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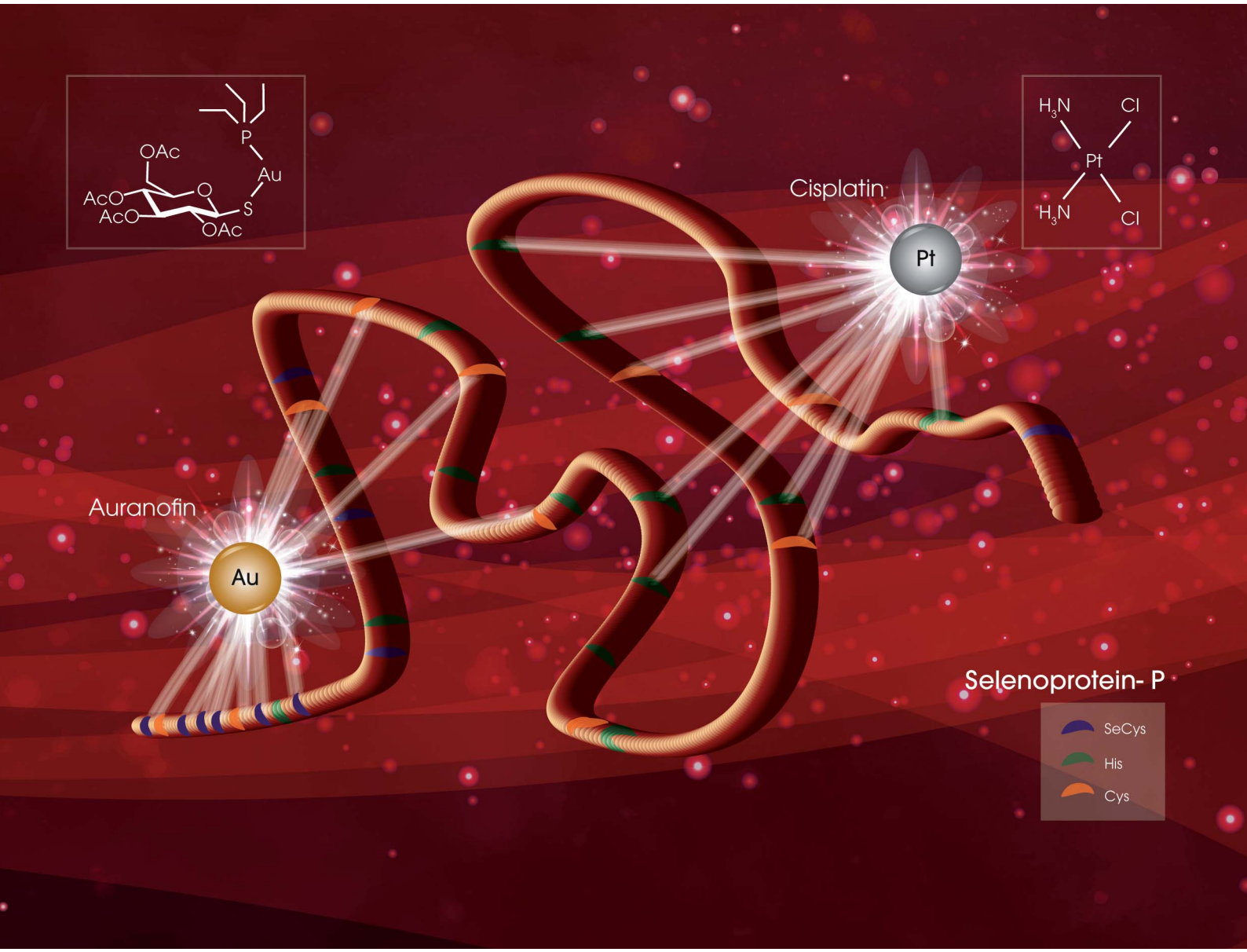

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Mass spectrometry insights into interactions of selenoprotein P with auranofin and cisplatin†

J. Lamarche,[a] K. Bierla,[a] L. Ouerdane,[a] J. Szpunar,[a] L. Ronga[a] and R. Lobinski*[ab]

The reactivity of selenoprotein P (a serum selenoprotein containing 10 selenocysteine (SeCys), 17 cysteine (Cys) and 14 histidine (His) residues) with two metallodrugs (auranofin and cisplatin) was investigated. The selenoprotein was purified from human serum by sequential affinity chromatography using immobilized metal (Co^{2+}) affinity chromatography (IMAC) and heparine affinity, followed by solid-phase extraction preconcentration. The purified selenoprotein P was sequenced by nanoLC-MS/MS to reach a complete sequence coverage. It eluted from SEC as two major peaks likely to correspond to the glycosylated and non-glycosylated forms and a minor one, probably a truncated form. Size-exclusion chromatography with the selective Se (^{78}Se) and metal (^{197}Au or ^{195}Pt) detection by ICP MS showed the co-elution of selenoprotein P forms with Au and with Pt. SEC-ICP MS of the tryptic digest showed a considerable shift of the elution of selenium towards the lower molecular masses while preserving the co-elution of selenium and the metal at some elution times. NanoHPLC – electrospray MS/MS analysis of the post-reaction mixture demonstrated the formation of peptides with the privileged binding to Cys and His residues for cisplatin and SeCys and Cys residues for auranofin.

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Introduction

Several noble metal compounds have long been used in drug therapies.[1] They include, among others, platinum compounds (cisplatin, oxaliplatin and carboplatin) used in half of cancer chemotherapies[2] and gold compounds *e.g.*, auranofin, originally employed as an antiarthritic drug[3] and more recently repurposed for its potential anticancer activity.[4,5] The identification of the metal-binding target sites in proteins is an important step towards the elucidation of the mechanisms of action of a metallodrug. Indeed, *in vitro* screening of metallodrug-protein interactions is one of the key areas for research prior to clinical approval.[6]

The selenol group (–SeH) has recently been extensively studied and reported as a privileged target site for auranofin and a series of drug candidate gold(III) compounds (Meier-Menches *et al.* 2016). In a theoretical study, the order of spontaneity of the reaction of auranofin with amino acids was Sec >> Cys >> Lys > His, corroborating the high affinity of gold(I), a soft Lewis acid, for selenium and sulfur (Dos Santos 2014). Among the proteins studied, thioredoxin reductase (TrxR) has enjoyed a particular interest as a target for anticancer drugs.[7] In studies of intact TrRx, the presence of multiple sites of interactions (other than SeCys) accessible to Au(I) has been suggested.[8] Peptide models of TrxR: 4-mer[9] and 11-mer peptides,[10,11] corresponding to the C-terminal SeCys-containing motif of human TrxR, were reacted with a panel of gold(I) complexes and the gold binding sites were identified as SeCys and Cys. In an attempt to study TrxR interaction with Pt-anticancer drugs, it was suggested that terpyridine-platinum(II) is bound to Cys in the GCCG motif of a TrxR mutant in which SeCys-498 was replaced by Cys (Lo *et al.* 2009).

Selenoprotein P (SelP) is unique amongst all the characterized human selenoproteins as it contains up to 10 SeCys residues whereas all the other selenoproteins contain only one.[12] Recent advances in the understanding of the role of SelP and its potential medical and pharmaceutical implications were reviewed (Tsutsumi & Saito 2020). The protein was attributed multiple functions including its involvement in the storage of Se in the brain and testis, defence against oxidative stress, and regulation of heavy metals concentrations; it was also reported to play a potential role in the development of various forms of cancer and type 2 diabetes.[13]

Because of its abundance as the principal form of selenium in serum and the number of SeCys residues, SelP may be worth investigating as a potential target for metallodrugs. In contrast to the previously studied selenol-containing model peptides, SelP is neither commercially available nor can be readily heterologously expressed, it has therefore to be purified from serum. The methods for SelP purification are usually based on the immunoaffinity precipitation or chromatography (using mono- and/or polyclonal antibodies) or on the chemical affinity

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to immobilized transition metal ions as discussed in a recent review.[23] The use of immunoaffinity methods is critically dependent on the quality and selectivity of antibodies, which privileges the chemical affinity methods.[14] As the concentration of SelP in human serum is barely at the 50 ppb level (as Se), the purification protocols are tedious. Also, cutting-edge, sensitive analytical methods are necessary to monitor the Se-metal interactions and provide molecular evidence of the binding sites.

