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A Mass Spectrometric and Molecular Modelling Study of Cisplatin Binding to Transferrin

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A combination of mass spectrometry, UV/Vis spectroscopy and molecular modelling techniques have been used to characterise the interaction of cisplatin with human serum transferrin (Tf). Mass spectrometry indicates that cisplatin binds to the hydroxy functional group of threonine 457, which is located in the iron(III)-binding site on the C-terminal lobe of the protein. UV/Vis spectroscopy confirms the stoichiometry of binding and shows that cisplatin and iron(III) binding are competitive. The binding of cisplatin has been modelled by using molecular dynamic simulations and the results suggest that cisplatin can occupy part of both the iron(III)- and carbonate-binding sites in the C-terminal lobe of the protein. Combined, the studies suggest that cisplatin binding sterically restricts iron(III) binding to the C-terminal lobe binding site, whereas the N-terminal lobe binding site appears to be unaffected by the cisplatin interaction, possibly allowing the iron(III)-induced conformational change necessary for binding to a Tf receptor.

Introduction

Human serum transferrin (Tf) belongs to the transferrin family of iron carrier proteins that also includes lactoferrin, a component of secretary fluids, and ovoferrin, found in egg white. The structures of the transferrin family of proteins reveal that all members adopt a similar fold, with a single polypeptide chain forming two distinct but structurally similar lobes, each containing around 330 residues and separated by a short peptide.[1] Although the crystal structures for both the N- and C-terminal lobes of Tf have been reported,[2,3] coordinates are only available for the N-terminal lobe.[3] By contrast, the structure of lactoferrin has been more comprehensively characterised and coordinates for both lobes in the apo form and with various metals and anions bound are available.[4–6]

The crystal structures of transferrins show that each lobe can be further divided into two similarly sized domains that are separated by a cleft where the iron(III) binds. On binding iron(III), the domains rotate relative to one another, thereby reducing accessibility of the active site for solvents. Thus, the apo-protein conformation is described as "open" and the more compact structure of the diferric complex as "closed" (Figure 1). The conformational change induced by iron(III) binding is more pronounced in the N-terminal lobe than the C-terminal lobe.[5,6] This structural difference is attributed to an additional disulfide bond in the C-terminal lobe, which prevents the cleft opening as far as that of the N-terminal lobe in the absence of iron(III) and may even influence the affinity of the protein for iron(III). Indeed, the C-terminal lobe binds metals preferentially over the N-terminal lobe and it is also the last lobe to release iron(III) in the lysosome in a process induced by reduced pH, either through the protonation of the bicarbonate anion or through a pH-sensitive interdomain interaction.[7]

Tf is found in abundance in blood serum, where it binds to iron(III) and delivers it to cells through the Tf receptor (TfR). As much as 60% of the Tf is in the apo form, thereby enabling the efficient uptake of circulating iron(III) and preventing its use by pathogenic microorganisms. The diferric Tf complex binds with considerably higher affinity to the TfR than the apo form of the protein,[8] although it is not clear whether iron(III) binding at the C-terminal lobe is essential for receptor recognition or is merely an event that precedes N-terminal lobe iron(III) binding.

Certain diseased cells have a high iron(III) requirement, in order to facilitate rapid cell growth, which is satisfied by increasing the number of TfRs on the cell surface[9] and thereby sequestering a greater amount of the circulating metal-loaded Tf. Consequently, Tf has been proposed as a potential drug transport and delivery vehicle.[10] The increase in the number of Tf receptors depends on the cell line, and in vivo radio-labelling studies typically show a 2–12-fold increase for certain cancers, compared to healthy tissues.[11] The increased number of TfRs on cancer cells might help to direct Tf-binding anticancer drugs to cancer cells, thereby increasing their selectivity. Such a mechanism has been postulated to account for the remarkably low toxicity of a ruthenium(III) anticancer drug that has recently entered phase I clinical trials.[12] In addition, several other metal ions have been shown be transported to cells by Tf,[13] including bismuth[14] and titanium,[15] both of which have therapeutic effects.

