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Characterisation of Cisplatin Binding Sites in Human Serum Proteins Using Hyphenated Multidimensional Liquid Chromatography and ESI Tandem Mass Spectrometry

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Cisplatin binding sites in human serum proteins have been characterised by using combined multidimensional liquid chromatography and ESI tandem mass spectrometry (MudPIT). Following incubation periods of 3 h for cisplatin–blood serum mixtures and subsequent trypsin digestion, MS–MS spectra were recorded for individual peptides that had been separated by SCX and RP liquid chromatography. Matching of the MS–MS spectra to theoretical sequences that were generated for human proteins in the SWISS-PROT database led to the identification of specific binding sites in human serum albumin (HSA), serotransferrin (Trfe) and other abundant serum proteins (A2mg, A1at, Apoa1, Apoa2). The cisplatin coordination sites in HSA and Trfe were confirmed by independent MudPIT studies on cisplatin reaction mixtures with the individual proteins. A total of five specific binding sites were identified for HSA, including the cysteine residue C34, two methionine sites (M329, M548) and the tyrosine and aspartate O-donor sites Y150 (or Y148) and D375 (or E376). Methionine-256 was established as a cisplatin coordination site for Trfe in addition to the O-donor sites E265, Y314, E385 and T457. Inspection of the protein structures indicates that the preferred residues belong either to peripheral α helices or to flexible loops within the protein-binding pockets. O-donor residues dominate as cisplatin binding sites for other abundant serum proteins.

Introduction

One day after its intravenous infusion, 65–98% of the anticancer drug cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) in blood plasma is protein bound.[1,2] Although many of its severe side effects have been attributed to protein binding,[3] the exact role of Pt–protein complexes in the mechanism of action of the metallodrug is still not well understood. It is possible that major serum proteins such as albumin and transferrin might take over a transport and delivery function for cisplatin[4] and other antitumour metal complexes and thereby influence their overall distribution and efficacy.[2,5] Albumin, for instance, is taken up by tumour cells at increased levels in comparison to normal cells and has been exploited as a carrier protein for organic anticancer drugs[6] and more recently for an arene–ruthenium(II) (RAPTA) drug.[7]

Despite numerous studies of cisplatin interactions with serum proteins, the exact nature of the drug-binding sites remains unclear.[5] A recent LA-ICP-MS (laser ablation inductively coupled plasma mass spectrometry) study of Pt-binding plasma proteins that had been separated by SDS-PAGE has confirmed human serum albumin (HSA), serotransferrin (Trfe) and α-2-macroglobulin (A2mg) as major serum targets for cisplatin following a 12 h incubation period.[8] ESI-Q-TOF mass spectrometry has been employed to detect an intact HSA adduct that contained approximately four cisplatin fragments.[9] A 1:1 stoichiometry was observed in the case of Trfe. Efficient binding of cisplatin to HSA is also indicated by the reported reaction rate and binding constants of $k = 3.2\ \text{M}^{-1}\,\text{min}^{-1}$[10,11] and $K = 7500\ \text{M}^{-1}$,[12] and 10.2 moles of bound Pt per mole of protein was established by CE–ICP-MS (CE = capillary electrophoresis) after an incubation period of 48 h for a 20:1 cisplatin–albumin molar ratio.[10] The free SH group in C34 was identified as an important cisplatin binding site by Gronias and Pizzo,[13] who observed a 4–5-fold decrease in Pt coordination after carboxyamidomethylation of the thiol function. This residue was confirmed by Momburg et al.,[14] and by the IR spectroscopic studies of Neault and Tajmir-Riahi,[15] who also provided evidence for the possible participation of HSA tyrosine residues in cisplatin binding. A detailed $^1H,^{15}N$ HSQC NMR spectroscopy study by Sadler et al.[16] established a probable methionyl $\kappa^2 S_M, N_M$ chelate as the major HSA sulfur-binding site after incubation of a 1:1 cisplatin–protein mixture for 17 h at 310 K. Monofunctional adducts involving C34 and one or two other methionine residues were also observed. On the basis of its accessibility on the HSA surface, M298 was proposed to be the most probable major binding site.

The same group has also reported an NMR spectroscopic study of cisplatin binding to human Trfe,[17] in which they observed that only the ^{13}C resonances that were previously assigned[18] to the δ-CH_3 groups of M256 and M499 were influenced by adduct formation. In contrast, the T457 was established as a specific cisplatin binding site by Dyson et al.[19,20]

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using ESI-MS–MS after trypsin digestion of a cisplatin–Trfe reaction mixture. Although these results appear to be contradictory, it is important to note that very different reaction conditions and incubation times were employed. Whereas T457 was identified for a 10:1 cisplatin–Trfe mixture following incubation for 15 or 30 min at 293 K,[19,20] the $^{1}H,^{13}C$ HSQC NMR spectra that led to the assignment of M256 and M499 were measured on a 1:1 reaction mixture after 24 h at 310 K.[17] This suggests that residues that contain oxygen donor atoms (D, E, S, T, Y) could offer kinetically preferred binding sites for cisplatin in serum proteins, but that methionine residues might gain in importance after prolonged incubation.

Hyphenated multidimensional LC–LC–MS–MS techniques now offer a powerful and rapid means of determining the presence of individual proteins in complex mixtures. We have recently described the use of an automated method for shotgun proteomics called the multidimensional protein identification technology (MudPIT)[21] to establish protein targets for [(η^6-*p*-cymene)$RuCl_2$(DMSO)] and cisplatin in *E. coli*.[22,23] MudPIT combines 2D SCX (strong cation exchange) and RP (reversed-phase) chromatography with ESI-MS–MS, and allows up to 1,500 proteins to be characterised in a 24 h period. We now report the application of this technique to identify cisplatin binding sites in serum proteins. MudPIT exhibits a linear dynamic range of 10^4–1 between the most and least abundant proteins in a complex mixture, and allows the detection of tryptic peptides down to a sensitivity limit of ~10 fmol.[21] This means that MudPIT could allow the detection of specific cisplatin binding sites in less abundant serum proteins together with those in the major carrier proteins, HSA and Trfe. To validate the results that were obtained for blood serum samples from two healthy volunteers, individual LC–MS–MS studies were also performed for reaction mixtures of cisplatin with the major proteins, Human serum albumin (HSA), Trfe and α-2-macroglobin.

Results and discussion

As we have discussed in previous articles,[22,23] effective MudPIT analyses of metal-binding sites in complex protein mixtures (e.g., whole-cell systems, blood serum) is only feasible when the binding sites exhibit a high kinetic stability during both the tryptic digestion at pH 7.8 and the subsequent biphasic chromatographic separation at ~pH 2.3. In addition, the metal fragment must remain intact and coordinated to a significant number ($\geq$35%) of the predicted b^+ and/or y^+ peptide ions during MS–MS conditions, even when neutral loss of co-ligands (e.g., NH_3 from cisplatin) can accompany peptide fragmentation. The characteristic isotopic distribution of the metal should allow its presence to be visually confirmed in appropriate peptide fragment ions. Although collision-induced translocation of the Pt fragment to generate regioisomers that contain noncovalently bonded metal cations cannot be fully ruled out under MS–MS conditions, it would lead to a series of Pt-containing b^+ and y^+ ions of much lower intensities than those of the covalently bonded fragment. As depicted in Figure 1 for the model peptide GALTNVSMAK following incubation with cisplatin (1:1 molar ratio, 310 K, 24 h), MS peaks are typically observed for platinated peptide ions that contain different Pt fragments, in this case $\{Pt\}^{2+}$, $\{(NH_3)Pt\}^{2+}$ and $\{(NH_3)_2Pt\}^{2+}$. The peptide was chosen to study possible competition between the methionine sulfur atom and the serine and threonine oxygen atoms in Pt-binding sites. Observation of platinated b_8^+ and b_9^+ ions together with the platinated y_3^+ ion in the MS–MS spectrum of the most intense ion (*m/z* = 1201) is in accordance with Pt binding to the methionine sulfur atom. Because the three most intense MS ions are selected for full MS–MS scans during MudPIT runs, peptide fragmentation data can often be obtained for different platinum fragments.