The metallodrug-protein *in vitro* interactions can be readily investigated by soft ionization (electrospray or MALDI) mass spectrometry which allows precise measurements of the molecular mass of proteins and their metal adducts.[15] However, ionization of the metal-complexes may be insufficient in the presence of complex matrices at low concentration levels. Alternatively, the formation of the metallodrug-selenoprotein bond can be demonstrated by the co-elution of selenium and the metal in different HPLC mechanisms. This can be possible by the use of multielement isotope specific detection by inductively-coupled plasma mass spectrometry (ICP MS).[16,17]

The molecular insight into the exact binding site is more difficult. The observation of the metal–selenium bond is possible by synchrotron radiation X-ray absorption spectroscopy (XAS).[18] An alternative is breaking down the adduct into fragments while preserving the Se(S)–metal bond to a point that they can be unambiguously identified by mass spectrometry. Such breakup can be carried out by fragmentation in the gas phase or by enzymatic (usually tryptic) digestion. Successful examples of the use of this approach to metal–selenoprotein binding have been rare[19] and none of them has shown an MS/MS characterization of the binding site. To our best knowledge, no studies of the determination of SelP–metal binding sites have been reported so far.

The objective of this study was to investigate the reactivity of SelP with the two most popular metallodrugs, auranofin and cisplatin. For this purpose, the purification of selenoprotein P from human serum was revisited and the purified protein was characterized by mass spectrometry. Size-exclusion chromatography with specific detection of Se, Au and Pt was adapted to study the formation of the metal-protein adducts and their behaviour during tryptic digestion. Finally, tandem mass spectrometry was investigated to sequence metallopeptides in order to identify the metal biding sites.

Experimental

Sample, reagents, solutions

Human blood serum was purchased from Pan™ Biotech (Aidenbach, Germany). The serum collection was done aseptically; the collected and coagulated blood was centrifuged, pooled and frozen. After defreezing, the serum was filtered through a succession of filters (the last of which had a porosity of 0.2 μm) and packaged in PETG bottles. Upon reception, the serum was divided in aliquots of 20 mL and stored at −80 °C. Each of the aliquots was used to prepare a batch of SelP for a series of experiments.

Analytical reagent grade chemicals and LC-MS grade solvents were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) and Honeywell (Seelze, Germny), respectively. Deionized water (18 Ω cm) from a MilliQ system (Millipore, Bedford, MA, USA) was used throughout.

Instrumentation

A peristaltic pump (ISMATEC®) was used for the IMAC column loading. A Visiprep™ SPE Vacuum Manifold DL (Disposable Liner) from Supelco (Bellefonte, PA) was used for solid phase extraction. A SpeedVac Concentrator Plus (Eppendorf, Hamburg, Germany) was used for solvent (80% MeOH) evaporation.

The ICP MS instrument was Agilent Model 7700s (Agilent, Tokyo, Japan) equipped with a collision cell. The ICP system consisted of a 400 μL min^{-1} micro-mist U nebulizer (Glass Expansion), a Scott type spray chamber, and a 2.5 mm i.d. injector torch. A set of nickel (sampler and skimmer) cones were used for total analysis and in the case of coupling with. An Agilent 1200 Series (Agilent, Tokyo, Japan) pump with a UV-module, automated sampler, and fraction collection module, was used for the IMAC, heparin and SEC separations. A SEC column Superdex-75 (300 × 3.2 mm × 9 μm) using cross-linked agarose and dextran as stationary phase was employed.

The electrospray ionization mass spectrometer was Orbitrap LUMOS Fusion (Thermofisher Scientific, San Jose, USA) usually fitted with an ESI Ion Max NG source was operated in positive ionisation mode. For the coupling with nanoUPLC, the ESI source was replaced by a TriVersa Nanomate (Advion, Ithaca, NY) using an injection chip with a coupler. Data processing was carried out using the Thermo XCalibur, Thermo Free Style, Proteome Discoverer and Expasy softwares. For the ESI MS and MS/MS characterization, peptides and their metal adducts were separated by reversed phase HPLC using a C18 Acclaim PepMap RSLC column (50 cm × 75 μm, Thermo Scientific) and a nano-UPLC Dionex Ultimate 3000 RS pump (ThermoFischer Scientific, Germering, Germany).

Procedures

ICP MS and ESI MS conditions. The experimental parameters, such as plasma power, torch position, the voltage on extraction and focusing lenses were optimized daily by using a tune solution containing 1 μg L^{-1} each of Li, Mg, Y, Ce, Tl and Co in 2% HNO_3 according to the manufacturer's protocol. Hydrogen at 4.5 mL min^{-1} was used as reaction gas to reduce spectral interferences.[20] For ESI MS, the resolution (m/z 200) was 120 000 for MS and 15 000/30 000 for MS^2 (CID). The scan range was 350–1800. The TriVersa NanoMate system (Advion, NY, USA) was used as a nano ESI source for HPLC experiments.

Purification of SelP by IMAC. The IMAC column was loaded with 25 mL of IMAC Chelating Sepharose (Cytiva, Marlborough, MA) and kept at 4 °C in 20% of ethanol until use. Before each experiment, the column was washed with 0.2 M EDTA (pH 7), followed by 5 column volumes of water (at 4 mL min^{-1}). A freshly prepared solution of 0.1 M of $Co(NO_3)_2$ was then loaded, followed by the washing of the non-retained Co^{2+} with water.

The column was finally conditioned with the running buffer (0.5 M ammonium acetate, pH 7).

Serum samples were thawed prior to experiment, and centrifuged. Then, 7.5 mL aliquots were diluted twice with water and loaded onto the column at 2 mL min^{-1} by means of the peristaltic pump. The column was then integrated in the 1100 Agilent HPLC system coupled on-line with ICP-MS. Buffer A was 0.5 M ammonium acetate at pH 7 and buffer B was 0.5 M ammonium acetate at pH 4.1. The elution was carried out at 5 mL min^{-1} with the following gradient: 0–20 min 0% B, 20–40 min 15% B, 40–55 min 15% B, 55–70 min 25% B, 70–90 min 25% B, 90–100 min 35% B, 100–105 min 100% B, 105–140 min 100% B, 140–141 min 0% B, 141–145 min 0% B. The eluate was split into (*i*) a 4.5 mL min^{-1} flow directed to the fraction collector (Agilent), and (ii) 0.5 mL min^{-1} flow directed to UV-ICP MS. The latter flow was 1 : 1 diluted on-line with water to reduce the salt content and avoid clogging the nebuliser; ^{59}Co, ^{78}Se and ^{80}Se isotopes were monitored. The fractions collected between 100 and 115 min were combined and kept at 4 °C till the next purification step – heparin affinity separation.

Heparin affinity chromatography. The heparin column was washed prior to each experiment using water and 0.1 M ammonium acetate buffer (5 column volumes each) fed by means of a peristaltic pump at 1 mL min^{-1}. The combined fraction collected after IMAC separation was diluted 10 times with MilliQ water, pH was adjusted to 5.8 with ammonium hydroxide and the solution was then loaded onto a Heparin HitrapTM 5 mL column (Cytiva). The column was then integrated into the HPLC-ICP MS system. Buffer A was 0.1 M ammonium acetate (pH 5.8) and buffer B was 1.5 M ammonium acetate (pH 5.8). The elution was carried out at 5 mL min^{-1} with the following gradient 0–30 min 0% B, 30–35 min 70% B, 35–40 min 100% B, 40–55 min 100% B, 55–60 min 0% B, 60–70 min 0% B. The eluate was split into (i) a 4.5 mL min^{-1} flow directed to the fraction collector, and (ii) 0.5 mL min^{-1} flow directed to UV-ICP MS. The latter flow was 1 : 1 on-line diluted with water to reduce the salt content and to avoid the clogging of the nebuliser; ^{59}Co, ^{78}Se and ^{80}Se isotopes were monitored. The fractions eluted between 34 and 38 min (total volume of 20 mL) were combined and kept at 4 °C till the next steps of the procedure.