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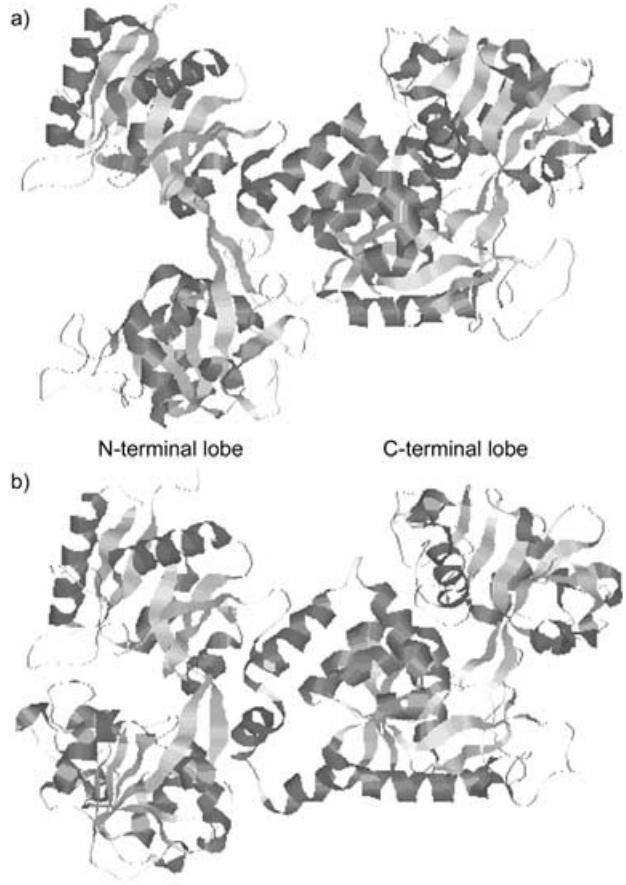

Figure 1. Ribbon representations of the crystal structure of a) free and b) iron(III)-bound lactoferrin, with the N-terminal lobes aligned towards the left of the figure and the C-terminal lobes to the right.

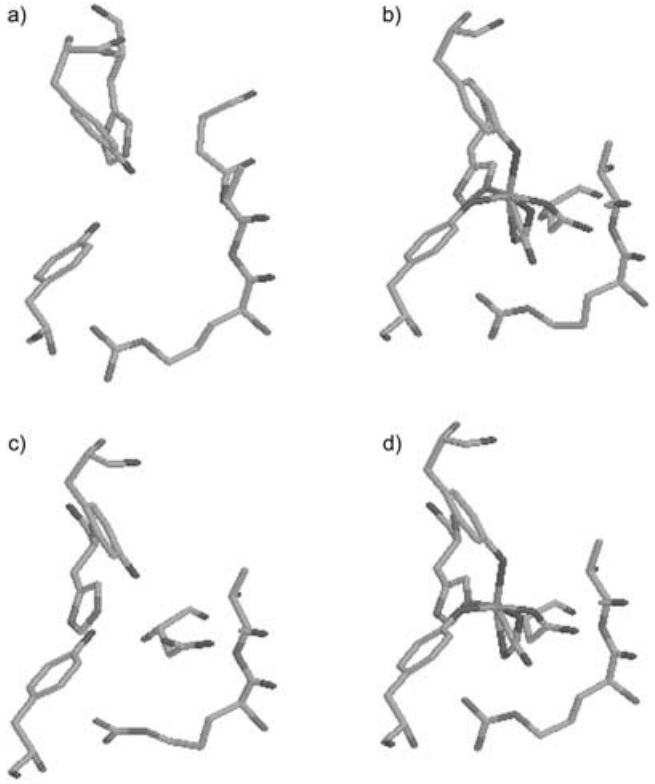

Figure 2. Stick representations of the metal-binding site of lactoferrin, as solved by X-ray crystallography: a) free N-terminal lobe, b) iron(III)-bound N-terminal lobe, c) free C-terminal lobe, d) iron(III)-bound C-terminal lobe.

In this paper we expand on our earlier work[16] by proposing a mechanism for the binding of cisplatin to transferrin, a mechanism which may have implications concerning the effectiveness of this unique metallodrug in chemotherapy.

Results and Discussion

Each lobe of Tf has an independent metal-binding site composed of a histidine, an aspartate and two tyrosine residues, as well as a bicarbonate anion that binds in an adjacent pocket, all of which coordinate to give an iron(III) complex with a distorted octahedral geometry (Figure 2).[17] Spectroscopic methods readily identify iron(III) binding involving the tyrosine residues, as electron transfer between the metal ion and the delocalised ring electrons results in distinctive bands in the UV/Vis spectrum with, for example, iron(III) binding resulting in an absorption band at 460 nm.[18] By contrast, binding of platinum(II) compounds, including cisplatin, does not induce this spectroscopic change.[19]