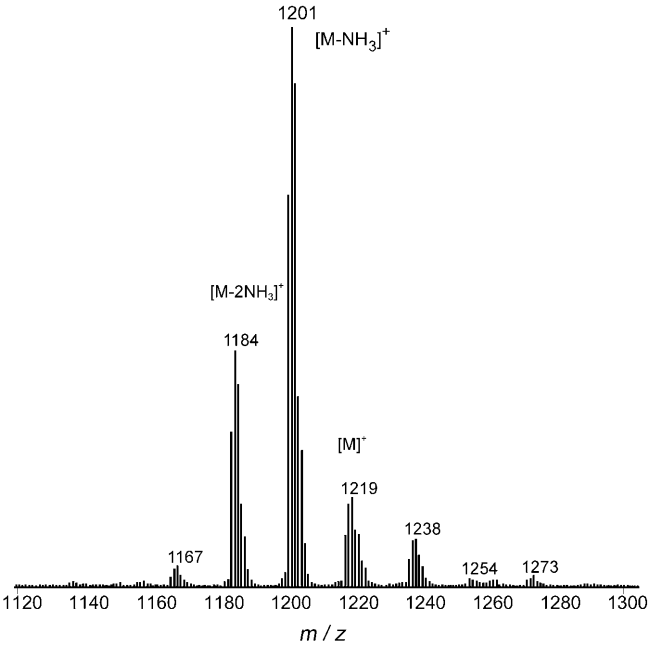


Figure 1. ESI-MS spectrum for the platinated ions of the G-A-L-T-N-V-S-M-A-K peptide after incubation for 24 h at 310 K with an equimolar quantity of cisplatin; $[M]^+$ at *m/z* 1219 corresponds to $[(NH_3)_2Pt(peptideH_{-1})]^+$.

An example of an MS–MS spectrum (peptide sequence 3(b), Table 1) of a blood serum target that fulfils all of the above criteria is presented in Figure 2 and corresponds to HSA residues D324–A335. Of the possible b^+ and y^+ ions, 16 could be successfully assigned, including 8 b^+ and 8 y^+ ions of which 6 and 3 are platinated, respectively. The typical isotopic patterns for the platinated b_{10}^+ and y_8^+ ions are highlighted in Figure 2. The fragment ion b_6^+ and heavier b^+ ions are platinated, as are y_7^+, y_8^+ and y_9^+. Because ions y_2^+–y_6^+ are not platinated, M329 can be clearly assigned as a Pt coordination site despite the very small ΔCn of only 0.01, relative to the alternate D324 assignment. In general, when the *n*th residue is platinated, all of the observed b_j^+ ions with $j \geq n$ will be mass modified by the coordinating Pt fragment. For a peptide with $n+m$ residues (Scheme 1), all observed y_k^+ ions with $k > m$ will likewise be mass modified in this case. The MS–MS spectrum of Figure 2 also contains platinated peaks for the molecular ions $[b_j-NH_3]^+$ ($6 \leq j \leq 10$) that might result from neutral loss of ammonia from the coordinated $\{(NH_3)Pt\}^{2+}$ fragment.

Table 1. Platinated peptide sequences in HSA of blood serum samples.

Peptide sequence[a]	Pt fragment mass[b]	Sample no.	Charge	SEQUEST parameters Xcorr	ΔCn	Ions[c]	Alternative binding sites[d]
1. Residues **C34**/E37/D38/H39							
(a) K·[21]ALVLIAFAQYLQQ**C**PFEDH@V[40]·K	210	1	2	4.20	0.25	18/38	E37,D38,**C34**(0.05)
(b) K·[21]ALVLIAFAQYLQQ**C**@PFED[38]·H	263	1	3	3.95	0.17	23/68	D38(0.00),E37(0.01)
(c) K·[21]ALVLIAFAQYLQQ**C**PFED@HV[40]·K	210	2	2	4.31	0.18	16/38	H39,**C34**,E37(0.04)
2. Residues Y148/**Y150**							
(a) R·[146]H@PYF**Y**APELLFFA[158]·K	263	1	2	4.13	0.40	19/24	Y148(0.11),**Y150**(0.34)
(b) R·[145]RHPY@F**Y**APELLFFA[158]·K	263	1	2	2.99	0.23	14/26	**Y150**(0.06)
(c) P·[148]Y@F**Y**APELLFFAKR[160]·Y	263	1	2	2.82	0.17	18/24	Y150(0.30)
3. Residues D324/**M329**							
(a) K·[324]DVFLG**M**@FLYEYAR[336]·R	210	1	3	4.10	0.24	21/48	D324(0.27)
(b) K·[324]DVFLG**M**@FLYEYA[335]·R	210	1	2	3.03	0.34	16/22	D324(0.01)
4. Residues **D375**/E376							
(a) K·[373]VF**D**@EFKPLVEEPQNLIK[389]·Q	210	1	2	4.06	0.12		
(b) F·[375]**D**E@FKPLVEEPQNLIK[389]·Q	227	1	2	4.66	0.40	19/28	**D375**(0.01),K378(0.18)
(c) K·[373]VF**D**@EFKPLVEEPQNLIK[389]·Q	210	2	2	3.88	0.12	16/32	E376(0.10)
(d) F·[375]**D**E@FKPLVEEPQNLIK[389]·Q	227	2	2	3.64	0.26	16/28	**D375**(0.01),K378(0.14)
(e) F·[375]**D**@EFKPLVEEPQNLIK[389]·Q	263	2	3	3.77	0.11	26/56	E376(0.0),K378(0.11)
5. Residues **M548**/D549/D550							
(a) K·[546]AV**M**@DDFAAFVE[556]·K	210	1	2	2.97	0.18	10/18	D549(0.24),D550(0.26)
(b) K·[546]AV**M**@DDFAAFVE[556]·K	227	1	2	2.85	0.12	12/20	D550(0.07),D549(0.07)

[a] The assigned binding site in the listed platinated peptide is designated by an @ symbol following the residue. The most probable binding site on the basis of all analysed peptide sequences in this range is given in bold type. [b] $\{(NH_3)Pt\}^{2+}=210$, $\{(NH_3)_2Pt\}^{2+}=227$, $\{(NH_3)_2PtCl\}^{+}=263$. [c] Ratio of assigned b^+ and y^+ ions to the total number of possible ions. [d] Alternative neighbouring binding sites with ΔCn values given in parentheses.

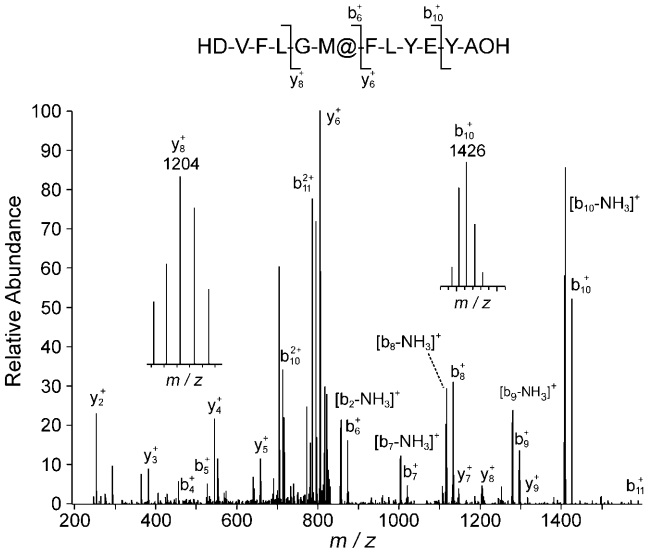
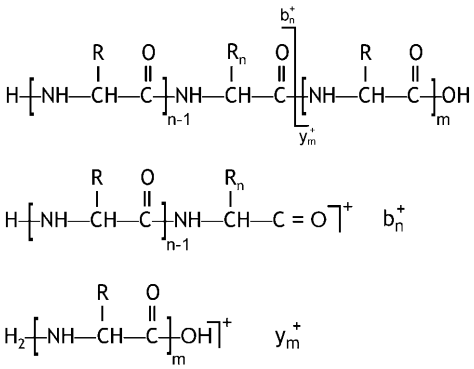


Figure 2. MudPIT MS–MS spectrum of 3(b) (Table 1, Xcorr = 3.03, ΔCn = 0.34 for M329) for the molecular ion $[DVFLGM@FLYEYA]^{2+}$ (@ = $\{(NH_3)Pt\}^{2+}$) containing residues D324–A335 of HSA.



Scheme 1. The b_n^+ and y_m^+ fragment ions for a peptide with $m+n$ amino acid residues.

