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Isolation, Renaturation and Partial Characterization of Recombinant Human Transferrin and its Half Molecules from *Escherichia coli*

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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 here. 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 transferrin. Renaturation of the recombinant proteins is described, resulting in water-soluble iron-binding molecules. Iron binding was confirmed by ^{59}Fe labelling, absorption spectrophotometry and EPR spectrometry. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

Transferrin is an 80 kDa glycoprotein functioning as a ferric iron carrier in higher eukaryotes. Being one of the major serum proteins, it has been studied extensively since its first description (Schade and Caroline, 1946).

The amino acid sequence of human transferrin has been known for several years now (MacGillivray *et al.*, 1982; Yang *et al.*, 1984). The protein can be divided into two globular domains each containing one high affinity binding site for iron. These two globular domains show a high degree of internal homology.

The C-terminal domain (residues 337-679) carries two branched N-linked glycans. These two glycan chains are linked at the amide group of the asparagine residues at positions 413 and 611 through a N-glycosidic linkage (Jamieson, 1964). No specific function has been ascribed to the carbohydrate moieties of transferrin so far. It has been reported that the glycan chains play no role in the binding of transferrin to its receptor (Mason *et al.*, 1993), neither do they influence the secretion rate of transferrin by hepatoma cells (Bauer *et al.*, 1985). Carbohydrate-deficient transferrin has been described in several fish species of the Cyprinidae family (Strati1 *et al.*, *1983*, 1985).

During certain physiological and pathological conditions, such as pregnancy, rheumatoid arthritis, malignancies and alcohol abuse (van Eijk *et al.*, 1987; de Jong *et al.*, 1990, 1992; Léger *et al.*, 1989; Yamashita *et al.*, 1989; Stibler *et al.*, 1978), characteristic changes in the glycan

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetra-acetic acid; IPTG, isopropyl-β-D-thiogalacto-pyranosidase; PAGE, polyacrylamide gel electrophoresis; rhTf, recombinant human transferrin; rhTf/2C, recombinant human C-terminal half-transferrin; rhTf/2N, recombinant human N-terminal half-transferrin; hTf/2N, human N-terminal half-transferrin; hTf, human serum transferrin; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; NTA, nitrilotriacetic acid.

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structures occur. The significance of this variation in microheterogeneity is not elucidated totally. *In vitro* experiments employing transferrins differing in their glycan composition have shown subtle differences in the processing of these transferrins by the organs most intimately involved in iron homeostasis in man. These results have been fitted in a model that explains the redistribution of iron between iron stores in iron deficiency, pregnancy and states of chronic inflammation (de Jong, 1993).

Studying the biochemical, structural and physiological properties of human transferrin lacking the carbohydrates could give more insight into the function of the glycans. Initial attempts to remove the glycans chemically or enzymatically turned out to be unsuccessful. Either transferrin was damaged irreversibly or the carbohydrates were only partially removed (de Jong *et al.*, 1990, 1993). Another problem with enzymatic deglycosylation is that it can be applied only to small amounts of denatured transferrin, although successful use of enzymes under non-denaturing conditions has been reported (Padda and Schrijvers, 1990). The production of non-glycosylated transferrin in eukaryotic cells using site-directed mutagenesis has been described previously (Mason *et al.*, 1993).

Large quantities of aglycotransferrin can be produced in a bacterial expression system (de Smit *et al.*, 1995). Bacteria lack the rough endoplasmic reticulum and the Golgi system and, therefore, are not able to attach N-linked glycans to their products. For this reason, our group has directed its efforts at developing a system in which recombinant human transferrin DNA could be expressed in *E. coli* and recently has published the results (de Smit *et al.*, 1995).

This bacterial expression system has been employed as our source for aglycotransferrin. Synthesized recombinant transferrin (rhTf) is deposited in a denatured state in large amorphous particles called inclusion bodies. This is true also for the N- and C-terminal half-transferrins (rhTf/2C and rhTf/2N, respectively). Therefore, studying biochemical properties of these proteins requires previous renaturation.

Here, we report on the isolation, renaturation and partial characterization of rhTf, rhTf/2N and rhTf/2C from *E. coli* cultures expressing these proteins. Following purification, we determined the amino acid composition of the recombinant proteins in order to compare this with the theoretical composition that 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.

We renatured the recombinant transferrins into water soluble proteins and determined their iron-binding capacity.

MATERIALS AND METHODS

Production of rhTf, rhTf/2N and rhTf/2C in E. coli

Production of recombinant transferrin and the recombinant half-molecules was performed as described by de Smit *et al.* (1995).