The binding of cisplatin to Tf has been investigated by using UV/Vis spectrophotometry and the results suggest that, in contrast to the two high-affinity sites per Tf monomer observed for iron(III) binding,[20] cisplatin preferentially occupies a single binding site,[21] in line with the binding of ruthenium(III) complexes to the protein, although binding of additional cisplatin units elsewhere on the protein cannot be discounted. Accordingly, mass spectrometry (MS) has been used to provide further information and the results show that several cisplatin units can bind to Tf. The mass spectra of free Tf in 10 mM bicarbonate buffer and of the same sample incubated with a 10-fold molar excess (with respect to the number of binding sites) of cisplatin after 20 min and 3 h incubation times are shown in Figure 3. The theoretical molecular weight of transferrin calculated from its amino acid sequence is 75143 Da. In Figure 3, the deconvoluted spectrum of transferrin alone shows three peaks, each with a higher molecular weight than that based purely on the amino acid sequence, namely, the peaks at 78935, 79228 and 79519 Da, which are due to glycosylation. The amino acid sequence of transferrin possess two *N*-glycosylation sites,[22] a fact which accounts for the difference. The most prominent peak is at 79519 Da, which is close in value to the literature mass,[23] and the other two glycoforms indicated at 79228 and 78938 Da are of much lower relative intensity. From the difference in mass of 291 Da between the peaks, it is possible to conclude that the glycan present is *N*-acetylneuraminic acid (sialic acid).

The spectrum of transferrin incubated with cisplatin for 20 min exhibits, in addition to the peaks of the free protein, 4 new peaks at 79819, 80121, 80420 and 80719 Da (Figure 3d). The difference in mass between these peaks is 299 Da, which corresponds to the mass of intact cisplatin. After an in-

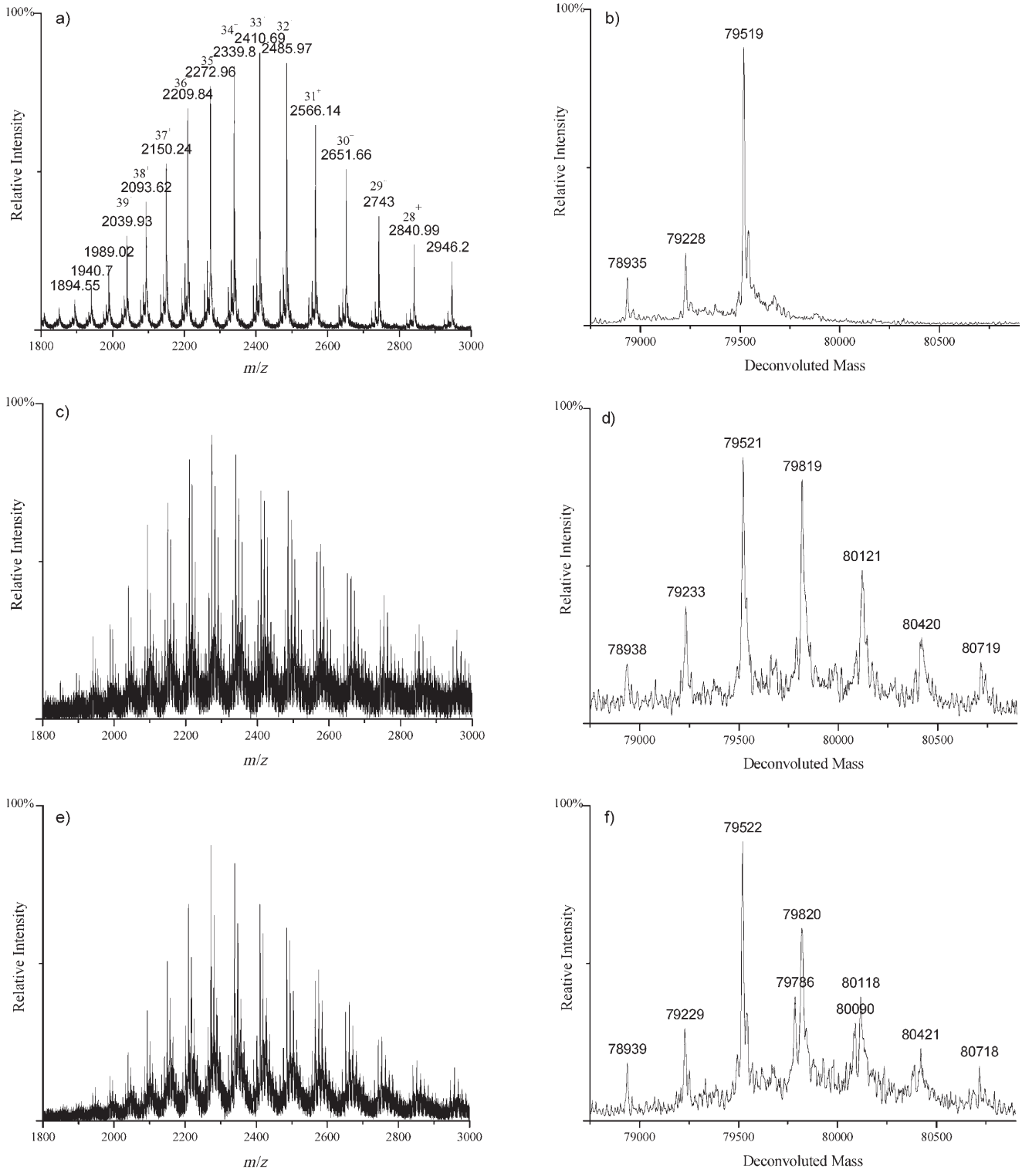

Figure 3. Mass spectra of a) free transferrin and b) its deconvoluted spectrum, c) cisplatin-bound transferrin after 20 min incubation and d) its deconvoluted spectrum, e) cisplatin-bound transferrin after 3 h incubation and f) its deconvoluted spectrum.

cubation period of 3 h, the spectra exhibits a new peak at 79 786 Da (Figure 3 f) that gives a difference of 264, a value indicative of the loss of chloride from cisplatin; this result suggests that the platinum species is covalently bound to the protein.