Cisplatin binding sites in serum albumin

A total of five cisplatin binding sites were identified for HSA by MudPIT analyses after a 3 h incubation of the metallodrug with blood serum samples in a 10:1 molar ratio at 310 K (Table 1). The binding site with the best Xcorr (cross correlation score) value (@ after residue) and the most probable binding site on the basis of all the identified platinated peptide sequences in the same range (bold) are given in Table 1. As discussed below, the probable binding site is not necessarily identical to the designated binding site in the listed peptide. Two of these sites (1 and 4, Table 1) were found in both samples, whereas the remaining sites (2, 3 and 5) were only detected in the first sample. The differences between the results for the individual blood serum samples are presumably due to small variations in sample composition and preparation conditions, but all of the established sites were, however, confirmed by the LC–MS–MS analyses of individual cisplatin–HSA reaction mixtures. This finding suggests that all five binding sites are characteristic for the interaction of cisplatin with HSA, the most abundant pro-

tein found in human plasma (~52%). It is important to note that MudPIT spectral sampling generally favours the identification of high-abundance proteins, and only a relatively small fraction of low-abundance proteins in complex mixtures will typically be observed. A recent study by Yates III et al.[24] has shown that whereas 71.6% of all highly abundant proteins (~10^4–10^5 copies per cell) in *S. cerevisiae* could be identified in a single MudPIT run, this percentage fell to only 6.5% of the proteins in the range of 10^2–10^3 copies per cell. The number of amino acid residues and their specific characteristics can also bias the observation of tryptic peptides and their resulting fragment ions in the mass spectrometer.[25] For instance, whereas a sequence coverage of 54% was achieved for HSA in blood sample 1, a much lower value of 28% was obtained for Trfe. Similar coverage levels of 54 and 51% were, however, observed for the LC–MS–MS studies on the individual proteins. These values suggest that about one half of all HSA and Trfe binding sites are detectable by ESI-MS–MS after tryptic digestion. Residue platination with its associated localised +1 or +2 charge can lead to the preferred cleavage of neighbouring backbone amide bonds during initial MS ionisation, and the resulting peptide ions might belong to the three most intense molecular ions that are selected for full MS–MS scans.[23] As a result, many of the peptide ions that are listed in Table 1 are only partially tryptic. The absence of b^+ and y^+ ions that result from upstream cleavage of peptides that are platinated close to the N-terminal residue, or alternatively, downstream cleavage of peptides that are platinated close to the C-terminal residue can render assignment of the specific binding site more difficult.

A case in point is given by site 1 (C34/E37/D38/H39), which contains three neighbouring possible coordination positions in addition to the free thiol function of C34. An example for an MS–MS spectrum of a platinated peptide with site 1 is illustrated in Figure 3 for the 20-membered peptide sequence 1(a) of Table 1 (A21V40, Xcorr = 4.01, ΔCn = 0.20 for C34) which exhibits 8 b^+ and 10 y^+ ions. It is apparent that only b_j^+ ions with $j \leq 13$ are present and this is also the case for all the other MS–MS spectra of this platinated sequence listed in Tables 1 and 2. Table 2 presents a compilation of the Pt-containing peptide sequences that were first identified for a 1:1 reaction mixture of cisplatin with HSA (incubation time 3 h, 310 K) or in the case of sites 1 and 3 for 3:1 or 5:1 reaction mixtures. Repeat observations at higher cisplatin–albumin ratios are not listed. In addition to the 10 y_k^+ ions that are present in MS–MS spectrum 1(a), a number of possible neutral loss ions such as $[y_{15}-CO_2H]^+$ (*m/z* 1962), $[y_9-NH_3]^+$ (*m/z* 1296) and $[y_8-NH_3]^+$ (*m/z* 1168) can also be identified in Figure 3. The higher Xcorr score of 4.20 for the proposed platinated-H39 peptide (Table 1) in comparison to that of 4.01 for the C34 site is due to the assignment of y_2^+ and y_5^+ ions at *m/z* 465 and 856, respectively. It is clear, however, that these are of very low relative abundance (<4%) and also that fragment ions that do not belong to either the b^+ or y^+ series appear in the *m/z* range of 400–1000. These y^+ ion assignments must, therefore, be treated with caution, in particular in view of the fact that peptide sequence 1(b) of Table 1 does not even contain the

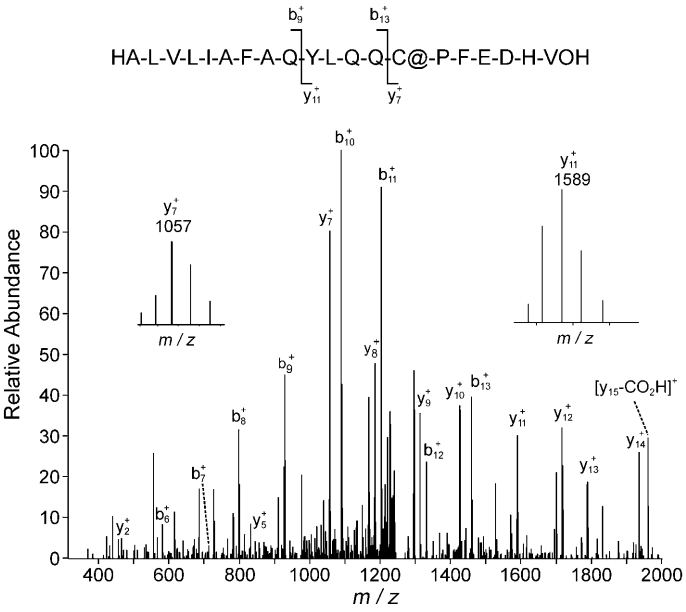


Figure 3. MudPIT MS–MS spectrum 1(a) (Table 1, Xcorr = 4.01, ΔCn = 0.20 for C34) for the molecular ion $[ALVLIAFAQYLQQC@PFEDHV]^{2+}$ (@ = $\{(NH_3)Pt\}^{2+}$) containing HSA residues A21–V40. A higher SEQUEST Xcorr value (4.20) was obtained for a designated H39-binding site, based on the assignment of low-intensity peaks to the appropriate y_2^+ and y_5^+ ions.

H39 residue. The first y^+ ion of high abundance in sequence 1(a) is the platinated y_7^+ ion, which corresponds to the C34–V40 sequence. On taking this aspect and, in particular, the absence of b^+ ions for sequences longer than A21–Q33 for all MS–MS spectra of the platinated peptide A21–V40 into account, it is reasonable to identify C34 as the most probable binding site. C34 does, indeed, also give the highest Xcorr value for the sequence 1(b) in Table 1 and the sequences 1(a) and 1(e) in Table 2. This assignment is also in agreement with previous studies,[13–16] which have established the free thiol group of C34 as a preferred cisplatin binding site in HSA.

Although the Xcorr values are slightly higher for H146 as a possible binding site for the MS–MS spectrum 2(a), which is listed in Table 1, the observation of a platinated Y148–R160 peptide as the parent ion for the third MS–MS spectrum 2(c) indicates that the histidine residue may not be involved in Pt coordination. This conclusion is supported by the registration (Table 2) of two Y148–K159, two F149–K159 and one Y150–K159 sequence for platinated peptides from the 1:1 cisplatin–HSA reaction mixture. The tyrosine residue Y150 is clearly established as a cisplatin binding site on the basis of these observations. The large ΔCn differences between Y148 and Y150 for the MS–MS spectra 2(a) and 2(c) in Table 1 and 2(d) in Table 2 suggest, however, that Y148 might also participate in Pt coordination. Whereas only one of the tyrosine residues can be involved for the $\{(NH_3)_2PtCl\}^+$ fragment of sequences 2(a)–2(c) in Table 1, simultaneous coordination by both Y148 and Y150 would be possible for the $\{Pt\}^{2+}$ fragment of sequence 2(a) in Table 2. Figure 4 depicts the MS–MS spectrum for sequence 2(b) of Table 1 for HSA in the blood serum sample 1. The higher Xcorr value for Y148 as the proposed binding site

Table 2. Platinated peptide sequences in individual HSA samples.

