Synthesis and properties of aglyco transferrin

Productie en eigenschappen van aglyco transferrine

Proefschrift

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Voor Ellen, mijn ouders en grootouders Marteijn.

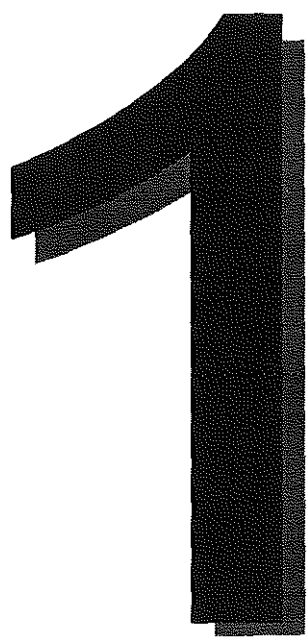
Contents

List of abbreviations

DNase	:deoxyribonuclease
DTT	:dithiothreitol
EDTA	:ethylenediaminetetra acetic acid
EPR	:electron paramagnetic resonance
Gal	:galactose
GlcNac	:N-acetylglucosamine
GSH	:glutathione, reduced form
GSSG	:glutathione, oxidized form
IEF	:isoelectric focusing
IPTG	:isopropyl-ß-D-thiogalacto-pyranosidase
K_d	:dissociation constant
kDa	:kilodalton
LMW	:low molecular weight
Man	:mannose
Mw	:molecular weight
NeuAc	:N-acetylneuraminic acid (sialic acid)
NTA	:nitrilotriacetic acid
p-TSA	:p-toluenesulfonic acid
PAGE	:polyacrylamide gel electrophoresis
PBS	:phosphate buffered saline
pI	:isoelectric point
rhTf	:recombinant human transferrin
rhTf/2N	:recombinant human N-terminal half-transferrin

rhTf/2C	:recombinant human C-terminal half-transferrin
SDS	:sodium dodecylsulphate
Tf	:transferrin
TFA	:trifluoroacetic acid
TfR	:transferrin receptor
Tris	:tris(hydroxymethyl)-aminomethane
v/v	:volume/volume
w/v	:weight/volume

Introduction



1.1 Introduction

Iron is the most abundant metal in the universe and the fourth most common element on earth (1). It is essential for all known life forms, with the possible exception of a few micro-organisms. Depending on the ligand it is linked to, iron has access to a wide range of redox potentials. It is involved in the redox reactions of the cell, especially in those reactions implying molecular oxygen formation and the release of chemical energy for use in the energy-requiring processes of life.

Iron-containing proteins can be subdivided into 3 classes (1,2):

1 Iron-sulphur proteins

These proteins contain one or more iron atoms ligated by sulphur. The proteins are all involved in electron transfer, although this is by no means their only function. Examples of the iron-sulphur proteins are: succinate dehydrogenase, NADH-Q reductase, aconitase.

2 Haem proteins

Haem proteins are distinguished by protoporphyrin prosthetic groups containing a central iron atom. Haem proteins have a variety of properties and can be found throughout the whole animal and plant kingdom. Examples are: haemoglobin, myoglobin, cytochromes, peroxidases.

3 Non haem and non iron-sulphur proteins

Many iron containing proteins can not be classified in one of the two groups mentioned above. Some examples are: transferrin (Tf), ferritin, superoxide dismutase, dioxygenases.

In spite of the profusion of the element in the biosphere, the availability of iron for living cells is strongly diminished by the poor solubility of Fe(III). In an aqueous and aerobic environment the solubility product of $Fe(OH)_3$ is $4x10^{-38}$. This mediocre solubility of iron has made it necessary for nature to evolve systems capable of binding

iron, to provide for relatively high iron concentrations in body fluids of living organisms.

Disregarding a few exceptions such as *Lactobacillus* (3), all micro-organisms need iron in order to survive. Bacteria therefore have developed a range of low-molecular-weight iron chelators known as siderophores (4). These siderophores have a high affinity for Fe(III) and chelate and solubilize the metal out of minerals and organic substrates. Bacteria synthesize and secrete siderophores according to their iron requirement. Specific receptors on the bacterial surface bind the iron-siderophore complex and either the complex is absorbed by the cell, or the iron is channeled across the membrane.

Higher plants on the other hand have developed a variety of methods to acquire iron from the environment. Like bacteria they also secrete low-molecular-weight iron chelators known as phytosiderophores. The affinity for iron of the phytosiderophores is lower than of their bacterial equivalent. Plants, however, also secrete acid or reducing substances into the soil, both increasing the solubility of iron (5).

Vertebrates have access to a far more soluble and available iron; as a result of their diet they do not need to secrete low-molecular-weight iron chelators. On the other hand, these large and complex higher organisms do face another problem i.e. how to transport the potentially insoluble iron in the body to the different compartments of utilisation and/or storage. For this purpose all vertebrates possess large quantities of an iron-transporting protein in their blood plasma. This protein, transferrin (Tf), is a member of the siderophilin family (2).

1.2 The siderophilins

The siderophilins, also known as transferrins, are monomeric iron binding proteins dependent on concomitant binding of a suitable anion, generally bicarbonate. This cooperative binding between anion and metal ion is an almost absolute requirement, as neither of them reacts strongly without the other (6).

In general the transferrins have two iron-binding sites and a molecular weight of approximately 80 kDa, although this may vary between 40 kDa for *Pyura stolonifera* Tf (7) and 150 kDa for crab Tf. Predominantly they have two domains, the N-terminal and the C-terminal domain, the latter bearing one or more N-linked glycans. A unique feature

of most of the siderophilins is the display of a salmon-pink color as a result of iron binding. Consequently the proteins show an absorbance maximum at 465nm. The major siderophilins are:

1 Transferrin

Transferrin (Tf) has been isolated and partially characterized by Schade and Caroline (8) from human plasma and by Laurell and Ingelman (9) from pig plasma. Tf was found in the plasma of all vertebrates investigated, including amphibia, birds, fish, mammals and reptiles (10,11,12). Tf was also detected in a variety of biological fluids like lymph, amniotic fluid and cerebrospinal fluid. Tf is mainly synthesized in the liver, but also in the central nervous system, testis, ovary, placenta, thymus and lymphocytes. The main function of Tf, as the name indicates, is the transport of iron. Moreover, it has antimicrobial properties.

2 Ovotransferrin

Ovotransferrin was identified in 1900 by Osborne and Campbell (13) and initially named conalbumin, the iron binding capacity of ovotransferrin has been discovered at the end of the Second World War by Schade and Caroline (14). Ovotransferrin can be found in the egg white of birds and reptiles, where it accounts for 10% of the proteins found in the egg white. Within these species the composition of ovotransferrin and Tf only differs in the composition of the glycans, the proteins are a product of the same gene locus. This similarity was first demonstrated in chickens (15).

3 Lactoferrin

Lactoferrin has been described first by Johansson in 1958 (16). Lactoferrin can be found in milk. In human milk it can account for up to 30% of the total protein content, in cow milk for approximately 10%. Human Tf and lactoferrin are not related like chicken ovotransferrin and chicken Tf (17). Lactoferrin, like Tf, bears two N-linked glycans, but lactoferrin bears one on the C-terminal domain and one

on the N-terminal domain, whereas Tf bears both glycans on the C-terminal domain (18). Another difference is found in the affinity of both proteins for iron, the affinity of lactoferrin being 300 times higher than that of Tf. The function of lactoferrin probably is the transport of dietary iron from mother to child. Lactoferrin also has a strong antimicrobial quality.

4 Melanotransferrin

In 1982 a glycoprotein (p97) has been isolated and partly sequenced from melanoma tumour cell membranes (19). The sequence showed homology with Tf, moreover antibodies directed against p97 cross-reacted with Tf. The protein has the ability to bind iron. It has a molecular weight slightly over 80 kDa and it has an extra sequence of 25 hydrophobic aminoacids at the C-terminal end compared to Tf.

All known transferrins are glycoproteins, with the exception of the serum transferrins of some fish species belonging to the *Cyprinidae* (20,21).

1.3 General considerations on glycoproteins

Glycoproteins are a diverse group of complex macromolecules, best defined as conjugated proteins containing a prosthetic group of one or more covalently bound heterosaccharides. They are present in nearly all life forms. Bacteria, however, do not contain glycoproteins, with the exception of the genus *Halobacterium* and *Bacillus stearothermophilus NRS 2004/34* (22).

Glycoproteins cover several classes of proteins such as transport proteins (e.g. transferrin), immunoglobulins, hormones and enzymes.

Although known for nearly one and a half century, the glycan moieties on proteins have been ignored until the 1960s. The discovery that protein-bound carbohydrates play a role in biological recognition such as in host-pathogen, cell-cell and cell-molecule interactions has focused an enormous interest on glycoproteins. The finding that the glycans could vary in different developmental or pathological states also has contributed to

an increasing scientific interest in glycoproteins (22,23,24).

Presumably the glycan moieties of glycoproteins can encode biological information that can be decoded by other proteins known as lectins (25).

The majority of the glycoproteins secreted by eukaryotic cells contain N-linked oligosaccharides. These asparagine-linked glycans can be divided into 3 types; the high mannose-, the hybrid- and the complex type (fig 1). Serum Tf contains two glycans of the complex type.

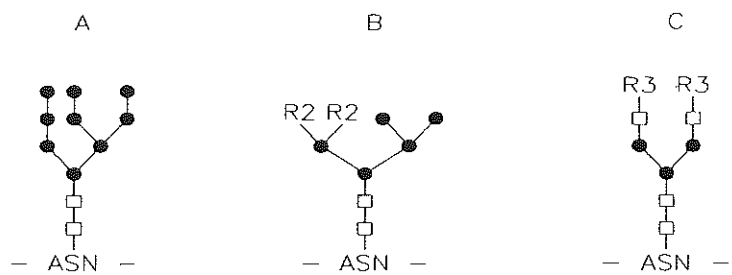


Figure 1 The structures of the typical asparagine-linked glycans: the high mannose (A), the hybrid (B) and the complex (C) type of oligosaccharides. The symbols represent: □ N-acetylglucosamine, ● mannose.
R2 ± N-acetylglucosamine ± galactose ± sialic acid.
R3 ± galactose ± sialic acid.

All N-linked oligosaccharides are synthesized via a common pathway. This pathway can be subdivided into 3 subsequent parts, i.e. the rough endoplasmic reticulum (RER), the Golgi apparatus and the *trans*-Golgi network (26,27). The first step in the biosynthesis of asparagine-linked oligosaccharides is the cotranslational transfer of an oligosaccharide by oligosaccharyltransferase from the dolichol-linked oligosaccharide $Glc_{0-3}Man_{5-9}GlcNac_2$-$(PO_4)_2$-dolichol to asparagine residues in nascent polypeptides in the rough endoplasmic reticulum (28,29). In fact, the oligosaccharide is linked to asparagine residues in the tripeptide -Asn-X-Ser/Thr. However, less than half of the known tripeptide sequences in secreted glycoproteins are glycosylated, possibly due to differences in accessibility.

Once the oligosaccharide is transferred to the protein it becomes subject to removal of glycoside residues by a number of glycosidases. This trimming mainly occurs in the ER, transitional ER and the *cis*-Golgi apparatus. In both the *cis*- and *medial*-Golgi apparatus the processed oligosaccharide is elongated by addition of other glycoside residues. This elongation with additional glycosides and other constituents is continued in the *trans*-Golgi apparatus and the *trans*-Golgi network. The pathway for the biosynthesis of Asn-linked oligosaccharides is shown in figure 2.

1.4 Transferrin

Transferrin (Tf) is the fourth most abundant plasma protein in man. Except in blood the protein can also be found in several other body fluids including lymph, cerebrospinal fluid, tears, saliva, amniotic fluid and seminal fluid. Tf is a monomeric protein consisting of 679 aminoacids and a calculated molecular mass of 79,570 Da. As discussed earlier, the major function of Tf is transporting iron in the circulation. The protein consists of 2 domains, the N-terminal- and the C-terminal domain. Both domains are capable of binding 1 Fe(III) atom. The affinity constant is $1x10^{22}$ M^{-1} and $6x10^{22}$ M^{-1} for the N- and the C-terminal domain respectively (30). Tf is also capable of binding a whole range of other metals like aluminium, gallium, chromium, manganese, cobalt, nickel, copper, zinc, cadmium and even plutonium (31-38). With the binding of iron, the concomitant binding of an anion is also required (39,40). In a few cases Tf might play a physiological role in the transport and binding of a nutritionally essential element other than iron, such as zinc and manganese. In other cases Tf may participate in the accumulation of toxic metals.

In a healthy person serum Tf is about 30% saturated with iron. Under pathological conditions such as haemochromatosis this can raise to 100% (41).

The aminoacid sequence of Tf has been determined (42,43). A striking feature of this sequence is the similarity between the N-terminal and C-terminal domain of the protein. Approximately 40% of the aminoacids in the N-terminal domain (residues 1-336) have identical counterparts in the C-terminal domain (residues 337-679). It has been suggested that the Tf gene is the product of an intragenic gene duplication (44).

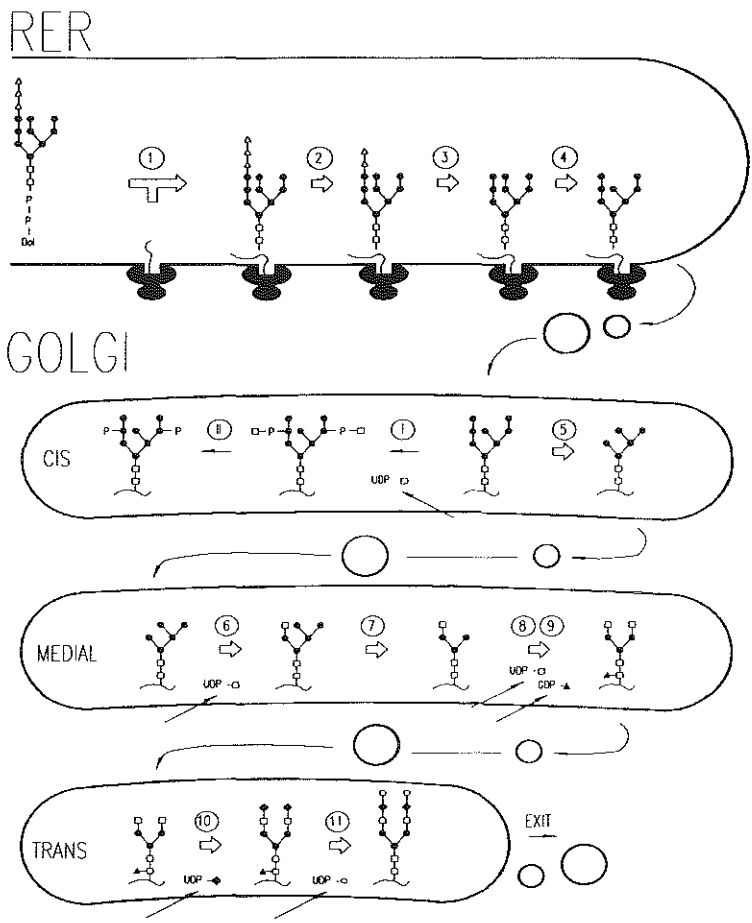


Figure 2 Schematic pathway of the biosynthesis of Asn-linked oligosaccharides. The biosynthesis is initiated in the rough endoplasmic reticulum (RER) and proceeds through the cis-, medial- and trans-Golgi apparatus. The reactions are catalyzed by the following enzymes: (1) oligosaccharyltransferase, (2) α-glucosidase I, (3) α-glucosidase II, (4) ER α1,2-mannosidase, (I) N-acetylglucosaminylphosphotransferase, (II) N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase, (5) Golgi α-mannosidase I, (6) N-acetylglucosaminyltransferase I, (7) Golgi α-mannosidase II, (8) N-acetylglucosaminyltransferase II, (9) fucosyltransferase, (10) galactosyltransferase, (11) sialyltransferase. The symbols represent: □ N-acetylglucosamine, ● mannose, Δ glucose, ▲ fucose, ◆ galactose, O sialic acid. Adapted from Kornfeld (26)

The advantage is obvious, enlarging the protein prevents losing both iron and protein through glomerular ultrafiltration. Two other members of the human siderophilin family, lactoferrin and melanotransferrin, also show a similar degree of internal homology between the N-terminal and C-terminal domain.

The C-lobe carries two N-linked oligosaccharide chains attached to the asparagine residues 413 and 611. These "complex type" glycans are highly variable and contribute to the so called microheterogeneity of Tf. The glycans can differ in the degree of branching; biantennary, triantennary and even tetraantennary structures have been shown to exist (fig 3, 124).

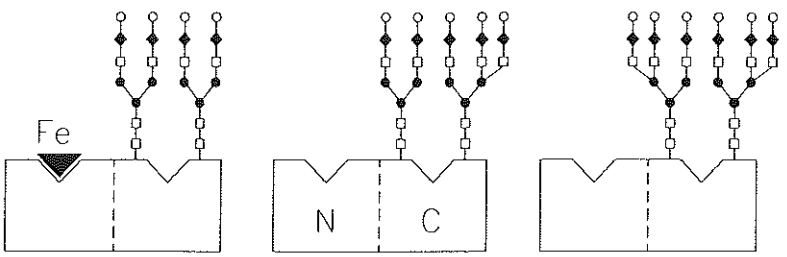


Figure 3 Schematic representation of the human Tf molecule. The N-terminal- and C-terminal domain are both capable in binding 1 Fe(III) atom. The C-terminal domain carries two glycans. Bi-bi-, bi-tri- and tri-tri antennary Tf are shown.

The sialic acids on the glycans contribute substantially to the isoelectric point of Tf. Therefore Tf can be separated according to the amount of sialic acid by a technique called isoelectric focusing (IEF). Subfractions of Tf isolated by means of IEF can contain Tf bearing all possible branched glycan variants, because the glycans can be partly desialylated. The isoelectric point of Tf varies between pH 5 to 6.

1.5 The significance of transferrin glycans

Specific variations in the oligosaccharide moieties of Tf are known to occur in several (patho)physiological conditions, for instance pregnancy, rheumatoid arthritis, malignancies and alcohol abuse (45-54).

During pregnancy a shift towards the higher branched (and higher sialylated) glycans has been reported in humans and in guinea pigs (45,48,49). An increased affinity of the transferrin receptor (TfR) for Tf bearing higher branched glycans was reported by Rudolph (55). However this could not be confirmed by other autors (45,46,56). Regarding guinea pigs, Van Dijk *et al.* (46) report, the changes seen in the Tf glycans during pregnancy do not seem to be functionally related to maternal or fetal erythropoiesis.

The changes in the Tf glycans due to malignancies are more variable. Increased branching, fucosylation and bisecting of the glycan chains (fig. 4) have been reported.

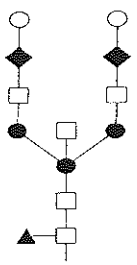


Figure 4 Biantennary complex-type glycan with fucosylation and bisecting N-acetylglucosamine. The symbols represent: □ N-acetylglucosamine, ● mannose, ▲ fucose, ◆ galactose and o sialic acid.

In alcoholism, the 2-sialylated Tf subfraction increases at the expense of the higher sialylated Tf subfractions. Figure 5 shows the normal distribution of the sialo-transferrin subfractions.

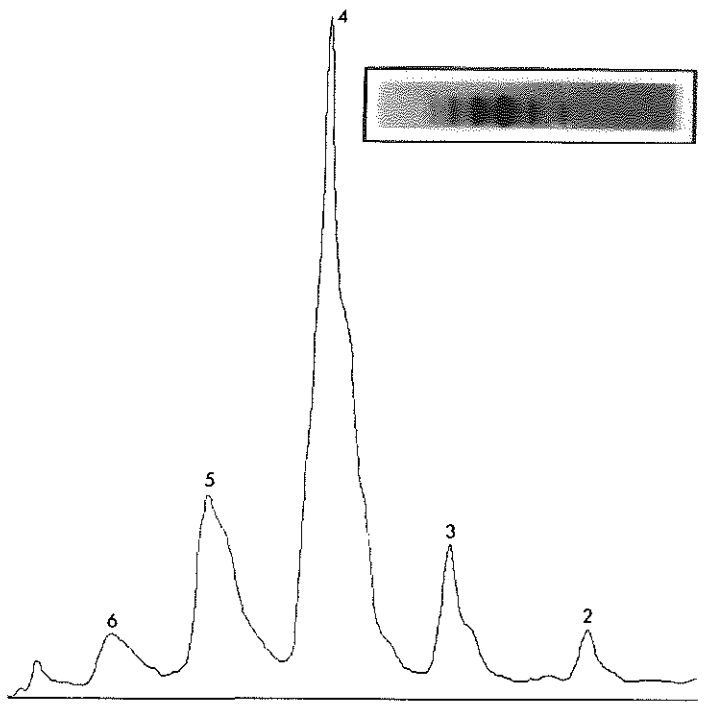


Figure 5. The normal distribution of the sialo-transferrin subfractions. The insert shows the separation of Tf into different sialo-transferrin subfractions on an IEF gel.

The shift of biantennary glycans towards tri- and tetra-antennary glycans on Tf in humans has not only been reported during pregnancy, but also during the course of liver cirrhosis (57,58). In case of the latter (pathological) situation, a similar shift has been described for alpha-1-acid glycoprotein (59). Since cirrhosis of the liver renders this organ chronically hypoxic (60), it was suggested that hypoxia affected the glycosylation of Tf. However, this could not be demonstrated in rats kept under hypoxic conditions (61).

Rat Tf bears only one glycan. Removal of this glycan results in reduced hepatic

iron uptake compared to normal Tf. The affinity for iron and the kinetics of iron release from the N-lobe and C-lobe however, is not affected by the deglycosylation of rat Tf (62).

The oligosaccharide moieties of glycoproteins can be of importance to the intracellular transport of these proteins during synthesis. Tf is one of the exceptions, in which absence of the glycans does not influence the rate of intracellular transport during synthesis (63).

1.6 Production of aglycotransferrin

To tackle the intriguing question whether or not Tf glycans serve any physiological function, it is necessary to compare Tf with nonglycosylated Tf. Theoretically nonglycosylated Tf can be obtained by deglycosylation of Tf. In general protein deglycosylation is either performed by chemical methods with anhydrous hydrogen fluoride or by enzymatical methods with endo- or exoglycosidases.

Treatment of nonglycosylated lysozyme and RNase as well as glycosylated ovomucoid with anhydrous hydrogen fluoride has shown that these proteins can retain their biological function after such a treatment. Unfortunately GlcNac-Asn linkages are not cleaved by anhydrous hydrogen fluoride treatment (64,65,66) making this method not applicable for Tf.

The use of endoglycosidases for enzymatically cleaving the glycan chains at their base, is widespread. Best results are obtained with proteins after denaturation by sodium dodecyl sulfate (SDS)(67,68,69), rendering these proteins unsuitable for biological studies. However, some authors have reported enzymatical deglycosylation without prior denaturation (70,71). In any case endoglycosidases are very expensive, therefore the production of a fair quantity of deglycosylated Tf would be a very valuable enterprise.