Identifying the cisplatin-binding sites

UV/Vis spectroscopy was used to determine the stoichiometry and affinity of iron(III) citrate binding to the apo-protein and to the Tf–cisplatin complex. The results in Table 1 indicate that

Table 1. Dissociation constants (k_d) and absorbance coefficients (A) at 465 nm for the iron(III)-bound complex.

	k_d [μM]	A [$cm^{-1}\,\mu M^{-1}$]
Free protein	3.5 ± 0.30	3.1 ± 0.23
Tf/cisplatin (1:1)	8.1 ± 1.4	2.9 ± 0.13
Tf/cisplatin, (1:5)	9.1 ± 1.2	3.5 ± 0.65
Tf/cisplatin, (1:10)	9.0 ± 1.6	3.8 ± 0.60

cisplatin increases the dissociation constant (k_d) for iron binding but does not significantly alter the absorbency coefficient of the bound complex, a result suggesting a competitive mode of binding. However, as there are two nonequivalent iron(III)-binding sites per protein monomer, these data do not give an indication of whether the increased k_d value observed in the presence of cisplatin is due to the need for iron(III) to displace cisplatin before binding or to the possibility of iron(III) no longer binding at the higher affinity C-terminal lobe binding site and only occupying the N-terminal lobe binding site. There was no evidence to suggest cisplatin binding involved tyrosine residues, a fact in accordance with the literature data.[19]

Apo-Tf and Tf incubated with cisplatin were digested with trypsin and the resulting peptides were analysed by MS/MS (see the Experimental Section for full details). Difference spectra of the free and cisplatin-bound digest patterns showed that a parent ion carrying a triple charge with an m/z value of 614 was absent from the apo-protein digest. Further analysis showed that the parent ion with $m/z = 614$ had an isotope distribution characteristic of platinum. The difference in molecular weight between this peptide fragment and the equivalent peptide with $m/z = 789$ in the apo-protein sample was 263 Da, a value which corresponds to ciplatin having lost a chloride ligand (Figure 4), which is in agreement with the MS results for the intact protein after 3 h incubation with cisplatin (see above).

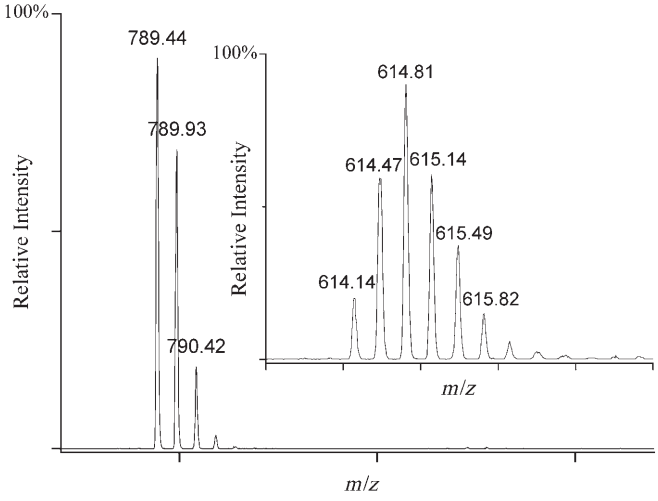

Figure 4. Mass spectrum of the doubly charged (789.44 Da) apo-transferrin fragment ^{457}TAGWNIPMGLLYNK^{470}, with that of the same peptide observed as triply charged species after incubation of the intact protein with cisplatin shown in the inset.