Peptide sequence[a,b]	Pt fragment mass[c]	[cisplatin]/[protein]	Charge	SEQUEST parameters		Ions[d]	Alternative binding sites[e]
				Xcorr	ΔCn		
1. Residues **C34**/E37/D38/H39							
(a) K·[21]ALVLIAFAQYLQQ**C**@PFEDH[39]·V	193	5 (7 d)	2	4.53	0.75	15/36	E37, D38, H39(0.09)
(b) K·[21]ALVLIAFAQYLQQ**C**PFED@H[39]·V	210	5 (7 d)	3	4.19	0.47	23/72	H39, E37, **C34**(0.05)
(c) K·[21]ALVLIAFAQYLQQ**C**PFEDH@VK[41]·L	210	5 (7 d)	3	3.80	0.79	26/80	D38, E37, **C34**(0.06)
(d) K·[21]ALVLIAFAQYLQQ**C**PFE@DHV[40]·K	227	3 (3 h)	3	4.34	0.66	34/76	D38, H39, **C34**(0.10)
(e) K·[21]ALVLIAFAQYLQQ**C**@PFEDHV[40]·K	263	3 (3 h)	3	3.35	0.65	24/76	E37, D38, H39(0.11)
2. Residues Y148/**Y150**							
(a) P.[148]Y@F**Y**APELLFFAK[159]·R	193	1 (3 h)	2	3.39	0.82	17/22	**Y150**(0.03)
(b) Y.[149]F**Y**@APELLFFAK[159]·R	193	1 (3 h)	2	3.11	0.71	17/20	
(c) Y·[149]F**Y**@APELLFFAK[159]·R	210	1 (3 h)	2	3.15	0.53	16/20	
(d) P.[148]Y@F**Y**APELLFFAK[159].R	210	1 (3 h)	2	2.79	0.81	16/22	**Y150**(0.23)
(e) F·[150]**Y**@APELLFFAK[159]·R	263	1 (3 h)	2	2.60	0.77	14/18	
3. Residues D324/**M329**							
(a) K.[324]DVFLG**M**@FLYEY[334]·A	210	1 (3 h)	2	2.54	0.65	11/20	D324(0.15)
(b) K·[324]DVFLG**M**@FLYEYAR[336]·R	210	5 (3 h)	3	4.07	0.74	23/48	D324(0.15)
4. Residues **D375**/E376							
(a) K·[373]VF**D**@EFKPLVEEPQNLIK[389]·Q	210	1 (3 h)	2	5.35	0.54	20/32	E376(0.08)
(b) V·[375]F**D**@EFKPLVEEPQNLIK[389]·Q	210	1 (3 h)	2	4.25	0.69	29/60	E376(0.08)
(c) F·[375]**D**E@FKPLVEEPQNLIK[389]·Q	227	1 (3 h)	2	3.85	0.67	25/56	**D375**(0.02)
(d) K.[373]VF**D**@EFKPLVEEPQNLIK[389].Q	227	1 (3 h)	2	3.71	0.65	32/64	E376(0.01)
(e) F.[375]**D**@EFKPLVEEPQNLIK[389]·Q	263	1 (3 h)	2	3.66	0.51	16/28	E376(0.0)
5. Residues **M548**/D549/D550							
(a) A·[547]V**M**@DDFAAFVEK[557]·C	210	1 (3 h)	2	2.48	0.77	16/20	D549(0.18), D550(0.33)

[a] Specific platinated peptides are only listed once and are given for the reaction solution with the lowest [cisplatin]/[protein] ratio or the shorter incubation time (for 5:1 solutions). [b] The assigned binding site in the listed platinated peptide is designated by an @ symbol following the residue. The most probable binding site on the basis of all analysed peptide sequences in this range is given in bold type. [c] $\{Pt\}^{2+}=193$, $\{(NH_3)Pt\}^{2+}=210$, $\{(NH_3)_2Pt\}^{2+}=227$, $\{(NH_3)_2PtCl\}^{+}=263$. [d] Ratio of assigned b^+ and y^+ ions to the total number of possible ions. [e] Alternative neighbouring binding sites with ΔCn values given in parentheses.

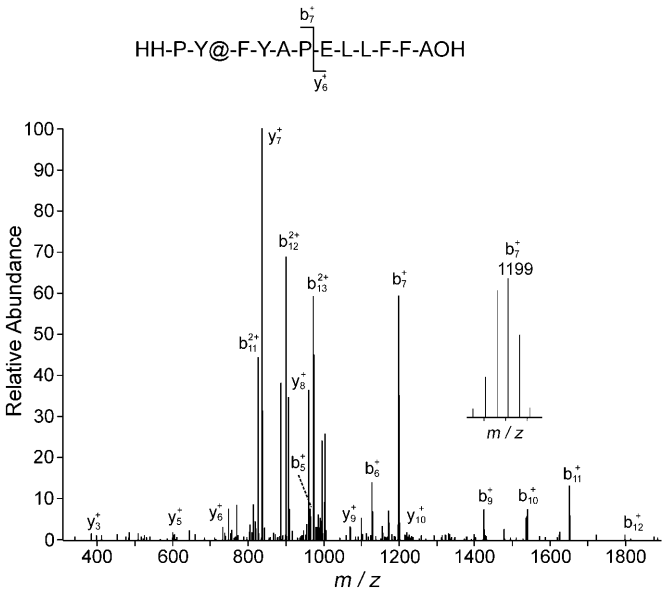


Figure 4. MudPIT MS–MS spectrum 2(b) (Table 1, Xcorr = 2.99, ΔCn = 0.23 for Y148) for the molecular ion $[RHPY@FYAPELLFFA.K]^{2+}$ (@ = $\{(NH_3)_2PtCl\}^{+}$) containing HSA residues R145–A158.

results from two low peaks of low relative abundance (< 4%) at *m/z* 1071 and 1219, which are assigned to y_9^+ and y_{10}^+. When these peaks are ignored and the absence of high abundance y_k^+ ions for $k > 8$ is taken into account, it is possible that Y150 might be an alternative binding site in this case. It is worth noting that Sadler et al. found no NMR spectroscopic evidence for Pt coordination by histidine residues in the course of their extensive studies of the cisplatin–HSA system.[16] The characterisation of Y150 and possibly Y148 as cisplatin-coordination sites in HSA is in accordance with the results of Neault and Tajmir-Riahi,[15] who concluded on the basis of IR studies that at least one tyrosine residue must be involved in Pt binding.

HSA contains a total of six methionine residues, M87, M123, M298, M329, M446 and M548, whose relevant tryptic peptides exhibit 12, 19, 27, 13, 21 and 12 amino acid residues, respectively. Only three of these residues (M123, M329 and M548) belong to the 54% of peptide sequences that are covered by the MudPIT analyses of HSA in this work. The ESI-MS–MS analyses can, therefore, provide no evidence as to whether the remaining sites (M87, M298 and M446) participate in Pt coordination. The lack of MS–MS coverage for M298 might be due to the length of the relevant tryptic peptide S287–K313 (27 residues). Peptides containing 6–21 residues are generally most

amenable to extensive fragmentation into b^+ and y^+ ions. The MudPIT analyses did, however, allow the clear-cut identification of the methionyl residues M329 and M548 as characteristic cisplatin binding sites in HSA. The MS–MS spectrum 3(b) of Table 1 for the M329-containing peptide D324 A335 is depicted in Figure 2 and has already been discussed in detail. Figure 5 illustrates the MS–MS spectrum 5(a) for the short tryptic peptide A546–E556 with 11 residues; 14 of the 20 possible ions can be equally assigned to members of the b^+ and y^+ series and these include seven platinated b^+ ions (b_3^+, b_4^+, $b_6^+ - b_{10}^+$) and seven nonplatinated y^+ ions (y_k^+, $2 \le k \le 8$). The assignment of the platinated b_3^+ ion and the nonplatinated y_8^+ ion clearly confirm M548 as the Pt-binding site.

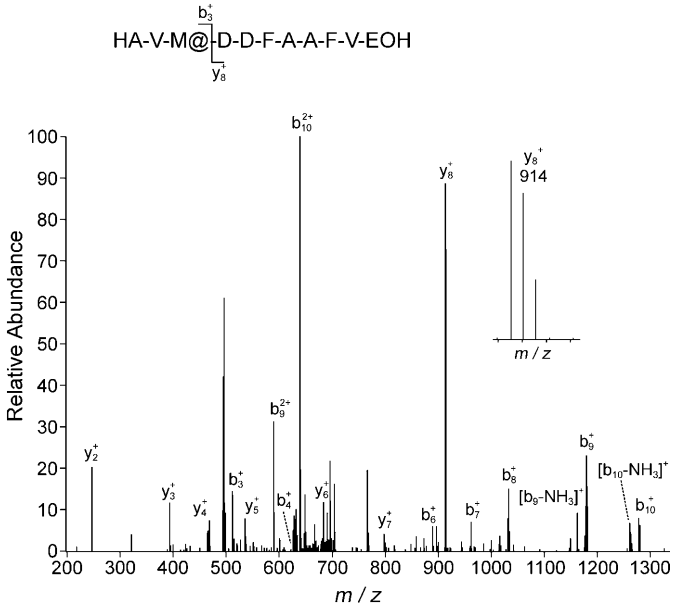


Figure 5. MudPIT MS–MS spectrum 5(a) (Table 1, Xcorr = 2.97, ΔCn = 0.18 for M548) for the molecular ion $[AVM@DDFAAFVE]^{2+}$ (@ = $\{(NH_3)Pt\}^{2+}$) containing HSA residues A546–E556.