Solid-phase extraction. A *ca.* 20 mL aliquot obtained after the heparin purification was concentrated on an Isolute C4 SPE column (Biotage, Uppsala, Sweden). The column was washed using 20 mL of 50% MeOH, and 20 mL of 0.1% TFA in 5% MeOH. After loading, the column was washed with 5 mL of 5% MeOH. Selenoprotein P was then eluted with 1 mL of 80% MeOH. The three sub-samples were subsequently treated. The solution was concentrated at 30 °C for 1 h. The final volume of the SelP solution was 150 μL.

Characterization of selenoprotein P by a bottom-up proteomics approach. A 150 μL aliquot of selenoprotein P solution was buffered to pH 7.5 with 100 mM Tris–HCl to fall within the reactivity pH range of trypsin, treated with dithiotreitol (DTT, 1 : 10 eq.) and iodoacetamide at 37 °C prior to overnight incubation at 37 °C. The reaction was stopped by an addition of 1 μL of formic acid. The obtained tryptic digests were kept at −80 °C prior to analysis.

The tryptic digest was analyzed by nanoUPLC - ESI-MS. The column was kept at 40 °C. The elution was performed at 300 nL min^{-1}. The mobile phases were: A – 0.1% formic acid, and B, 0.1% formic acid in 90% acetonitrile. The elution gradient was: 0–5 min 0% B, 5–10 min 10% B, 10–110 min 25% B, 110–120 min 40% B, 120–121 min 90% B, 121–130 min 90% B, 130–131 min 5% B, 131–140 min 0% B. Sample injection volume was 3 μL.

Reactivity of selenoprotein P with metallodrugs. Stock solutions at 0.6 mM of auranofin and 0.1 mM cisplatin were prepared by dissolving the corresponding amounts of metallodrugs powders in dimethyl sulfoxide (DMSO). An aliquot of SelP (10 μL) solution was diluted (2-fold) with 2 mM ammonium acetate buffer (pH 7.0). An amount of 10 equivalents of DTT was added to the protein solution followed by 3 to 10 equivalents of a metallodrug (auranofin or cisplatin) and the mixture was stirred overnight at 37 °C in a water bath. The adducts formed were eluted from a SEC column using 0.1 M ammonium acetate (pH 7) at 0.7 mL min^{-1}. The injection volume was 5 μL. ^{78}Se, ^{80}Se, ^{195}Pt and ^{197}Au were monitored.

Analysis of tryptic digests of selenoproteins P adducts with metallodrugs. The mixture after the reaction of selenoprotein P with a metallodrug (auranofin or cisplatin) was incubated under stirring overnight at 37 °C in the presence of 1 : 50 eq. of trypsin. The reaction was stopped with 1 μL of formic acid and the digest was frozen and kept at −80 °C until analysis. An aliquot of the digest was analysed by SEC-ICP MS in the conditions as above and another one by nanoUPLC-ESI MS/MS using the same conditions as described for the characterization of the digest of purified SelP.

Results and discussion

Isolation of SelP

The principle of purification is based on the affinity of SelP to heparin or metal ions (Co^{2+}, Ni^{2+}) conferred by two His-rich regions: the first region consists of 9 histidines out of 14 residues, and the second one a 4 consecutive histidines.[21] The presence of these histidine-rich regions, in conjunction with the presence of Cys and SeCys, makes it possible to retain SelP on an IMAC-sepharose column loaded with Co^{2+} [14] and on a heparin column.[22] The protocols described in literature are long, tedious and produce large volume of solutions.[23] The optimization was based on the literature reports summarized in a recent review[23] and closely followed by monitoring the selenium content throughout the procedure; the final product was concentrated by SPE to obtain a small volume of relatively high concentrated preparation suitable for further experiments.

Fig. 1a (blue line) demonstrates that SelP can be separated from the other selenium-containing proteins (glutathione peroxidase, selenoalbumin) by IMAC. It also shows (green line) that a large part of the matrix is not retained and elutes in the void. The separation takes about 2 h and produces a fraction of 51 mL containing 35% of the total selenium present in serum. This percentage matches well the literature values[23] of the

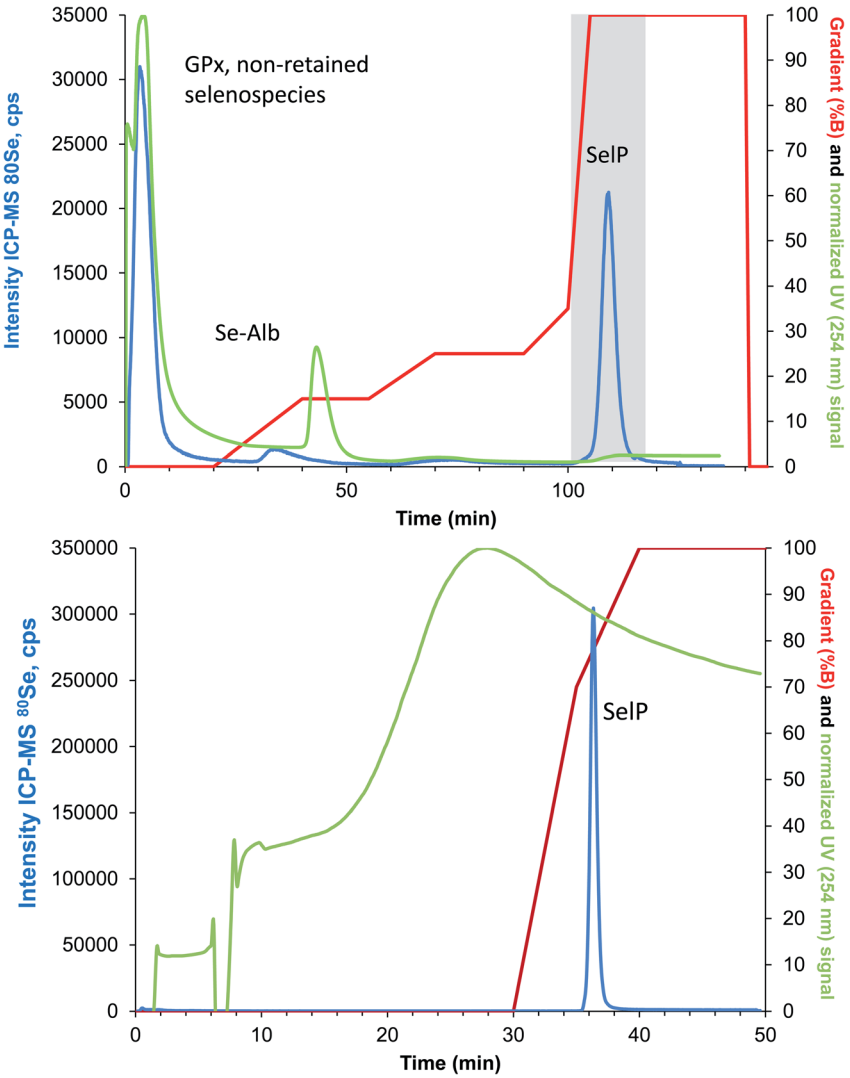

Fig. 1 The 2-dimensional purification of SelP from human plasma: (a) immobilized metal affinity chromatography (IMAC), (b) heparin affinity chromatography. Red line: gradient used (% B); green line: normalized UV (254 nm) intensity; blue line: ^{78}Se ICP MS intensity.

fraction of selenium present in serum as selenoprotein P. The collected SelP was then retained on the heparin column and eluted as a very narrow peak (blue line) at 36 min (Fig. 1b) to complete the separation from matrix components (UV trace, green line). This peak corresponds to a volume of 24 mL which contains 71% of selenium introduced on the heparin column. The collected SelP-containing fraction was ultimately concentrated using solid-phase extraction resulting in a volume of 150 μL solution containing 1.5×10^{-5} nM of protein (as determined by Bradford essay[24]).

Sequencing of the isolated SelP

The SelP-containing fraction collected after the IMAC-heparin separation was digested with trypsin and analysed by nanoHPLC – ESI MS/MS to identify the produced peptides and to reconstruct of the SelP sequence (bottom-up protein identification approach). A full coverage of a protein sequence corresponding to the full-length SelP consisting of 381 amino acids (Fig. 2)[13,23] was achieved.