An alternative for deglycosylation of Tf is the production of nonglycosylated Tf. This can either be performed by blocking the glycosylation of Tf during synthesis with tunicamycin (18,72), by producing nonglycosylated Tf in eukaryotic cells using site-directed mutagenesis (73) or by production of the human protein in a bacterial expression system (74-77). Bacteria lack the rough endoplasmic reticulum and the Golgi system and are therefore not able to attach N-linked glycans to their products. The two first-mentioned

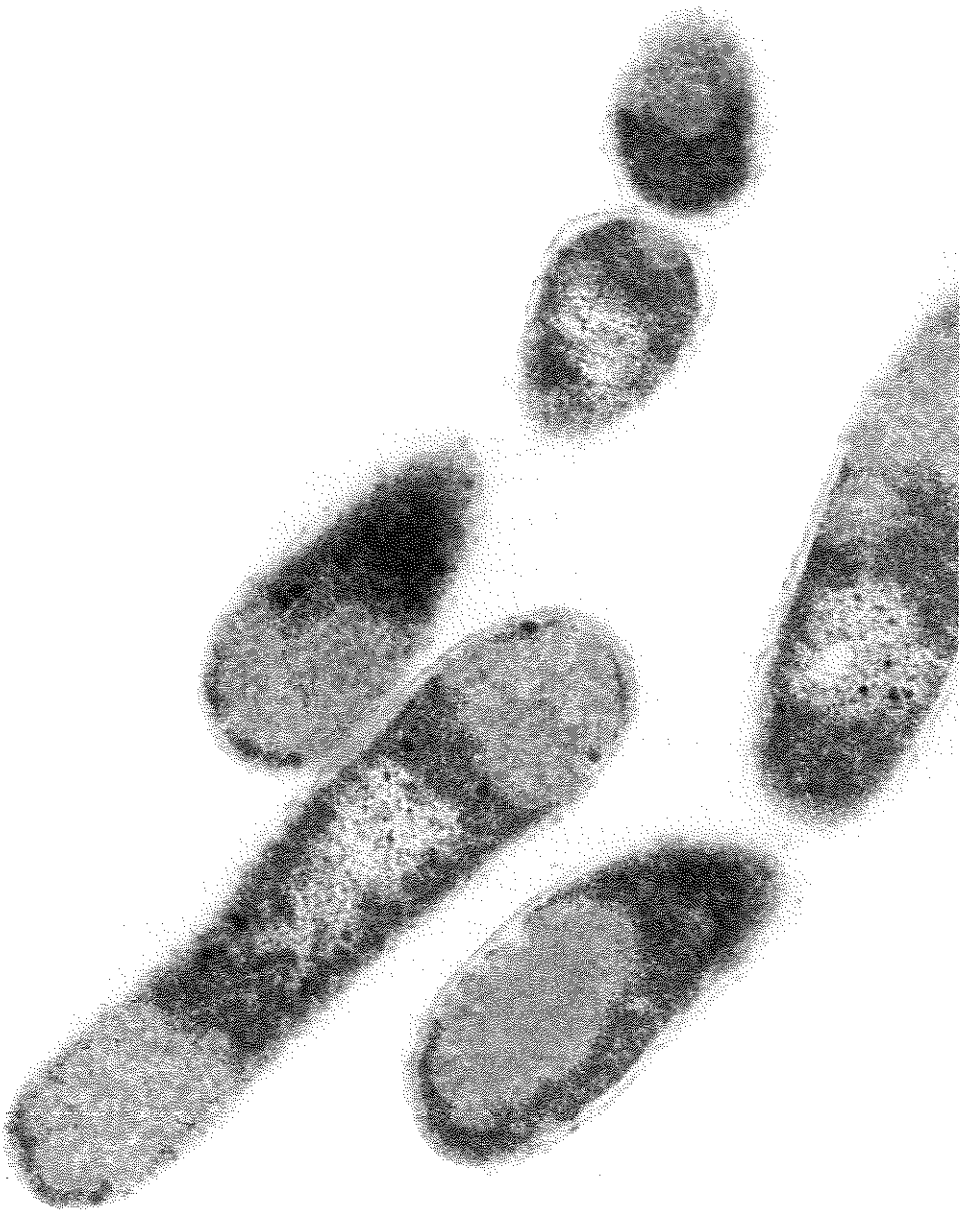
methods are not suitable to obtain large quantities of nonglycosylated Tf in contrast to the third method.

Gene expression in the cytoplasm of *Escherichia coli* characteristically results in the accumulation of levels of recombinant protein ranging up to 25% of the total cell protein (64). A drawback of overexpressing proteins in prokaryotic organisms is that the polypeptide chains are often produced in an insoluble and biologically inactive form, clustered in inclusion bodies (fig. 6).

The exact mechanism of inclusion body formation remains to date unknown. Initially this formation was attributed to the generation of an incorrectly transcribed or translated sequence (65). However, it turned out that native active proteins could be obtained from inclusion bodies by completely unfolding these proteins with strong denaturants and subsequently searching for conditions in which refolding occurs (reviewed in 64). Furthermore normal *Escherichia coli* proteins synthesized to high levels using recombinant DNA techniques can also accumulate in insoluble forms (66,78,79). Therefore inclusion body formation is neither simply a response to "foreign" proteins, nor just a consequence of proteins synthesized to concentrations above their solubility as proteins expressed at very low levels also form inclusion bodies (80).

Inclusion bodies are dense and can be sedimented by centrifugation as a first step to purify the recombinant proteins from *Escherichia coli* cell debris after lysis of the cells. Subsequently inclusion bodies can be dissolved for instance in 6 M guanidine (81,82,83) or 8 M urea (75,84,85).

In the *Escherichia coli* cytoplasm reducing conditions do exist (86). When inclusion bodies are isolated that contain proteins able to form disulphide bonds, this formation is likely to occur as a result of air oxidation. These disulphide bonds can stabilize the inclusion bodies and this is undesirable when isolation of proteins from the inclusion bodies is intended. Therefore the inclusion bodies should be dissolved under reducing conditions, e.g. in the presence of ß-mercaptoethanol or dithiothreitol (DTT).



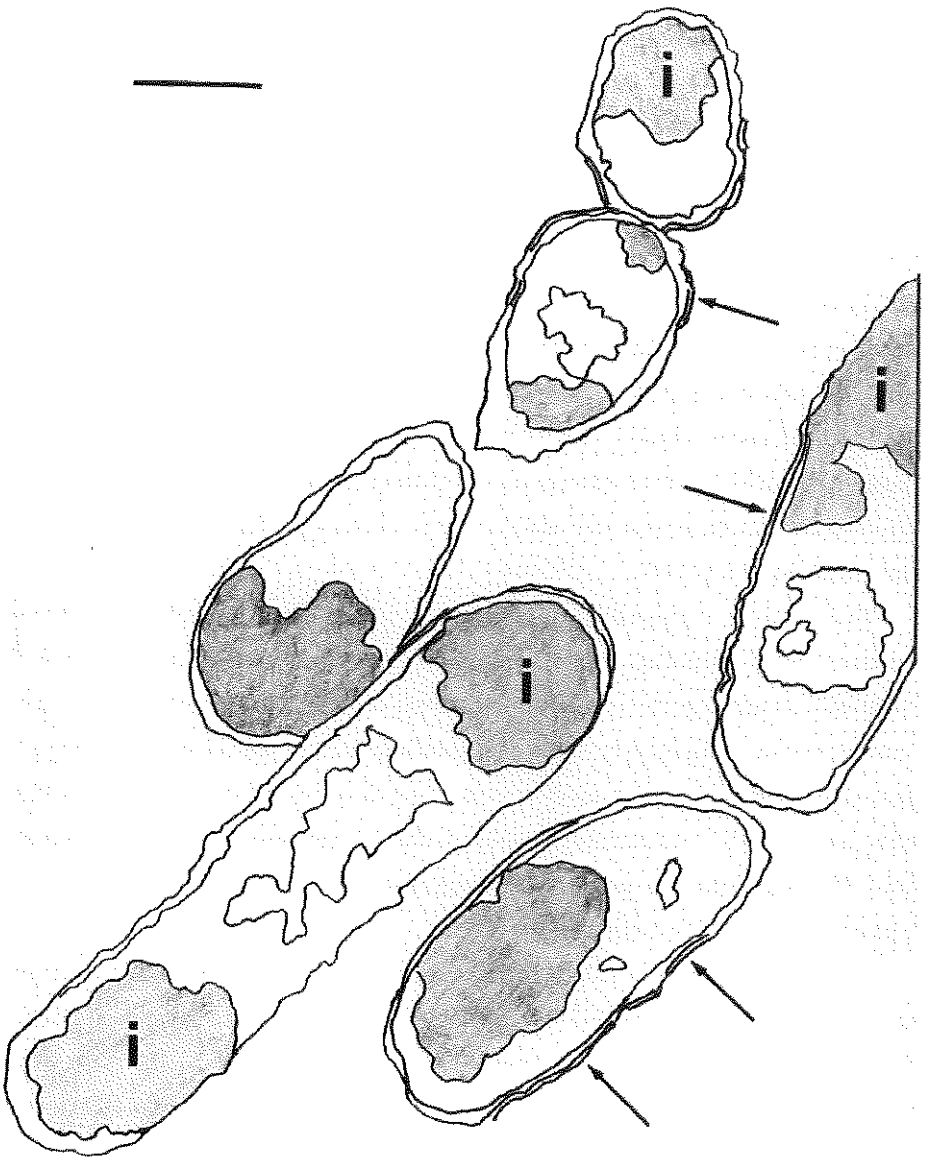


Figure 6. Inclusion bodies (i) in recombinant human transferrin producing *Escherichia coli.* No membrane can be seen around the inclusion bodies. Arrows indicate the double layer membrane of the bacteria. The bar represents 0.6μm.

1.7 Renaturation of recombinant proteins

To be biologically active, proteins have to adopt a specific three-dimensional structure. The genetic encoding for a protein however, contains only information on the lineair amino acid sequence of that particular protein, but no information for the three-dimensional conformation. It is believed that the primary structure of proteins contains the necessary information for folding into the correct state, as was first suggested by Anfinsen (87). During the late 1950s he showed that completely unfolded RNase A with its four disulphide bonds cleaved in a 8 M urea solution containing ß-mercaptoethanol, could be renatured on a quantitative basis by dialyzing away the urea and exposing the solution to oxygen. The resulting protein showed nearly 100% enzyme activity and was indistinguishable from native RNase, suggesting the protein renatured spontaneously under physiological conditions. This spontaneous renaturation is not simply a random search for a conformation with the lowest free energy, as this would, even for small proteins, take an immense amount of time (88).

Thus proteins overexpressed in prokaryotes and deposited in inclusion bodies can be renatured after dissolving the proteins using chaotropes such as urea or guanidine hydrochloride. Removing the chaotropes by means of dilution or dialysis should initiate the correct folding compelled by the amino acid sequence of the proteins. Although this method gives good yields with small proteins such as lysozyme and RNase, with bigger proteins the yield is considerably diminished due to aggregation during the refolding process. This aggregation is ascribed to hydrophobic interactions (65,89,90) and to covalent disulphide linkage formation (91).

Additional factors can reduce or even prevent correct refolding of overexpressed proteins. Prokaryotes are unable to perform N-glycosylation of proteins, lack of carbohydrates can diminish the refolding of these proteins (92,93). The lack of any prosthetic groups if necessary can also impair the refolding (93). Furthermore expression of mature proteins that normally are synthesized in eukaryotic cells as larger precursors and subsequently are processed after folding, e.g. insulin, can possibly not be refolded after production (94).

More recently biological "factors" have been discovered facilitating the (re)folding

of proteins such as protein-disulphide isomerase (PDI) (95,96) that catalyzes disulphide interchange reactions and several so called chaperones (97). These chaperonins can be found in all types of cells, in the cytoplasm, the endoplasmic reticulum and mitochondria. They are believed to catalyse protein folding and to prevent aggregation (98,99,100). Although the intrinsic information of the protein structure in the polypeptide sequence remains the basis of present renaturation experiments, the use of chaperonins in quantitative refolding procedures can become important in the near future since it has been shown that the yield of renaturation experiments increases *in vitro* (101,102,103).

1.8 Iron donation by transferrin

Tf is the major source of iron for human cells. Iron transfer from Tf to the cell is initiated by the binding of Tf to the transferrin receptor. After this interaction two iron-uptake mechanisms are suggested; iron is released from Tf and transported through the cell membrane (104,105,106), or the Tf-TfR complex is endocytosed by the cell. In both mechanisms the TfR has a central role. The first indications of the existence of a TfR were described by Jandl in 1959 (107).

The TfR is a class II receptor consisting of two identical subunits of 95 kDa each (108-111). The subunits are transmembrane glycoproteins containing at least three asparagine linked oligosaccharides; complex type glycans as well as high mannose type glycans are found on the mature receptor. The function of these glycans is unknown, but it has been suggested that they are involved in the proper folding of the molecule during biosynthesis and assembly in the endoplasmic reticulum (112,113,114) as well as the transport to the cell surface. The oligosaccharides linked at the TfR also influence the affinity for Tf (115,116).

The bulk of the TfR molecule is exposed on the cell surface and can be cleaved off the cell surface with a low concentration of trypsin. The soluble fragment generated this way is still able to bind Tf. The TfR subunits are linked by disulphide bonds. Both subunits are able to bind one Tf molecule. Tf binds to its receptor with high affinity, the dissociation constant of the TfR varies from 5-20 x 10^{-9} M. The serum concentration of Tf is 1.5-2.5 x 10^{-5} M, so the transferrin receptors should be continuously saturated with

its ligand. Figure 7 shows a schematic representation of a TfR.

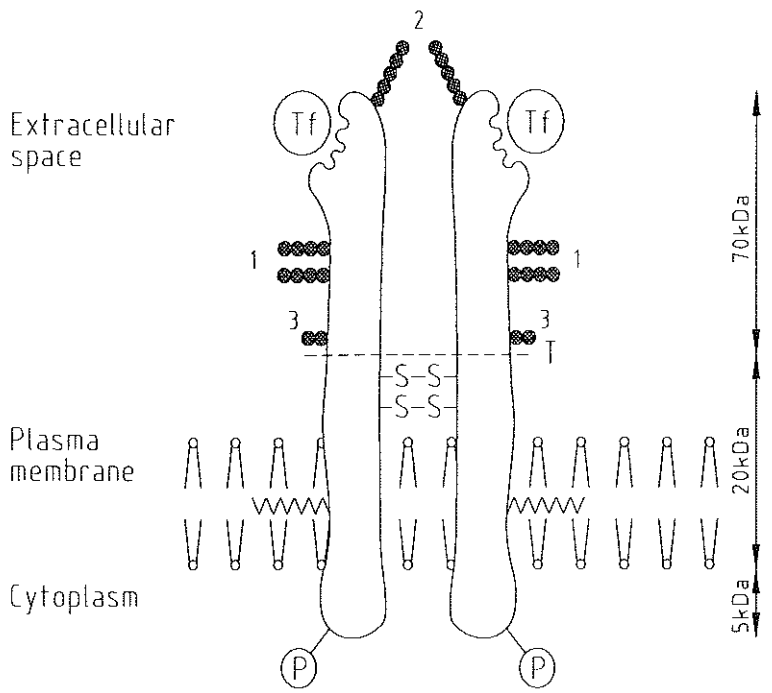


Figure 7 Schematic representation of the transferrin receptor The transferrin receptor is a dimer formed by two identical 95 kDa polypeptide structures and linked by disulphide bonds. Each monomer can bind one transferrin molecule.
Legend: 1: N-linked glycans of the high mannose type; 2: N-linked glycans of the complex type; 3: O-linked glycans; P: phosphorylation site; T: trypsin cleavage site; /\/\/\/\/ : palmitic acid, covalently bound to the receptor.

Several authors have suggested that iron can be released from Tf by a reductive process at the cell surface (117,118,119). More important is the iron uptake by means of "receptor mediated endocytosis". This process is initiated by the binding of Tf to its receptor. It is believed that the primary receptor-recognition site is situated in the C-terminal lobe of Tf (120). The transferrin receptors are concentrated in clathrin-coated pits. At physiological pH the affinity of the TfR is much higher for diferric Tf than for monoferric- or apotransferrin, ensuring an effective use of the transferrin receptors. The clathrin-coated pit folds into a vesicle and becomes internalized into the cytoplasm. Once internalized, the coated vesicles lose their clathrin coat and become rapidly acidified by means of a proton-pump. Lowering the pH to values ranging between 5 and 6 serves several purposes.

Firstly, the acidification facilitates the release of iron from Tf. However, it takes several hours before total release of iron from Tf is achieved in an environment with a pH of 5.5. Since the endocytosis/exocytosis cycle of Tf is only a matter of minutes (121), it has been suggested by several authors that the TfR plays a crucial role in the release of iron from Tf under these conditions (122,123). The released iron is subsequently removed from the endosome by unknown mechanisms.

Secondly, lowering the pH creates reversible conformational changes of the TfR (124). These changes could induce protease insensitivity and could be of importance of segregating the TfR from other receptors.

Finally the acidification also increases the affinity of the TfR for apotransferrin ensuring the binding and subsequent transfer of this carrier protein to the extracellular cavity. This is an important mechanism in the protection of Tf against lysozomal degradation. Once exposed to the neutral pH of the extracellular environment, the apotransferrin-TfR complex rapidly falls apart, leaving the apotransferrin for another quest for iron.

Regarding the function of the Tf glycans, one other mechanism of iron uptake should be considered, i.e. uptake by means of asialoglycoprotein receptors (ASGR). This C-type lectin (125) has been described by Pricer Jr. *et al.* in 1974 (126) and can be found on hepatocytes. It has a high affinity for galactose and N-acetylgalactosamine (127,128)

terminating oligosaccharides of desialylated glycoproteins including partially desialylated Tf. Hepatocytes are able to obtain iron through ASGR (129,130).

Initially it was assumed that Tf which entered the cell via the ASGR was degraded. However, it was demonstrated that partially desialylated Tf was exocytosed after entering hepatocytes through the ASGR (131). The TfR also plays an important role in this so called "diacytosis" (132). Tf even can be resialylated during this process (133,134,135).

1.9 Aims of this thesis

Although initially neglected, the presence of oligosaccharides on glycoproteins are now a source of intriguing questions. Constantly new data on the microheterogeneity of Tf becomes available but the biological significance of the glycans is still not elucidated. With this work we hope to acquire more insight in the influence of the glycans on the structure and biological function of Tf.

Changes in the microheterogeneity of Tf are used as a clinical marker for (gross) alcohol abuse. We have investigated the usefulness of this marker for detection of a more limited degree of alcohol abuse.

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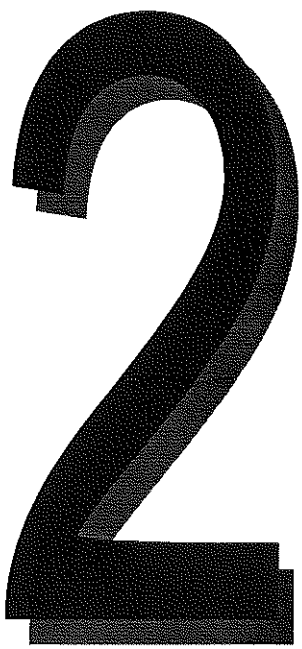
Materials and methods



2.1 Introduction

This chapter gives an overview of the general techniques used to obtain the results described in this thesis.

All chemicals used were of analytical grade.

2.2 Preparation and isolation of different transferrin glycovariants

2.2.1 Introduction

The bi-bi antennary transferrin (bi-bi Tf) subfraction was purified from commercially obtained transferrin (Tf) (Behringwerke). Bi-bi Tf was step-wise partially deglycosylated by the proper exoglycosidases. Following each enzyme incubation, Tf was purified by using a Sepharose-bound anti-transferrin IgG column (1) or by means of preparative isoelectric focusing (2,3) and the effect of the deglycosylation was determined by carbohydrate analysis (4) or amino acid analysis (5,6).

Furthermore aglyco Tf was isolated from a patient suffering from the Carbohydrate Deficient Glycoprotein syndrome.

2.2.2 Isolation of bi-bi antennary transferrin

The bi-bi Tf subfraction was isolated from the bi-tri- and tri-tri Tf subfractions by means of a Sepharose ConA (Pharmacia) column (7). The binding of bi-tri Tf to ConA is not as strong as the binding of bi-bi Tf to this lectin. Tri-tri Tf does not bind to ConA at all. The ConA column was equilibrated with buffer containing 0.05 M Tris/HCl, 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$ (pH 7.6). This buffer will further be referred to as ConA buffer. The binding of bi-tri Tf to a Sepharose ConA column can be broken by eluting the column with ConA buffer containing 10 mM glucose. Bi-bi Tf can be eluted from the column by elution with buffer containing 150 mM α-methyl-glucopyranoside. Figure 1 shows a separation of commercially obtained Tf into tri-tri-, bi-tri- and bi-bi Tf subfractions.

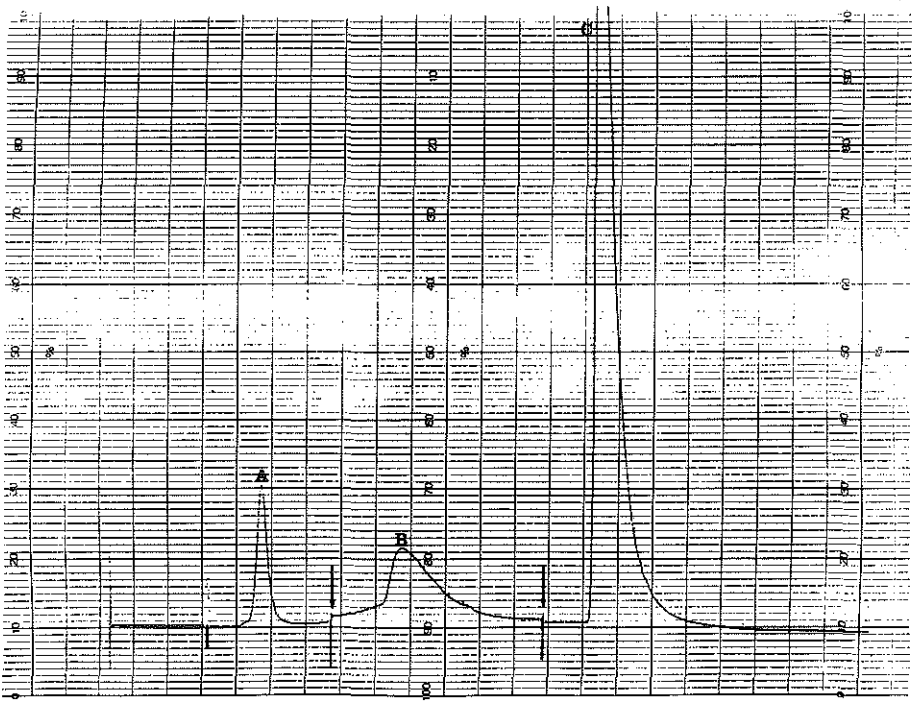


Figure 1. Fractionation of commercially obtained Tf into tri-tri- (A), bi-tri- (B), and bi-bi Tf (C) subfractions. Arrows indicate buffer changes to respectively ConA buffer containing 10 mM glucose and ConA buffer containing 150 mM α-methyl-gluco-pyranoside.

2.2.3 Stepwise enzymatic deglycosylation of bi-bi antennary transferrin

2.2.3.1 Neuraminic acid removal

For this purpose, bi-bi Tf was incubated at 37°C with *Clostridium perfringens* neuraminidase EC 3.2.1.18 (type X, Sigma) in sodium-citrate buffer (50 mM, pH 5.0) for at least 24 hours in a ratio of 1 mU enzyme/mg Tf, giving rise to partially and totally desialylated Tf subfractions.

The asialo Tf subfraction was isolated from this mixture by preparative isoelectric focusing (2) on an Ultrodex gel (Pharmacia, LKB) as described below (2.2.5).

2.2.3.2 Galactose removal

The asialo Tf subfraction was incubated with *Diplococcus pneumoniae* ß-galactosidase (EC 3.2.1.23, Boehringer Mannheim) for at least 24 hours. In our experiments longer incubation times with a lower enzyme/Tf ratio yielded better results. Dialysis of the enzyme/Tf mixture during incubation also improved the results. Incubations were performed in sodium-citrate buffer (50 mM, pH 6.5) at 37°C, using 2.5 mU enzyme/mg Tf.

Following incubation the agalacto Tf was isolated by preparative isoelectric focusing (see 2.2.5) and the galactose content was determined. A decrease in the galactose content of 95% or more compared to bi-bi Tf was considered successful, otherwise the enzyme incubation and isolation were repeated.

2.2.3.3 Removal of the distal glucosamine

The agalacto Tf subfraction was incubated with *Diplococcus pneumoniae* N-acetyl-ß-D-glucosaminidase (EC 3.2.1.30, Boehringer Mannheim) at 37°C in sodium-citrate buffer (50 mM, pH 4.8) for at least 24 hours. As with ß-galactosidase, longer incubations yielded better results. The incubation was performed with an agalacto Tf concentration of approximately 20 mg/ml and 30 mU enzyme/mg Tf.