The MudPIT analyses on blood serum samples and for HSA alone also allowed the identification of the carboxylate function of D375 or possibly its neighbour E376 as a preferred cisplatin binding site. An unequivocal assignment is not possible, because the residues are close to the N-terminal residue for all the observed peptide sequences. D375, however, might be selected as the more probable binding site on the basis of the larger ΔCn differences compared with E376 in several cases of D375 assignment, that is, 0.14 for sequence 4(a) of Table 1 and 0.08 for sequences 4(a) and 4(b) of Table 2. The five established cisplatin binding sites in HSA are depicted in Figure 6, which indicates that the participating residues are well distributed over the subdomains of the heart-shaped transport protein. HSA contains predominantly α-helical motifs to which the residues M329, D375 and M548 belong.[26] Their side chain donor atoms should be readily accessible for rapid cisplatin binding. In contrast to these residues, the other established binding sites, C34 and Y150, are located in flexible loops,

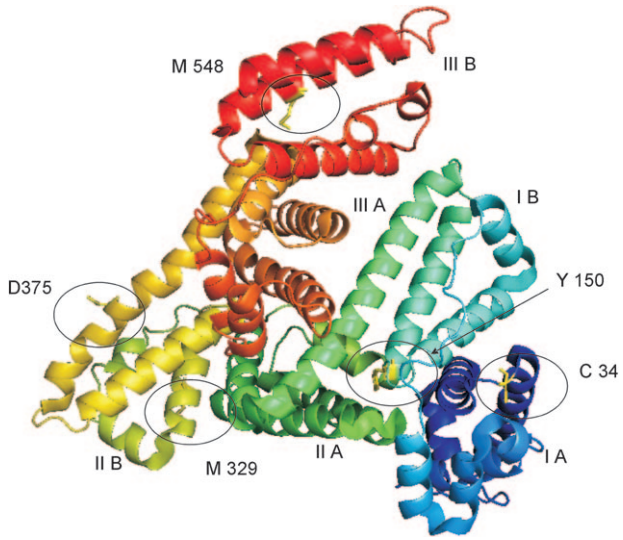


Figure 6. Cisplatin binding sites in HSA (PDB ID: 1n5uA).[26]

which belong in the first case to subdomain IA, and in the second case, lie at the interface between the subdomains IA and IIA. The latter location is close to a binding site that is occupied by unsaturated fatty acids. Figure 7 illustrates the environments of their thiol and phenol functions in more detail, and shows that both Y148 and Y150 will be accessible for cisplatin binding.

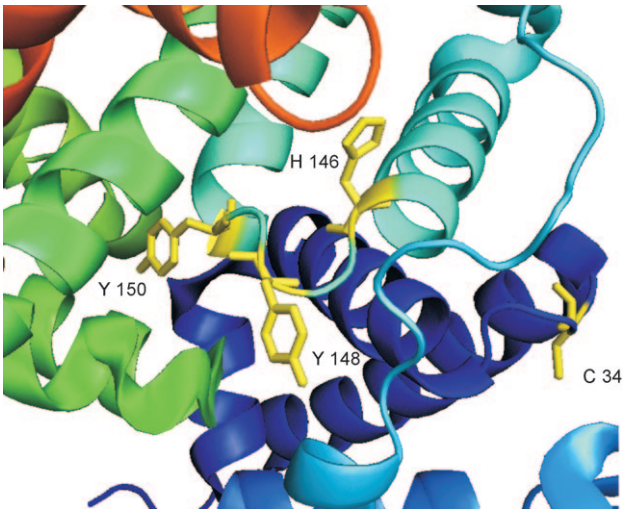


Figure 7. Environments of the C34 and Y148–Y150 binding sites in HSA (PDB ID: 1n5uA).

Cisplatin binding sites in serotransferrin

Table 3 lists the cisplatin binding sites that were identified for the abundant serum proteins Trfe, A2mg, α-2-antitrypsin (A1at), apolipoprotein A-I (Apoa1) and apolipoprotein A-II (Apoa2) after a 3 h incubation of the metallodrug with the blood serum samples at a 10:1 molar ratio (310 K). Individual Pt-bindings sites were also located for ceruloplasmin and a

Table 3. Platinated peptide sequences of other abundant proteins of blood serum samples.

Peptide sequence[a]	Pt fragment mass[b]	Sample no.	Charge	SEQUEST parameters Xcorr	SEQUEST parameters ΔCn	Ions[c]	Other possible binding site(s)[d]
1. Serotransferrin (Trfe): Residues **Y314/E385**							
(a) K·^{313}M^{ox}**Y**@LGYEYVTAIR^{324}·N	210	2	2	2.86	0.10		15/22
(b) K·^{381}IM@NG**E**ADAMSLDGGFVYIAGK^{401}·C	193	1	2	4.49	0.48	22/40	**E385**(0.11), D387(0.31)
(c) K·^{381}IM@NG**E**ADAMSLDGGFVYIAGK^{401}·C	227	2	2	5.14	0.50	19/40	**E385**(0.18), D387(0.33)
(d) M·^{383}NG**E**@ADAM^{ox}SLDGGFVYIAGK^{401}·C	263	2	2	4.57	0.64	21/36	D387(0.18)
2. α-2-Macroglobulin (A2mg): Residues **E300/S532**							
(a) K·^{297}LHT**E**@AQIQEEGTVVELTGR^{315}·Q	227	1	3	3.71	0.29	24/72	T299(0.05), H298(0.05)
(b) R·^{517}LLIYAVLPTGDVIGD**S**@^{532}·A	227	2	2	2.88	0.33	22/30	D531(0.08)
3 α-1-Antitrypsin (A1at): Residues **D107/K368**							
(a) L·^{104}NQP**D**@SQLQLTTGNGLFLSEGLK^{125}·L	210	1	2	3.12	0.28	15/42	S108(0.09)
(b) L·^{04}NQP**D**@SQLQLTTGNGLFLSEGLK^{125}·L	210	2	2	3.44	0.34	16/42	S108(0.09)
(c) R·^{102}TLNQP**D**S@QLQLTTGNGLFLSEGLK^{125}·L	227	1	3	4.12	0.30	30/92	**D107**(0.11)
(d) K·^{366}FN**K**@PFVFLMIEQNTK^{380}·S	193	1	3	4.67	0.16		24/56
(e) N·^{368}**K**@PFVFLM^{ox}IEQNTK^{380}	263	2	2	4.41	0.23		20/24
4. Apolipoprotein AI (Apoa1): Residues **D48/D73/S228**							
(a) K·^{46}LL**D**@NWDSVTSTFSK^{59}·L	210	2	2	3.50	0.15	16/26	D51(0.11)
(b) K·^{60}LREQLGPVTQEFW**D**@N^{74}·L	227	1	2	3.34	0.22		16/28
(c) K·^{227}V**S**@FLSALEEYT^{337}·K	263	1	2	3.08	0.29		15/20
5. Apolipoprotein AII(Apoa2): Residues **C6/E8**							
(a) P·^{6}**C**@VESLVSQYFQTVTDYGK^{23}·D	193	2	2	4.54	0.49	22/34	**E8(0.07)**, S9(0.08)
(b) E·^{5}P**C**@VESLVSQYFQTVTDEYGK^{23}·D	210	1	2	3.94	0.26	17/36	**E8(0.03)**, S9(0.14)
(c) V·^{8}**E**@SLVSQYFQTVTDYGK^{23}·D	227	1	2	5.46	0.52	22/30	S9(0.04)
(d) P·^{6}**C**@VESLVSQYFQYFQTVTDYGK^{23}·D	263	1	2	3.65	0.25	21/34	**E8(0.01)**, S9(0.06)

[a] The assigned binding site in the listed platinated peptide is designated by an @ symbol following the residue. The most probable binding site on the basis of all analysed peptide sequences in this range is given in bold type. [b] $\{Pt\}^{2+}=193$, $\{(NH_3)Pt\}^{2+}=210$, $\{(NH_3)_2Pt\}^{2+}=227$, $\{(NH_3)_2PtCl\}^{+}=263$. [c] Ratio of assigned b^+ and y^+ ions to the total number of possible ions. [d] The ΔCn values of alternative neighbouring sites are given in parentheses.

number of IgGs (Ighg1–Ighg3) and complement proteins (Co3–Co5), but in each case in only one of the serum samples.