The Cys and SeCys-containing peptides were detected as their carbamidomethylated derivatives (CAM) as a result of the reaction with DTT (intended to unfold the protein) and iodoacetamide (intended to protect Cys and SeCys from oxidation). Some peptides were observed as their glycosylated forms (SelP in serum is known to undergo glycosylation.[23] Many *O*-glycosylation sites (highlighted in blue in Fig. 2) were identified by successive loss of a hexose (Hex), *N*-acetylneuraminic acid (NeuNAc) and *N*-acetylhexosamine (HexNAc) summing up the observed mass difference to 663.319. All the six *N*-glycosylation sites (N-46, 83, 119, 128, 174 and 338) already reported[25] as well as three unreported so far (N90, 323 and 371) were found, they are highlighted in green in Fig. 2). The *N*-glycosylation involved also the sequence of *N*-acetylneuraminic acid, *N*-acetylneuraminic acid galactose and two *N*-acetylhexosamine moieties (summing up the observed mass difference to 947.32029). The glycosylations were reported to increase the original molecular weight of rat SelP 43 kDa of SelP up to 57 Da.[26] Note that the

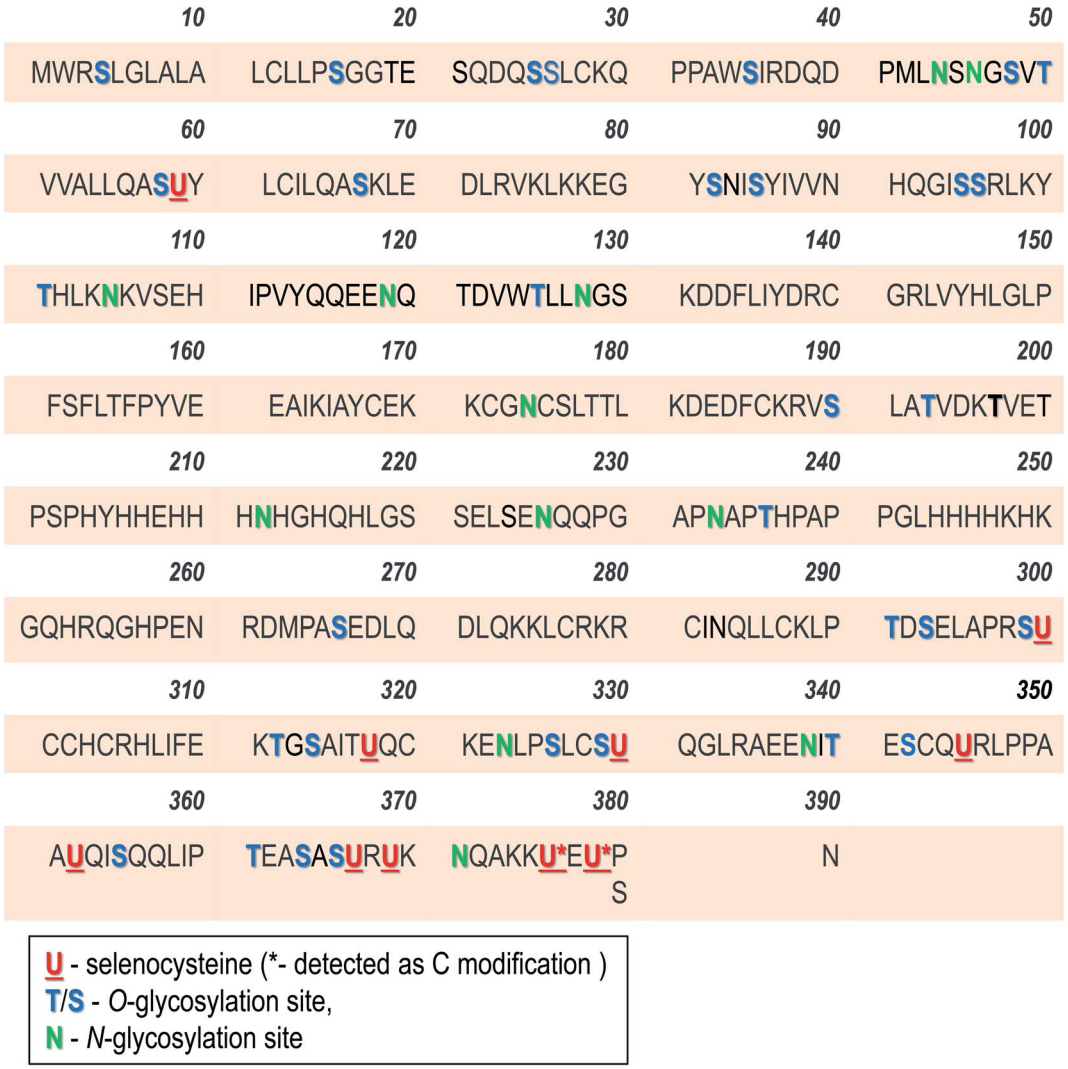

Fig. 2 Sequence coverage of the purified SelP together with the details of its glycosylation.

tryptic digests contained both glycosylated and non-glycosylated forms of the same peptides.

Also, multiple acetylation modifications have been detected. Acetylation of tryptic peptides by a reaction with acetic anhydride is a means of improving the quality of mass spectra by increasing the occurrence and abundance of fragment ions.[27] Although no intentional acetylation was carried out, the presence of these modifications can be attributed to a prolonged contact of the sample with high concentrations of acetate during the SelP purification step followed by acidification with (*ca.* 1% formic acid just after tryptic digestion) which could catalyze the observed reaction. In particular, acetylation has been demonstrated by MS/MS data for the lysine-containing peptides; such as ^{306}LIFEKTGSAITCQCKENLPSLCSCQGLR^{334}, ^{280}RCINQLLCKLPT^{290}, ^{348}PAACQISQQLIPTEASASCR^{368} and ^{38}DQDPMLNSNGSVTVVAL^{54} peptides.

Table 1 summarizes the list of the SeCys-containing peptides. It has been rare in the literature that sequences of selenopeptides were demonstrated experimentally by mass spectrometry[23] because of their instability due to oxidation and dehydroalanination[28] and this is the first time that the full sequence coverage of human SelP is accounted for by mass spectrometry. Interestingly, in parallel to selenopeptides, their sulfur analogues (containing cysteine in the place of selenocysteine) have been detected. Indeed, for the last two theoretical SeCys (U376 and U378) moieties, only their cysteine analogues were detected. This phenomenon was already observed by Turanov *et al.*[29] who estimated that 8% Cys was inserted in place of SeCys in SelP isolated from blood plasma of healthy volunteers. This percentage was estimated on the basis of 4 tryptic selenopeptides (^{299}SUCCHCR^{305}, ^{312}TGSAITUQCK^{321}, ^{322}ENLPSLCSUQGLR^{334} and ^{335}AEENITESCQUR^{346}) and depended on the SeCys position.[29] It was postulated that a change in selenium status might result not only in lowering SelP concentration or the presence of truncated forms, but also in SelP SeCys content due to replacement of SeCys with Cys.[29] In our work, the substitution rate estimated on the basis of the intensity of ^{322}ENLPSLCSUQGL^{333}/^{322}ENLPSLCSCQGLR^{334}, was around 4%.

Table 1 SeCys-containing peptides and their Cys analogues identified in the tryptic digest of purified SelP