The platinum-modified peptide was sequenced by MS/MS and the results were mapped onto the complete sequence of the protein, thereby allowing the platinum-bound residue to be identified as threonine 457. In the data collected, there was no evidence to suggest platinum binding at any other site. The mass spectrometry experiments presented herein are not quantitative and, although cisplatin was found to modify threonine 457, it is not possible to estimate the fraction of the Tf in the digested sample that is modified in this way. A previous report has postulated that the cisplatin-binding site involves methionine 256, with this conclusion being drawn from NMR spectroscopy data that show a substantial chemical shift of the ^{13}C-methyl-methionine resonance, tentatively assigned to this residue when the protein is incubated with cisplatin, which is not observed when the protein is incubated with iron.[19] The triply charged peptide observed at $m/z = 844$ with the amino acid sequence 255–276, which includes methionine 256, was found and sequenced; no interaction with platinum was detected. It is possible that cisplatin binds weakly to methionine 256 and that the sulfur–platinum bond is broken during the preparation and MS analysis of the protein fragments. There is no structural basis for mutually exclusive binding between the methionine 256 and threonine 457 sites. Indeed, the competitive mode of cisplatin binding with respect to iron(III) binding, identified by spectroscopic methods, supports threonine 457 as being the major residue involved in the cisplatin interaction.

Molecular modelling studies

Threonine 457 lies close to the ligand-binding sites on the C-terminal lobe of the protein. Crystal structures of the lactoferrin–iron(III)–bicarbonate complex show that the hydroxy functional group of the equivalent threonine residue in the N- and C-terminal lobe binding pockets is within hydrogen-bonding distance of the bound bicarbonate, a fact suggesting a role in ligand binding.[24] In the N-terminal lobe of Tf the equivalent residue to threonine 457 is substituted by a serine residue. This substitution could affect the affinity of the cisplatin binding at the different lobes because, although the difference in the binding energies of cisplatin to threonine and serine residues is likely to be small, the additional methyl group on the threonine residue reduces the acidity of the hydroxy functional group, compared with that of serine, thereby favouring binding of the platinum(II) complex to this latter residue. In addition, the binding affinity of cisplatin to each binding site of Tf is also likely to be affected by the structural differences between the two lobes. As mentioned above, the C-terminal lobe iron(III) binding site is less exposed to solvent than the N-terminal lobe binding site and it is therefore likely to be more hydrophobic. The hydrophobicity could promote the binding of metal compounds that are of somewhat lower solubility in aqueous solution, including certain iron(III) compounds and cisplatin.[11]

By using the coordinates from the RSCB protein databank for the N-terminal lobe of Tf,[3] a homology model for the C-terminal lobe of Tf has been generated. A ribbon representation of the model of the platinum-bound C-terminal lobe of Tf is shown in Figure 5, together with the crystal structures of the iron(III)-bound N-terminal lobe of Tf and the N- and C-terminal lobes of lactoferrin. The overall fold of the model is similar to that of the crystal structures, with equivalent structural differences between the crystal structures of the N- and C-terminal lobes of lactoferrin to those between the crystal structure of the N-terminal lobe of Tf and the homology model of the C-terminal lobe. The cisplatin-modified threonine 457 residue does not cause disruptions to the structure of the protein, as shown in Figures 6 and 7b, as it occupies the bicarbonate-anion-binding pocket and part of the iron(III)-binding pocket. Although some residues in the cisplatin-bound model of the C-terminal lobe are in slightly different positions to those in the iron(III)-bound N-terminal lobe (Figure 7a), there are equivalent differences in the positions of the amino acid residues in the crystal structures of the active sites of iron(III)-bound N- and C-terminal lobes of lactoferrin (Figure 2b and d, respectively).

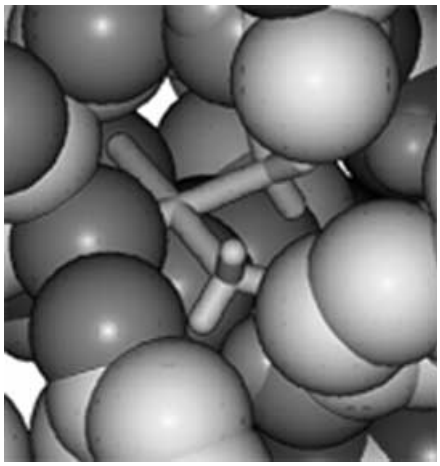

Figure 6. Surface representation of the homology model of the C-terminal lobe of Tf with cisplatin (green sticks) bound.

The model indicates that cisplatin and iron(III) binding at the C-terminal lobe binding site are mutually exclusive. This mode of binding ties in with the ligand-binding studies which