After the incubation this glucosamine deficient Tf was purified by sepharose-bound anti-transferrin IgG affinity chromatography (see 2.2.5.) and is henceforward referred to as aglucosamine Tf. The effectivity of the incubations was examined by determining the glucosamine content.

2.2.4 Isolation of carbohydrate deficient transferrin

Aglyco Tf was isolated from a patient suffering from Carbohydrate Deficient Glycoprotein syndrome by means of Sepharose-bound anti-transferrin IgG affinity chromatography and preparative isoelectric focusing (see 2.2.5.). Complete lack of glycans

in this isolated subfraction was demonstrated by carbohydrate analysis.

2.2.5 Purification of partially deglycosylated transferrin

The partially deglycosylated transferrins were purified using several methods: preparative isoelectric focusing in an Ultrodex gel (Pharmacia) or in an Immobiline gel (Pharmacia) or by means of affinity chromatography. The application of these different purification methods was merely based on the disparity in the amount of protein that could be purified in one time.

2.2.5.1 Preparative isoelectric focusing in an Ultrodex gel

A 4% Ultrodex gel was prepared with a 5% Ampholine solution (Ampholine Carrier Ampholytes 5-8) and poured into a tray placed on an exactly horizontal table. The excess of water was evaporated from this suspension using a small blow-drier and this process was carefully monitored by regularly weighing of the gel. Thereupon the gel was placed on a Multiphor cooling plate (Pharmacia) and the electrode strips were placed on the gel, the anodic strip soaked in 1 M phosphoric acid and the cathodic strip soaked in 1 M sodium hydroxide (8). The gel was prefocused for 90 minutes. The maxima of the power supply were set on 1200 V, 45 W and 75 mA (2). Following the prefocusing an electrode strip soaked in Tf/enzyme solution was placed into the gel approximately 1 cm above the cathode and this material was focused for approximately 120 minutes. Subsequently the asialo Tf containing zone of the gel was transferred into a column with a spatula and the protein was eluted from the gel. Using this method several hundred mgs of asialo Tf preparations could be purified in one run.

2.2.5.2 Preparative isoelectric focusing using Immobiline DryPlates

For the purification of smaller amounts of Tf Immobiline DryPlates (pH 5.6 - 6.6) were used (3). Prior to the isoelectric focusing, a piece of DryPlate was rehydrated in distilled water for 90 minutes. Subsequently the gel was carefully dried using filter paper and placed on the cooling bed of a Multiphor (Pharmacia). Two electrode strips were applied on the gel, the strip on the cathodic site was soaked in 10 mM sodium hydroxide

and the strip on the anodic site was soaked in 10 mM phosphoric acid. The Tf/enzyme solution was applied on the gel near the cathode and the material was focused for several hours. When purifying small amounts of Tf subfractions, the zone containing e.g. asialo Tf was cut out and the protein was washed out of the gel by placing it in a tube containing 10 mM sodium bicarbonate. When loading the gel more heavily, the asialo Tf even exuded out of the focusing zone (fig.2) and could easily be collected by soaking it into electrode strips.

2.2.5.3 Purification of transferrin subfractions using CNBr-activated Sepharose 4B

Rabbit anti-human transferrin IgG was coupled to CNBr-activated Sepharose 4B (Pharmacia) as described by Van Eijk (1). This gel was used to perform affinity chromatography to separate the Tf from the enzyme solution. The Tf/enzyme solution was eluted through the column with phosphate-citrate buffer pH 8.0 containing 0.25 M NaCl. Subsequently coupled Tf was washed from the column with phosphate-citrate buffer pH 2.8 containing 0.25 M NaCl.

2.2.6. Carbohydrate analysis

To monitor the result of the enzymatic incubations using ß-galactosidase and N-acetyl-ß-D-glucosaminidase, carbohydrate analyses were performed after each enzymatic step on purified Tf subfractions.

For the determination of the galactose content, 100 to 400 µl aliquots of agalacto Tf solution with a known protein concentration were hydrolysed at 100°C in 2 M TFA for 24 h at pressures < 1mm Hg. The hydrolysed mixture was evaporated at room temperature, the residue then was dissolved in 100 µl distilled water and mixed with 900 µl 100% ethanol. The hydrolysate was cleaned up with 6 µl analytical resin suspension (Ultra pac 11, Pharmacia/LKB), followed by partition chromatography with a cation-exchange resin using 90% ethanol as eluent and tetrazolium chloride as reagent (4).

N-Acetylglucosamine was determined as glucosamine after special hydrolysis in 3 M pTSA during 24 h at 110°C and P < 1mm Hg, using a slightly adapted amino acid analysis program (Biochrom 20, Pharmacia/LKB, Cambridge, UK) (5,6).

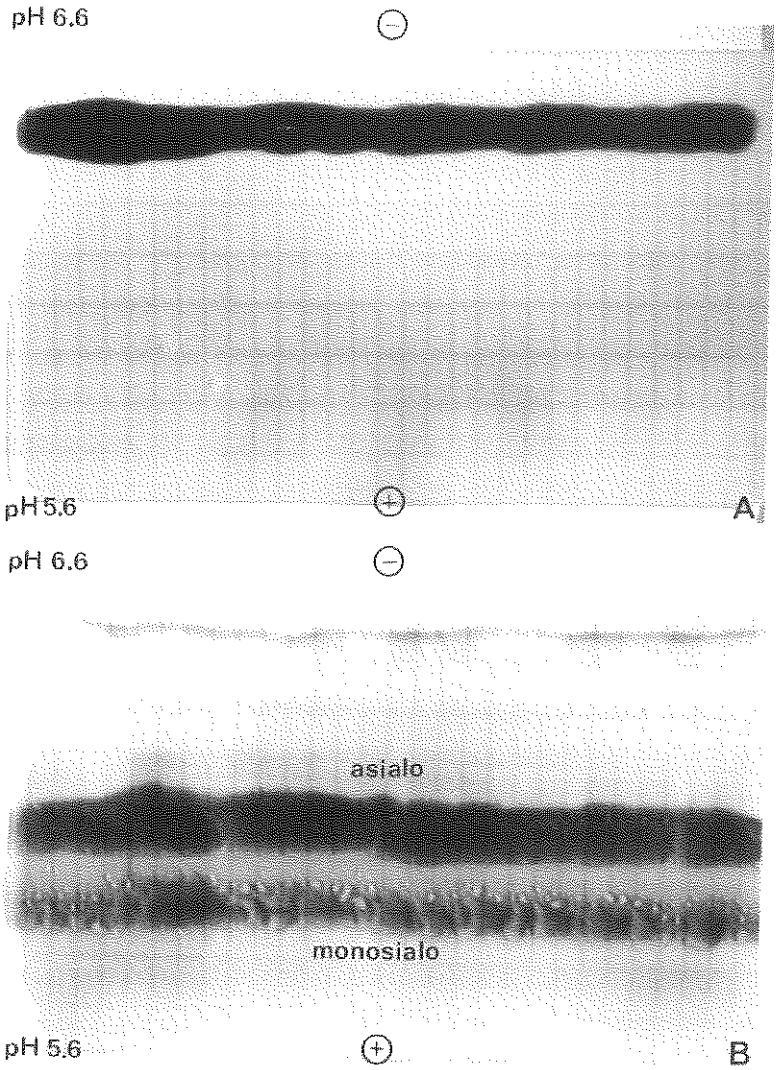


Figure 2. Isolation of asialo fraction from a bi-bi Tf solution after incubation with neuraminidase. The Tf solution is loaded on an Immobiline gel (pH 5.6 - 6.6, A). The asialo and monosialo Tf subfractions exude out of the gel during focusing (B) and can easily be collected.

2.3 Production, isolation and renaturation of recombinant human aglycotransferrin and its half-molecules

2.3.1 Introduction

In the following section the production, isolation and renaturation of recombinant aglyco Tf and recombinant N-terminal and C-terminal half aglyco Tf is described. The recombinant proteins are produced by *Escherichia coli*.

The construction of the recombinant transferrins producing *E. coli* strains has been described by us (9). In short, for expression purposes the *E. coli* K12 strain LC137 was used. This bacterial strain is deficient in heat-shock response (*htpR*) and in La-protease (*lon*). A transferrin expression plasmid *ptac*RRTF3101 was constructed using the following plasmids: pPLc2833 (10), pMS2-7 (11) and pTfR27A (12). For the production of the N-terminal half-transferrin, codon 338 was mutated to a termination codon. For the production of C-terminal half-transferrin, the N-terminal half of the coding sequence was removed. Transformation of LC 137 required electroporation, and was performed using a CellJect Basic apparatus at 2500 V (Eurogentec, Liège).

Bacteria are not able to attach N-linked glycans to their proteins and could serve therefore as a good source of aglyco Tf. The recombinant transferrins are deposited in a denatured state in large amorphous particles; inclusion bodies.

2.3.2 Production of the recombinant aglycotransferrins

We inoculated 25 ml Luria Complete (LC) medium containing ampicillin (50 µg/ml) and spectinomycin (100 µg/ml) with one of the recombinant transferrins producing *Escherichia coli* strains. Incubation was performed at 30°C in an air-shaker for approximately 22 hours. Subsequently this culture was added to 1 L LC medium and incubated for another 20 hours under similar conditions. After 2½ hours however, the p_{tac}-promotor was induced by adding 0.2 g isopropyl-ß-D-thiogalactopyranoside (IPTG,9).
Finally the cells were collected by centrifugation and stored at -20°C until further processing.

2.3.3 Isolation of the inclusion bodies from Escherichia coli

Bacteria harvested from 1 L LC medium were resuspended in 10 ml lysis buffer (see below), 4 mg lysozyme was added and the cells were incubated at room temperature for 30 minutes. Subsequently the bacteria were snap-frozen in a mixture of solid CO_2 and ethanol. The sample was thawed and incubated with approximately 300 U DNase I for 45 minutes at room temperature. The produced recombinant transferrins are deposited in so called inclusion bodies in the bacteria. These inclusion bodies were isolated from the cell lysate by centrifugation at 12,000 g for 15 minutes. The pellet was washed 3 times in washing buffer (see below) and dissolved in 10 ml dissolving buffer (see below).

Composition of the used buffers

Lysis buffer:	50 mM Tris/HCl, 1 mM EDTA, 250 mM NaCl, 5 mM DTT, 5 mM sodium bisulfite, pH: 8.0
Washing buffer:	50 mM Tris/HCl, 1 mM DTT, 1% Triton X-100, pH: 8.0
Dissolving buffer:	8 M urea, 1 mM DTT, 40 mM Tris/HCl, 10% glycerol, 0.02% NP-40, pH: 7.6

2.3.4 Renaturation of the recombinant transferrins

In general, renaturation was acquired by reoxidizing the reduced recombinant proteins in a concentration ranging from 10-20 µg/ml at 6°C using a glutathione redox couple (13-16). Briefly, the protein concentration of the inclusion body solution was determined according to the method described by Bradford (17). Next, renaturation was initiated by diluting this inclusion body solution in renaturation buffer (0.1 mM Tris/HCl, 0.1 mM Na-EDTA, 1.0 mM GSH, pH 8.2, 6°C) to a final protein concentration of approximately 20 µg/ml. The demineralized water used to prepare the renaturation buffer was degassed by means of boiling and it was stored under an atmosphere of nitrogen. The total volume of renaturation buffer used in one experiment mounted up to 60 L. Next, the mixture was incubated for 15 minutes and subsequently GSSG was added to a final concentration of 0.5 mM. This solution was incubated for another 22 hours at 6°C.

After this step the solution was concentrated and dialyzed against 10 mM $NaHC0_3$

using an Ultrasette with an omega 10 kDa membrane (Filtron). Subsequently the solution was filtered over 5 µm low ash polyvinyl chloride membranes (GelmanSciences) and concentrated further using successively an Amicon Stirred Cell with a PM 10 membrane and Centriflo CF 25 cones (Amicon).

2.3.5 Purification of the recombinant transferrins

Due to the fact that the inclusion bodies contain other proteins next to the recombinant aglycotranferrins, the latter proteins have to be purified after renaturation. For this purpose the renatured proteins were dialyzed against 0.05 M phosphate buffer (pH 5.5) for 24 hours. As a result a substantial part of the (unwanted) proteins precipitated. The suspension was centrifuged at 12,000 g for 15 minutes and the supernatant was collected. The pH of the supernatant was adjusted to 8.2 and the recombinant aglyco Tf was saturated by adding an excess of iron ($FeCl_3$/NTA complex) and 5% $NaHCO_3$ (w/v). Recombinant aglyco Tf was isolated from this solution by means of Sepharose-bound anti-transferrin affinity chromatography (see 2.2.4).

2.4 Labeling of the transferrin subfractions

The different Tf subfractions (enzymatically partially deglycosylated or from recombinant origin) used for binding studies were labeled with ^{125}I and also with ^{59}Fe (Amersham) when used for uptake studies. Labeling with ^{59}Fe and ^{125}I was performed as described by Starreveld (18). For iodination, 1 mg of Tf subfraction was incubated with ^{125}I (0.83 mCi) for 10 minutes at room temperature in a glass vial coated with 100 µg Iodo-Gen (Pierce). Free ^{125}I was removed with a Sephadex PD-10 column (Pharmacia) followed by extensive dialysis against a 10 mM Tris/HCl buffer (pH 8.2) for 3 x 24 hours. When doubly labeled Tf subfractions were required, the Tf subfractions were labeled with ^{59}Fe prior to iodination.

For the labeling with ^{59}Fe, apo-Tf subfractions are required. Apo-Tf was prepared by dialyzing Tf against 0.1 M acetate buffer (pH 5.0) containing 0.04 M EDTA. Subsequently apo-Tf was dialyzed against distilled water and 10 mM Tris/HCl (pH 8.2). 5% $NaHCO_3$ (w/v) was added before incubating the protein with ^{59}Fe/NTA in an iron to Tf

molar ratio of 3 to 1 for 30 minutes at 37°C. The excess of iron was removed with a Sephadex PD-10 column.

2.5 Cell culture conditions and cell related experiments

2.5.1 Culturing human hepatoma cells

The human hepatoma cell line PLC/PRF/5 was obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.). The cells were cultured in cell culture flasks (Costar) in a Salvis Biocenter 2001. The cells were grown at 37°C in humidified 5% CO_2/95% air in Dulbecco's Modification of Eagle's Medium (DMEM, ICN Biomedicals) containing 20 mM Hepes. Prior to use we added 10% (v/v) Fetal Bovine Serum (FBS, ICN Biomedicals), 4 mM L-Glutamine (Merck) and 0.05 mg/ml Gentamycin (Pharmachemie B.V.) to this medium. Culture medium was changed every third or fourth day.

2.5.2 Transferrin binding determination

Approximately 4 days before starting a binding experiment the cells were harvested from the culture flasks and disseminated in 35 mm cell culture cluster dishes (Costar). Experiments were performed as soon as the cells formed a continuous monolayer. The culture medium was removed and the cells were washed twice with PBS of 4°C. To minimize the expression of transferrin receptors saturated with endogenous Tf, the cells were incubated with PBS of 37°C for 15 minutes and subsequently washed 3 times with icecold PBS. The PBS was replaced by incubation medium (DMEM containing 20 mM Hepes and 4 mM L-Glutamine). A concentration range from 0.25 µg/ml to 3 µg/ml of the ^{125}I labeled diferric Tf subfractions of interest was added to the dishes and the cells were incubated 90 minutes at 0°C.

Nonspecific binding was determined by the following experiment: PLC/PRF/5 cells were incubated with ^{125}I labeled transferrins with a 50 times excess of unlabeled Tf, the latter being of the same subfraction as the type investigated. Following the incubations, the cells were washed 3 times with PBS (4°C), lysed by addition of distilled water and

collected with a rubber "Policeman". The dishes were rinsed with 0.1% Triton X-100. The samples were homogenized by sonication in melting ice for 20 seconds and samples were taken for protein determination according to the method described by Bradford (17). Surface bound radioactivity was measured with a Packard 500C autogamma spectrometer.

2.5.3 Transferrin and iron uptake determination

Iron uptake studies were performed in similar culture dishes as the binding studies. The cells, forming a continuous monolayer, were washed twice with PBS (37°C) and incubated with incubation medium as used in the binding studies. At t=0 doubly labeled Tf was added to a final concentration of 3 µg/ml. At preset times the incubation medium was removed and the cells were washed 3 times with icecold PBS. To remove all surface bound Tf, the cells were incubated in acetate buffer (25 mM sodium acetate, 0.15 M NaCl, 20 mM $CaCl_2$, pH 4.5, 4°C) for 8 minutes followed by a 2 minute incubation in PBS (4°C). This procedure was repeated once. Subsequently the cells were lysed and collected as described in 2.5.2. Intracellular ^{59}Fe and ^{125}I were determined with a Packard 500C autogamma spectrometer.

2.6 Protein determination

Protein concentrations in the samples collected after the different cell incubations as described above, and in the inclusion body dissolving buffer (see 2.3.3.) were determined according to Bradford (17). Bovine serum albumin in different concentrations was used as a standard.

2.7 Analytical electrophoresis techniques.

2.7.1 Native and SDS polyacrylamide gel electrophoresis.

Analytical electrophoresis was either performed on a PhastSystem (Pharmacia) using PhastGels (12.5% homogeneous or 8-25% gradient) or on a Mini Protean system II (Bio-Rad) using Ready Gels (Bio-Rad). No additional sample preparations are needed for performing a native polyacrylamide gel electrophoresis (PAGE) on the PhastSystem. Prior

to performing a native PAGE on the Mini Protean system, the samples were mixed with 20% glycerol (v/v) and 0.005% Bromophenol Blue (w/v). The settings for performing a native 12.5% homogeneous PAGE or a native 8 - 25% gradient PAGE with the PhastSystem are summarized below. When the Mini Protean system was used, the power supply was limited to 200 V and electrophoresis was terminated as soon as the tracking dye (Bromophenol Blue) reached the bottom of the gel.

Prior to performing a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the samples were mixed with an equal volume sample buffer (0.1 M Tris, 0.04 M DTT, 0.14 M SDS, 20% glycerol (v/v), 0.005% Bromophenol Blue (w/v), pH 6.8) and boiled for 4 minutes. Subsequently the samples were applied on the gel.

The settings for performing a 12.5% SDS-PAGE with the PhastSystem are summarized below. The running conditions used for a SDS-PAGE on the Mini Protean system were similar as for a native PAGE (see above).

Homogeneous 12.5% SDS PAGE

Sample applicator down at			x.2	0Vh		
Sample applicator up at			x.3	0Vh		
Sep x.1	250V	10.0mA		3.0W	15°C	1Vh
Sep x.2	250V	1.0mA		3.0W	15°C	1Vh
Sep x.3	250V	10.0mA		3.0W	15°C	70Vh

Homogeneous 12.5% Native PAGE

Sample applicator down at			x.2	0Vh		
Sample applicator up at			x.2	2Vh		
Sep x.1	400V	10.0mA		2.5W	15°C	10Vh
Sep x.2	400V	1.0mA		2.5W	15°C	2Vh
Sep x.3	400V	10.0mA		2.5W	15°C	120Vh

8 - 25% gradient Native PAGE

Sample applicator down at			x.2	0Vh		
Sample applicator up at			x.3	2Vh		
Sep x.1	400V	10.0mA		2.5W	15°C	10Vh
Sep x.2	400V	1.0mA		2.5W	15°C	2Vh
Sep x.3	400V	10.0mA		2.5W	15°C	268Vh

2.7.2 Analytical isoelectric focusing

Analytical isoelectric focusing was performed on a PhastSystem using PhastGel IEF (pH: 4 - 6.5).

Isoelectric focusing on PhastGel pH 4 - 6.5

Sample applicator down at			x.2	0Vh		
Sample applicator up at			x.3	0Vh		
Sep x.1	2000V	2.0mA		3.5W	15°C	485Vh
Sep x.2	200V	2.0mA		3.5W	15°C	15Vh
Sep x.3	2000V	5.0mA		3.5W	15°C	150Vh
Sep x.4	200V	2.0mA		2.0W	15°C	500Vh

For the determination of the microheterogeneity of transferrin in serum samples (see chapter 6) a piece of Immobiline gel, ranging from pH 5 - 6, was cut out from an Immobiline gel (pH 4 - 7, Pharmacia) to the same size as a PhastGel. Prior to isoelectric focusing this gel was rehydrated for 90 minutes by applying 1 ml 20% (v/v) glycerol on the surface of the gel. Tf in the serum samples was saturated by adding 2 µl 0.5 M $NaHCO_3$ and 2 µl 10 mM Fe(III)-citrate to 50 µl serum.

After focusing the gel was incubated with 200 µl rabbit anti-human Tf for 20 minutes at room temperature. Subsequently the gel was washed in 0.15 M NaCl during 48 hours before being stained. During this interval, the washing solution was changed several

times. After staining (see 2.7.3), the gels were quantitated with an Ultroscan XL densitometer (He/NeLaser, λ = 633 nm, Pharmacia) connected to a personal computer with GelScan^{TM} XL software (version 2.1, Pharmacia).

Isoelectric focusing on Immobiline pH 5 - 6

Sample applicator down at			x.2	0Vh		
Sample applicator up at			x.3	0Vh		
Sep x.1	2000V	1.5mA		1.5W	15°C	485Vh
Sep x.2	200V	0.3mA		0.3W	15°C	15Vh
Sep x.3	2000V	1.5mA		1.5W	15°C	1800Vh

2.7.3 Staining of the gels.

The gels were incubated with 20% TCA for 20 minutes prior to staining them with Coomassie Brilliant Blue R (1 g/L) at 50°C for 10 minutes as described in the PhastSystem Users Manual (Pharmacia). Destaining was performed in 30% methanol (v/v), 10% acetic acid (v/v) at room temperature.

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Influence of transferrin glycans on receptor binding and iron donation

3.1 Introduction

In chapter 1 (1.5) specific variations in the oligosaccharide moieties of transferrin (Tf) in several (patho)physiological conditions such as pregnancy, rheumatoid arthritis, malignancies and alcohol abuse (1-10) were discussed. As stated, the significance of these variations as well as the function of the Tf glycans remain unclear.

In the Carbohydrate Deficient Glycoprotein (CDG) syndrome, first reported by Jaeken *et al.* (11), strong deviations of the carbohydrate content of serum glycoproteins (e.g. transferrin) can be found. Even a subfraction of Tf completely lacking carbohydrates has been described (12,13). The consequence of these variations on the function of Tf remains unknown. Since the transferrins are not homogeneously affected, and all other glycoproteins are also affected, it is impossible to assign any of the clinical manifestations of the CDG syndrome merely to the Tf deviations.

Recently, Hu *et al.* reported (14) that in comparison to its fully glycosylated form, rat aglyco Tf showed a decreased iron donating capacity towards rat hepatocytes. This was not ascribed to a decreased affinity of the transferrin receptor (TfR) for the deglycosylated protein, but to a decreased interaction of aglyco Tf with "low-affinity binding sites" on the rat hepatocytes. His conclusions, however, were not based on binding experiments. The author also reported that no significant differences were found between rat aglyco Tf and rat Tf with respect to the affinity for iron, and with respect to the kinetics of the release of this metal from the N- and C-lobe.

Mason *et al.* (15) ran some experiments in a human cell test system. He reported that no difference could be found regarding receptor binding and iron donating capacities between human aglyco Tf (obtained by site-directed mutagenesis) and commercially available human Tf. However, as he conducted his experiments at 37°C, he unwillingly restricted himself to uptake studies.

We decided to run proper binding experiments at 4°C to compare the affinity of normal human bi-bi Tf to 3 partially deglycosylated subfractions and to human aglyco Tf. Subsequently we performed Tf and iron uptake studies at 37°C with bi-bi Tf, with the 3 subfractions and with the human aglyco Tf. The experiments were run with the human hepatoma cell line PLC/PRF/5, otherwise known as the Alexander cell line. This cell line

was chosen because of the absence of the asialoglycoprotein receptor (16), in this way avoiding one of the difficulties of non TfR mediated binding and uptake.

3.2 Materials and Methods

3.2.1 Partial deglycosylation of bi-bi-antennary Tf

In general we used human bi-bi Tf as a standard and as starting material for deglycosylation. Bi-bi Tf was prepared from commercially obtained human Tf by means of a Sepharose ConA column (see chapter 2).