Peptide	Theoretical mass	Experimental mass	Observed ions, m/z
^{38}DQDPMLNSNGSVTVVALLQAS^{59}**U**YLCILQASK^{68}	3329.5658	3443.4220	1147.8073 (z=3) 1147.8048 (z=3)
^{58}S^{59}**U**YLCILQASK^{68}	1390.59381	1390.59404	695.8007 (z=2)
^{59}**U**YLCILQASK^{68}	1303.56178	1303.56182	652.2846 (z=2)
*^{56}QAS^{59}**C**YLCILQASKLEDLR^{73}*	2111.06237	2111.07895	704.3645
^{299}S^{300}**U**CCHCR^{305}	1085.26244	1085.26334	363.0975 (z=3)
*^{299}S^{300}**C**CCHCR*HLIFEKTGSAIT^{318}**C**QC^{320}	2656.1016	2656.1034	886.0393 (z=3)
^{312}TGSAIT^{318}**U**QCK^{321}	1059.0404	1174.4543	587.2271 (z=2)
*^{307}LIFEKTGSAIT^{318}**C**QCK^{321}*	1797.8874	1797.8929	899.4501 (z=2)
^{322}ENLPSLCS^{330}**U**QGLR^{334}	1467.6163	1582.6653	791.3326 (z=2) 791.3334 (z=2)
^{307}LIFEKTGSAITUQCK*ENLPSLCS^{330}**C**QGLR^{334}*	3255.5629	3255.5836	814.6513 (z=4)
*^{321}ENLPSLCS^{330}**C**QGLR^{336}*	1533.71482	1533.71428	767.3608 (z=2)
^{335}AEENITESCQ^{345}**U**R^{346}	1430.5119	1545.5609	772.7805 (z=2)
^{335}AEENITESCQ^{345}**C**R^{346}	1845.8106	1845.8163	615.9436 (z=3)
^{347}LPPAA^{352}**U**QISQQLIPTEASAS^{367}**U**R^{369}	2380.0312	3152.3212	1050.7737 (z=3) 1050.7753 (z=3)
*^{350}AA^{352}**C**QISQQLIPTEASAS^{367}**C**RCK^{370}*	2265.07843	2265.09404	1133.0506 (z=2)
^{369}**U**KNQAK^{374}	-	-	-
^{358}LIPTEASAS^{367}**C**R^{369}**C**KNQAK^{374}	1918.94734	1918.94512	959.97620 (z=2)
^{376}**U**E**U**PSN^{390}	-	-	-
^{375}K^{376}**C**E^{378}**C**PS^{380}	792.28960	792.29025	396.6484 (z=2)

Purity of the isolated SelP

SDS PAGE (Fig. SI-1†) produced two bands at 55 and 75 kDa preceded by another band identified by LC MS/MS as α-2-macroglobulin (data not shown), obviously an impurity. Based on the previous discussion in literature,[26] it is reasonable to assume that they correspond to the glycosylated and non-glycosylated forms of SelP that coexist.

The presence of two distinct forms of SelP varying by about 20 kDa is also demonstrated by SEC-ICP MS of purified SelP (Fig. 3a). In addition, a third (minor) peak, likely to correspond to the truncated isoform,[23] was observed in the lower molecular mass range by SEC-ICP MS.

Probing the SelP interactions with cisplatin and auranofin by SEC-ICP MS

The reactivity of selenoproteins P with metallodrugs was investigated by size-exclusion chromatography with the simultaneous ICP MS detection of selenium and the metal, as proposed elsewhere [16,17]. This approach should permit to trace the intact SelP, a SelP-metal adduct and free metallodrug. However, free auranofin does not elute from SEC columns (recovery lower than 3%, data not shown).

Fig. 3b shows the co-elution of Pt with Se at two elution volumes, corresponding to the peaks 2 and 4. Which can be tentatively attributed to SelP-metallodrug adducts. The ^{197}Pt

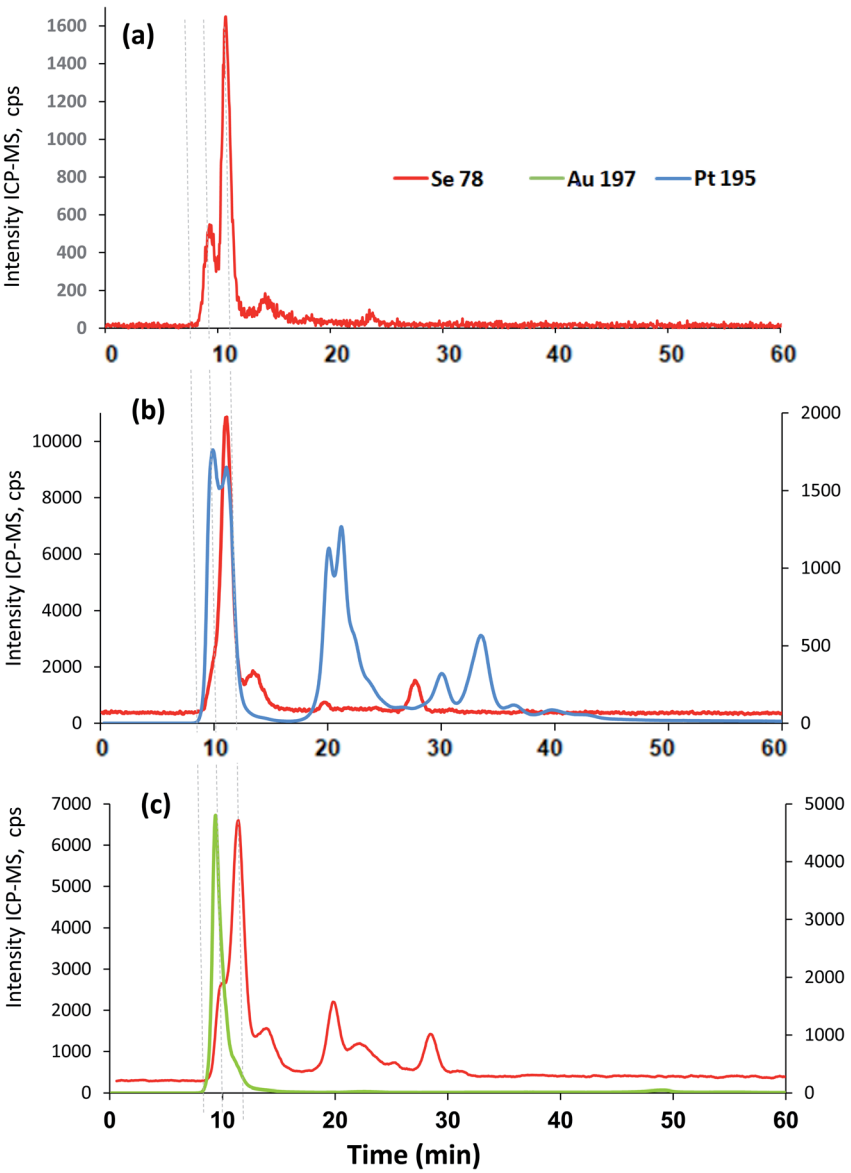

Fig. 3 Size-exclusion chromatography – ICP MS of (a) selenoprotein P preparation, (b) post-reaction mixture of SelP with cisplatin, (c) post-reaction mixture of SelP with auranofin.

specific chromatogram shows also other peaks that do not co-elute with selenium; two of them (at *ca.* 23 and 35 min) seem to correspond to the excess of unreacted cisplatin (chromatogram not shown). In the case of gold (Fig. 3c) the co-elution is much less evident and is observed only at the elution volume of the heavier SelP species.

The stability of the adducts formed was investigated during tryptic digestion with the purpose of reducing them in size while preserving the metal–selenium (–sulfur, –nitrogen) bond for further investigations of the binding site by MS/MS. The chromatogram in Fig. 4a shows a considerable shift of the elution of both Pt and Se toward the smaller molecular masses. The co-elution of Pt and Se is maintained showing the preservation of the bond. The Pt/Se ratio changes showing that the dominant binding peptide may not be a selenium one. Indeed, cisplatin was reported to bind covalently to His and Met[30] and Cys[31,32] which would explain such a behaviour.

In the case of auranofin (Fig. 4b), the digestion is apparently more difficult. It can be explained by the fact that the presence of Au may inhibit trypsin.[33] Nevertheless, a shift towards low molecular mass is seen, even if the intensities of the Au/Se adducts are lower than in the case of cisplatin. In contrast to Pt, a large amount of Au elutes de-correlated from selenium which may suggest a strong binding to other ligands. Note that the unbound auranofin would not elute from the column at these conditions.