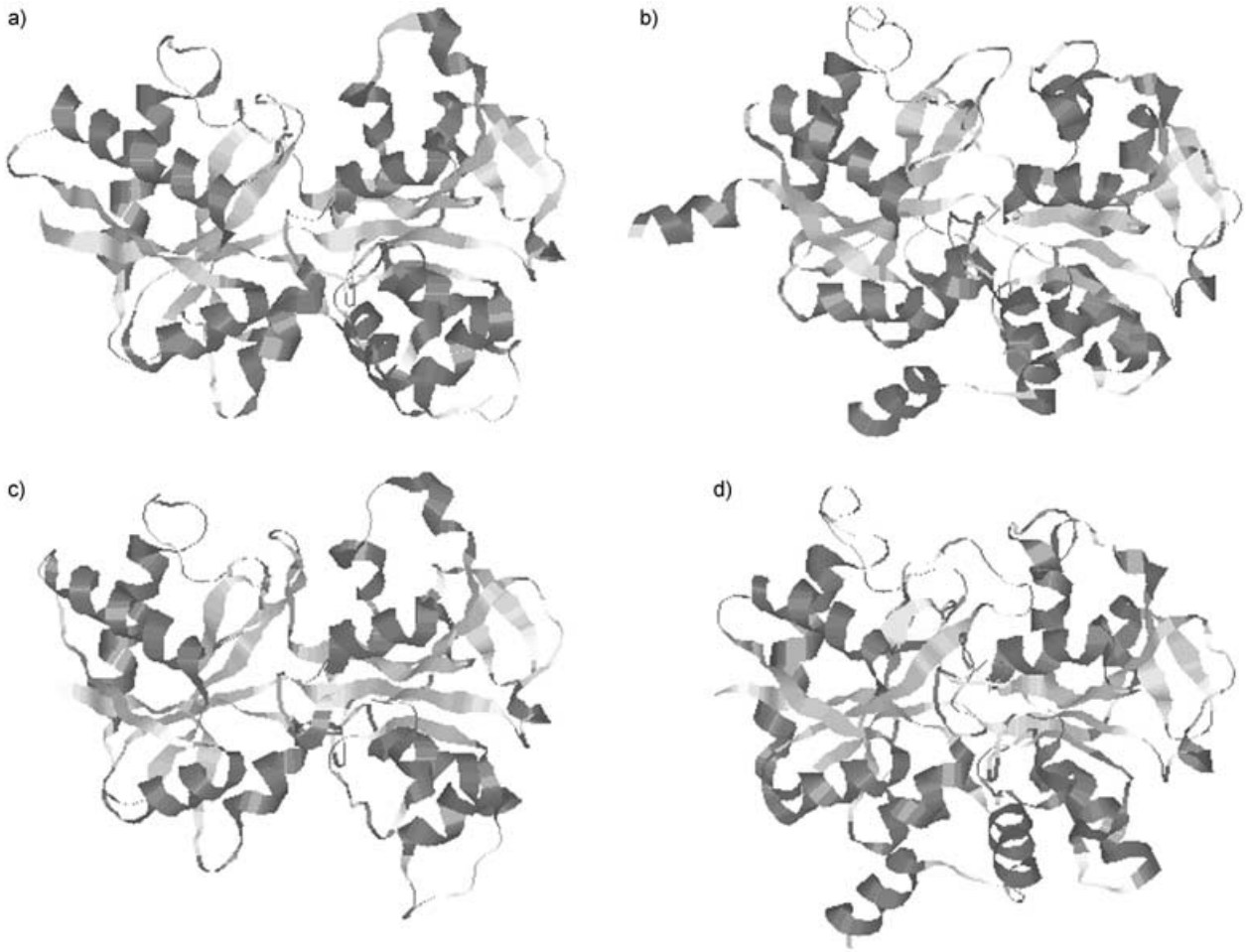

Figure 5. Ribbon representation of a) the crystal structure of the iron(III)-bound N-terminal lobe of Tf, b) the platinum-bound model of the C-terminal lobe of Tf, c) the crystal structure of the N-terminal lobe of iron(III)-bound lactoferrin, and d) the crystal structure of the C-terminal lobe of iron(III)-bound lactoferrin.

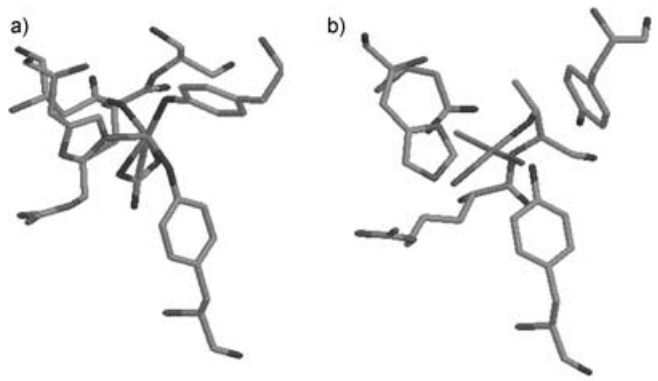

Figure 7. Stick representation of the Tf metal-binding sites of the a) N-terminal and b) C-terminal lobes with iron(III) and cisplatin bound, respectively.

show that cisplatin binding reduces the affinity of iron(III) binding in a competitive manner (see Table 1). However, the binding of cisplatin to the C-terminal lobe of Tf is unlikely to have an effect on iron(III) binding at the N-terminal lobe of the protein, a fact suggesting that cisplatin may indeed be targeted to tumour cells with elevated levels of TfRs as a Tf–iron(III)–cisplatin complex. Presumably, the other cisplatin units that bind to Tf (Figure 3) only involve weak interactions, probably involving S-, O- or N-containing residues on the surface of the protein, which could also be delivered to the TfRs; however, under physiological conditions multiple cisplatin binding is unlikely to occur.