Partial deglycosylation was performed step-wise up to the mannose bifurcation (fig. 1) by the proper exoglycosidases (neuraminidase, ß-galactosidase and N-acetyl-ß-D-glucosaminidase), resulting in 3 partially deglycosylated Tf subfractions. In the following text the subfractions will be referred to as asialo Tf, agalacto Tf and aglucosamine Tf. Regarding aglucosamine Tf, please note that 1 mole aglucosamine Tf still contains 4 moles of glucosamine in the chitobiose core. Attempts to deglycosylate aglucosamine Tf any further by alpha-mannosidase (Boehringer Mannheim) failed several times although conditions were varied. Removing $Man_3GlcNac_1$ from aglucosamine Tf following the method described by Muramatsu (17) using endoglycosidase D (Seikagaku, Tokyo, Japan) also failed for unknown reasons.

Each enzymatical incubation step was followed by a purification of the incubated Tf as described in detail in 2.2.5.

3.2.2 Isolation of aglyco Tf

Aglyco Tf was obtained from a patient suffering from Carbohydrate Deficient Glycoprotein syndrome. It was isolated by means of Sepharose-bound anti-transferrin IgG affinity chromatography and preparative isoelectric focusing as described in 2.2.4.

3.2.3 Carbohydrate analysis

Carbohydrate analyses were performed on the purified Tf and on the aglyco Tf samples as described in 2.2.6. Complete lack of glycans on this isolated aglyco Tf was

demonstrated by carbohydrate analysis.

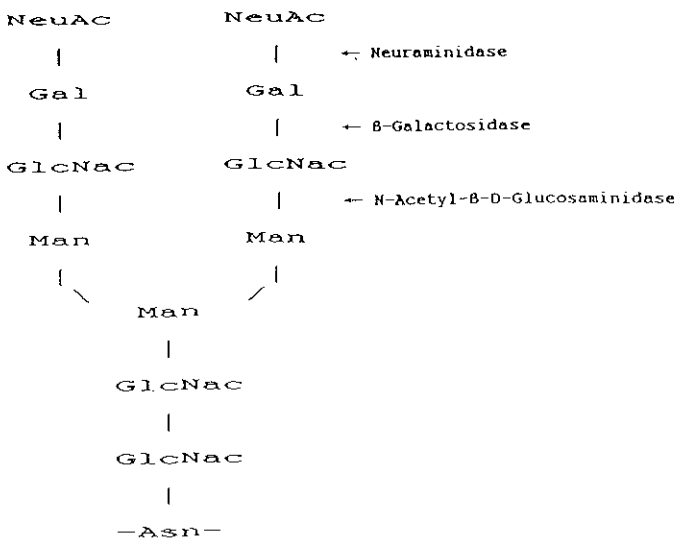


Figure 1. Biantennary glycan chain of the complex type showing the cleavage sites of the exoglycosidases used for partial deglycosylation.

3.2.4 Electrophoresis and isoelectric focusing

Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) and isoelectric focusing were performed as described in 2.7 on a PhastSystem (Pharmacia, LKB).

3.2.5 Labeling of the transferrin subfractions

Radio-labeling with ^{125}I and ^{59}Fe of bi-bi Tf, the 3 Tf subfractions and aglyco Tf was performed as described in 2.4.

3.2.6 Cell culturing conditions

The human hepatoma cell line PLC/PRF/5 (European Collection of Animal Cell Cultures, Salisbury, U.K.) was cultured in cell culture flasks (Costar) at 37°C in humidified 5% CO_2/95% air. Details are described in 2.5.1.

3.2.7 Binding studies

Binding to hepatoma cells of ^{125}I labeled partially deglycosylated Tf subfractions was compared simultaneously in one experiment to the binding of ^{125}I labeled bi-bi Tf. Hepatoma cells were incubated (90 minutes, 0°C) with ^{125}I labeled Tf in a concentration ranging from 0.25 µg to 3 µg per ml incubation medium. All binding experiments were run in duplicate. Nonspecific binding was determined in a parallel experiment by adding to the ^{125}I labeled Tf a 50 times excess of unlabeled Tf of the same subfraction as the type investigated.

In a second experiment the binding of ^{125}I labeled aglyco Tf was compared to the binding of ^{125}I labeled bi-bi Tf. Unfortunately it was not possible to determine nonspecific binding for the aglyco Tf in the presence of a 50 times excess of unlabeled aglyco Tf, because of its minute availability. Instead of an excess of unlabeled aglyco Tf, an excess of unlabeled bi-bi Tf was used.

After incubation, the cells were washed, lysed and collected. Surface bound radioactivity was measured with a Packard 500C autogamma spectrometer and the protein concentration of the different samples was determined according to the method described by Bradford (18). See for a more detailed description of the Tf binding assays chapter 2 (2.5.2).

3.2.8 Uptake studies

Uptake studies were performed as described in detail in 2.5.3. Briefly, at t=0 the hepatoma cells were incubated with ^{59}Fe and ^{125}I labeled Tf in a concentration of 3 µg/ml. At indicated times the incubation medium was removed and the cells were washed with icecold PBS. Surface bound Tf was removed by incubating the cells with acetate buffer (25 mM sodium acetate, 0.15 M NaCl, 20 mM $CaCl_2$, pH 4.5, 4°C) for 8 minutes

followed by a 2 minute incubation in PBS (4°C). This procedure was repeated once.

Subsequently the cells were lysed and collected. Intracellular ^{59}Fe and ^{125}I was determined with a Packard 500C autogamma spectrometer. As in the binding studies, the experimental results of the partially deglycosylated Tf subfractions were compared simultaneously to bi-bi Tf and the results of the aglyco Tf were compared to one of the transferrins. All experiments were run in duplicate.

3.2.9 Mathematical analysis of the transferrin binding experiments

A nonlinear curvefit program (Statgraphics, Statistical Graphics Corporation) was used to evaluate the data obtained in the binding assays according to the Langmuir equation for equilibrium binding: $B = (B_{max}*x)/(x+K_d)$ + nonspecific binding. B represents the fraction of Tf (subfraction) that is bound, B_{max} is the saturation level for the specific binding, x is the concentration of the free Tf (subfraction) and K_d is the concentration of Tf (subfraction) at which the specific binding has reached half its maximum. The nonspecific binding is a linear function of x.

3.3 Results

Partial deglycosylation results in a slight decrease in molecular mass, as can be seen on the SDS polyacrylamide gel shown in figure 2. Figure 3 shows that the isoelectric point (IEP) changes v 'len sialic acid is removed. Further deglycosylation has no influence on the IEP.

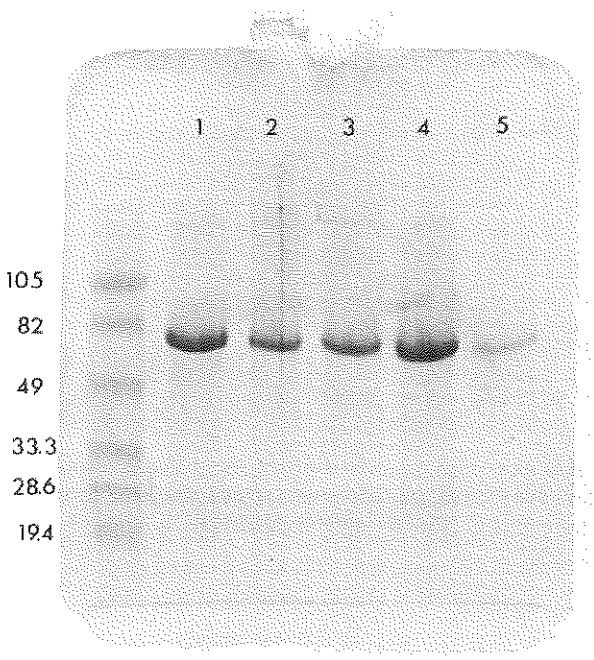


Figure 2. SDS PAGE (PhastGel Homogeneous 12.5) showing the different isotransferrin fractions. Lane 1: fully glycosylated transferrin; lane 2: asialo transferrin; lane 3: agalacto transferrin; lane 4: aglucosamine transferrin; lane 5: aglycotransferrin.
Markers are indicated in kDa. Staining was done with Coomassie Brilliant Blue R250.

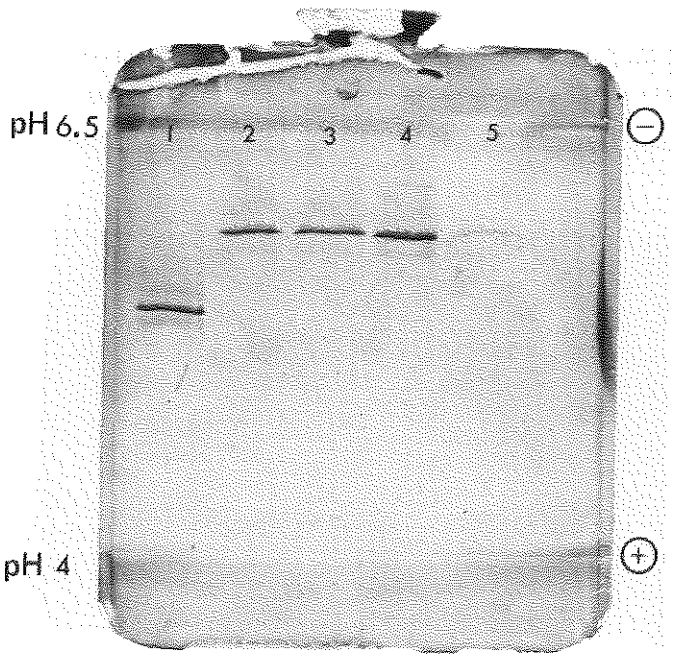


Figure 3. IEF (PhastGel IEF 4 - 6.5) showing the different isotransferrin fractions. Lane 1: fully glycosylated transferrin; lane 2: asialo transferrin; lane 3: agalacto transferrin; lane 4: aglucosamine transferrin; lane 5: aglycotransferrin. Staining was done with Coomassie Brilliant Blue R 250.

Carbohydrate analysis data of the Tf subfractions and the isolated aglyco Tf are shown in table 1 and 2. Tabel 1 shows the almost total absence of galactose in agalacto Tf and the almost total absence of galactose and mannose in aglyco Tf. Table 2 depicts a decrease of 50% in the N-acetylglucosamine content of aglucosamine Tf and a nearly total absence of N-acetylglucosamine in aglyco Tf.

As to the binding experiments; no significant difference as determined by a one way analysis of variance at $p < 0.05$ (Statgraphics, Statistical Graphics Corporation) could be revealed between the dissociation constants (K_d) of the investigated transferrins. The mean K_d was calculated at 4.2×10^{-9} M/L (SD 3.1×10^{-9} M/L). The transferrin binding curves are shown in figure 4.

	reference values serum transferrin	agalacto transferrin	aglycotransferrin
Mannose	5.49	5.51	0.52
Galactose	4.31	0.14	0.42

Table 1. Galactose and mannose determination of agalacto transferrin and aglycotransferrin. Rhamnose was used as an internal standard for the quantification of mannose and galactose in the aglycotransferrin fraction.

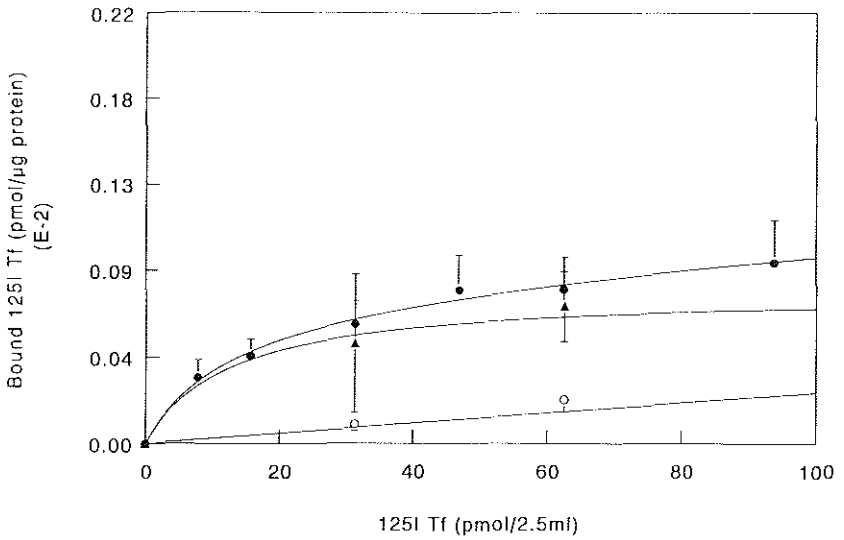
	reference values serum transferrin	aglucosamine transferrin	aglycotransferrin
N-Acetyl glucosamine	8.0	4.1	0.8
Threonine	30.0	29.8	30.7
Serine	41.0	40.1	40.6
Glutamate + NH_2	59.0	57.9	60.0
Glycine	50.0	50.3	48.8
Alanine	57.0	49.4	57.3
Valine	45.0	44.9	46.8
Methionine	9.0	7.2	8.6
Isoleucine	15.0	14.7	15.4
Leucine	58.0	56.1	59.3
Tyrosine	26.0	24.9	26.2
Phenylalanine	28.0	27.3	28.4
Aspartate +NH_2	79.0	75.3	81.5
Histidine	19.0	18.6	18.8
Lysine	58.0	57.3	62.3
Arginine	26.0	24.4	26.4

Table 2. N-Acetylglucosamine determination of aglucosamine transferrin and aglycotransferrin.

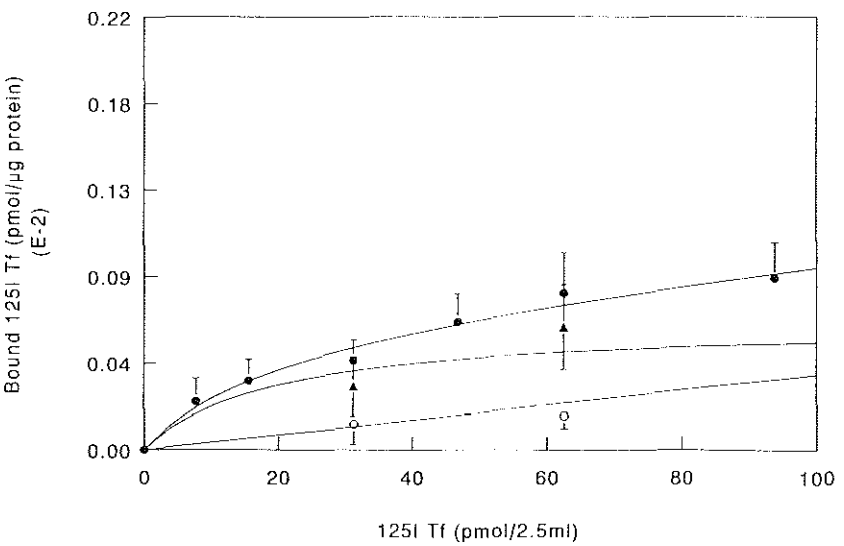
The uptake of bi-bi Tf is compared to the partially deglycosylated Tf subfractions in fig. 5. The iron uptake from these transferrins is also compared in fig. 5. Regarding the Tf uptake, bi-bi Tf and the 3 partially deglycosylated Tf subfractions all reach a similar steady state in the hepatoma cells. The iron uptake rates from these transferrins by the hepatoma cells are also similar. In fig. 5 the results of one experiment being representative for all five experiments performed are shown.

Fig. 6 depicts the results of an uptake experiment in which we compare bi-bi Tf with aglyco Tf. The steady state reached by the aglyco Tf is clearly lower than the steady state reached by bi-bi Tf. The iron donation rate by aglyco Tf is also distinctly lower compared to the iron donation rate by bi-bi Tf.

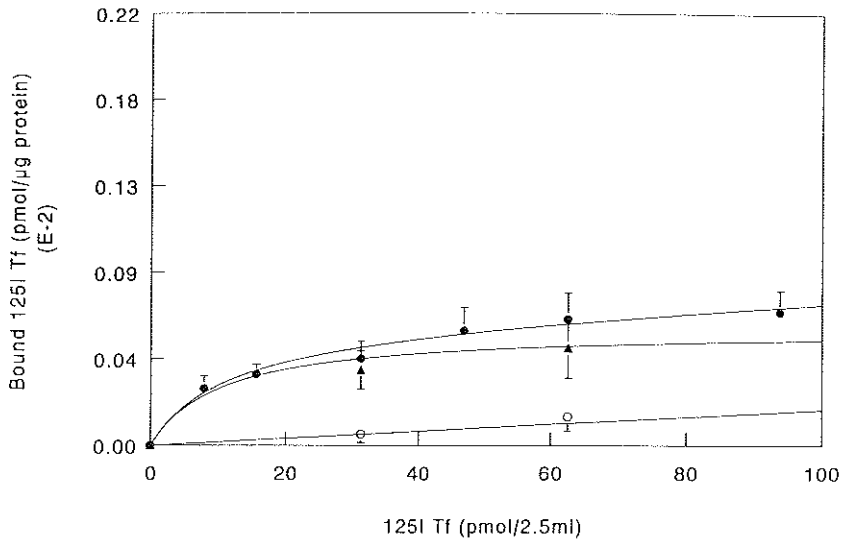
Bi-bi transferrin



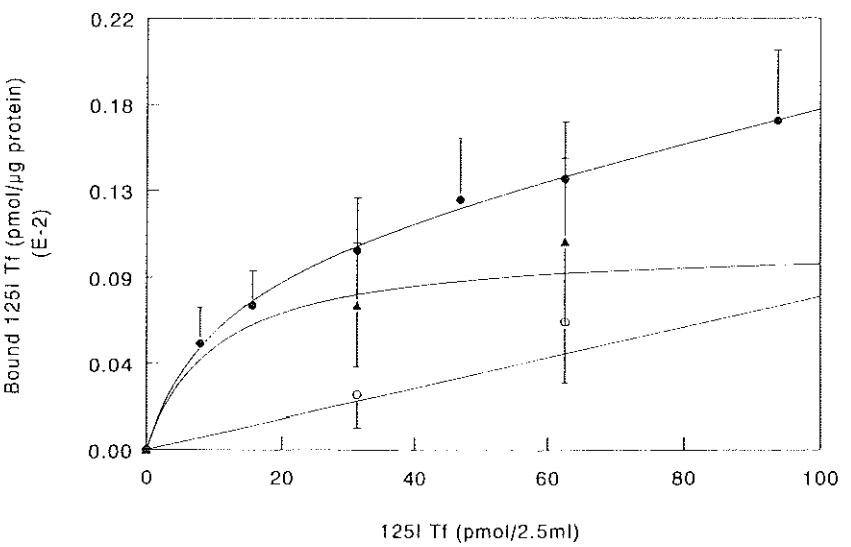
Asialo transferrin



Agalacto transferrin



Aglucosamine transferrin



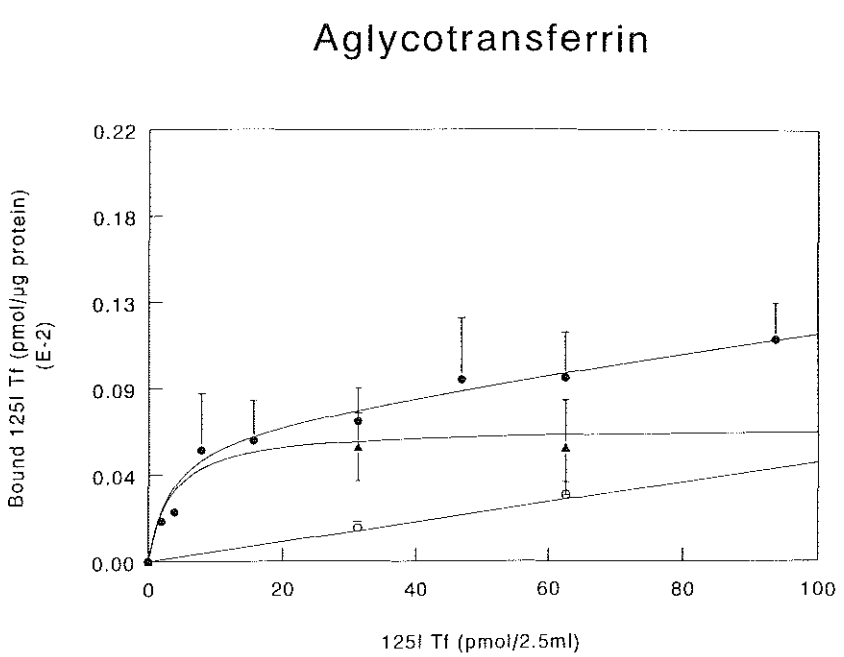


Figure 4. Total binding (•), specific binding (▲) and nonspecific binding (○) of ^{125}I labeled bi-bi transferrin, asialo transferrin, agalacto transferrin, aglucosamine transferrin and aglycotransferrin by PLC/PRF/5 cells.
The total binding curve is fitted to the obtained data points using the function $y=(B_{max}*x)/(x+ K_d)+$ nonspecific binding.
The nonspecific binding for each Tf subfraction was determined by incubating the PLC/PRF/5 cells with the ^{125}I labeled Tf subfraction in the presence of a 50 times excess of the same (unlabeled) subfraction. Due to the minute availability of aglyco Tf, the nonspecific binding of this subfraction was determined using a 50 times excess of bi-bi Tf. The nonspecific binding curve was fitted to the obtained data points using the function $y=a*x$.
The specific binding curve is calculated by subtracting the nonspecific binding from the total binding. In other words, the specific binding is represented by $y=(B_{max}*x)/(x+K_d)$.
The mean values of the obtained data points are given in the figures.

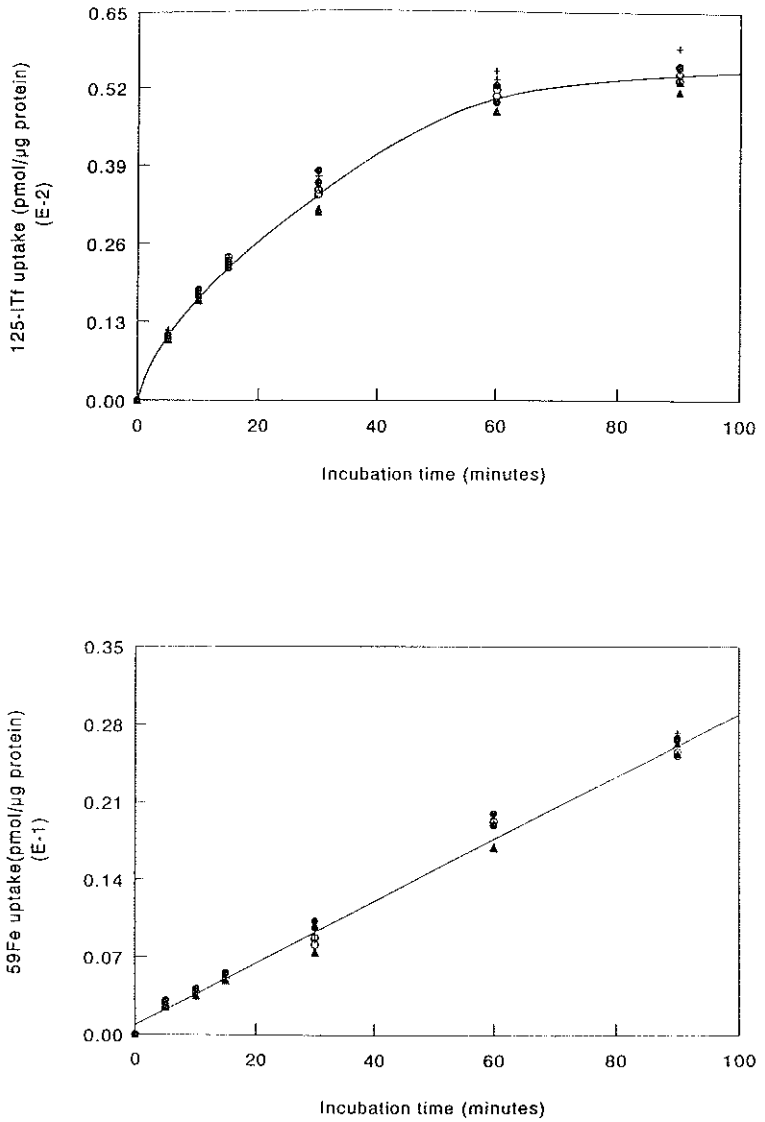


Figure 5. ^{125}I-Tf and ^{59}Fe uptake by PLC/PRF/5 cells.
Legend: +: fully glycosylated transferrin; ▲: asialo transferrin; ○: agalacto transferrin; •: aglucosamine transferrin.