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

To obtain cell lysates, the cultured bacteria were harvested using a MSE Coolspin centrifuge at 2600 g for 15 min. 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 min at room temperature, the sample was snap-frozen by submersing it in a mixture of solid CO, and ethanol. The thawed sample was sonicated. Approximately 300 U DNase I was added and the sample was incubated for 45 min at room temperature.

The inclusion bodies were isolated from the cell lysate by centrifugation at 12 000 g for 15 min. The pellet was washed three 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.

Preparative SDS electrophoresis was used to purify rhTf, rhTf/2N and rhTf/2C. This was performed with a model 392 Bio-Rad Prep Cell. The rhTf, rhTf/2N or rhTf/2C solution (600 μl) was mixed with an equal volume of 8% SDS (w/v), 24% glycerol (v/v), 50 mM Tris/HCl, 4% β-mercaptoethanol (v/v), 0.01% Serva blue G (w/v), pH 6.8 and incubated for 30 min at 40°C. This sample was applied to the polyacrylamide gel (9% T, 3.3% C) of the Prep Cell. The purities of hTf and its half-molecules were examined on 9% SDS polyacrylamide gels after being concentrated with Centriflo CF 25 cones (Amicon). The samples were dialysed against 40 mM $NaHCO_3$. The molecular cutoff was approximately 10 kDa.

Amino acid analysis

The recombinant proteins were analysed using an Alpha Plus 4151 amino acid analyser (Pharmacia LKB Biochrom Ltd, Cambridge, U.K.). The proteins were hydrolysed by mixing 50 μl (0.5-1.0 g/l) protein solution with 150 μl 4 M paratoluene sulphonic acid and 2% 3-(2-amino ethyl) indole HCl (van Eijk and van Noort, 1986). This mixture was incubated for 24 hr 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. This mixture (25 μl) was analysed. During the analysis, the eluent was mixed with a 0.2% (w/v) *ortho*-phthal aldehyde (OPA) 'reagent in 1 M borate. The signal was measured with a fluorometer F 1000 (Merck Hitachi, Amsterdam, The Netherlands). As a reference, we used a very pure human transferrin preparation (4-sialo-bibi-antennary transferrin of the genetic C, type) isolated from serum obtained from adult healthy volunteers as described by van Noort *et al. (1994).*

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

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

Renaturation experiments were performed with 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 (Bradford, 1976). 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. The proteins were incubated for 15 min. Subsequently, GSSG was added to a final concentration of 0.5 mM. This solution was incubated for another 22 hr at 6°C. Renaturation buffer was degassed thoroughly prior to use and renaturation was performed under an atmosphere of nitrogen. After incubation, 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 further using an Amicon Stirred Cell with a PM 10 membrane and finally Centriflo cones, CF 25 (Amicon).

After concentration rhTf, rhTf/2C or rhTf/2N was saturated with iron using a $FeCl_3$/NTA solution and separated from the other proteins using a Sepharose bound anti-transferrin column (van Eijk and van Noort, 1976).

To demonstrate the iron-binding capacity of the recombinant transferrins we saturated rhTf, rhTf/2C and rhTf/2N with iron using a $^{59}FeCl_3$/NTA solution following standard procedures ($^{59}FeCl_3$; Amersham Life Science). The iron-saturated samples 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. Also, we determined the molecular weight of the proteins in the different samples by running them on a 12.5% homogeneous SDS polyacrylamide gel.

Spectrophotometry and EPR

An absorption spectrum and an $A_{280/470}$ ratio of the iron-saturated recombinant transferrins was determined using an Ultrospec III spectrophotometer and application software (Pharmacia, LKB).

The EPR spectra were determined using a Bruker EPR 200 D spectrometer as described by Pierik and Hagen (1990).

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-PAGE (Fig. 1). As expected, rhTf migrated slightly faster then hTf. The lack of oligosaccharide chains reduces the Mw by approximately 4 kDa. The rhTf/2N and rhTf/2C showed bands just above the 32.5 kDa marker, which corresponds to the calculated Mw of respectively 37.3 and 38.3 kDa.

In Table 1, the amino acid composition of rhTf, rhTf/2N and rhTf/2C is compared with hTf. The composition of the recombinant transferrins corresponds very well to the references. Most amino acids show less then 5% deviation from the expected values. In Table 2, the first 10-15 amino acids sequenced from the N-terminal side of the recombinant proteins are compared with the expected amino acids (Yang *et al.*, 1984). These sequences match the references exactly, except for one N-terminal extra methionine at 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 explained in the discussion.