The O-donor side chains of tyrosine Y314 and glutamate E385 participate in platinum coordination by Trfe. Whereas the former site was only detected in the first blood serum sample, the latter site was identified in both samples. Both sites were also confirmed by LC–MS–MS analyses of individual cisplatin–Trfe reaction mixtures at 1:1 (3 h, 310 K) and 5:1 (3 h, 310 K; 7d, 310 K) molar ratios (Table 4). Although the SEQUEST analyses generate slightly higher Xcorr values for M313 in the MS–MS spectra 3(a), 3(b), 3(d) and 3(f) of Table 4, Y314 is clearly identified as a cisplatin binding site by the MS–MS spectra 3(c) and 3(e) for the platinated sequence Y314–R324. The presence of a platinum fragment that is coordinated to Y314 presumably leads to cleavage of the M313–Y314 bond during the initial MS ionisation of the tryptic peptide M313–R324 in these cases. It is important to note, that $^{1}H,^{15}N$ and $^{1}H,^{13}C$ HSQC NMR studies by Sadler and co-workers have demonstrated that M313 is not a significant cisplatin binding site in the apo and metal-bound forms of Trfe.[17] M313 is buried in the interlobe contact region of the intact transport protein, but becomes the preferred methionine-binding site after removal of the C-lobe.[17]

Figure 8 depicts the MS–MS spectrum of peptide sequence 1(d) of Table 3, which belongs to the platinated peptide N383–K401. A total of twenty-one of the thirty-six possible b^+ and y^+ ions could be assigned; these comprised nine platinated b^+ and twelve nonplatinated y^+ ions. The presence of the non-platinated ions y_{15}^+ and y_{16}^+ in good relative abundancies is in accordance with E385 platination, and this glutamate residue is also clearly identified by the MS–MS spectra of peptide sequences 4(c) and 4(d) in Table 4. Significantly higher Xcorr values, however, are observed for M382 as an alternative coordination position in the tryptic I381–K401 peptide sequences in Tables 3 and 4, which suggests that this methionine residue should not be ruled out as an additional or competing cisplatin binding site.

Trfe contains a total of nine methionine residues, M26, M109, M256, M309, M313, M382, M389, M464 and M499, whose tryptic peptides exhibit 4, 10, 5, 4, 12, 21, 21, 14 and 15 amino acid residues, respectively. Six of these residues (M109, M256, M313, M382, M389, M464) belong to the 51% of peptide sequences that are covered by the MudPIT analyses of Trfe in this work. The doubly and triply charged peptide ions of the short sequences that contain M26, M256 and M309 are not detectable in the experimental range of *m/z* 350–2000. The residue M256 was identified as a preferred binding site and the residue M499 as an additional site by the NMR spectroscopic studies of Sadler and co-workers.[17] It is interesting to note that only the threonine residue T457 was located as a cisplatin coordination site in a previous LC–MS–MS study by Dyson et al.[19,20] These latter authors employed much shorter incubation times (15 or 30 min) and a much higher cisplatin–

Table 4. Platinated peptide sequences in individual serotransferrin (Trfe) samples.

Peptide sequence[a, b]	Pt fragment mass[c]	[cisplatin]/[protein]	Charge	SEQUEST parameters		Ions[d]	Other possible binding site(s)[e]
				Xcorr	ΔCn		
1. Residues S255M**256**/E260/D261							
(a) R·^{255}S**M**@GGKEDLIWELLNQAQEHFGK^{276}·D	193	5 (7 d)	3	5.19	0.76	32/84	S255–K259, E260, D261(0.01)
(b) R·^{255}S**M**@GGKEDLIWELLNQAQEHFGK^{276}·D	210	5 (7 d)	3	4.84	0.48	33/84	S255–K259, E260, D261(0.09)
2. Residue **E265**							
(a) D·^{262}LIW**E**@LLNQAQEHFGK^{276}·D	227	1 (3 h)	2	4.04	0.61	19/28	
3. Residues M313/**Y314**							
(a) K·^{313}M@**Y**LGYEYVTAIR^{324}·N	193	1 (3 h)	2	3.80	0.65	20/22	**Y314**(0.06)
(b) A·^{312}KM@**Y**LGYEYVTAIR^{324}·N	193	5 (3 h)	2	2.83	0.58		
(c) M·^{314}**Y**@LGYEYVTAIR^{324}·N	193	5 (7 d)	2	2.67	0.68	17/20	
(d) K·^{313}M@**Y**LGYEYVTAIR^{324}·N	210	5 (3 h)	2	2.65	0.83	14/22	**Y314**(0.03)
(e) M·^{314}**Y**@LGYEYVTAIR^{324}·N	210	5 (7 d)	2	3.11	0.70	13/20	
(f) K·^{313}M@**Y**LGYEYVTAIR^{324}·N	227	5 (7 d)	2	2.64	0.68	14/22	Y314**(0.00)**
4. Residues M382/**E385**/D387							
(a) K·^{381}IM@NG**E**ADAMSLDGGFVYIAGK^{401}·C	193	5 (3 h)	2	4.21	0.72	26/40	**E385**(0.07), D387(0.18)
(b) K·^{381}IM@NG**E**ADAMSLDGGFVYIAGK^{401}·C	210	1 (3 h)	2	2.96	0.74	17/40	**E385**(0.13), D387(0.32)
(c) M·^{383}NG**E**@ADAMSLDGGFVYIAGK^{401}·C	227	1 (3 h)	2	6.25	0.79	27/36	D387(0.14)
(d) M·^{383}NG**E**@ADAMSLDGGFVYIAGK^{401}·C	263	1 (3 h)	2	4.65	0.73	19/36	D387(0.16)
5. Residue **T457**							
(a) R·^{457}**T**@AGWNIPM*GLLYNK^{470}·I	193	5 (3 h)	2	2.72	0.62	15/26	
(b) R·^{457}**T**@AGWNIPM*GLLYNK^{470}·I	210	5 (3 h)	2	3.73	0.69	15/26	

[a] Specific platinated peptides are only listed once and are given for the reaction solution with the lowest [cisplatin]/[protein] ratio or the shorter incubation time (for 5:1 solutions). [b] The assigned binding site in the listed platinated peptide is designated by an @ symbol following the residue. The most probable binding site on the basis of all analysed peptide sequences in this range is given in bold type. [c] $\{Pt\}^{2+}=193$, $\{(NH_3)Pt\}^{2+}=210$, $\{(NH_3)_2Pt\}^{2+}=227$, $\{(NH_3)_2PtCl\}^{+}=263$. [d] Ratio of assigned b^+ and y^+ ions to the total number of possible ions. [e] The ΔCn values of alternative neighbouring sites are given in parentheses.

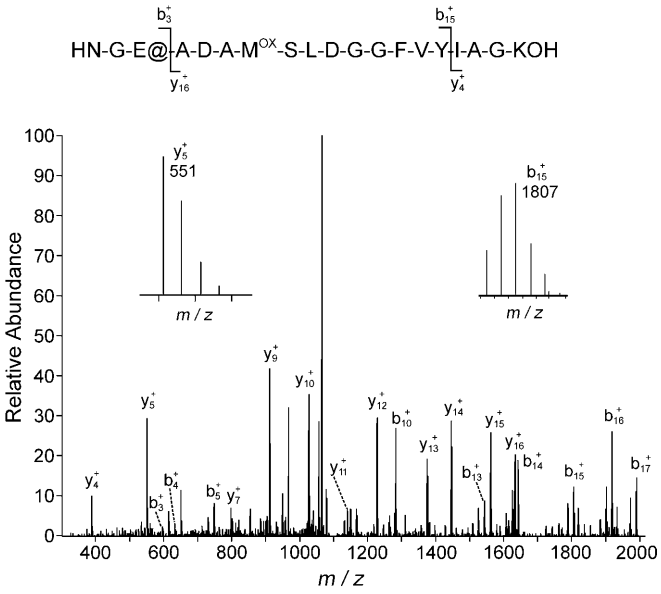


Figure 8. MudPIT MS–MS spectrum 1(d) (Table 3, Xcorr = 4.57, ΔCn = 0.64 for E385) for the molecular ion [NGE@ADAM^{ox}SLDGGFVYIAGK] (@ = $\{(NH_3)_2PtCl\}^+$) containing Trfe residues N383–K401.

Trfe ratio (10:1) than those used for the MudPIT analyses of the blood serum samples in this study. We did not identify T457 for a 1:1 cisplatin–Trfe reaction mixture after an incubation period of 3 h, but increasing the molar ratio to 5:1 did allow us to confirm this binding site (Table 4). Employment of this higher cisplatin–Trfe ratio and a longer incubation period of 7 d also led to the confirmation of M256 as a cisplatin coordination site, albeit for the 22-membered tryptic peptide S255–K276 with a missed cleavage site at K259. Because this longer peptide is only present in low concentrations in comparison to the shorter tryptic peptides S255–K259 and E260–K276, it is reasonable to assume that M256 is a preferred cisplatin binding site.