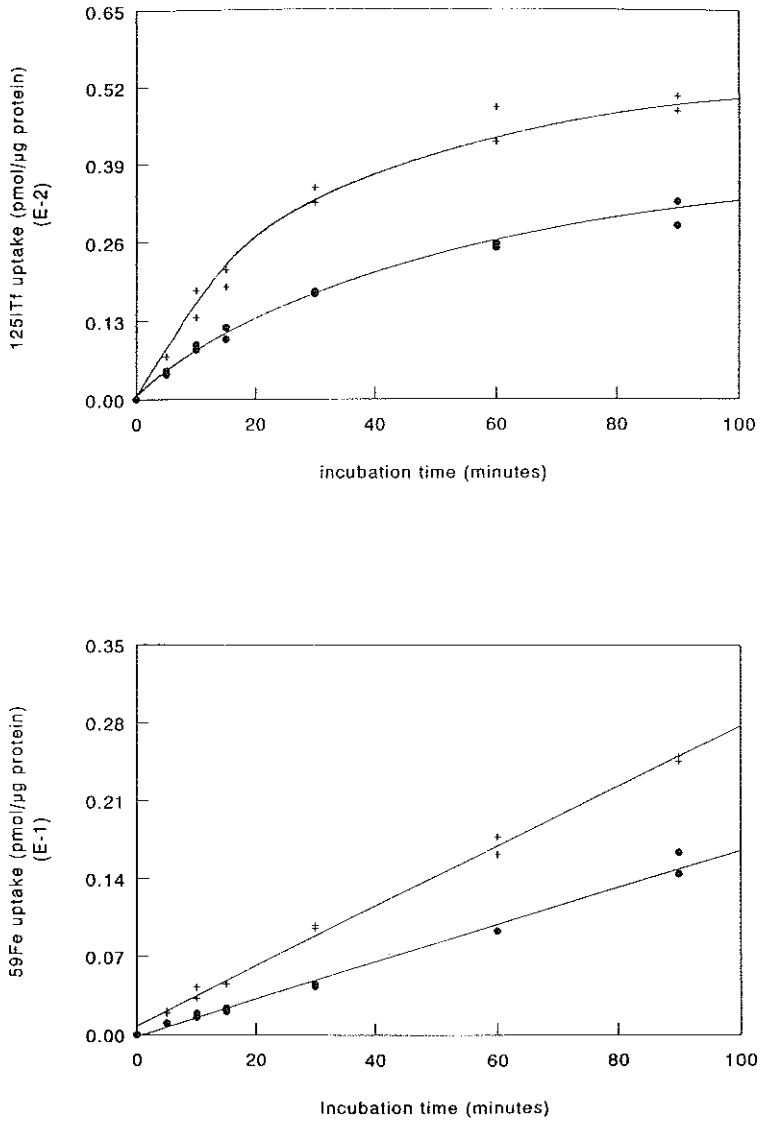


Figure 6. ^{125}I-Tf and ^{59}Fe uptake by PLC/PRF/5 cells.
Legend: +: fully glycosylated transferrin; •: aglycotransferrin.

3.4 Discussion

In this chapter the effect of the Tf glycan chains on receptor binding and iron-donating capacity of Tf is described. Fully glycosylated Tf was compared with partially deglycosylated Tf subfractions and aglyco Tf for the qualities mentioned above.

Table 1 shows that agalacto Tf contains less than 5% of the galactose found in bi-bi Tf. Aglucosamine Tf contains approximately 50% of the amount of N-acetyl glucosamine found in bi-bi Tf, indicating that all distal N-acetyl glucosamine has been removed (Table 2). Only small amounts of sugar can be found in aglyco Tf (Table 1 and 2), approximately 10% of the normal values for all determined sugars. This can be easily explained by a slight contamination of the aglyco Tf with Tf bearing normal glycans. We therefore can conclude that the partial deglycosylation of the three transferrin subfractions has been successful and that the isolated aglyco Tf is deficient of carbohydrates.

From our binding experiments we conclude that the oligosaccharides of Tf have no influence on the affinity of the TfR for its ligand. The calculated K_d values of the different binding experiments showed, unfortunately, from experimental run to run a high degree of variability (Table 3). This explains, when averaged, the high SD of the mean.

The lack of oligosaccharides does not influence the iron binding of Tf as seen on an EPR spectrum (19). However the carbohydrates of Tf do affect the uptake of iron, and also the uptake of Tf itself by PLC/PRF/5 cells (fig. 6).

Tf with oligosaccharides trimmed up to the mannose bifurcation has the same iron donating characteristics as its fully saturated counterpart, also the uptake of Tf itself remains unaffected (fig. 5). A similar uptake pattern of the partially deglycosylated Tf subfractions as bi-bi Tf (as shown in fig. 5), indicates that partially deglycosylated Tf is processed like normal Tf by the hepatoma cells. Regarding the uptake of aglyco Tf, this reaches a lower steady state than bi-bi Tf (fig. 6) or the Tf subfractions. Our results indicate that the lower part of the glycan chains i.e. up to and including the mannose bifurcation, is of importance for the uptake of both iron and Tf.

transferrin subfraction	K_d
bi-bi transferrin	11.5
	7.2
	2.3
	3.4
asialo transferrin	4.2
	0.7
	4.2
agalacto transferrin	5.5
	3.4
	7.0
	2.0
aglucosamine transferrin	1.8
	9.9
	5.0
aglycotransferrin	2.7
	0.5
	3.7
	0.7

Table 3. The calculated K_d values for the different transferrin binding experiments. The values are given in 10^{-9} M/L.

A reduced steady-state of aglyco Tf can be explained by a decreased endocytosis rate or by an increased exocytosis rate. The latter explanation is not very likely, because the iron uptake of aglyco Tf is diminished compared to the partially and fully glycosylated transferrins.

The steady-state reached by the aglyco Tf also shows that this protein takes part in an endocytosis/exocytosis cycle, i.e. is not degraded in the cell.

In summary, our experiments indicate that removing the oligosaccharides from Tf

does not influence the affinity of Tf for its receptor. Removing the 3 distal sugars from the Tf glycan chains does not alter the uptake of Tf (or iron) by PLC/PRF/5 cells. However, a total lack of glycans on Tf reduces the uptake of this protein (and iron). Therefore the basal part of the Tf glycans seems to have an important function in the endocytosis of the Tf-TfR complex.

3.5 Summary

Human bi-bi-antennary transferrin (bi-bi Tf) was partially deglycosylated enzymatically by step-wise incubation with one or more of the following exoglycosidases: neuraminidase, ß-galactosidase or N-Acetyl-ß-D-glucosaminidase. Aglyco Tf was isolated from serum of a patient suffering from the Carbohydrate Deficient Glycoprotein syndrome. Receptor binding and iron donating capacities of bi-bi Tf, of partially deglycosylated Tf subfractions and of aglyco Tf were compared using the human hepatoma cell line PLC/PRF/5. There was no difference in binding capacity between bi-bi Tf, the Tf subfractions and aglyco Tf. The iron donating capacity of aglyco Tf, however, was significantly reduced compared to bi-bi Tf and the other Tf subfractions.

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Renaturation of recombinant human transferrin and its half-molecules

4.1 Introduction

The growing interest in the function of oligosaccharides of glycoproteins has led to an increasing amount of studies in this field. Transferrin (Tf) does not constitute an exception and numerous studies have been published regarding this protein in relation to its glycan chains.

Nowadays a lot of data are available, specifically on the variations in the Tf glycans during certain diseases like hepatocellular carcinoma, rheumatoid arthritis and Parkinson's disease (1-5), as well as on changes that occur during pregnancy (6-11). Characteristic changes in the glycan moieties of Tf are also reported to occur in excessive alcohol intake (12,38, see also chapter 6).

Although subject to speculation, the physiological significance of these variations in the glycan composition remains unknown, but several facts are already ascertained. For instance, the Tf glycans do not influence the intracellular transport of the protein during synthesis (13-16), a well known function of glycans of several other glycoproteins. Another reputed fact is, that glycosylation does not influence the affinity of Tf for iron or for the transferrin receptor (TfR), but lack of the glycan chains reduces the iron donation of Tf to hepatocytes (17,18,19).

Studying the biochemical, structural and physiological properties of human Tf lacking the glycans could give more insight in the function of the glycans. To run experiments it is necessary to obtain fair amounts of aglyco Tf. As discussed in chapter 1 (section 1.6), there are several options for producing aglyco Tf: removing the glycans of Tf either chemically or enzymatically, inhibition of glycosylation of *de novo* synthesized Tf, producing nonglycosylated Tf in eukaryotic cells using site-directed mutagenesis or finally, producing Tf in organisms unable to glycosylate proteins (e.g. *Escherichia coli*). For producing high quantities of aglyco Tf the latter method seems to be the most preferable (see chapter 1).

Although the high productivity of and the low culture requirements for *E. coli* are of advantage, the accumulation of the overexpressed proteins as insoluble protein aggregates (better known as inclusion bodies) is a major drawback. As discussed in chapter 1, recombinant proteins can be refolded after solubilizing the inclusion bodies

(20,21,22). This is an absolute requirement in studying the biochemical properties of the produced recombinant proteins.

The bacterial expression system as described by us (23) has been employed as source for aglyco recombinant human transferrin (rhTf) as well as C-terminal (rhTf/2C) and N-terminal (rhTf/2N) half-transferrins.

In this chapter the isolation, renaturation and partial characterization of rhTf, rhTf/2N and rhTf/2C from *E. coli* cultures expressing these proteins is described. Following purification the amino acid compositions of these recombinant proteins were determined in order to compare these compositions with the theoretical one which is known from the primary structure. In addition the first 10 - 15 amino acids from the N-terminal side of the three recombinant products were sequenced. The recombinant transferrins were refolded into water soluble proteins and subsequently their iron-binding capacity was determined.

4.2 Materials and Methods

4.2.1 RhTf, rhTf/2N and rhTf/2C production in E. coli

Production of rhTf and the recombinant half-molecules in *E. coli* was performed as described by us (23, see also 2.3.1).

4.2.2 Purification of rhTf, rhTf/2N and rhTf/2C

The cultured bacteria were harvested using a MSE Coolspin centrifuge at 2600 g for 15 minutes. The cells were resuspended in 10 ml 50 mM Tris/HCl, 1 mM EDTA, 250 mM NaCl, 5 mM DTT, pH 8.0 and disrupted by adding approximately 100,000 U lysozyme. After incubating for 30 minutes at room temperature the sample was snap-frozen by submersing it in a mixture of solid CO_2 and ethanol. The thawed sample was sonicated. Approximately 300 U DNase I was added and the sample was incubated for 45 minutes at room temperature.

We isolated the inclusion bodies from this cell lysate by centrifugation at 12,000 g for 15 minutes. The pellet, containing the inclusion bodies, was washed 3 times with 50

mM Tris/HCl, 1 mM DTT, pH 8.0 and dissolved in 10 ml 8 M urea, 1 mM DTT, 40 mM Tris/HCl, 10% glycerol (v/v), pH 7.6.

We used preparative SDS electrophoresis to purify rhTf, rhTf/2N and rhTf/2C. This was performed with a model 392 Bio-Rad Prep Cell. We mixed 600 µl of rhTf, rhTf/2N or rhTf/2C solution with an equal volume of 8% SDS (w/v), 24% glycerol (v/v), 50 mM Tris/HCl, 4% ß-mercaptoethanol (v/v), 0.01% Serva blue G (w/v), pH 6.8 and we incubated for 30 minutes at 40°C. This sample was applied to run on the polyacrylamide gel (9% T, 3.3% C) of the Prep Cell. The separated proteins were eluted from the gel at very low concentrations. Due to these very low concentrations the isolated proteins were very difficult to distinguish on the chromatogram. Therefore the collected fractions were concentrated using Centriflo CF 25 cones (Amicon) and run on analytical 9% SDS polyacrylamide gels. The fractions containing the pure recombinant Tf were collected.

4.2.3 Amino acid analysis

The recombinant proteins were analysed using an Alpha Plus 4151 amino acid analyser (Pharmacia LKB Biochrom Ltd., Cambridge, England). The proteins were hydrolysed first by mixing 50 µl (0.5 - 1.0 g/L) protein solution with 150 µl 4 M Para-toluene sulfonic acid and 2% 3-(2-amino ethyl) indole HCl (24). This mixture was left for 24 hours at 110°C. After hydrolysis the mixture was titrated with 1 M NaOH to pH 2.0, and 0.2 M lithium citrate was added to a final volume of 2 ml. We analyzed a sample of this mixture. During the analysis the eluent was mixed with a 0.2% (w/v) ortho-phthal aldehyde (O.P.A.) reagent in 1 M borate. The signal was measured with a fluorimeter F 1000 (Merck Hitachi, Amsterdam). As a reference we used a very pure human Tf preparation (4 sialo-bibi-antennary Tf of the genetic C_1 type) isolated as described in van Noort *et al.* (25).

For protein sequencing a model 473A Protein Sequencer (Applied Biosystems) was used, performing Edman degradation and phenylthiohydantoin amino acid analysis.

4.2.4 Renaturation of rhTf, rhTf/2C and rhTf/2N

Renaturation experiments were performed with the inclusion body solution without further purification of the recombinant proteins. Prior to renaturation the protein concentration of the inclusion body solution was determined according to the method of Bradford (26). Renaturation was performed by diluting the proteins in a renaturation buffer (0.1 mM Na-EDTA, 0.1 mM Tris/HCl, 1.0 mM GSH, pH 8.2, 6°C) to a concentration of 20 µg/ml. This mixture was left for 15 minutes. Subsequently GSSG was added to a final concentration of 0.5 mM. This solution was left for another 22 hours at 6°C. Renaturation buffer was thoroughly degassed prior to use and renaturation was performed under an atmosphere of nitrogen. After renaturation the solution was concentrated and dialysed against 10 mM $NaHCO_3$ using an Ultrasette with an omega 10 kDa membrane (Filtron). Subsequently the solution was filtered and concentrated using first an Amicon Stirred Cell with a PM 10 membrane, and finally Centriflo cones, CF 25 (Amicon). After this concentration step the acquired proteins (rhTf, rhTf/2C or rhTf/2N) were separated from the other proteins by a Sepharose-bound anti-transferrin column (27). Finally the purified recombinant transferrins were saturated with iron using a $FeCl_3$/NTA solution, hoping in this way to stabilize our product.

To determine whether the recombinant transferrins bind iron, rhTf, rhTf/2C and rhTf/2N were saturated with radio labeled iron using a $^{59}FeCl_3$/NTA solution ($^{59}FeCl_3$, Amersham Life Science) following standard procedures as described in 2.4. The different recombinant transferrin samples saturated with ^{59}Fe were loaded onto two 12.5% homogeneous polyacrylamide PhastGels (Pharmacia). One gel was used to illuminate an X-ray film and the other gel was stained for proteins using Coomassie Brilliant Blue R-250. We also determined the molecular weight of the proteins in the different samples by running them on a 12.5% homogeneous SDS polyacrylamide gel.

4.2.5 Spectrophotometry and EPR

Absorption spectra and $A_{280/465}$ ratios of the iron saturated recombinant transferrins were determined using an Ultrospec III spectrophotometer provided with the appropriate software (Pharmacia, LKB).

EPR spectra were determined using a Bruker EPR 200 D spectrometer as described by Pierik and Hagen (28).

4.3 Results

The rhTf, rhTf/2C and rhTf/2N samples purified from inclusion bodies by means of preparative SDS electrophoresis each showed one protein band on a 9% SDS-polyacrylamide gel (fig. 1). As expected, rhTf migrated slightly faster than human Tf. The lack of oligosaccharide chains reduces the Mw by approximately 4 kDa. RhTf/2N and rhTf/2C showed bands just above the 32.5 kDa marker, which corresponds with the calculated Mw of respectively 37.3 kDa and 38.3 kDa.

In table 1 the amino acid composition of rhTf, rhTf/2N and rhTf/2C is compared to that of human Tf. The composition of the recombinant transferrins corresponds very well with the composition of human Tf. Most amino acids show less then 5% deviation from the expected values. In table 2 the sequence of the first 10 - 15 amino acids from the N-terminal side of the 3 recombinant proteins is compared to the sequence of the amino acids as determined for Tf (29). These sequences match the references exactly except for one N-terminal extra methionine in each recombinant protein and a glutamic acid replacement by glutamine in rhTf/2C. The latter was introduced during construction of the expression-plasmid and will be discussed later on.

An absorption spectrum of the iron saturated recombinant transferrins was determined in the range from 280 nm to 600 nm. Normal diferric human Tf has an absorption maximum at 465 nm. The recombinant transferrins showed spectra similar to that of diferric human Tf with an absorption maximum at 462 nm, 459 nm and 464 nm for rhTf, rhTf/2C and rhTf/2N respectively. Normal diferric Tf has an $A_{280/465}$ ratio equivalent to 21, this value has also been reported for iron-saturated human Tf/2N (30). For rhTf, rhTf/2C and rhTf/2N we calculated values of 30, 28 and 24. Assuming these ratios can be converted into iron-saturation values as can be done with normal Tf, these figures correspond with 70% and 90% iron-saturation for rhTf and rhTf/2N respectively. Assuming the $A_{280/465}$ ratio for iron-saturated human Tf/2C is equal to that of human Tf and human Tf/2N the calculated value for rhTf/2C corresponds with 75% iron-saturation.

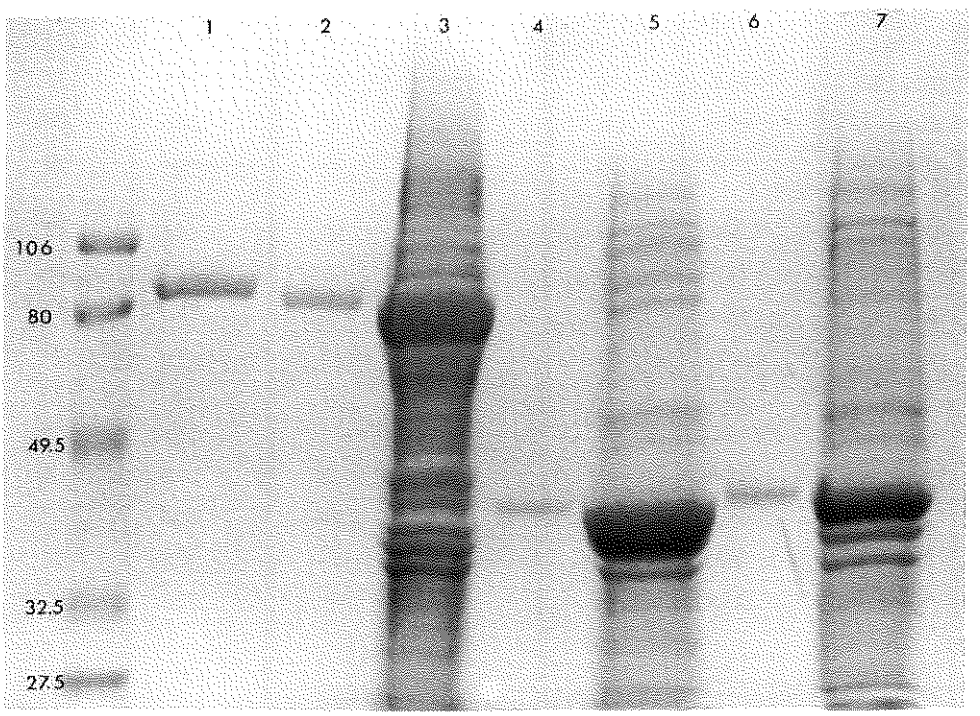

Figure 1: SDS-Page (9%) showing the purified recombinant proteins and the solubilized inclusion bodies from which these proteins were isolated.
Lane 1: Tf; lane 2: rhTf; lane 3: inclusion body solution containing rhTf; lane 4: rhTf/2N; lane 5: inclusion body solution containing rhTf/2N; lane 6: rhTf/2C; lane 7: inclusion body solution containing rhTf/2C.
Markers are indicated in kDa. Staining was done with Coomassie Brilliant Blue G250.

	recombinant human transferrin (rhTf)	reference values serum transferrin	N-terminal half-transferrin (rhTf-/2N)	reference values N-terminal part of serum transferrin	C-terminal half-transferrin (rhTf-/2C)	reference values C-terminal part of serum transferrin
Lysine	53.7	58	25.9	27	26.9	31
Histidine	18.0	19	9.2	9	8.7	10
Arginine	24.9	26	10.6	12	14.7	14
Aspartate + NH_2	74.0	79	35.3	36	42.2	43
Threonine	30.2	30	12.9	13	16.3	17
Serine	40.4	41	20.2	21	20.6	20
Glutamate + NH_2	57.8	59	28.5	29	32.4	30
Proline	n.d.	32	n.d.	20	n.d.	12
Glycine	51.5	50	25.2	24	27.3	26
Alanine	58.4	57	31.7	32	25.3	25
Cysteine	n.d.	38	n.d.	16	n.d.	22
Valine	44.7	45	22.7	22	22.3	23
Methionine	9.4	9	5.8	5	5.3	4
Isoleucine	14.6	15	7.8	8	7.6	7
Leucine	55.2	59	30.7	29	29.5	30
Tyrosine	26.0	26	15.2	14	12.0	12
Phenyla-lanine	25.8	28	15.4	16	13.1	12
Tryptophan	7.5	8	2.9	3	4.2	5
Total		679		336		343
Total minus Cys and minus Pro	592.1	609	300	300	308.4	309
deviation from predicted values	3.6 %		3.3 %		5.5 %	

Table 1. Amino acid analysis of recombinant transferrins (mol amino acid/mol protein). Sums of Asparagine and Aspartate and sums of Glutamine and Glutamate are given because of interconversion of these amino acids in the hydrolysis procedure.
The colour reactant used in the analysis procedure yields low absorbance products for Proline and Cysteine and are therefore not determined (N.D.).
The deviation represents the cumulated difference between determined numbers of separate amino acids in the transferrin species compared to the reference values derived from figures based on the transferrin characterization by Yang *et al.* (29).

serum Tf	rhTf	rhTf/2N	serum Tf	rhTf/2C
	Met	Met		Met
01 Val	Val	Val	337 Asp	Asp
02 Pro	Pro	Pro	338 Glu	Gln
03 Asp	Asp	Asp	339 Cys	Cys
04 Lys	Lys	Lys	340 Lys	Lys
05 Thr	Thr	Thr	341 Pro	Pro
06 Val	Val	Val	342 Val	Val
07 Arg	Arg	Arg	343 Lys	Lys
08 Trp	Trp	Trp	344 Trp	Trp
09 Cys	Cys	Cys	345 Cys	Cys
10 Ala	Ala	Ala	346 Ala	Ala
11 Val	Val	Val	347 Leu	Leu
12 Ser	Ser	Ser		
13 Glu	Glu	Glu		
14 His	His	His		

Table 2. Amino acid sequence of the recombinant transferrins, sequenced from the N-terminus, compared with the expected sequence according to Yang *et al.* (29).

The renatured recombinant transferrins were saturated with ^{59}Fe using a $^{59}FeCl_3$/NTA solution. This sample was run on a native polyacrylamide gel (fig. 2A). The ^{59}Fe saturated recombinant proteins all show more than one band on the autoradiograph (fig. 2B). The difference in migration pattern between rhTf/2C and rhTf/2N on the autoradiograph and the native polyacrylamide gel is remarkable. This difference is not seen on the SDS polyacrylamide gel (fig. 3).

The EPR spectra of the recombinant transferrins are nearly identical with the spectrum of native Tf (fig. 4).

The final efficiency of the renaturation experiments are estimated at approximately 5% based on the measured absorption at 465 nm of the renatured and with iron saturated recombinant transferrins .