An absorption spectrum of the iron-saturated recombinant transferrins was determined ranging from 280 to 600 nm. Normal diferric human transferrin has an absorption maximum at 465 nm. The recombinant transferrins showed spectra similar to those of diferric human transferrin with an absorption maximum at 462, 459 and 464 nm for rhTf, rhTf/2C and rhTf/2N, respectively. Normal diferric transferrin has an $A_{280/465}$ ratio equivalent to 21; this value also has been reported for iron-saturated hTf/2N (Funk *et* al., 1990). 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 transferrin, these figures correspond with 70 and 90% iron-saturation for rhTf and rhTf/2N, respectively. Assuming the $A_{280/465}$ ratio for iron-saturated hTf/2C is equal to that for hTf and hTf/2N, the calculated value for rhTf/2C corresponds with 75% iron-saturation.

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

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. The ^{59}Fe saturated recombinant proteins all show more than one band on the autoradiograph [Fig. 2(B)]. 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).

DISCUSSION

Here, we report on the purification and renaturation of human transferrin, C-terminal half-transferrin and N-terminal half-transferrin, produced in *E. coli* as described by de Smit *et al.* (1995). After purification, the amino acid compositions of the recombinant transferrin and the recombinant N- and C-terminal half-transferrins were determined. These compositions match very well with the ones to be expected, not only proving that the samples contain the proteins of interest to us, but also that they are very pure.

The N-terminal sequencing shows that the recombinant transferrins all have an extra methionine at the N-terminus (Table 2). The

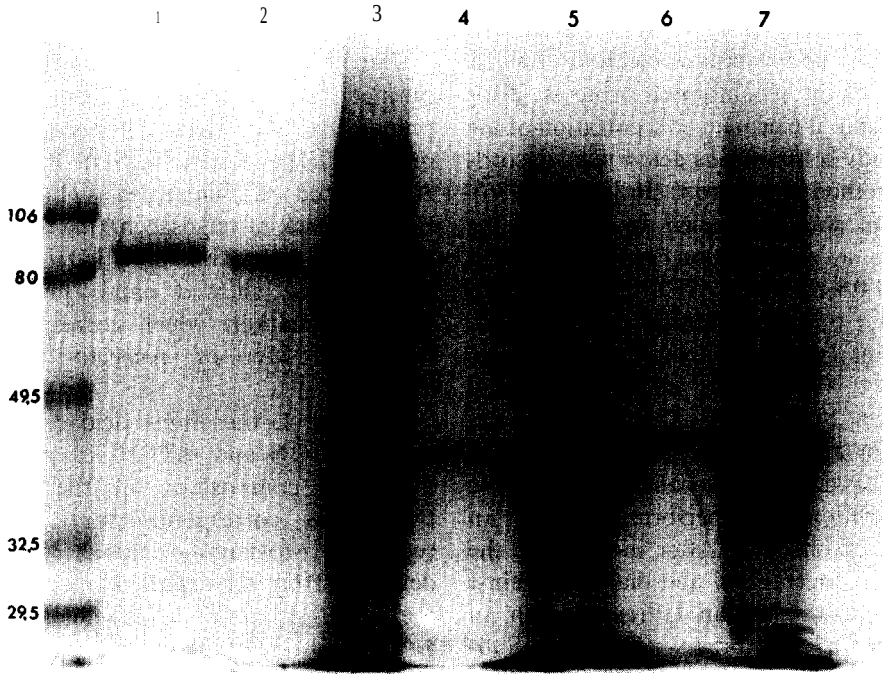

Fig. 1. The SDS-PAGE (9%) showing the purified recombinant proteins and the solubilized inclusion bodies from which these proteins were isolated. Lane 1: hTf; lane 2: rhTf; lane 3: inclusion body solution containing rhTf; lane 4: rhTf/2N; lane 5: inclusion body solution containing rhTf/2N; lane 6: rhTf/2C;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

Table 1. Amino acid analysis of recombinant transferrins (mol amino acid/mol protein)*

	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,*	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
Phenylalanine	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%	

*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, which therefore are 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. (1984).

difference in the amino acid sequence of rhTf/2C (amino acid 338) is due to the introduction of a *Kpn*I-site in the transferrin cDNA (de Smit *et al.*, 1995). Apart from this, the amino acid sequences of the recombinant transferrins match the expected sequences exactly (Yang *et al.*, 1984).