In accordance with our findings for HSA, O-donor side chains (Y314, E265, E385, T457) are also kinetically preferred binding sites for cisplatin in Trfe. As depicted in Figure 9, these sites are readily accessible together with M256, and are distributed over both the *N*-(V1–T336) and *C*-(N337–P679) lobes of the protein.[27] The environments of the adjacent residues M256 and Y314 in the former domain are illustrated in more detail in Figure 10. Although the Fe^{III}-binding sites (D63, Y95, Y188, H249) and (D392, Y426, Y517, H585) are not directly involved in Pt^{II} binding, the T457 site does belong to the bicarbonate-binding pocket and is also close to the Fe^{III} pocket. UV/Vis spectroscopic studies have demonstrated that cisplatin and Fe^{III} binding are competitive in Trfe.[20]

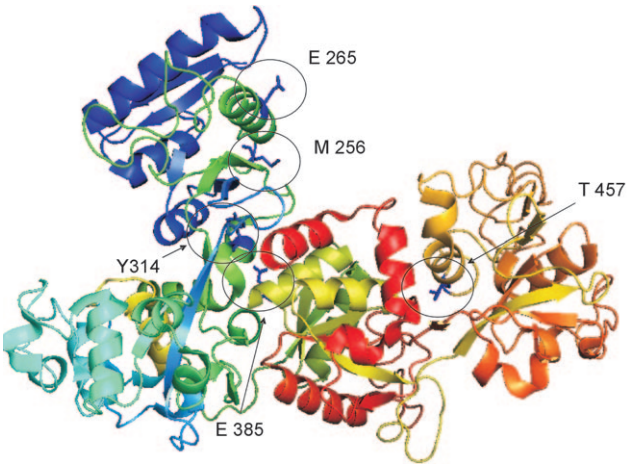


Figure 9. Cisplatin binding sites in Trfe (PDB ID: 2HAU).[27]

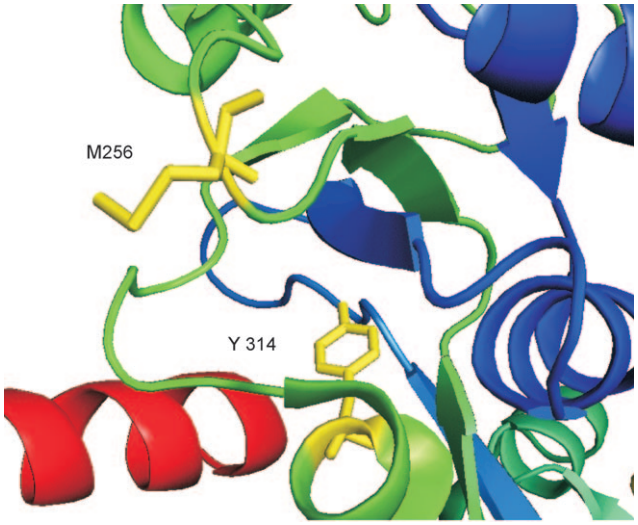


Figure 10. Environment of the M256 and Y314 residues in Trfe (PDB ID: 2HAU).

Specific cisplatin binding sites in other abundant serum proteins

The donor residues E30 and S532 were characterised as probable platination sites in A2mg belonging to the first and second blood serum samples, respectively (Table 3).These platinated residues, however, were not detected for individual 1:1 and 5:1 cisplatin–A2mg reaction mixtures, for which E75 (or E76) and T1409 were also identified as binding sites. Whereas a sequence coverage of only 25% was observed for A2mg in the blood samples, this rose to 42% for the LC–MS–MS analyses of individual cisplatin–A2mg reaction mixtures. Because peptide sequences that contain E30 and S532 were covered for the individual A2mg samples, this suggests that the large globular protein (1451 residues) might lack specific platination sites. In contrast, identical binding residues were found for the abundant serum proteins α-1-antitrypsin (D107 and K368) and apoliprotein A-II (C6 or E8) in both of the blood serum samples. Both proteins exhibited relatively high sequence coverage values of 62 and 63%, respectively, for the blood serum samples. The aspartate residues D48 and D73 were identified together with S228 for apolipoprotein A-I in the second (D48) and first blood sample (D73 and S228), respectively, and are highlighted in Figure 11. The sequence coverage was 69% for this protein in the blood serum samples. Apolipoprotein A-I contains six long α helices[28] and might, therefore, be expected to offer a range of nonspecific binding sites for cisplatin. It is interesting to note that Apoa1 makes up only about 2% of the total protein mass in human plasma, which means that the effective cisplatin–Apoa1 molar ratio in the blood samples was about 1:5.

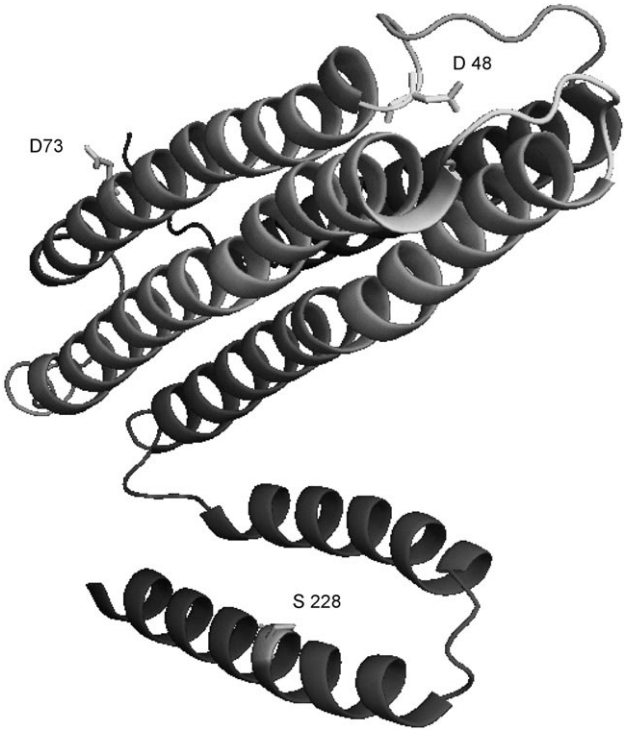


Figure 11. Cisplatin binding sites in apolipoprotein A1[28] (PDB ID: 2a01A).

Conclusions

Our present studies confirm the ability of MudPIT LC^2–MS^2 analyses to identify a significant number cisplatin binding sites for macromolecules of differing concentrations within a complex mixture of proteins. Although LC–ICP-MS evidence has very recently been provided that Pt-containing peptides remain intact after tryptic digestion of HSA,[9] no previous report has appeared on the use of MS proteomic strategies to identify the specific binding sites in the serum protein. The characterisation of the same binding site for proteins that belong to different blood samples or cisplatin–protein reaction mixtures underlines the potential of the MudPIT method for investigating metallodrug–protein interactions. The number of characterisable binding sites in a particular protein however, is

restricted by the sequence coverage that is accessible by ESI-MS–MS fragmentation, for example, 54% for HSA and 51% for Trfe after tryptic digestion. It should be possible to significantly extend this coverage in future work by combining fragmentation data from peptide mixtures that were obtained for other proteases or combinations of proteases.

Our present results indicate that the hard aspartate or glutamate carboxylate oxygen atoms and the hydroxy functions of serine, threonine and tyrosine are kinetically attractive coordination sites for cisplatin at neutral pH. This finding is in accordance with MudPIT analyses of the cisplatin/*E. coli* system, which identified seventeen O-donor residues for a total of thirty-one characterised protein targets.[23] Previous studies of cisplatin binding to HSA[13–16] and other proteins[29–31] have emphasised the important role of the softer cysteine and methionine sulfur atoms as preferred targets, and our present investigation does, of course, confirm C34 and establish M329 and M548 as specific binding residues in HSA, and M256 in Trfe. O-donor atoms, however, are known to be kinetically preferred[32–34] as initial binding sites for cisplatin, and inspection of the $^{1}H,^{15}N$ HSQC NMR spectrum taken for a cisplatin–HSA mixture (310 K, pH 6.4) after 2 h in the presence of added chloride (Figure 2a in ref. [15]) confirms the presence of a number of intense cross peaks that are attributable to Pt–O binding *trans* to an ammine ligand (NH_3). After 9 h these peaks disappeared, and only cross peaks that were due to Pt–S binding (C34 + 2–3 M residues) were observed. Our MudPIT analyses have now demonstrated that Y150 (or Y148) and D375 (or E376) are characteristic binding sites for cisplatin in HSA, and that these, and possibly other O-donor sites, can exhibit a remarkable degree of kinetic stability. Because coordination by these residues is more rapid than for S-donor residues,[32–34] it is reasonable to assume that they play an important role in the initial transport of the metallodrug. The relative thermodynamic weakness of Pt–O bonds means, however, that κO-coordinated cisplatin should be capable of slowly migrating to softer binding sites (e.g., DNA nucleobases) on reaching a target tumour cell. In contrast, the slow formation of stable $\kappa^2 S,N$-chelates for the C34 or M residues might prevent migration of the metallodrug from HSA. Trfe contains Y314 and E385 (together with E265 and T457) as characteristic κO-binding sites, which could also play an important role for cisplatin transport in the immediate time period after intravenous administration. The establishment of characteristic O-donor residues for other abundant serum proteins (A1at, Apoa2) is likewise in accordance with a dominant role for initial κO binding for cisplatin.