Approaches to the identification of binding sites

Even if the demonstration of a co-elution of Se and the metal indicates the existence of metal-peptide bonds, it does not prove

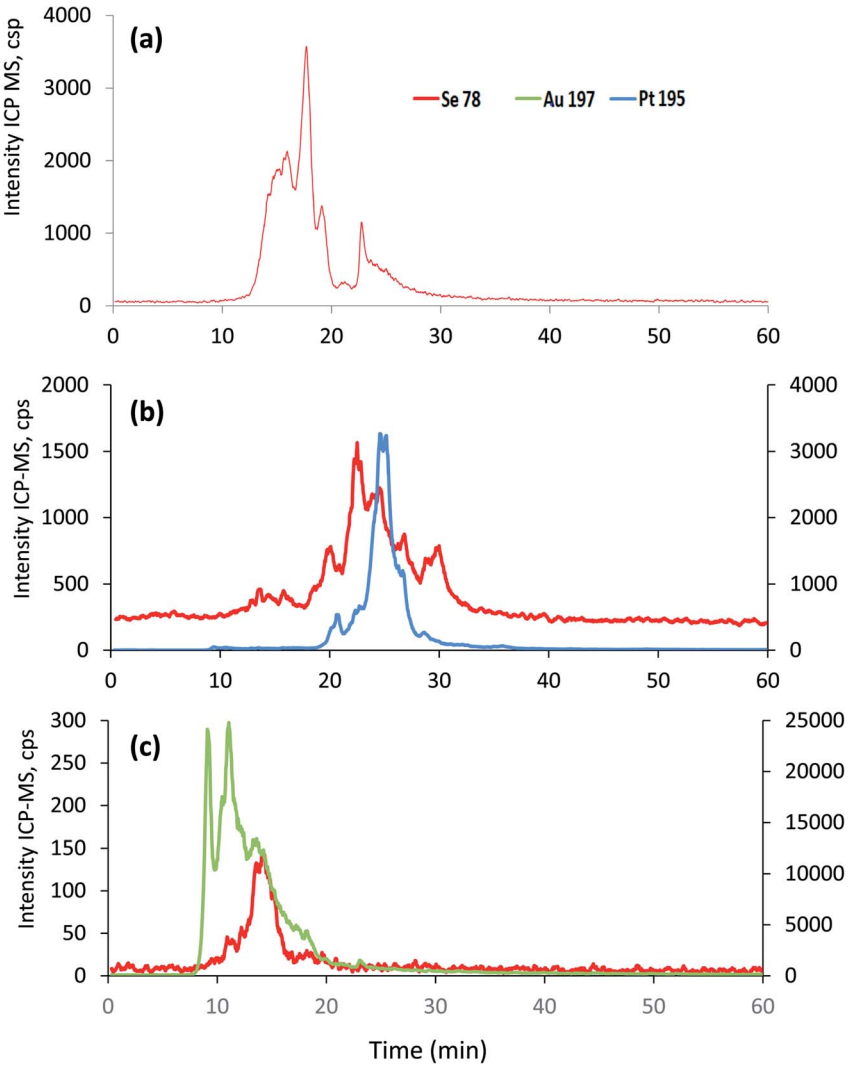

Fig. 4 Size-exclusion chromatography – ICP MS of tryptic digests of: (a) SelP preparation, (b) SelP-cisplatin adduct, and (c) SelP-auranofin adduct.

the direct involvement of selenium in the binding as other binding sites (*e.g.*, Cys, His) can exist in the same peptide sequence. An insight into the binding site can be obtained by a gas-phase fragmentation of the metal–peptide complex formed, and the association of the metal with the particular amino acid at the particular position in the protein sequence. Examples of such an approach for selenoproteins have been rare and concerned model peptides.[9,10,34] Here, the digest was analyzed by 2D-nanoHPLC – ESI MS/MS in order to identify the presence of putative metal-containing peptides by high

Table 2 Potential modifications of the masses of peptides resulted from the digestion of SelP-metallodrug adducts taken into account in the search of nanoHPLC-ESI MS data sets for the presence of metallopeptides

Modification	Structure	Monoisotopic mass	Resulted mass modification	Ref.
Cisplatin fragments				
Pt	$[Pt]^{2+}$	194.9637	192.9491	41 and 38
Pt1	$[Pt\ (NH_3)]^{2+}$	211.9902	209.9756	41 and 38
Pt2	$[Pt\ (NH_3)_2]^{2+}$	229.0168	227.0022	41, 38 and 42
Pt3	$[Pt\ (NH_3)_2Cl]^{+}$	263.9862	262.9789	38 and 40
Pt4	$[Pt\ (NH_3)(H_2O)^{2+}$	280.0008	277.98626	39, 40 and 42
Auranofin fragments				
Au	Au^{+}	196.9659	195.9559	8–10 and 45
Au1	$[AuP(C_2H_5)_3]^{+}$	315.05708	314.0498	8–10 and 45

resolution accurate mass (HRAM) spectrometry using an Orbitrap mass analyzer, characterize them by MS/MS and obtain an insight into the exact position of the metal-containing moiety.

A key step is data mining. Selenium, with its 6 isotopes, has a characteristic isotopic pattern which is often used for the search of Se-containing species.[35,36] This would also apply to Pt, which possesses 5 stable isotopes, so its pattern can be observed either alone or in combination with that of selenium. However, in contrast to low-molecular weight compounds and model systems, the size of the peptides of concern (1000–5000 Da) and the usual presence of multicharged (up to 5+) ions render the mass spectra complex. Furthermore, the mass accuracy of the Orbitrap mass analyzer (1 ppm and higher for less intense ions) is not enough for the unambiguous assignment of the empiric formulas and efficient machine search. Therefore, data mining had to be carried out based on hypotheses regarding the investigated metallodrug fragments, supposed to bind to proteins, already mentioned in the literature. The list of potential modifications of peptides resulting from the digestion of SelP cisplatin and auranofin adducts used for the mining of nanoHPLC-ESI MS data sets are summarized in Table 2.

Probing of the cisplatin binding sites by MS/MS

The list of Pt containing peptides detected, separated by nanoHPLC and identified by MS/MS after the tryptic digestion of the SelP-cisplatin adduct is given in Table 3. The binding was

Table 3 Pt-binding peptides in the tryptic digest of SelP. The binding site is highlighted in bold red

Peptide Sequence	Modifications	Charge	Ion m/z, [Da]	*peptide m/z MH⁺, Da*
^{1}MWRSLGLALALCLLPSGGTESQDQSSL**C**K^{29}	N-term(ac); **C28**(Pt)	4	825.6307	3299.501[a] 3299.486[b]
^{1}MWRSLGLALAL^{12}**C**LLPSGGTE^{20}	M1(ox); **C12**(Pt1); S16(ac); T19(ac)	3	800.03723	2398.097[a] 2398.0897[b]
^{4}SLGLALAL^{12}**C**LLPSGGTESQDQSSLCKQPPAWSIRDQ^{39}	**C12**(Pt2); S25(glu)	5	931.4293	4653.118[a] 4653.119[b]
^{30}QPPAWSIRDQDPMLNSNGSVTVVALLQAS**U**YL^{62}**C**ILQASKLE^{70}	C62(Pt4)	4	1184.547	4735.177[a] 4735.177[b]
^{38}DQDPMLNSNGSVTVVALLQAS**U**YL^{62}**C**ILQASKLEDLRVK^{75}	M43(ox); **C62**(Pt)	5	879.2005	4391.973[a] 4392.012[b]
^{42}MLNSNGSVTVVALLQAS^{59}**C**YLCILQASKLEDLRVK^{75}	N-term(acetyl); **C59**(Pt1)	3	1311.313	3931.925[a] 3931.945[b]
^{45}SNGSVTVVALLQAS^{59}**C**YL**U**ILQASK^{68}	S45(glu); S48(acetyl); **C59**(Pt1)	4	856.8711	3424.463[a] 3424.447[b]
^{47}GSVTVVALLQAS**U**YLCILQASKLEDLR^{73}	S48(acetyl); **C62**(Pt1); S67(glu)	3	1283.913	3849.724[a] 3849.711[b]
^{47}GSVTVVALLQAS**U**YL^{62}**C**ILQASKLEDLRVK^{73}	T50(acetyl); **C62**(Pt)	4	851.6653	3403.639[a] 3403.620[b]
^{50}TVVALLQAS^{59}**C**YL**U**ILQASK^{68}	T50(acetyl); **C59**(Pt1); Y11(acetyl)	3	789.351	2366.038[a] 2366.033[b]
^{53}ALLQASCYL^{62}**C**ILQASKLEDLRVK^{75}	**C62**(Pt); S67(glu)	4	857.653	3427.590[a] 3427.586[b]
^{104}KNKVSE^{110}**H**IPVYQQEENQTDVWTLLNGSK^{131}	N-term(acetyl); **H110**(Pt2)	4	889.1758	3553.682[a] 3553.3553[b]
		3	1185.229	3553.674[a] 3553.355[b]
^{131}DDFLIYDR^{140}**C**GRLVYHLGLPFSFLTFPY^{158}	N-term(acetyl); **C140**(Pt2)	3	1179.558	3536.660[a] 3536.646[b]
^{139}CGRLVY^{146}**H**LGLPFSFLTFPYVEEAIKIA^{166}	N-term(acetyl); Y145(acetyl); **H146**(Pt); T155(acetyl)	4	851.41107	3402.6225[a] 3402.6235[b]
^{143}LVY^{146}**H**LGLPFSFLTFPYVEEAIKIAYCEK^{170}	**H146**(Pt3)	3	1185.229	3553.674[a] 3553.700[b]
^{146}HLGLPFSFLTFPYVEEAIKIAY^{168}**C**EKK^{171}	S152(acetyl); T155(acetyl); Y158(acetyl); **C168**(Pt)	4	841.3933	3362.5514[a] 3362.5810[b]
^{223}LSENQQPGAPNAPTHPAPPGL^{244}**H**HHHK^{248}	N-Term(acetyl); **H244**(Pt2)	3	1013.458	3038.360[a] 3038.384[b]
^{226}NQQPGAPNAPTHPAPPGLH^{245}**H**HHKHKGQHR^{254}	**H245**(Pt1)	3	1131.864	3393.577[a] 3393.583[b]
^{347}LPPAA^{352}**C**QISQQLIPTEASASCRCKNQAK^{374}	**C352**(Pt4)	4	796.8759	3184.482[a] 3184.466[b]