For our first renaturation experiments, we used SDS/urea-denatured reduced hTf. All attempts to renature transferrin after such treatment failed. Although total removal of SDS from proteins has been reported (Kapp and Vinogradov, 1978; Hager and Burgess, 1980; Suzuki and Terada, 1988), we were not

Table 2. Amino acid sequence of the recombinant transferrins, sequenced from the N-terminus, compared with the expected sequence according to Yang et al. (1984)

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		

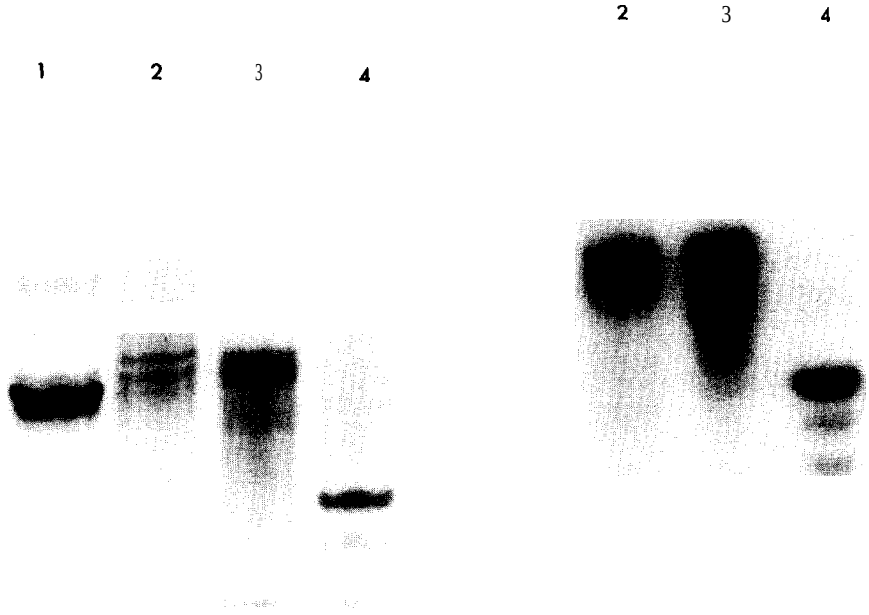

Fig. 2. (Left panel) Native PAGE (12.5%) showing the renatured recombinant proteins. Lane 1: hTf (Behringwerke); lane 2: rhTf; lane 3: rhTf/2C; lane 4: rhTf/2N. (Right panel) Autoradiograph of a comparable gel to that shown in the left panel, except the recombinant proteins were saturated with ^{59}Fe.

able to remove this detergent from denatured transferrin without complete precipitation of the protein of interest. Hence, renaturation experiments were performed with inclusion body solution following a procedure derived from Hirose (Hirose *et* al., 1989; Hirose and Yamashita, 1991) and adapted to our experimental system; i.e. prior isolation of the recombinant proteins by means of preparative SDS electrophoresis was abandoned.

After renaturation and purification, the recombinant transferrins all show a few bands on a native polyacrylamide gel [Fig. 2(A)]. This phenomenon is not caused by differences in molecular weight, because the same samples all show one single band on an SDS gel (Fig. 3),

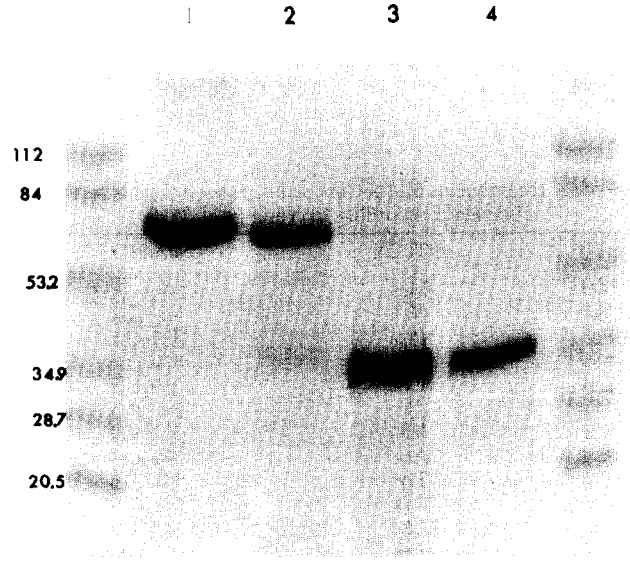

Fig. 3. The SDS-PAGE (12.5%) showing the renatured recombinant proteins. Lane 1: hTf (Behringwerke); lane 2: rhTf; lane 3: rhTf/2C; lane 4: rhTf/2N. Markers are indicated in kDa. Staining was done with Coomassie brilliant blue R250.