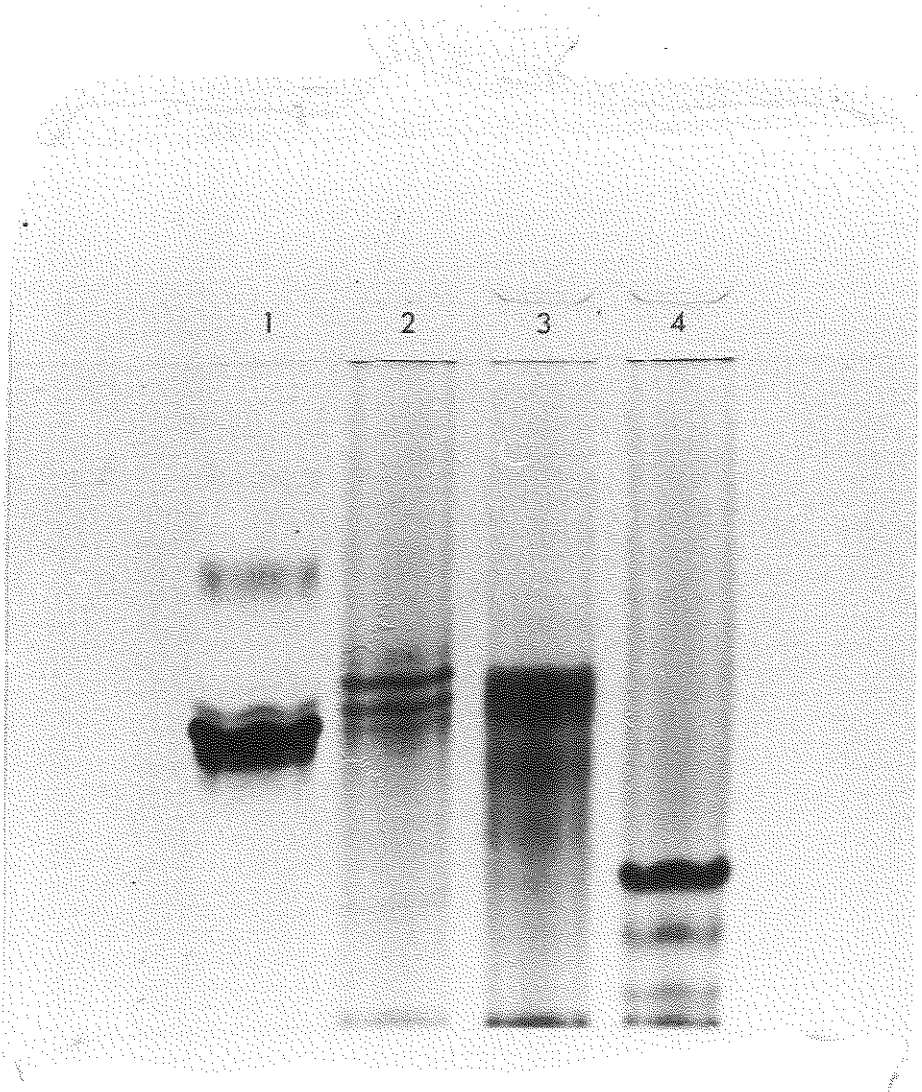

Figure 2A. Native PAGE (12.5%) showing the renatured recombinant proteins. Lane 1: Tf (Behringwerke); lane 2: rhTf; lane 3: rhTf/2C; lane 4: rhTf/2N.

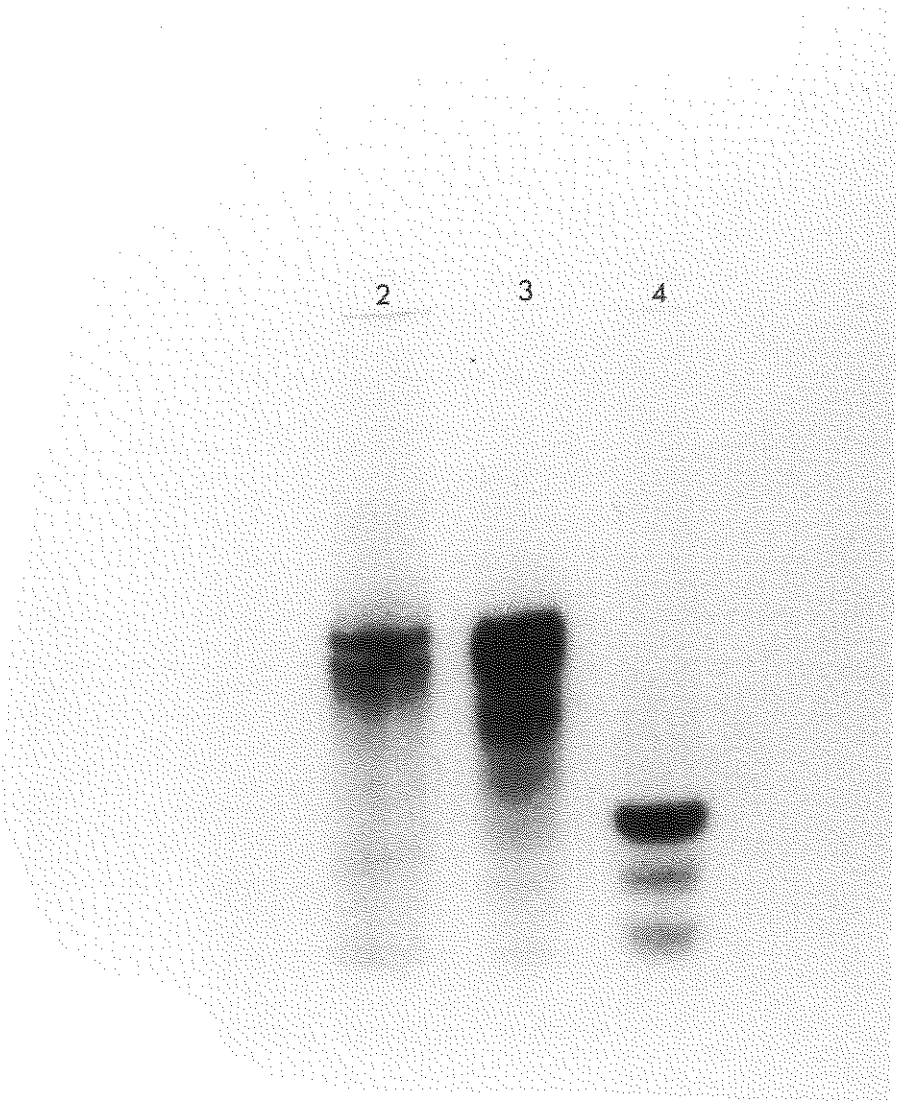

Figure 2B. Autoradiograph of a comparable gel as shown in figure 2A.
Only the recombinant proteins were saturated with ^{59}Fe.
Lane 2: rhTf; lane 3: rhTf/2C; lane 4: rhTf/2N.

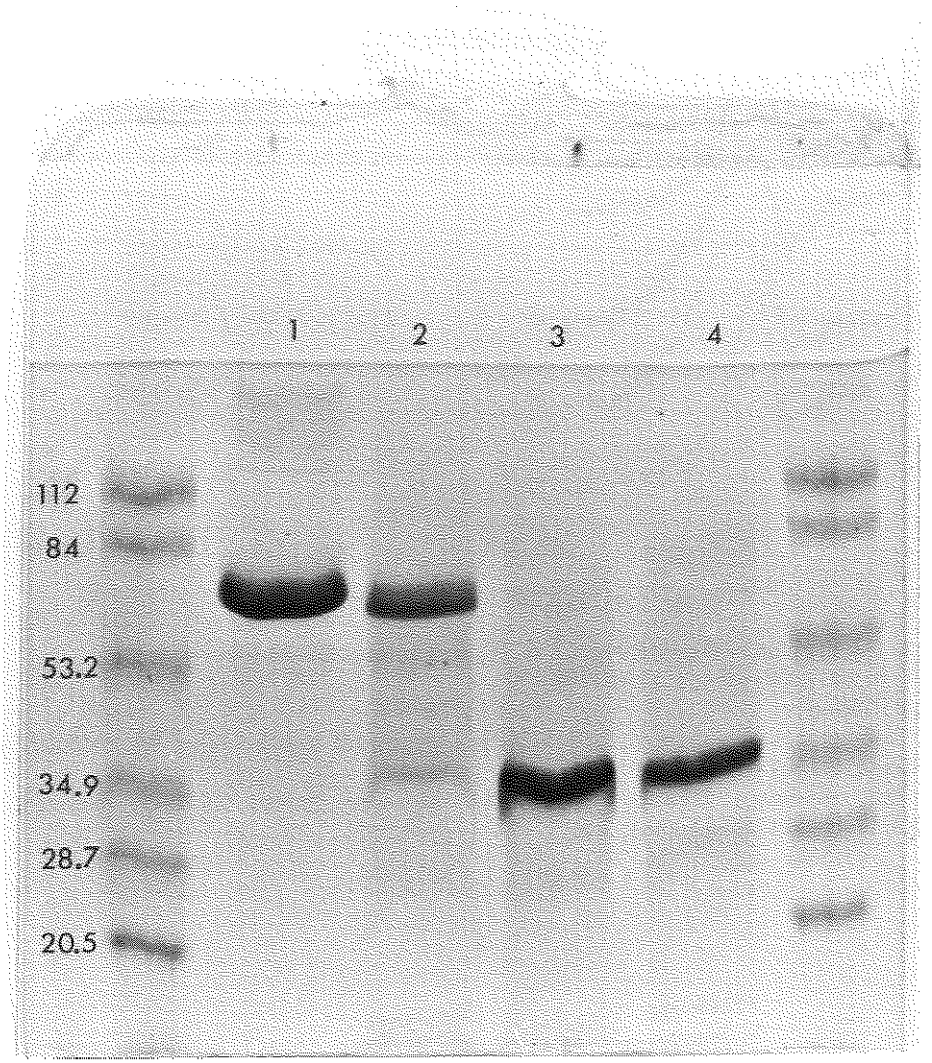

Figure 3. SDS PAGE (12.5%) showing the renatured recombinant proteins.
Lane 1: Tf (Behringwerke); lane 2: rhTf; lane 3: rhTf/2C; lane 4: rhTf/2N.
Markers are indicated in kDa. Staining was done with Coomassie Brilliant Blue R250.

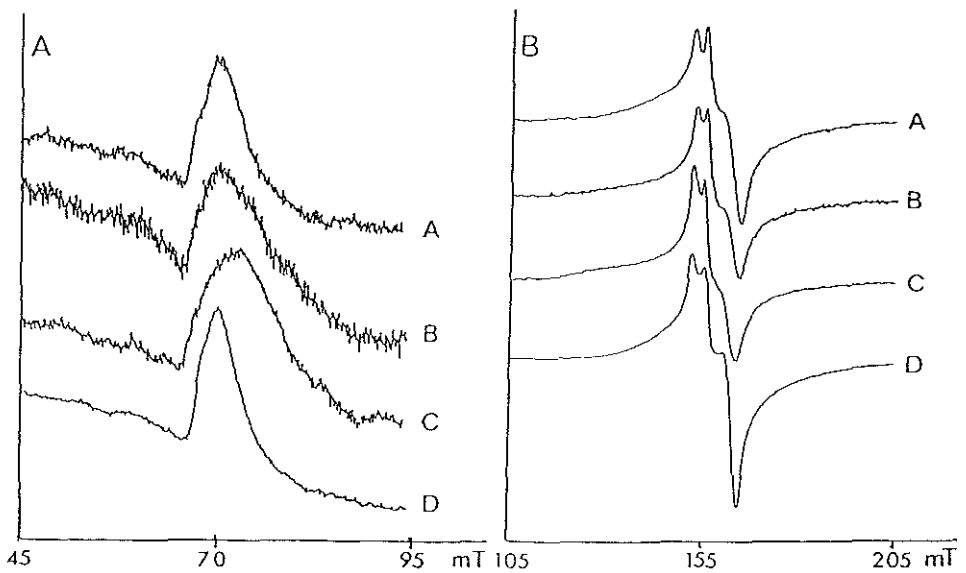

Fig. 4. Comparison of the EPR spectra of Tf and the recombinant transferrins.
A: Tf; B: rhTf; C: rhTf/2N; D: rhTf/2C.
EPR conditions:
Fig. 4A: microwave frequency: 9315 MHz; microwave power: 80 mW; modulation amplitude: 0.8 mT; temperature: 9 K.
Fig. 4B: microwave frequency: 9315 MHz; microwave power: 0.8 mW; modulation amplitude: 0.5 mT; temperature: 9 K.

4.4 Discussion

Several authors have reported the production of recombinant Tf in E. coli (36,37). Hershberger *et al.* (36) also reported on renaturation of recombinant Tf, although no data were shown.

In this chapter we report on the purification and renaturation of rhTf, rhTf/2C and rhTf2/N (39), produced in *E. coli* as described by us (23). We reported the production of the different recombinant transferrins amounts up to 60 mg/L cell culture. Our renaturation procedure has a final efficiency of approximately 5%. This estimation is based on the measured absorption at 465 nm of renatured and with iron saturated recombinant transferrin and on the assumption that only correctly folded recombinant transferrin binds iron. The measured absorption at 465 nm is indicative for the iron content of the renatured transferrins.

After purification the amino acid composition of rhTf, rhTf/2C and rhTf/2N were determined. Their compositions match very well the one to be expected from human Tf. This does not only prove that our samples contain the proteins of our interest, but also confirms that they are very pure.

N-terminal sequencing shows that the recombinant transferrins all have an extra methionine at the N-terminus (table 2). The difference in the amino acid sequence of rhTf/2C (amino acid 338) is due to the introduction of a *Kpn*I-site in the Tf cDNA (23). Apart from this, the amino acid sequences of the recombinant transferrins match the expected sequences exactly (29).

In our first renaturation experiments we used reduced Tf, that was denatured by SDS and urea. Attempts to renature Tf after such a treatment all failed. Although total removal of SDS from proteins has been reported (31,32,33), we were not able to remove this detergent from denatured Tf without complete precipitation of our protein of interest. Hence, renaturation experiments were performed with our inclusion body solution following a procedure derived from Hirose (34,35). We adapted this method to our experimental system; i.e. we abandoned prior isolation of the recombinant proteins by means of preparative SDS electrophoresis.

After our renaturation and purification procedure the recombinant transferrins all

show a few bands on a native polyacrylamide gel (fig 2A). This phenomenon can not be caused by differences in molecular weight, because the same samples all show one single band on a SDS gel (fig.3), except for a weak second band in lane 2, corresponding with the position of half-transferrin. The generation of half-molecules of rhTf can easily be explained to be the result of proteolytic activity of *E. coli* enzymes.

An explanation for the appearence of more than one band on the native gel could be that they represent different conformations of the protein, for example due to variations in disulphide bond formation. These conformational differences could affect the iron-binding sites, resulting in a decreased iron saturation as shown in our results.

On the native 12.5% homogeneous polyacylamide gel one of the major rhTf/2C bands was positioned approximately at the same level as the rhTf bands (Fig. 2A). On the other hand, on a gradient polyacrylamide gel (PhastGel 8-25%) this band was found more at the level of the rhTf/2N bands (results not shown). Regarding these two findings it seems most unlikely that this band represents a dimer of rhTf/2C. Moreover, dimerization as a result of intermolecular disulphide bridge formation was excluded, since none of the recombinant proteins showed any extra bands on a 12.5% homogeneous polyacrylamide SDS gel run under non-reducing conditions compared to a SDS gel run under reducing conditions (results not shown).

The recombinant transferrins are all capable of binding iron as shown on the autoradiograph (fig. 2B). This capability is also indicated by the similarity of the different recombinant transferrins absorption spectra and the spectrum of iron saturated Tf. All spectra have an absorption maximum near 465nm.

Moreover, the EPR spectra of the recombinant proteins are nearly identical with the EPR spectrum of human Tf. Thus the iron atoms in the recombinant proteins and in the native Tf are bound in a similar way. This strongly suggests that the iron-binding sites of the recombinant transferrins are intact. The minor differences seen in the EPR spectra are possibly a result of differences between the transferrins, e.g. presence or absence of oligosaccharides.

4.5 Summary

Recombinant human transferrin as well as N- and C-terminal half-transferrins, produced in *Escherichia coli* are deposited in inclusion bodies by the bacteria. The isolation and purification of the recombinant proteins from these inclusion bodies are described in this chapter.

The amino acid compositions and N-terminal sequences of the proteins were determined, and found to be in agreement with the known protein structure of human serum Tf. Renaturation of the recombinant proteins is described, resulting in water soluble iron-binding molecules. Iron binding was confirmed by ^{59}Fe labeling, absorption spectrophotometry and EPR spectrometry.

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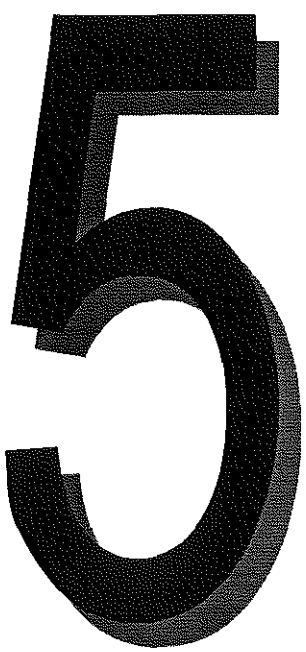
Receptor binding and iron donation by the recombinant transferrins



5.1 Introduction

In this chapter the biological activity of refolded recombinant transferrin (rhTf), recombinant C-terminal half-transferrin (rhTf/2C) and N-terminal half-transferrin (rhTf/2N) was studied. Transferrin binding and iron uptake of these proteins were determined.

As shown in the preceeding chapter recombinant transferrin and both its half-molecules isolated from inclusion bodies, can be renatured in a water soluble form capable of binding iron. The iron binding capacities of both rhTf and rhTf/2C compared to Tf were estimated at approximately 70% on the basis of measured $A_{280/465}$ ratios. For rhTf/2N this value was estimated to be 90% (1).

The inability to saturate our recombinant proteins totally with iron suggests that not all the proteins are refolded into their native conformation. Nevertheless it can be very interesting to determine the receptor binding and iron-donating ability of the refolded proteins.

5.2 Materials and Methods

5.2.1 Cell culture

For the uptake and binding experiments PLC/PRF/5 cells were used. This human hepatoma cell line was obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.). Cells were cultured as described in detail in 2.5.1.

5.2.2 Labeling of the transferrins

Labeling of the 3 recombinant transferrins and bi-bi antennary Tf (bi-bi Tf) with ^{125}I and ^{59}Fe was performed as described in detail in 2.4. Regarding the labeling with ^{59}Fe, this procedure was slightly adjusted for both recombinant half-transferrins. A half Tf molecule only binds one iron atom, so recombinant half-transferrin was incubated with ^{59}Fe in a molar ratio of 1 to 1.5. Bi-bi Tf and rhTf were incubated with ^{59}Fe in a molar ratio of 1 to 3 (see 2.4).

5.2.3 Determination of recombinant transferrin binding

Recombinant transferrin binding studies were performed as described in detail in 2.5.2. In short, approximately 4 days before the actual experiment the cells were disseminated in either 35 mm cell culture cluster dishes (Costar) or 40 mm glass wells. The binding experiments were performed at 0°C. PLC/PRF/5 cells were incubated with a concentration range of ^{125}I labeled recombinant Tf for 90 minutes. In a parallel experiment the nonspecific binding was determined by measuring the binding of ^{125}I labeled recombinant transferrin in the presence of a (50 times) excess of commercially obtained human serum transferrin (Sigma, further referred to as normal Tf). Following the incubation the cells were washed with PBS, collected and homogenised by sonication. Samples were taken for protein determination (Bradford, 2) and surface bound radioactivity was measured. Binding of the recombinant transferrins was compared to the binding of bi-bi Tf.

5.2.4 Determination of recombinant transferrin and iron uptake

Uptake studies were performed as described in section 2.5.3. In short, cells were incubated with doubly labeled rhTf at concentrations of 3 µg or 9 µg per ml incubation medium and with doubly labeled recombinant half-transferrins at concentrations of 5 µg per ml incubation medium. Uptake experiments were performed at 37°C. At predetermined times the incubation was terminated, the cells were washed with PBS and acetate buffer, collected and homogenised. Samples were taken for protein determination. Finally ^{59}Fe and ^{125}I were determined in the samples.

Uptake of the recombinant transferrins was compared to the uptake of bi-bi Tf. Simultaneously the uptake of iron from the recombinant transferrins was compared to that of bi-bi Tf. In a parallel experiment the uptake of doubly labeled Tf was determined in the presence of a (50 times) excess of unlabeled normal Tf.

Uptake of rhTf and bi-bi Tf in the presence of 200 µM bathophenanthrolinedisulphonic acid disodium salt (BPS) was compared to the uptake of these transferrins in the absence of BPS. The iron uptake from both transferrins in the absence or presence of BPS was also compared.

5.3 Results

5.3.1 Binding experiments

Determination of the binding capacity of rhTf to its receptor on PLC/PRF/5 cells was not possible. The binding of ^{125}I labeled rhTf in the presence of an excess of normal unlabeled human transferrin was nearly identical to the binding of ^{125}I labeled rhTf alone.

Experiments employing plastic wells without cells showed that rhTf had a high affinity for this well material. In fact the binding to empty wells was higher than the binding to wells containing a monolayer of PLC/PRF/5 cells. The use of glass wells made no difference.

5.3.2 Uptake experiments

5.3.2.1 Uptake experiments with rhTf

Uptake experiments were done with doubly labeled rhTf in a concentration of 3 and 9 µg/ml medium. More ^{125}I/µg cell protein was detected in the experiment with the highest concentration (results not shown).

With respect to rhTf uptake, no appreciable difference was found in the amount of detected ^{125}I per µg cell protein, between cell incubations with rhTf (3 µg/ml) in the absence or presence of an excess of unlabeled normal Tf (results not shown).

With respect to iron uptake, incubation with 9 µg rhTf/ml medium compared to incubation with 3 µg rhTf/ml medium results in a higher iron uptake per µg cell protein (fig. 1). The iron uptake of bi-bi Tf, however, is many times higher. Fig. 1 also shows that the ^{59}Fe uptake from rhTf (3 µg/ml) is clearly much lower in the presence of an excess of normal diferric Tf.

PLC/PRF/5 cells incubated with rhTf (9 µg/ml) in the presence of 200 µM BPS still show a considerable iron uptake compared to PLC/PRF/5 cells incubated with rhTf (9 µg/ml) alone. We obtained similar results with bi-bi Tf (fig. 2).

5.3.2.2 Uptake experiments with rhTf/2C and rhTf/2N

With respect to half-transferrin uptake, no difference could be demonstrated in the detected amount of ^{125}I after incubation with the ^{125}I labeled half-transferrins compared to incubation with the ^{125}I labeled half-transferrins in the presence of normal unlabeled Tf (results not shown).

However, with respect to the uptake of iron, a difference was seen between the two recombinant half-transferrins. The amount of detected $^{59}Fe/\mu g$ cell protein after incubation with doubly labeled rhTf/2N is similar to the detected amount ^{59}Fe after incubation with rhTf/2N in the presence of an excess of normal Tf. Regarding rhTf/2C, the amount of detected $^{59}Fe/\mu g$ cell protein after incubation in the absence of an excess of normal Tf is much higher, compared to incubation with rhTf/2C in the presence of an excess of normal Tf. Furthermore, the amount of detected $^{59}Fe/\mu g$ cell protein after incubation with rhTf/2C is several times higher than after incubation with rhTf/2N (fig. 3).