ox- oxidation, acetyl - acetylation, glu: N-Hex-HexNAc-NeuAc
[a]- experimental, [b]- theoretical

predominantly identified on the Cys (C12, C28, C59, C62, C140, C168 and C352) and His (H110, H146, H244 and H245) residues. The Pt moieties were Pt^{2+}, Pt $(NH_3)^{2+}$, $Pt(NH_3)_2{}^{2+}$ $Pt(NH_3)_2(H_2O)^{2+}$ and Pt $(NH_3)_2Cl^+$, apparently non-dependent on the target amino acid. There was always a single Pt-containing moiety bound to a given peptide.

Pt binding was detected in 5 tryptic selenopeptides of *N*-terminal SelP region which contains only one SeCys residue (U59). The presence of both Se and Pt is confirmed by co-appearance of Pt and Se peaks in SEC-ICP MS chromatograms of the tryptic digest of Pt–SelP adduct (Fig. 4a) but, despite the presence of SeCys residues in these peptides, MS/MS fragmentation data did not reveal the involvement of selenium in the binding of metal. On the basis of MS/MS data, the exact binding location of Pt in three of these peptides (^{47}GSVTVVALLQASUYLCILQASKLEDLR^{73}, ^{47}GSVTVVALLQASUYLCILQASKLEDLRVK^{73} ^{38}DQDPMLNSNGSV TVVALLQASUYLCILQASKLEDLRVK^{75}) proved to be cysteine (C62). Moreover, in two other Pt-binding selenopeptides corresponding to this region of SelP, the anomalous inversion of the positions of SeCys (U59) and Cys (C62) was found so Pt attached to 59 amino acid residue was in fact bound to Cys, and not to SeCys. This is illustrated in Fig. 5 for the ^{47}GSVTVVALLQA-SUYLCILQASKLEDLRVK^{73} peptide. The MS spectrum indicates the presence of a mixed Se–Pt isotopic pattern, even if some to be expected minor ions are missing. The identity of the peptide was

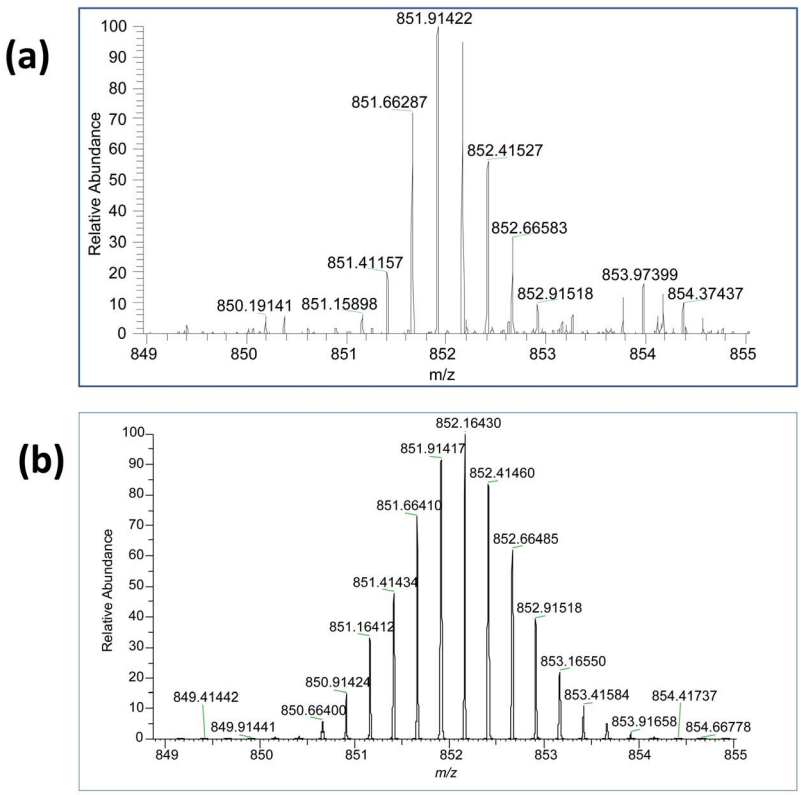

Fig. 5 LC-ESI MS/MS identification of cisplatin binding sites by SelP. (a) theoretical and found isotopic patterns of the putative Pt–Se containing peptide; (b) sequence of the peptide deduced from MS/MS fragmentation. The masses of the identified *b* and *y* ions (including PTMs) are given in ESI†.

Table 4 Au-binding peptides in the tryptic digest of SelP. The binding site is highlighted in bold red

Peptide sequence	*Modifications*	*Charge*	*Ion m/z , Da*	*Peptide m/z, Da*
^{1}MWRSLGLALAL^{12}**C**LLPSGGTESQDQSSLCKQPPAWSIR^{37}	C12[Au^{+}]; C28[ox]; S35[glu]	5	974.4522	4868.232[a] 4868.203[b]
^{41}PMLNSNGsVTVVALLQAS^{59}**U**YLCILQASKLEDLRVK^{75}	N-term[acetyl]; S48[glu]; U59[AuP(C_2H_5)$_3^+$]	5 4	968.2576 1210.068	4837.259[a] 4837.244[b]
^{307}HLIFEKtGSAIT**C**Q^{320}**C**KENLPSLCS**C**QGLR^{334}	N-term[acetyl]; T312[glu]; C318[ox]; C320[Au^{+}]	5	818.755	4089.746[a] 4089.736[b]
^{341}ESCQ**C**RLPPAA**C**QISQQLIPTEASAS**U**R**U**K^{370}	C352(SS); U367/369 [AuP(C_2H_5)$_3^+$]	5	789.9223	3945.582[a] 3945.562[b]

ox- oxidation, acetyl - acetylation, glu: N-Hex-HexNAc-NeuAc; SS: tetraacetatethioglucose
[a]- experimental, [b]- theoretical

confirmed by MS/MS that also proves that the Pt is bound to Cys and not to SeCys.