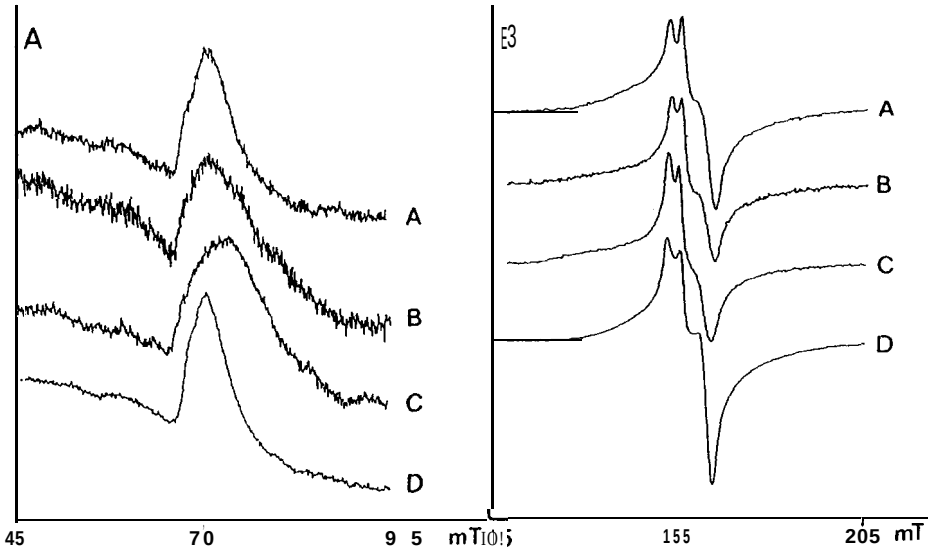

Fig. 4. Comparison of the EPR spectra of hTf and the recombinant transferrins. A: hTf; B: rhTf; C: rhTf/2N; D: rhTf/2C. EPR conditions for (A): microwave frequency, 9315 MHz; microwave power, 80 mW; modulation amplitude, 0.8 mT; temperature, 9 K. EPR conditions for (B): microwave frequency, 9315 MHz;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

except for a weak second band in lane 2, corresponding with half-transferrin. This could be explained easily as a result of proteolytic activity in *E. coli* during growth or during the inclusion body isolation procedure.

An explanation for the several minor bands on the native gel could be that different mobilities represent different conformations of the protein, e.g. due to variations in disulphide bond formation. These conformational differences could affect the iron-binding sites, resulting in a decreased iron saturation. One of the major rhTf/2C bands migrated approximately as far as the rhTf bands on the native 12.5% homogeneous polyacylamide gel [Fig. 2(A)] but, on a gradient polyacrylamide gel (PhastGel 8–25%), this band shifted more towards the rhTf/2N bands, indicating that this band is not a dimer. Dimerization as a result of intermolecular disulphide bridge formation was excluded for all the recombinant transferrins, since none of the recombinant proteins showed any extra bands on a 12.5% homogeneous polyacrylamide SDS gel run under non-reducing conditions compared with an SDS gel run under reducing conditions (not shown). In both cases, the recombinant proteins were saturated with SDS by incubating them in sample buffer (with or without β-mercaptoethanol) at 40°C for 30 min.

The recombinant transferrins all are capable of binding iron as shown on the autoradiograph [Fig. 2(B)]. This is indicated also by the similarity of the recombinant transferrin adsorption spectra with the spectrum of iron-saturated hTf. All the spectra have an adsorption maximum near 465 nm. The EPR spectra of the recombinant proteins are nearly identical with the EPR spectrum of hTf. Thus, the iron atoms in the recombinant proteins and in the native transferrin 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. lack of oligosaccharides.

As described by de Smit et al. (1995), production of recombinant transferrin in *E. coli* amounts to 60 mg/l. The renaturation procedure has a final yield of approximately 5%.

Several authors have reported the production of recombinant transferrin in *E. coli* (Hershberger *et* al., 1991; Steinlein and Ikeda, 1993). Hershberger *et al.* (1991) also reported on renaturation of recombinant transferrin, although no data were shown. Our work describes the production as well as the isolation, partial characterization, renaturation and iron-binding capacity of recombinant transferrin and both its half molecules. Renaturation of the recombinant transferrins results in water-soluble, iron-binding molecules.

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