Implications of cisplatin binding to Tf in cancer therapy

As much as 60% of circulating Tf is in the apo form, efficiently sequestering iron(III) and transporting it in a nontoxic form to cells according to the number of TfRs on the cell surface. Many diseased cells have a higher iron(III) requirement to satisfy the demand for rapid cell growth and one of the mechanisms of meeting this requirement is to increase the number of TfRs on the cell surface, thereby sequestering more of the iron(III)-loaded Tf circulating in the blood plasma.[9] Consequently, Tf has been the focus of several studies aimed at exploiting this natural iron(III)-delivery system to target drugs that can mimic iron(III) binding to diseased cells.[10]

Cisplatin has been shown to bind to Tf, but, in the presence of cisplatin alone, Tf does not adopt the closed conformation necessary for recognition by the TfRs on the cell surface, a fact suggesting that Tf-mediated cell targeting is not a mechanism of transport for this successful anticancer drug. When bound to diferric Tf, the cisplatin complex has been shown to interact with the Tf receptor according to the concentration of cisplatin in the incubation. Low molar excesses of cisplatin (up to 7-fold excess) allow recognition and uptake of the Tf–metal complex, but higher excesses (up to 15-fold) inhibit the Tf interaction.[21] These results suggest that at high concentrations of cisplatin there are multiple binding events (as established from the MS experiments described herein) or structural changes in the protein that could lead to denaturation. The physiological relevance of these secondary interactions of cisplatin with Tf is small because in vivo the drug could not be administered at the necessary level to produce these effects.

The conformational change induced by iron(III) binding to the C-terminal lobe is small compared to that of the N-terminal lobe (Figure 2) and it is possible that, in the event of the C-terminal lobe binding site being blocked by the binding of a competitive ligand, such that iron(III) only binds to the N-terminal lobe binding site, the conformational change induced is sufficient to allow the Tf complex to bind to the TfR. If the 'closed' conformation of the N-terminal lobe alone allows the Tf complex to bind to TfRs, then whether the C-terminal lobe is occupied by iron(III) or cisplatin would not affect the uptake of the complex. Thus, with the assumption that iron does not displace cisplatin binding in vivo, the monoferric–cisplatin–Tf complex may be targeted to some cancer cell types and Tf binding could be a key factor in dictating the anticancer activity of this type of metal-based drug.

Tf has been postulated as a delivery mechanism for several metal-based drugs, for example ruthenium(III) and titanium(IV) complexes, both of which are currently in clinical trials as anticancer therapies. Ruthenium(III) complexes, such as Hind-$[RuCl_4(Ind_2)]$ (Hind = indazolium, Ind = indazole), bind with the same stoichiometry as cisplatin, with a single drug molecule binding to each protein unit, whereas two binding sites are observed for the titanium(IV) compound $Ti(C_5H_5)Cl_2$. Crystallographic studies of $Hind[RuCl_4(Ind_2)]$ bound to Tf place the ligand in the N-terminal lobe iron-binding site, coordinated to histidine 253, with the displacement of a chloride ligand.[1] In contrast to platinum(II), ruthenium(III) has similar characteristics to iron(III) and thus, despite the larger ionic radius, is believed to mimic iron(III) binding including inducing the closed conformation of the protein necessary for TfR-mediated delivery. Therefore, despite the fact that the ruthenium(III) complex is binding to the more open N-terminal lobe of the protein, ruthenium(III) complexes can still be delivered to cells through the TfR interaction. Titanium(IV) also binds to the iron-binding sites in the protein.[25] In the case of both ruthenium(III) and titanocene derivatives, binding to Tf is reversible, with both metals being released at low pH values.

To conclude, cisplatin is a very important anticancer drug that is still used to treat approximately 70% of all cancer patients.[26] Accordingly, the interaction of cisplatin with various biomolecules has been extensively studied,[27] although knowledge of its interactions with Tf is still incomplete. Further characterisation of the Tf–cisplatin interaction, including monitoring the reversibility of binding and the interaction of iron–cisplatin–Tf complexes with TfR under physiological conditions, is necessary in order to evaluate the potential role of Tf in drug delivery. Comparison of the drug–Tf and drug–Tf–TfR interactions of successful anticancer drugs, such as cisplatin, with structurally similar compounds that do not have anticancer activity in vivo, may prove Tf to be an interesting candidate on which to base rational drug design for improved metal-based cancer therapies.