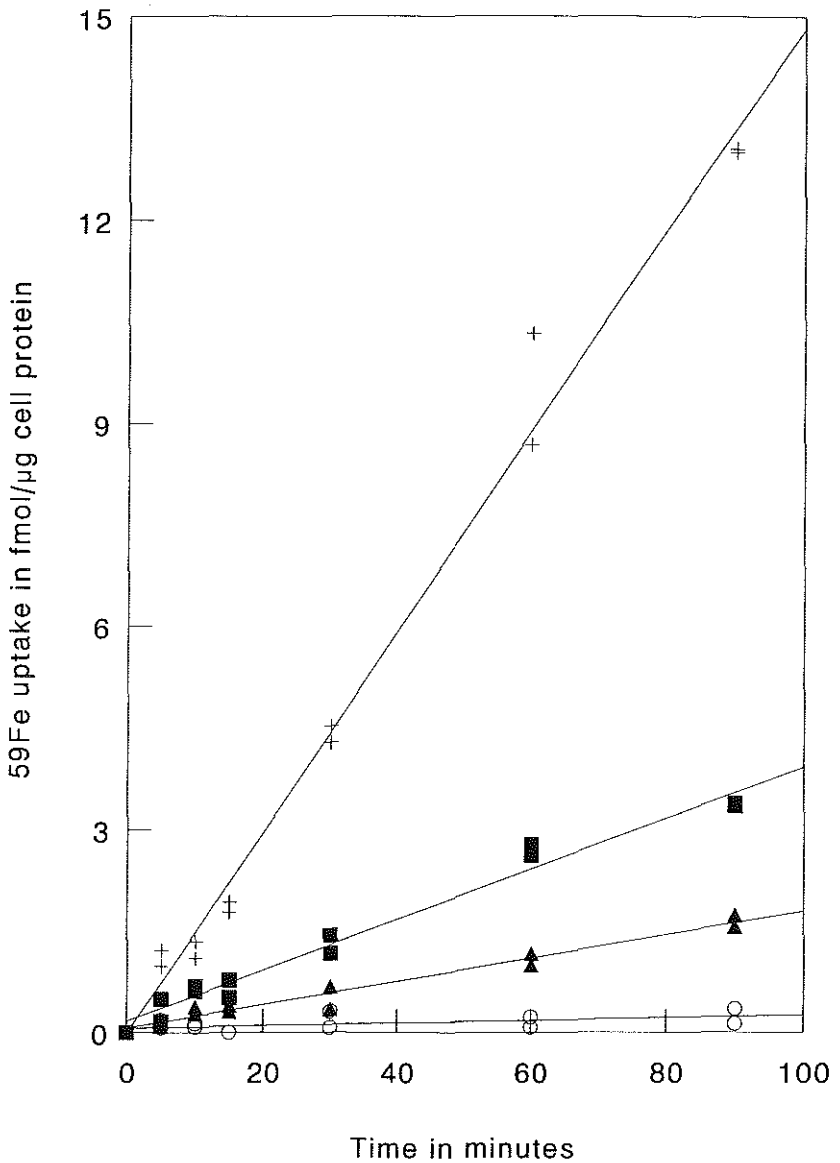


Figure 1. ^{59}Fe uptake by PLC/PRF/5 cells.
Legend: +: bi-bi Tf (3 µg/ml); ■: rhTf (9 µg/ml); ▲: rhTf (3 µg/ml); o: rhTf (3 µg/ml) + 50 times excess of normal Tf.

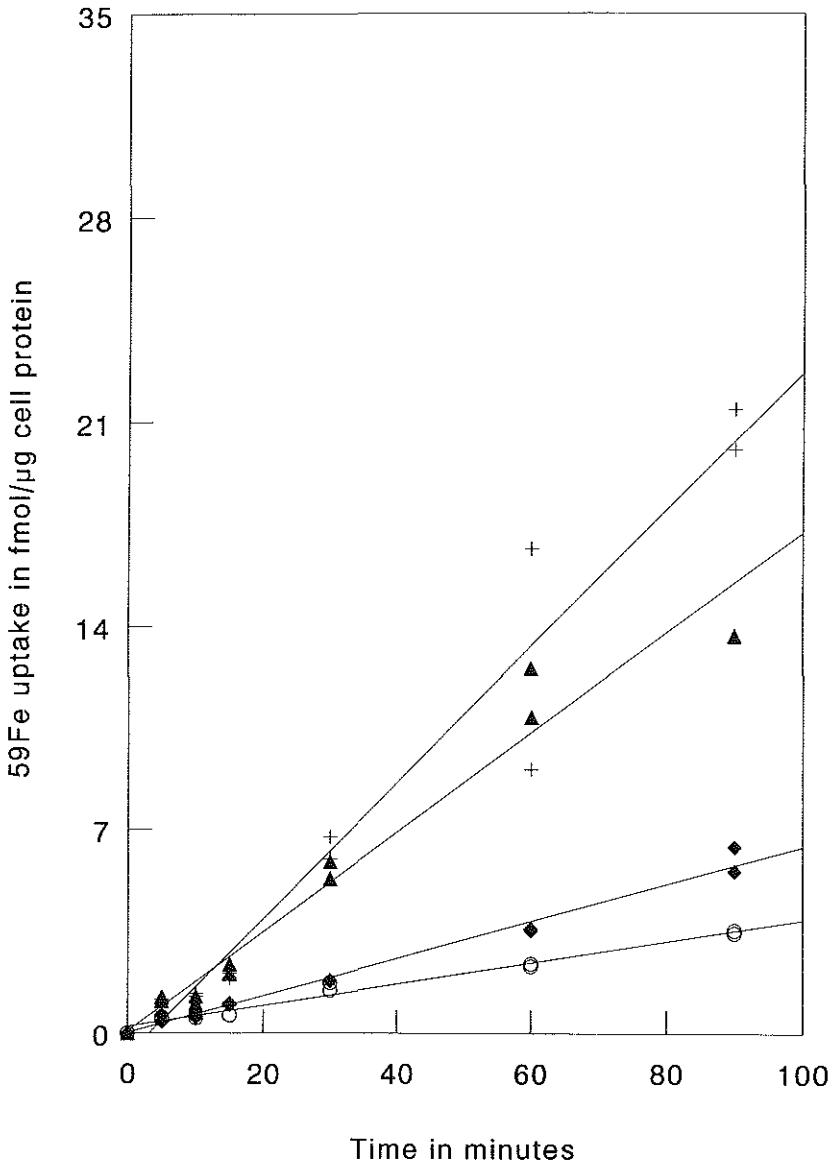


Figure 2. ^{59}Fe uptake by PLC/PRF/5 cells in the presence or absence of bathophenanthrolinedisulphonic acid disodium salt (BPS).
Legend: +: bi-bi Tf + BPS; ▲: bi-bi Tf - BPS; o: rhTf + BPS; ◆: rhTf - BPS.

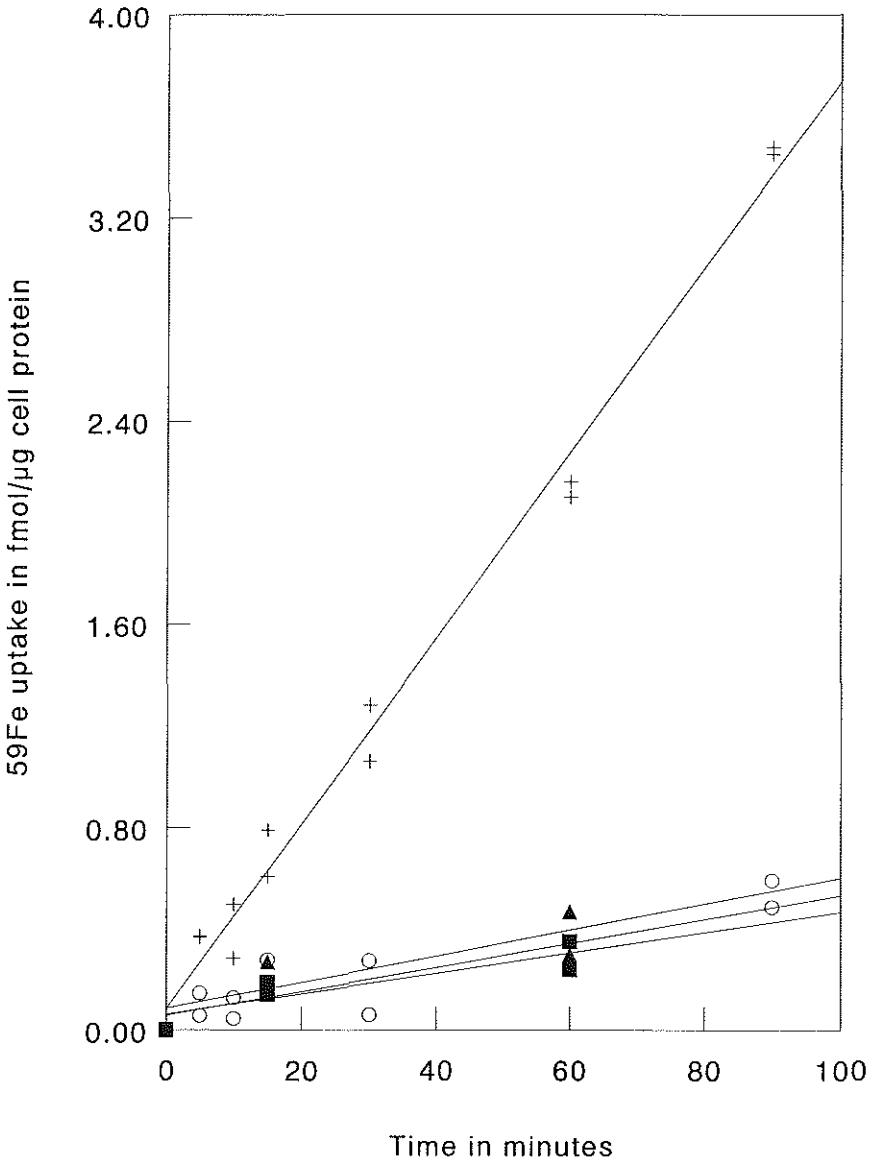


Figure 3. ^{59}Fe uptake by PLC/PRF/5 cells.
Legend: +: rhTf/2C; o: rhTf/2N; ▲: rhTf/2C + 50 times excess normal Tf; ■: rhTf/2N + 50 times excess normal Tf.

5.4 Discussion

The binding of ^{125}I labeled rhTf in the presence of a (50 times) excess of normal unlabeled human Tf being nearly identical to the binding of ^{125}I labeled rhTf alone, indicates a high aspecific binding of rhTf. This presumption was confirmed in our experiments with empty wells.

The high nonspecific binding of rhTf to the cells and the wall of the wells is an unusual feature. The lack of carbohydrates in rhTf being responsible for the increased nonspecific binding can not be ruled out in this context. On the other hand, it could be attributed to properties of subpopulations of recombinant transferrins with abnormal conformations (due to ineffective refolding) inside the proper population. This is in line with the observation that rhTf shows several bands on a native PAGE (fig. 2A and 2B, chapter 4), indicating that after refolding several subpopulations arise with different physiochemical characteristics. Since only a small portion of the refolded rhTf has a molecular mass deviating from the expected value (fig. 3, chapter 4), a difference in molecular weight can not account for all the differences seen on the native PAGE. Therefore it is very likely that these physiochemical differences are caused by various conformations of the refolded transferrin. Probably both refolded half-transferrins are similarly affected.

With respect to rhTf uptake, no difference was found between incubations with or without an excess of unlabeled normal Tf. This, like in our binding experiments, is an indication that rhTf shows a strong nonspecific adherence.

For running proper uptake experiments, it is of importance that the receptors on the cell surface are constantly saturated. Regarding Tf uptake experiments, concentrations of 3 µg Tf/ml incubation medium meet this requirement. Obviously, for our renatured rhTf, this concentration is bound to be higher, because we do not expect the total amount of the rhTf to be renatured in its native (receptor binding) conformation. Receptor saturating conditions can be derived from binding experiments. Unfortunately, rhTf binding studies showed inconclusive results (see 5.3.1).

Therefore we decided to perform an initial uptake experiment using 2 concentrations of doubly labeled (^{59}Fe and ^{125}I) rhTf viz., 3 µg/ml or 9 µg/ml. Iron uptake from the higher concentration (9 µg/ml) rhTf exceeded the iron uptake from the lower

concentration (3 µg/ml) rhTf by PLC/PRF/5 cells (fig. 1), indicating that a concentration of 3 µg/ml of rhTf indeed does not saturate the transferrin receptors continuously. By increasing the concentration it might have been possible to determine the concentration at which the receptors are continuously saturated, because theoretically the increase in iron uptake levels off when this concentration has been exceeded.

Unfortunately, due to shortage of recombinant transferrin it was not possible to incubate hepatoma cells with a series of concentrations higher then 9 µg/ml. At any rate, figure 1 also shows that iron uptake from recombinant transferrin is strongly decreased in the presence of an (50 times) excess of normal Tf, suggesting that the iron uptake of recombinant transferrin is a saturable process in line with receptor mediated endocytosis of rhTf.

Iron uptake from Tf by hepatocytes without internalization of the carrier protein has been described (3,4,5). It has been suggested that diferric Tf binds to its receptor in close proximity to NADH differic Tf reductase. The iron bound to Tf is reduced and the ferrous ions are transported through the plasma membrane. To determine whether iron uptake from rhTf by this mechanism plays any significant role, iron uptake in the presence of 200 µM BPS was defined. BPS forms a complex with ferrous iron, preventing it passing the plasma membrane. There is still a substantial iron uptake in the presence of BPS (fig. 2), so probably a major part of the iron uptake from rhTf is by means of receptor mediated endocytosis of rhTf.

With both our recombinant half-transferrins, no uptake of rhTf/2C or rhTf/2N could be demonstrated. The ^{125}I uptake measured after incubation with an excess of normal unlabeled Tf was nearly equal to the amount, measured after incubation without an excess of normal unlabeled Tf. So the nonspecific adherence of both rhTf/2C and rhTf/2N seems to be very high. In our work, this high nonspecific adherence can easily mask any uptake of the recombinant half-transferrins.

As can be concluded from figure 3, there is a striking difference in iron uptake from rhTf/2C and rhTf/2N, rhTf/2C being much higher. Incubating with rhTf/2N in the presence or without an excess of normal Tf gave similar results, so no specific uptake from rhTf/2N was demonstrated. However, the difference in iron uptake from rhTf/2C in

the presence or without an excess of normal Tf indicates a specific iron uptake from rhTf/2C. These results are in line with data from literature (6) in which experiments have been run with half-transferrins which were produced by digestion of Tf with thermolysin. The latter procedure probably leads to properly folded half-transferrins, in which the C-terminal half-transferrin is still glycosylated. As reported in this experiment, N-terminal half-transferrin is unable to bind to Tf receptors. The C-terminal half Tf, however, can bind to Tf receptors and can therefore enter cells by means of receptor mediated endocytosis (and donate iron).

In contrast, our results with rhTf/2N do not agree with a recent paper of Thorstensen *et al.* (7). These authors report that N-terminal half-transferrin (obtained the same way as described in (6)) is able to donate iron to rat hepatocytes through an unknown mechanism in which presumably pinocytosis plays a role without the interaction of transferrin receptors. We could not confirm iron donation from N-terminal half-transferrin using recombinant N-terminal half-transferrin.

In general, the few available studies examining iron uptake from aglyco Tf indicate that nonglycosylated Tf tends to decreased iron donation compared to normal transferrin (8,9). Before this can be confirmed using refolded recombinant aglycotransferrins, more data should be available on the different conformations that arise after refolding. When it is possible to perform studies with pure samples of correctly folded recombinant transferrins, it will be possible to investigate the influence of the transferrin oligosaccharides on the function of this protein.

At the present state of the art, we merely know that refolded rhTf can donate iron to hepatoma cells, probably after uptake by means of receptor mediated endocytosis. Regarding the recombinant half-transferrins, rhTf/2C is able to donate iron to hepatoma cells, and no iron donation from rhTf/2N could be demonstrated.

5.5 Summary

In this chapter the biological activity of recombinant transferrin, recombinant C-terminal and recombinant N-terminal half-transferrin after refolding is described. Insight in the functionality of the refolded recombinant proteins was obtained by performing transferrin binding- and iron uptake studies.

Receptor mediated Tf (and iron) uptake is a very specific process depending on a very specific conformation of the Tf molecule.

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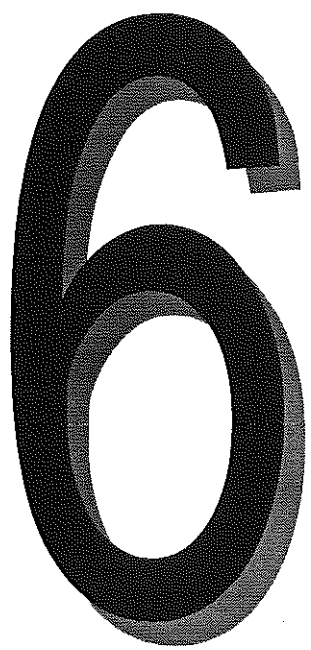
Evaluation of carbohydrate-deficient transferrin as a marker of alcohol abuse



6.1 Introduction

In Western society alcohol abuse has a prevalence of between 10 to 15% in the population. Within this small section there is a striking variability in an individual's response to the chronic effects of excess alcohol intake. A variety of symptoms will be exhibited by these subjects ranging from life threatening alcohol induced liver damage or brain damage, to cardiovascular disturbances, myopathy or merely skin disorders. Alcohol abuse is now recognized to precipitate or exacerbate certain cutaneous diseases in particular psoriasis (1) and these skin problems appear to occur as an early manifestation, often before evidence of classical hepatic toxicity is apparent. A recent survey of 100 psoriasis patients attending the Dermatology Out Patient Department of a London Teaching Hospital, showed that 39% of these patients were drinking in excess of recommended guidelines (2). Furthermore the psoriasis improved when the patient ceased alcohol intake. Similarly in patients with alcohol induced myopathy, which occurs in 70% of alcohol abusing subjects (3) there is amelioration of the myopathy on cessation of alcohol intake.

There have been extensive investigations to identify a biochemical marker of alcohol abuse which could be used firstly to assess the extent to which the patient is abusing alcohol, secondly recognise which patients are at particular risk of alcohol abuse (4) and perhaps more importantly whether continuing abuse of alcohol could progress to life threatening problems. Many biochemical markers have been studied e.g. HLA antigens, blood group antigens, platelet monoamine oxidase, erythrocyte macrocytosis and gamma-glutamyl transpeptidase (5), but each has been lacking in both precision and reliability for their ability to identify patients abusing alcohol.

Glycosylation of secreted proteins is often found to vary in disease conditions, although their mode of action and pathogenesis are not well understood. Alterations in the sialic acid content of transferrin (Tf) occurs in subjects severely abusing alcohol. The di- (2-Si), mono- (1-Si), and asialo forms of human Tf are increasing (6). These Tf subfractions with reduced sialic acid content are also referred as carbohydrate deficient transferrin (CDT). There is now an increasing scientific literature indicating that this index shows the necessary specificity and precision as a marker of gross alcohol abuse. However there

have been only a few studies of individuals who abuse alcohol to a limited degree, their intake being above the recommended guidelines, but nevertheless show an averse reaction which necessitates medical attention.

In this chapter we discuss the degree of sialylation of Tf in male and female subjects with psoriasis who have an intake of alcohol above the recommended guidelines, and compare this with another group of psoriasis patients with no evidence of abusing alcohol (7). Comparison of these results are made with patients with alcohol-induced myopathy and with two other groups, where gross tissue damage has been induced by excessive alcohol intake, namely liver disease and brain damage.

6.2 Materials and Methods

6.2.1 Selection of Patients

(i) Psoriasis patients

Patients (n=52) were recruited from the Dermatology Out-patients at King's College Hospital as part of a prospective study examining the relationship between alcohol consumption and skin disease. Drinking habits were assessed by means of a frequency/quantity questionnaire, as such structured questionnaires have been shown to be a more sensitive indicator of alcohol misuse than standard biochemical screening (8). Of these 52 patients, 4 female and 18 male patients were identified as heavy drinkers (mean intake of alcohol / week 35.6 units ± 16.7). One unit represents the amount of ethanol in one alcoholic drink (8-10 g).

(ii) Patients with alcohol induced liver cirrhosis or myopathy

Patients with alcohol induced cirrhosis (n=6) or alcoholic myopathy (n=6) which had been confirmed by liver and muscle biopsy, respectively, and were consuming alcohol up to the time of the blood sampling, (mean intake of alcohol / day 36.9 units ± 3.8) were selected as positive controls for alcohol abuse.

(iii) Patients with alcohol induced brain damage

Patients with alcohol induced brain damage (n=6), identified by biochemical (altered parameters for the erythrocyte transketolase enzyme) and cognitive test. Such subjects were not necessarily drinking at the time of the blood sampling.

(iv) Control subjects

Control group of subjects from healthy individuals was recruited for the study, with an age range comparable to the patient groups, who consumed only small quantities of alcohol per week (< 4 units).

6.2.2 Determination of the transferrin sialo-variants

Sera were prepared from the blood specimens collected from the different patient groups, and stored at -20^{o}C until analysis. Isoelectric focusing was run in PhastSystem with Immobiline Dry Plate pH 4-7 (Parmacia LKB, Uppsala, Sweden). A part of this gel was cut out to the same size as PhastGel, to obtain a pH-gradient between the electrodes of 5.0-6.0. Isoelectric focusing and staining of the gels were performed as described in chapter 2 (2.7.2 and 2.7.3).

6.2.3 Statistical Analyses

For the statistical analyses an analysis of variance (ANOVA) with protected Tukey 't'test was performed.

6.3 Results

Table 1 shows the results for the different isoforms of Tf in the sera of patients with psoriasis. There were changes in the percentages of isoforms of the highly sialylated forms of Tf present, particularly 5-Si, while there were significant increases in the percentage of the 2-Si forms present in the sera of psoriasis patients, both male and female, abusing alcohol, by comparison to psoriasis patients who did not. In addition the 3-Si form also increased in the psoriatic patients abusing alcohol. In each of the patient groups where excessive amounts of alcohol had induced liver, brain or muscle damage,

Isoforms of transferrin (%) in sera of psoriasis patients not abusing alcohol.

	7-Si	6-Si	5-Si	4-Si	3-Si	2-Si
Male n=20	0.7 ±0.4	3.9 ±1.1	20.9 ±2.9	63.9 ±4.6	7.0 ±2.3	2.7 ±1.3
Female n=18	0.5 ±0.2	4.9 ±1.1	23.6 ±3.3	60.8 ±4.0	7.1 ±2.3	3.0 ±1.1

Isoforms of transferrin (%) in sera of psoriasis patients abusing alcohol.

	7-Si	6-Si	5-Si	4-Si	3-Si	2-Si
Male n=10	0.6 ±0.4	4.2 ±0.9	20.4 ±2.2	63.9 ±5.5	7.5 ±3.1	3.4* ±2.7
Female n=4	n/d	4.5 ±1.8	23.8 ±3.8	56.9 ±6.8	10.2* ±1.8	4.7** ±0.7

Isoforms of transferrin (%) in sera of alcohol misusers with myopathy, liver or brain damage.

	7-Si	6-Si	5-Si	4-Si	3-Si	2-Si
Myopathy n=6	n/d	3.1 ±1.1	17.7 ±6.2	64.2 ±5.7	7.3 ±1.8	4.5** ±2.3
Liver damage n=6	n/d	5.2 ±1.4	21.0 ±3.9	59.1 ±5.3	7.7 ±2.3	6.6** ±4.9
Brain damage n=6	n/d	3.2 ±0.3	20.2 ±2.4	63.0 ±3.1	8.3 ±1.5	5.4** ±3.0

Isoforms of transferrin (%) in sera of control subjects.

	7-Si	6-Si	5-Si	4-Si	3-Si	2-Si
n=10	n/d	4.9 ±1.4	25.6 ±1.7	60.6 ±2.6	6.6 ±1.8	2.2 ±0.5

Table 1. Results are mean ± standard deviation.
Statistical significance by ANOVA with protected Tukey 't' test.
*P<0.05 **P<0.01
n/d: not determined

significantly increased percentages of di-sialo Tf were found. This increase in the 2-Si isoform of Tf appeared to be at the expense of the 6-Si and 5-Si isoforms of Tf which were both reduced.

6.4 Discussion

It is now well established that chronic ingestion of alcohol impairs the integrity of glycoproteins and glycolipids in liver, brain erythrocytes and intestine. This study confirmed the elevated levels of di-sialylated Tf in alcoholic patients with dependency, with either alcoholic liver disease or brain damage. In addition this study also showed that patients with either myopathy or psoriasis, in whom dependency on alcohol was not necessarily apparent, the 2-Si Tf isoform was increased.

