apo-rTf is consistent with a less compact state of the molecule, which is also observable by other methods (Charlwood 1973). When excited at 275 nm, there was a relative increase in emission by apo-rTf in the region over 340 nm (Fig. 5). As inferred from the unique reactivity of transferrin tyrosyls with iodine (Regoeczi 1983), this increase might have been due to tyrosinate emission (Lakowicz 1983). The spectra shown in Figs. 4 and 5 were not in-

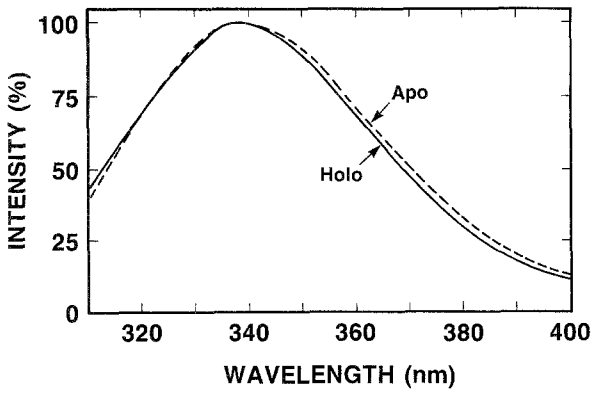

Fig. 4. Fluorescence emission spectra of apo-rTf and holo-rTf (10 μg/ml of each) when excited at 295 nm. For experimental conditions see Materials and methods. Each curve is the mean of three separate recordings

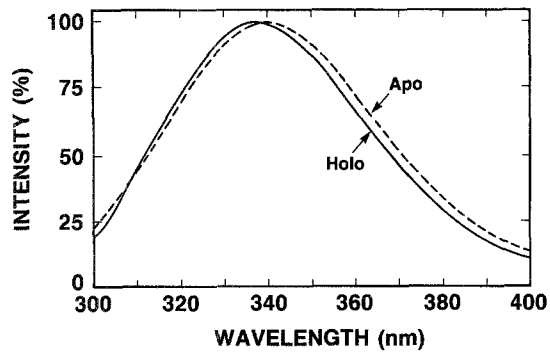

Fig. 5. Fluorescence emission spectra of apo-rTf and holo-rTf (10 μg/ml of each) following excitation at 275 nm. For experimental conditions see Materials and methods. Curves are the means of three separate recordings

fluenced by varying the concentration of rTf between 5–30 μg. Nor were the spectra of control and deglycosylated rTf distinguishable (not shown).

Discussion

The present work is the first comprehensive study with a completely deglycosylated Tf. Before, Kornfeld (1968) removed 9–47% of the carbohydrates (other than NeuAc) from human Tf by sequential treatment with glycosidases; however, the product gave inconsistent results when tested on rabbit reticulocytes.

Our results suggest that rAgTf binds and releases iron indistinguishably from its glycosylated counterpart. Furthermore, ^{59}Fe was cleared from the plasma normally when complexed to rAgTf, implying that rAgTf and rTf are comparable iron transporters.

Iron acquisition from Tf by erythroid cells can be completely inhibited by antibodies to the Tf-R (Morgan and Baker 1988), the inference being that access of Tf to these cells occurs only through specific receptors. From the data in Fig. 1 therefore, we conclude that the interaction of rTf with Tf-R is not impaired by either deglycosylation or desialylation. In studies using rabbit reticulocytes and rabbit AsTf, Hemmaplardh and Morgan (1976) came to similar conclusions.

Desialylation of rTf results in a markedly enhanced uptake of Fe by the liver (Regoeczi et al. 1984; Rudolph et al. 1986). The present study shows that rAgTf lacks this property (Fig. 2). Both rAsTf and rAgTf carry the same (reduced) charge. In addition, rAgTf is also devoid of terminal galactose residues. The different behaviour of the two proteins as hepatic iron donors therefore unequivocally establishes the importance of exposed galactose (in contrast to loss of sialyl carboxyl charges) as a mediator of the excessive Fe uptake by the liver from rAsTf. Further support is thus gained for the hypothesis (Rudolph et al. 1986) that Tf-R and Gal/GalNAc-specific lectin act synergistically in that process.

Significantly, removal of the whole glycan from rTf affected hepatic Fe uptake rates to become subnormal (Fig. 2). This novel observation prompts us to postulate a hitherto unsuspected role for the Tf glycan in liver/transferrin interactions. Three, simultaneously operating, mechanisms have been proposed for the hepatic acquisition of Fe from Tf, namely, specific receptors (Young and Aisen 1981), pinocytosis (Sibille et al. 1982) and low-affinity binding sites (Cole and Glass 1983; Page et al. 1984). The possibility of a reduced interaction of rAgTf with Tf-R may be discarded on the basis of data in Fig. 1. Alteration in the pinocytic rate of rTf due to deglycosylation seems an unlikely explanation. It remains conceivable therefore that the rTf glycan promotes the interaction of transferrin with low-affinity binding sites. This preliminary hypothesis clearly requires confirmation in future studies.

Glycoprotein glycans, by virtue of interacting with their protein backbones, can affect the conformation of the latter (West 1986). It was for this reason that the study was extended to protein fluorescence. There are no published fluorescence spectra of transferrin, to our knowledge. Measurements of Stokes radius (Charlwood 1973) as well as crystallographic studies (Bailey et al. 1988) have shown that the transferrin molecule becomes more compact on binding iron. The changes in fluorescence following the apo-holo transition (Figs. 4 and 5) were modest and no further changes appeared following deglycosylation. RTf contains 7 Trp and 19 Tyr residues (Schreiber et al. 1979). These residues could change their micro-environment in diverse ways as the protein assumes different conformations with the net effect that no larger spectral shifts take place.

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Note added in proof

According to a recent paper (Walsh MT, Watzlawick H, Putnam FW, Schmid K, Brossmer R [1990] Effect of the carbohydrate moiety on the secondary structure of β_2-glycoprotein I. Biochemistry 29:6250–6257), the circular dichroic (CD) spectrum of human plasma β_2-glycoprotein I (β_2I) changes following deglycosylation in such a way that parts of the random coiled segments assume β-turns. To see if similar changes occur in rTf, we examined, in aqueous buffer, rTf and rAgTf in a Jasco model J-600 spectropolarimeter. However, no differences were found in the CD spectra. A possible explanation is that β_2I has five N-glycosylation sites whereas rTf only one.