Our results, obtained for selenoprotein P and cisplatin are similar in terms of the demonstration of the formation of an adduct between a Cys-rich and SeCys containing protein and Pt-compounds. Indeed, MALDI MS was used to identify the ^{488}SGASILQAGCCG^{499}-terpyridine–Pt(II) adduct upon tryptic digestion of the TrxR adduct with terpyridine–platinum complex on the basis of its mass; the binding site was assumed to be on a cysteine but no MS/MS proof could be provided.[37] MS/MS has been used so far to evaluate cisplatin binding to commercially available proteins, such as human serum albumin,[38] cytochrome c[39 40], bovine insulin[41] and hen egg white lysozyme.[42] The formation of a mono adduct with hydrolized cisplatin form, $Pt(NH_3)_2(H_2O)^{2+}$ (reported earlier by Nygren *et al.*[43]), was shown for cytochrome c where $Pt(NH_3)_2Cl^+$ was not detected.[39] Four binding sites, including two Met and two His for both $Pt(NH_3)_2Cl^+$ and $Pt(NH_3)_2(H_2O)^{2+}$ were identified.[40] Cisplatin fragments $[Pt(NH_3)(H_2O)]^{2+}$ and $[Pt\ (NH_3)_2]^{2+}$ were detected and postulated to bind to cysteine;[42] four fragments Pt^{2+}, $Pt\ (NH_3)^+$ $[Pt\ (NH_3)_2]^{2+}$ and $[Pt\ (NH_3)_2Cl]^+$ were found to bind to a variety of amino acid residues (including Cys, Asp, Tyr and Met) in albumin.[38] Direct analysis of the tryptic peptides by nESI-LIT MS allowed the determination of Pt-binding sites in insulin; reactivity of free cysteines has been proved to prevail to N-donor groups, but when cysteines participated in disulfide bonds, their reactivity was found comparable to N-donor sites (N-terminus, His).[41]

Probing of the auranofin-binding sites by MS/MS

As mentioned above, the digestion yield of the auranofin adducts with SelP was much lower than that obtained for cisplatin. Also, as gold is monoisotopic, data mining, based on the isotopic pattern, was less straightforward than in the case of cisplatin. The charge on ions was higher (5+) which made the detection of the Se pattern, if present, difficult. Four candidate selenium-containing peptides, both containing $AuP(C_2H_5)_3^+$, could be observed (Table 4). Fig. 6 shows the comparison of its isotopic pattern with the theoretical one. The adduct was submitted to MS/MS fragmentation which gave the sequence ^{341}ESCQCRLPPAACQISQQLIPTEASASURUK^{370}, the identity of the bound gold moiety $AuP(C_2H_5)_3^+$ and determined that the binding occurred at a selenocysteine (U367) residue. The other peptide where SeCys-$AuP(C_2H_5)_3^+$ binding could be demonstrated was ^{41}PMLNSNGsVTVVALLQAS^{59}UYLCILQASKLEDL RVK^{75}.

SeCys is not the only observed binding site of auranofin. Table 4 shows two other peptides, containing Cys that also bind gold; the binding sites are C12 and C320 and the gold moiety Au^+. Our conclusions as to the binding sites are similar to those reported elsewhere for TrxR. Indeed, the bound Au-moiety corresponds to the loss of tetraacetatethioglucose by auranofin.[9,10] MS/MS analysis revealed that the SeCys and Cys residues are the preferential Au coordination sites; the presence of these coordinations sites was demonstrated.[10] The observation of Cys- and –SeCys as the only biding sites is in disagreement with the hypothesis formulated by Bindoli *et al.*[44] who postulated possible auranofin binding to methionine and histidine. In their work, up to four fragments of $AuPEt_3^+$were found to bind to TrxR (which possesses only one SeCys), indicating the presence of a large number of binding sites available for the Au(I). However, no molecular evidence of the biding sites location was provided.[44] The His as the target site was postulated on the basis of the detection by MALDI MS of peptide ^{236}IGEHMEEHGIK^{246}-gold adduct following the tryptic digestion of TrxR–Au adduct.[8] Also, Zoppi *et al.*[45] demonstrated that no auranofin was bound in the absence of accessible thiols, thereby excluding other amino acids as binding sites. Nevertheless, it has to be stressed that some observations are by definition not reproducible; glycosylation and SeCys–Cys

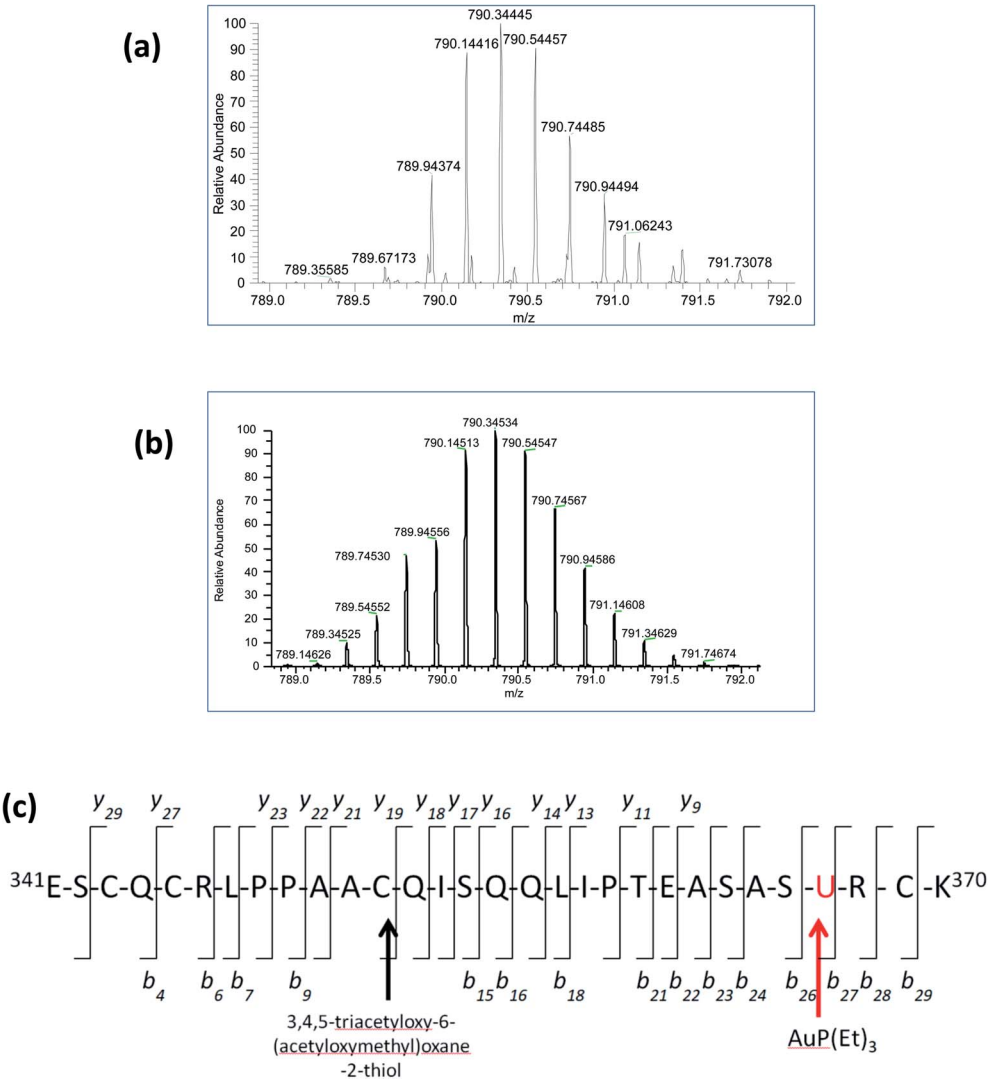

Fig. 6 LC-ESI MS/MS identification of auranofin binding sites by SelP. (a) theoretical and found isotopic patterns of the putative Pt–Se containing peptide; (b) sequence of the peptide deduced from MS/MS fragmentation. The masses of the identified *b* and *y* ions (including PTMs) are given in ESI†.

mutation probably depends on the serum donors and degradation of metallodrugs (the moieties lost and retained) as well as acetylation (its location) is procedure-dependent but not fully controllable at this point.

Conclusions

Selenoprotein P was demonstrated to react *in vitro* with auranofin and cisplatin. The study of metallodrug interactions with selenoprotein P by size-exclusion ICP MS combined with nanoHPLC- MS/MS of the tryptic digest of the adducts formed allowed a molecular insight into the metallodrugs binding sites indicating the potential of the technique for similar studies. This analytical approach excluded SeCys as the privileged target of cisplatin, the conclusion that could have be readily reached on the bases of co-elution of Se and Pt; cisplatin was fould to react preferentially with Cys and His residues resulting in the formation of Pt^{2+}, $Pt(NH_3)^{2+}$, $Pt(NH_3)_2{}^{2+}$, $Pt(NH_3)_2(H_20)^{2+}$, and $Pt(NH_3)_2Cl^+$ adducts. This study is also the first demonstration of the participation of SeCys in the binding of auranofin by a selenoprotein other than TrxR, with Cys as the alternative biding site.

Conflicts of interest

There are no conflicts to declare.

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