One of the principal criticisms of many of the reported studies of alcohol abuse has been the problem of retrospective self-reporting of alcohol consumption. In this present study we have attempted to correct this error of other studies by using a structured questionnaire.

Elevated amounts of carbohydrate deficient transferrin (CDT), notably the di- and tri-sialylated forms, were first identified by Stibler and Kjellin (9) in the cerebrospinal fluid and serum of alcohol misusers with cerebellar tremor. The explanation for the altered isoforms of Tf in alcoholic subjects with physical damage is possibly caused by alcohol interfering with the metabolism of glycoconjugates. Chronic alcohol misuse alters the N-acetyl-neuraminic acid (sialic acid) content of Tf resulting in increased serum concentrations of sialic acid-deficient Tf with isoforms with an isoelectric point (pI) of 5.7 and 5.9. The function of the sialic acid-deficient Tf in alcohol misusers remains unclear. Transferrin, the major serum iron transport protein is metabolised more rapidly (although unlike all other glycoproteins, not by the liver) following alcohol abuse until the development of cirrhosis (10). Loss of the carbohydrate chain does not appear to alter its catabolism. CDT can bind to two sets of receptors, the hepatic asialoglycoprotein receptors as well as to transferrin receptors. The former leads to degradation while the latter does not, suggesting that there is some compensatory mechanism activated by alcohol to restore Tf turnover to normal.

Carbohydrate deficient transferrin (CDT) has been suggested to show a high sensitivity and specificity (>70%) for alcohol misuse, particularly in patients with liver cirrhosis or recently consuming large amounts of alcohol. The most critical unresolved question is exactly what parameters are needed to alter the CDT levels, firstly how much alcohol needs to be consumed, what is the duration of the drinking history needed and lastly does there have to be associated tissue damage. In the few studies where CDT values have been assayed in healthier and younger alcohol misusers with shorter drinking histories, CDT has been shown to have a sensitivity of less than 30% for alcohol misuse (11). Furthermore only 20% of normal subjects consuming 60 g alcohol/day for 3 weeks in another study, showed CDT levels that exceeded the upper normal level (12). Such results have led to the belief that there must be some degree of physical damage before CDT is elevated (11,13).

CDT was shown to have a lower specificity and sensitivity for alcohol abuse by females compared to alcohol abuse by males (14), although this was not confirmed in this present study. The few psoriasis patients abusing alcohol showed highly elevated amounts of 2-Si.

A study of 439 treated hypertensives showed that there was a positive relationship between CDT and high alcohol intake, the sensitivity of the test being 87%, but there were a considerable number of false positives (15). Serum CDT concentrations were also associated with insulin sensitivity (16) indicating that factors related to insulin may be operative.

It remains unclear as to whether excessive alcohol consumption actually alters iron homeostasis. It is clear that alcohol will increase gut permeability, and possibly increase absorption of iron into the enterocyte (17). Whether this iron is actually utilised internally is unknown. There was a two fold increase in the mean concentration of serum ferritin in the psoriasis group associated with increased alcohol intake by comparison to the non-drinking psoriasis group, (165.9 ± 146 v 87.8 ± 67.5 ng/l), although only five of the former group had values for ferritin increased to the normal ranges (unpublished results). Other diseases where there are perturbations of iron metabolism have been investigated to ascertain whether changes also occur in the Tf isoforms. Patients with haemochromatosis

(increased serum ferritin levels) showed marginally increased CDT values, particularly during phlebotomy (18) although the values were not necessarily elevated above the reference ranges. Iron deficiency caused either as a normal physiological response to pregnancy (decreased serum ferritin) or as a result of chronic inflammation (increased serum ferritin) as observed in rheumatoid arthritis patients, showed a tendency for an increase in the highly sialylated transferrins, rather than the di-sialo Tf which maintained a constant value (19).

Earlier methods used non-quantitative methods of isoelectric focusing as reported in this present communication, while more recently quantitative methods have been developed to separate Tf components above pI 5.65 by micro-anion exchange chromatography (6) followed by RIA to evaluate CDT. This method has been further modified by adoption of a different elution buffer that achieves a more stable anion exchange chromatography of isotransferrins. Studies which have compared isoelectric focusing and anion exchange chromatography have found a somewhat higher sensitivity and specificity with isoelectric focusing immunofixation than with anion exchange chromatography-RIA method (e.g. 20-23). In addition the analysis of CDT by anion exchange chromatography will also suffer from the fact that total Tf is somewhat variable among individuals regardless of whether or not they are alcoholic; furthermore the presence of anticoagulants in the sample may interfere with the chromatography method.

These studies have indicated that CDT is a sensitive marker of alcohol intake in subjects not necessarily showing gross physical damage. CDT may therefore prove to be an effective marker of early alcohol misuse, possibly before the onset of irreversible organ damage. Although other markers of alcohol abuse show a high specificity and sensitivity either alone or in combination (such as the determination of apo A-II, plasma γ-GT, plasma uric acid and MCV (24)), the simplicity of a single test to assess alcohol abuse remains attractive. Further studies are required to identify what role these different isoforms of Tf may play within biochemical systems.

6.5 Summary

The different isoforms of Tf have been quantitated by isoelectric focusing of the sera of psoriasis patients with and without a history of abusing alcohol. In both male and female subjects abusing alcohol there were significant increases, $P<0.05$ and $P<0.01$ respectively, in the 2-sialylated forms by comparison to either psoriasis subjects not abusing alcohol or control subjects. Other groups of patients who showed alcohol induced tissue damage, i.e. liver, brain or muscle, used as positive controls, similarly showed significant increases in the 2-sialylated forms, $P<0.01$ in each group, by comparison to controls. These results substantiate the current use of carbohydrate deficient Tf as a sensitive marker of alcohol abuse.

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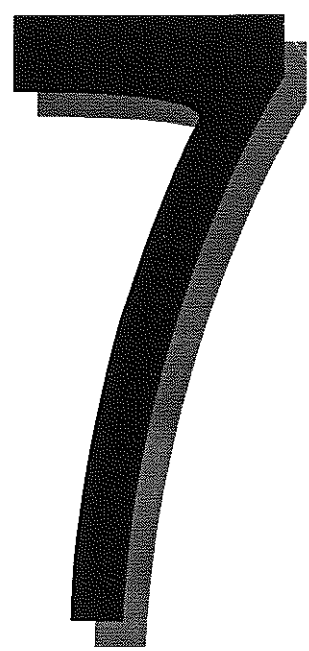
Conclusions



7.1 Introduction

During the last decade, there has been an increasing interest in the significance of protein glycans. Protein glycosylation is the most common post-translational modification of newly synthesized proteins. All membrane proteins of higher organisms and most secretory proteins (e.g. transferrin, Tf) are glycosylated. Glycoproteins can also be found in the cytoplasm and nucleus of cells (1).

The conservation of carbohydrate units on glycoproteins during evolution indicates that these structures are of vital importance for a normal function of these proteins. However, no universal function could be attributed to the glycans, but it is generally accepted that the oligosaccharide moieties of glycoproteins can alter the physiochemical properties of the molecules. These alterations can result in an increased solubility and in an increased stability of the glycoproteins, against proteolysis as well as thermal stress (2-5). Protection of glycoproteins against proteolytic enzymes by the glycans can be effected either by increased rigidity of the protein (due to carbohydrate-carbohydrate or carbohydrate-protein interactions) or by sterically shielding susceptible sites. Glycans can nearly completely cover proteins as can be seen with human alpha-1-acid glycoprotein (6). Another general function of the glycans could be to support the folding of the nascent polypeptide chain and to stabilize the conformation of the glycoprotein (2). In fact, any function depending on the conformation of the glycoproteins may be affected by changes in the glycan chains. Glycans can also increase the solubility of glycoproteins, especially through the effect of the negative charges of the sialic acid residues and sulphate groups.

Glycans are also known to influence the biological properties of glycoproteins, such as the regulation of intracellular transport (7,8) and the determination of the lifetime of glycoproteins in the circulation (9,10). Modulation of the activity of enzymes, hormones and receptors has also been described for several glycoproteins (2,11,12). However, in some cases these changes in activity could merely be a direct result of changes in the protein conformation as already discussed above.

The importance of glycans for the function of glycoproteins has been reviewed in several publications (2,3,4,13). It can be concluded from these publications that no univocal function can be attributed to the glycans of glycoproteins. Clearly the

oligosaccharide moieties have different effects on different glycoproteins, and in many cases even no effect at all can be detected.

7.2 The importance of the glycans for the function of transferrin

No function could be attributed to the Tf glycans so far. The glycans play no role in the transport of Tf during synthesis (7,8), as has been reported for other glycoproteins. The glycans of Tf can not be of vital importance for the folding of Tf, otherwise aglyco Tf would not be found occurring in the serum of CDG syndrome patients, but would probably form aggregates during synthesis. Presumably the glycans of Tf are not of essential importance for Tf, given that Tf of several fish species of the family *Cyprinidae* is nonglycosylated (14,15).

7.3 The clinical significance of the transferrin glycans

The Tf glycans play a significant role in the diagnosis of the Carbohydrate Deficient Glycoprotein (CDG) syndrome (16). Excessive alcohol intake changes the distribution of the different sialo variants of Tf in the serum towards an increase of the lower sialylated transferrin subfractions. First described by Stibler (17), numerous studies have since been published on the use of Tf as a marker for alcohol abuse in certain subpopulations (for instance in combination with certain clinical signs or symptoms, 18-23). Most of these studies, however, concentrated on gross alcohol abuse.

7.4 Production of aglycotransferrin

The purpose of the research described in this thesis was to obtain some insight in the significance of the glycans on Tf for the function of this protein. Lately, much attention has been directed towards the specific changes in glycan microheterogeneity of Tf during the course of certain diseases and pregnancy (24-35). Assuming these changes represent an adjustment of the Tf function to altered (patho)physiological changes, it would be very interesting to learn more about the primary function of the Tf glycans. Since the major function of Tf is the transport of iron to the various cell types requiring this metal, our efforts were focused on the influence of the Tf glycan chains on this

function. In order to assess the importance of the Tf glycans to receptor binding and uptake of Tf, the availability of aglyco Tf is essential.

Attempts were made to produce aglyco Tf by two different methods; by expression in *Escherichia coli* resulting in nonglycosylated rhTf and by step-wise digestion of the glycan chains using the proper enzymes. Both methods have been extensively described in the preceeding chapters.

In short, expression of human Tf in *E. coli* resulted in a nonglycosylated form of this protein, deposited by the bacterial metabolism in a denatured and reduced state in so called inclusion bodies. The inclusion bodies were isolated and dissolved. Subsequently the recombinant Tf (rhTf) was refolded under appropriate conditions. Recombinant C-terminal and N-terminal half-transferrins (rhTf/2C and rhTf/2N) were produced in a similar way.

Production of aglyco Tf by step-wise digestion of the Tf glycans using the proper enzymes, proved to be impossible. Attempts resulted in partially deglycosylated Tf, in other words Tf lacking only the three distal sugar residues of its glycans.

A third source of aglyco Tf proved to be serum obtained from a patient suffering from the Carbohydrate Deficient Glycoprotein (CDG) syndrome. Regretfully only minute amounts of aglyco Tf could be obtained from this source.

7.5 Biological properties of aglycotransferrin and partially deglycosylated transferrin

Binding and uptake studies were performed with aglyco Tf isolated from a patient suffering from the CDG syndrome. Tf binding studies comparing aglyco Tf with normal glycosylated Tf, revealed that the absence of the Tf glycans does not influence the affinity of this protein for its receptor (chapter 3, fig. 4).

Tf uptake studies showed that aglyco Tf reached a lower steady state in PLC/PRF/5 cells then fully glycosylated Tf (chapter 3, fig. 6). Likewise the iron donation by aglyco Tf was decreased compared to fully glycosylated Tf (chapter 3, fig. 6).

Enzymatical digestion of the three most distal sugar residues (sialic acid, galactose and N-acetyl glucosamine, see chapter 3, fig. 1) of the Tf glycan chains of normal glycosylated Tf did not influence the affinity of Tf for its receptor (chapter 3, fig. 4). This

partial deglycosylation did not have any influence on the Tf and iron uptake, when compared to fully glycosylated Tf.

With respect to aglyco Tf, it can be concluded that the Tf glycans do influence the uptake of Tf and iron by cells without affecting the affinity of the protein for its receptor. With respect to partially deglycosylated Tf, it can be concluded that the antennae of the Tf glycans do not influence the uptake of Tf and iron.

These results fit in a hypothetical model in which the glycan chains of Tf are involved in the regulation of the endocytosis of the Tf-transferrin receptor complex. The antennae of the glycan chains do not have any influence on this regulation, since their removal does not alter the endocytosis of Tf. This hypothesis implicates that Tf binding by the Tf receptor is necessary for the receptor to be endocytosed. Although this has been reported to be true (35), this matter is still a point of discussion.

7.6 Biological properties of recombinant aglycotransferrin and recombinant half-transferrins

Tf binding studies comparing rhTf to normal glycosylated Tf were inconclusive, because rhTf showed a very high aspecific binding. For the same reason Tf uptake studies were also impossible to assess.

Iron uptake studies comparing fully glycosylated Tf with rhTf showed a reduced uptake of iron from the latter protein. This reduction was much stronger than that seen with the iron uptake from aglyco Tf, isolated from serum of a CDG syndrome patient. It is very likely that the reduced uptake is due to the lack of the glycan chains and to the fact that a substantial part of the refolded rhTf has a misfolded conformation (chapter 5). Therefore rhTf will only be suitable for comparative studies as soon as more data are available on the conformations that arise during refolding, and as soon as the correctly folded rhTf can be isolated from the misfolded conformations.

Iron uptake studies comparing rhTf/2C and rhTf/2N to normal glycosylated Tf showed that of both recombinant transferrins, only rhTf/2C is capable of donating iron to hepatoma cells. As for rhTf, more data on the conformations of both half-transferrins after refolding are needed, before any comparative studies can be performed. Only then we can

try to elucidate the question whether the Tf glycans are of vital importance to the (function of the) protein.

7.7 Conclusions

It can be concluded from our research that:

(1) Absence of the Tf glycans on Tf does not influence the affinity of this protein for its receptor.

(2) Uptake of aglyco Tf by hepatoma cells (PLC/PRF/5 cell line) is decreased compared to fully glycosylated Tf.

(3) Iron uptake from aglyco Tf by PLC/PRF/5 cells is decreased compared with fully glycosylated Tf.

(4) Recombinant human Tf produced by *Escherichia coli* and deposited in inclusion bodies can be renatured to a water soluble and iron-binding form.

(5) This renatured recombinant human Tf is capable to donate iron to PLC/PRF/5 cells (human hepatoma cells).

(6) Renatured recombinant human C-terminal half-transferrin (rhTf/2C) is also capable to donate iron to PLC/PRF/5 cells. Renatured recombinant human N-terminal half-transferrin, however, is not capable to donate iron to PLC/PRF/5 cells.

(7) Carbohydrate-deficient Tf can be used as a marker for the detection of moderate alcohol abuse in psoriasis patients.

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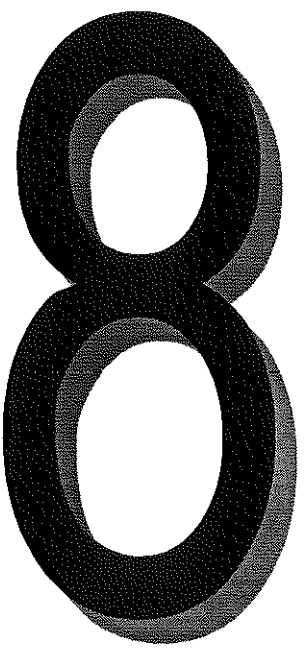
Summary
samenvatting



8.1 Summary

Within species there is an important difference in the glycosylation of serum transferrin (Tf). The number of glycans on Tf varies from zero (in certain fish species) to two (in humans).

In humans the Tf glycans can be of the bi-antennary, tri-antennary or even tetra-antennary type. These differences in the glycans contribute to the so called microheterogeneity of Tf. The distribution of transferrins, differing in their glycan composition, is considerably constant in humans. However, (patho)physiological changes (e.g. pregnancy, alcohol abuse, carcinomas, rheumatoid arthritis) result in characteristic changes in the glycan composition of Tf. No specific function could be attributed to the Tf glycans so far. In order to determine the biological significance of the Tf glycans, the function of normal Tf should be compared to the function of nonglycosylated Tf.

This thesis describes the isolation and renaturation of nonglycosylated recombinant Tf produced in *Escherichia coli* and the production of partially deglycosylated Tf by incubating Tf with the proper exoglycosidases. Nonglycosylated Tf was also obtained from serum of a patient suffering from the Carbohydrate Deficient Glycoprotein (CDG) syndrome. The binding of the abnormally glycosylated transferrins was compared to the binding of the normally glycosylated Tf. The uptake of these abnormally glycosylated transferrins by hepatoma cells was compared to the uptake of normally glycosylated Tf by these cells. Similarly the iron donation was compared.

Finally this thesis describes the possibility to use the specific changes of the Tf microheterogeneity as a marker for moderate alcohol abuse.

Chapter 1

This chapter gives an overview of:

(a) The function of iron.

(b) The siderophilins.

(c) General considerations on glycoproteins.

(d) Transferrin.

(e) The significance of transferrin glycans.

(f) Production of aglycotransferrin.

(g) Renaturation of recombinant proteins.

(h) Iron donation by transferrin.

Chapter 2

This chapter describes the general techniques that contributed to the experimental results discussed in this thesis.

Chapter 3

In this chapter the receptor binding of partially deglycosylated Tf and nonglycosylated Tf (obtained from serum of a patient suffering of the CDG syndrome) is compared to the receptor binding of normally glycosylated Tf. Uptake of these transferrins with abnormal glycosylation by hepatoma cells is compared to uptake of normally glycosylated Tf. Iron donation is compared in a similar way.

Chapter 4

The isolation and renaturation of recombinant human transferrin (rhTf), recombinant human C-terminal half-transferrin (rhTf/2C) and recombinant human N-terminal half-transferrin (rhTf/2N) produced in *Escherichia coli* is described in this chapter. The amino acid composition of the recombinant transferrins is compared to the amino acid composition of normal human Tf.

The iron binding capacities of the recombinant transferrins is determined by ^{59}Fe labeling, absorption spectrophotometry and EPR spectrometry.

Chapter 5

In this chapter receptor binding and uptake of rhTf, rhTf/2C and rhTf/2N are compared to receptor binding and uptake of normally glycosylated Tf. Iron donation by the recombinant transferrins is compared to iron donation by normal human Tf.

Chapter 6

The use of changes in the Tf microheterogeneity as a marker for moderate alcohol abuse is discussed in this chapter.

Chapter 7

In this chapter the conclusions of the experimental results described in this thesis are discussed. Furthermore a hypothesis on the function of the Tf glycans is proposed.

8.2 Samenvatting

De mate van glycosylering van serum transferrine (Tf) is duidelijk verschillend bij diverse diersoorten. Het aantal aan Tf gebonden glycanen varieert van geen enkele (bij bepaalde vissoorten) tot twee (bij mensen).

Aan humaan Tf kunnen zowel bi-antennaire, tri-antennaire als tetra-antennaire glycanen gevonden worden. Deze verschillen in glycosylering dragen bij tot de zogenaamde micro-heterogeniteit van Tf. De verdeling van Tf met een verschillende mate van glycosylering is vrij constant. Bij bepaalde (patho)fysiologische veranderingen (bijv. zwangerschap, alkoholmisbruik, bepaalde carcinomen, rheumatoïde arthritis) worden specifieke veranderingen in deze verdeling van glycanen gezien. Het is nog niet bekend of deze glycanen een funktie vervullen. Om dit te onderzoeken, is het noodzakelijk te beschikken over Tf zonder deze glycanen (aglyco Tf).

In dit proefschrift worden de zuivering en renaturatie van recombinant Tf beschreven. Dit recombinant Tf (geproduceerd in *Escherichia coli* bacteriën) bevat geen glycanen. Tevens wordt de gedeeltelijke afbraak van de glycanen van humaan Tf met behulp van enzymen beschreven in dit proefschrift. Aglyco Tf werd ook verkregen door dit eiwit te isoleren uit serum van een patiënt met het Carbohydrate Deficient Glycoprotein (CDG) sydroom.

De receptor binding van de hierboven genoemde transferrines werd vergeleken met de receptorbinding van normaal geglycosyleerd Tf. De opname van deze transferrines (en ijzer) door hepatoma cellen werd vergeleken met de opname van normaal geglycosyleerd Tf door hepatoma cellen.

Tenslotte wordt het gebruik van de specifieke verandering in de Tf micro-heterogeniteit als een marker voor alkoholmisbruik beschreven.

Hoofdstuk 1

In dit hoofdstuk worden achtereenvolgens beschreven:

(a) De funktie van ijzer.

(b) De siderophilines.

(c) Algemene opmerkingen betreffende glycoproteinen.

(d) Transferrine.

(e) Het belang van de transferrine glycanen.

(f) Productie van aglycotransferrine.

(g) Renaturatie van recombinant eiwitten.

(h) IJzer afgifte door transferrine.

Hoofdstuk 2

In dit hoofdstuk worden de technieken vermeld die toegepast zijn en geleid hebben tot de onderzoeksresultaten beschreven in dit proefschrift.

Hoofdstuk 3

In dit hoofdstuk wordt de receptor binding van gedeeltelijk gedeglycosyleerd Tf en aglyco Tf (verkregen uit serum van een patient met het CDG syndroom) vergeleken met de receptor binding van normaal geglycosyleerd Tf. De opname van abnormaal geglycosyleerde transferrines wordt vergeleken met de opname van normaal geglycosyleerd Tf. Tevens wordt de ijzer afgifte door de verschillende transferrines vergeleken.

Hoofdstuk 4

In dit hoofdstuk worden de zuivering en renaturatie van in *Escherichia coli* geproduceerd recombinant humaan transferrine (rhTf), recombinant humaan C-terminaal half-transferrine (rhTf/2C) en recombinant humaan N-terminaal half-transferrine (rhTf/2N) beschreven.
De aminozuur samenstelling van de recombinant transferrines wordt vergeleken met die van het normale humane Tf.
Het ijzerbindend vermogen van de recombinant transferrines werd aangetoond met behulp van ^{59}Fe, absorptie spectrofotometrie en EPR spectrometrie.

Hoofdstuk 5

In dit hoofdstuk worden receptor binding en opname van rhTf, rhTf/2C en rhTf/2N vergeleken met receptor binding en opname van normaal Tf. Tevens wordt de ijzer afgifte door de recombinant transferrines vergeleken met de ijzer afgifte door normaal

transferrine.

Hoofdstuk 6

De mogelijkheid om veranderingen in de Tf micro-heterogeniteit te gebruiken voor het opsporen van alkoholmisbruik wordt beschreven in dit hoofdstuk.

Hoofdstuk 7

In dit hoofdstuk worden de conclusies uit de bovengenoemde hoofdstukken samengevat en is een hypothese over de functie van de Tf glycanen opgesteld.

Publications

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Curriculum vitae

Peter Hoefkens werd geboren op 22 februari 1965 te Oostkapelle. In 1983 behaalde hij het VWO diploma aan de RSG Scheldemond te Vlissingen. Tot 1984 studeerde hij aan het HBO-V te Goes. In dat jaar begon hij aan de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens zijn studie liep hij stage aan het Queensland Institute of Medical Research te Brisbane, Australië. In 1992 behaalde hij zijn artsexamen en kreeg een aanstelling als AIO op de afdeling Chemische Pathologie van de Erasmus Universiteit te Rotterdam.
Op deze afdeling werd onder leiding van Prof. Dr. H.G. van Eijk het in dit proefschrift beschreven onderzoek uitgevoerd.
Op 1 december a.s. start hij met de opleiding tot Klinisch Chemicus op de afdeling Klinische Chemie van het Academisch Ziekenhuis Rotterdam (opleider Dr. J. Lindemans).