Experimental Section

Tf was purchased from Sigma and cisplatin from Aldrich; both were used as received without any purification. For mass spectrometry, a solution of iron-free Tf (100 μM) in ammonium bicarbonate (10 mM, pH 8.5) was prepared and incubated at 20 °C with cisplatin (1 mM) for 15 or 30 min. Sequencing-grade trypsin (Promega, USA) was added to a final concentration of 1.2 ng μL^{-1} and the mixture was incubated at 37 °C for a further 45 min.

Q-TOF mass spectrometry: Electrospray-ionisation MS and MS/MS data were acquired on a Micromass Q-Tof Ultima mass spectrometer fitted with a Z-spray nanoflow electrospray ion source. The mass spectrometer was operated in the positive-ion mode with a source temperature of 80 °C, with a countercurrent gas flow rate of 40 L h^{-1} and with a potential of 2600 V applied to the Nanospray continuous LC probe. All data were acquired with the mass spectrometer operating in an automatic data-dependent switching mode. The instrument was calibrated with a fourth-order calibration by using selected ions from Glu-fibrinopeptide-B. The trypsin-digested samples were separated by using a Micromass modular Cap LC system connected directly to the Z-spray source of a Q-Tof Ultima instrument. Each sample was loaded on to a C18 precolumn (5 mm length, 320 μm diameter) at a flow rate of 30 μL min^{-1} and desalted for 3 min with a solution of 0.1 % formic acid. The samples were then eluted from the C18 precolumn and directed onto a C18 Picofrit column (5 cm length, 75 μm diameter) by using 95 % solution A (95 % water, 5 % acetonitrile, 0.01 % formic acid) and 5 % solution B (5 % water, 95 % acetonitrile, 0.01 % formic acid) at a flow rate of 200 nL min^{-1}. The sample was eluted from the C18 Picofrit by using a stepped gradient up to 80 % solution B including a 3 min stationary phase of the same buffer. All data were processed by using ProteinLynx software and protein idenitification was achieved by analysis with ProteinLynx Global Server Version 1.0. The cisplatin-modified peptide was identified by the platinum isotope pattern and sequenced by using MS/MS.

Determination of equilibrium dissociation constants and stoichiometry by using UV/Vis spectroscopy: Equilibrium dissociation constants of ligands binding to Tf were determined by exploiting the changes in absorbency at 465 nm due to iron(III) binding. Small volumes of ligand solution were titrated into a cuvette containing iron-free Tf and, in the presence of cisplatin at molar ratios of 1, 5 and 10 to Tf-binding sites. The mixtures were dissolved in 50 mM phosphate buffer (pH 7.5) containing 100 mM sodium chloride and 25 mM sodium bicarbonate. The protein was equilibrated in this buffer at 25 °C for 30 min prior to the titrations. The spectra were corrected for the absorbency of iron(III) citrate at 465 nm. The dissociation constants (k_d) and maximum change in absorbency for the titration were determined by fitting a quadratic equation to the corrected data as a function of ligand concentration. The absorbency coefficient for the iron(III)–Tf complex was calculated by dividing the maximum change in absorbance at 465 nm by the number of binding sites in solution. Care was taken to ensure that the system had reached equilibrium before spectra were recorded and that the protein concentration was sufficiently low to ensure that accurate estimates of the k_d value could be made. The number of ligand-binding sites was determined by a similar method but with a higher protein concentration, so that stoichiometric binding could be observed.

Molecular modelling studies: The crystal structure of the N-terminal lobe of Tf[3] was used to generate a homology model of the C-terminal lobe, for which no structural data are available. Alignments and structure building were carried out by using the FUGUE[25,28] and Quanta/Modeller (MSI, San Diego) programs.[26,29] The FUGUE program makes a backbone model of the sequence of interest and this was used as a template to build side chains with the SQUIRL program.[27,30] By contrast, the Modeller program yields a set of complete structures. The structures generated from both methods were solvated with a shell of water molecules and refined by using a combination of energy minimisation and molecular dynamics[28–31] with the Charmm extended atom force field[29–32] and the TIP3 potential for water.[30–33] Cisplatin was docked onto threonine 457 and the charges for the complex were estimated from the change in point charges for cisplatin and threonine before and after complexation by using the ADF software (Cerius, MSI, San Diego). The same change was applied to the charges derived by using quanta charge template methods. A potential energy map was then calculated by rotating across the χ1 torsion of threonine and the torsion defining the threonine–platinum linkage. At each combination of the torsions, the whole system was subject to energy minimisations under gradually decreasing constraints until the change in slope of the potential energy surface was smaller than 10–3 kcal mol^{-1} A^{-1}. While both methods gave qualitatively similar results, we chose the structure from the FUGUE protocol because it yielded the most favourable conformation energetically.

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Keywords: cisplatin · mass spectrometry · molecular modelling · platinum · protein binding

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