The absence of histidine residues as cisplatin binding sites for serum proteins even after an incubation period of 7 d at 310 K is, at first sight, rather surprising. The NMR spectroscopic investigations of Sadler et al. provided, however, no evidence for histidine coordination even after long periods of incubation for HSA and Trfe with cisplatin.[16,17]

Experimental Section

Human blood serum samples from two healthy volunteers were provided by the Berufsgenossenschaftliches Universitätsklinikum Bergmannsheil GmbH (Bochum, Germany) after consent had been obtained. Human serum albumin (HSA), iron-free Trfe (Trfe) and α-2-macroglobulin (A2mg) were purchased from Sigma–Aldrich (Heidelberg, Germany) and used as received. Cisplatin, (*cis*-$[PtCl_2(NH_3)_2]$) was obtained from Chempur (Karlsruhe, Germany). The buffer components NH_4OAc, NH_4HCO_3 and formic acid were purchased from Merck (Darmstadt, Germany), as was acetonitrile (MeCN). Sequencing grade trypsin was obtained from Promega (Mannheim, Germany). The model peptide G-A-L-T-N-V-S-M-A-K was a product of PSL (Heidelberg, Germany).

Sample preparation: The blood serum samples contained a protein concentration of about 70 $\mu g\,\mu L^{-1}$ (~1 mM) as determined by a Bradford assay. Each sample (1 μL) was diluted to 500 μL using equilibration buffer, and incubated for 3 h at 310 K with a tenfold molar excess (20 μM) of cisplatin. Unbound cisplatin and other low-molecular-weight components with a mass of less than 3 kDa were subsequently removed with a 3 kDa cut-off filter. The remaining proteins were then digested with trypsin (1.4 μg) at 310 K for 12 h before 98% formic acid (1.5 μL) was added to reduce the pH to 2.3. After filtration of the remaining high-molecular-mass components with a 10 kDa cut-off filter, the volume of the final reaction mixture of tryptic peptides was reduced to 20 μL. For the individual serum proteins, 1–5 μM solutions in an $(NH_4)_2CO_3$ buffer (10 mM, pH 7.8) were incubated at 310 K with cisplatin at a protein–Pt molar ratio of 1:1 for 3 h or 1:5 for 3 h and 7 d, respectively. An additional 1:3 molar ratio was employed for a 3 h incubation with HSA. The subsequent removal of unbound cisplatin and tryptic digestion of the protein were performed in a similar manner as for the blood serum samples.

ESI-MS–MS: ESI-MS and MS–MS data for the model peptide G-A-L-T-N-V-S-M-A-K were recorded on a Finnigan LCQ mass spectrometer (Thermo Electron Corp., San Jose, CA, USA), which was operated in the positive-ion mode with a capillary temperature of 200 °C and a spray voltage of 1.8 kV. Samples were prepared by treating the peptide with cisplatin for 24 h at 310 K and were delivered to the mass spectrometer by a syringe pump that was operating at a flow rate of 0.5–1.0 $\mu L\,min^{-1}$. The relative collision energy for collision-induced dissociation (CID) was set to 35–40%.

For the MudPIT analyses, a dual-gradient system HPLC pump (Dionex, Amsterdam, Netherlands), including a Famos autosampler and Switchos, was connected to a Finnigan LTQ ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA, USA). The LTQ was calibrated with a set of standard solutions prior to use (100 ppm mass accuracy) and operated by using Xcalibur Instrument Method files to acquire full MS scans in the range of *m/z* 350–2000 for individual chromatographic peaks, and these were followed by full MS–MS scans of the three most intense ions in the MS scan. A temperature of 180 °C was employed for the heated desolvation capillary. The relative collision energy for CID was set to 35% and dynamic exclusion was enabled with a repeat count of 1 and a 3 min exclusion duration window.

Clean samples of the blood serum tryptic peptides were loaded onto a triphasic microcapillary column.[21] This fritless 100 μm capillary was first packed with 12 cm of Eclipse XDB C_{18} (Hewlett–Packard, Palo Alto, CA, USA), then with 12 cm of a strong cation exchange (SCX) material (Whatman, Clifton, NJ, USA), and finally with 4 cm of the reversed-phase material (Hewlett–Packard). A column flow rate of 0.15–0.25 $\mu L\,min^{-1}$ was employed and the spray voltage was set to 1.8 kV. Four buffer solutions were employed for the chromatographic separations and these comprised 2% MeCN/

0.1% formic acid (buffer A), 80% MeCN/0.1% formic acid (buffer B), 250 mM NH_4OAc/0.1% formic acid/MeCN (buffer C), and 1.5 M NH_4OAc/0.1% formic acid (buffer D), respectively. After an initial 30 min wash with buffer A, peptides not bound to the SCX resin were eluted by applying a 180 min linear gradient up to 100% buffer B. Eluent compositions of 10–100% buffer C with increasing percentages in 10% steps, and eluents comprising 33, 50 and 75% buffer D were subsequently employed for periods of 2 min to displace fractions from the SCX resin onto the reversed-phase material. Each individual salt step was followed by a 7 min reversed-phase washing step with buffer A and a 70 min gradient up to 50% buffer B followed by an additional 20 min gradient up to 100% buffer B, and held at100% buffer B for a further 5 min before a final 20 min wash with 100% buffer D.

A monophasic microcapillary column was employed for the tryptic peptides from individual protein–cisplatin reaction mixtures. This fritless 100 μm capillary was packed with 12 cm of Eclipse XDB C_{18} and a column flow rate of 0.12 $\mu L\,min^{-1}$ and spray voltage of 1.8 kV were used. Only buffer solutions A and B were required for the reversed-phase chromatographic separation. Following an initial 5 min wash with buffer A, a 120 min linear gradient up to 55% buffer B followed by a 20 min linear gradient up to 100% buffer B was applied to elute the tryptic peptides.

SEQUEST analysis: The potentially coordinating residues C, D, E, H, K, M, S, T and Y were included in the search file as modified residues with mass gains of 193, 210, 227 and 263, respectively, for coordination by the possible cisplatin fragments $\{^{195}Pt\}^{2+}$, $\{(NH_3)^{195}Pt\}^{2+}$, $\{(NH_3)_2{}^{195}Pt\}^{2+}$ and $\{(NH_3)_2{}^{195}PtCl\}^{+}$. To allow for the net loss of two protons from a target peptide ion, two mass units were subtracted from the major ^{195}Pt isotope mass for the first three fragments. One mass unit was accordingly subtracted for the singly charged $\{(NH_3)_2{}^{195}PtCl\}^{+}$ fragment. Very conservative Xcorr and ΔCn criteria[22,23] were employed for SEQUEST matching[35] of MS–MS spectra to a serum protein in the Swiss-Prot database.[36] The following parameters were implemented for all peptides from the blood serum samples: Del Mass $\leq$ 2.5, Sp (preliminary score) $\geq$ 500, RSp (preliminary score rank) $\leq$ 6. Tryptic or partially tryptic peptides with a +3 charge were provisionally accepted when their Xcorr values were better than 3.50 and the difference from the next best peptide sequence (ΔCn) was $\geq$0.10 upon ignoring amino acid modification due to platination of an alternative residue in the same sequence. Analogous peptides with overall charges of 2+ were assigned to the initial target list when Xcorr $\geq$ 2.5, ΔCn $\geq$ 0.10 and at least 35% (ion ratio $\geq$ 0.35) of the observed MS–MS ions could be identified as b^+ or y^+-type peptide fragment ions (Scheme 1).[37] The minimum Xcorr values were set to 3.3 and 2.4 for peptides from the individual cisplatin–serum protein reaction mixtures with overall changes of respectively 3+ and 2+.

Following the SEQUEST analysis, MS–MS spectra were visually assessed[22,23] to positively confirm the presence of platinum in appropriate ion peaks on the basis of the characteristic broad isotopic pattern of platinated peptides (Figure 1). Peptide sequences whose MS–MS spectra did not conform to this criterion were removed from the final target list. Only platinated peptides with MS–MS spectra of good quality and with both platinated and non-platinated fragment ions whose relative abundances were clearly well above the baseline were retained in the final lists of cisplatin targets compiled in Tables 1–4.

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