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Exploiting Soft and Hard X-Ray Absorption Spectroscopy to Characterize Metallodrug/Protein Interactions: the Binding of [*trans*-$RuCl_4$(Im)(dimethylsulfoxide)][ImH] (Im = imidazole) to Bovine Serum Albumin

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The reaction of bovine serum albumin (BSA) with [*trans*-$RuCl_4$(Im)(dimethylsulfoxide)][ImH] (Im = imidazole) (NAMI-A), an experimental ruthenium(III) anticancer drug, and the formation of the respective NAMI-A/BSA adduct were investigated by X-ray absorption spectroscopy (XAS) at the sulfur and chlorine K-edges and at the ruthenium K- and L_3-edges. Ruthenium K and L_3-edge spectra proved unambiguously that the ruthenium center remains in the oxidation state +3 after protein binding. Comparative analysis of the chlorine K-edge XAS spectra of NAMI-A and NAMI-A/BSA, revealed that the chlorine environment is greatly perturbed upon protein binding. Only modest changes were observed in the sulfur K-edge spectra that are dominated by several protein sulfur groups. Overall, valuable information on the nature of this metallodrug/protein adduct and on the mechanism of its formation was gained; XAS spectroscopy turns out to be a very suitable method for the characterization of this kind of systems.

Introduction

There is great interest today in the analysis of the interactions of metal-based drugs with serum proteins in view of their relevant biological and pharmacological implications. Extensive evidence has been gathered so far showing that serum proteins, in particular serum albumin and serum transferrin, are crucially involved in the transport of a variety of metal ions and metallodrugs through the blood stream ensuring their transfer from absorption to utilization sites.[1,2] These interactions greatly impact the bioavailability, biodistribution, and pharmacokinetics of metallodrugs and merit therefore a careful characterization. The state of the art on these topics has been recently described.[1]

Although substantial work has been directed, during the last two decades, to the biophysical characterization of metallodrug adducts with either serum albumin[2] or serum transferrin,[3−5] a full elucidation of the resulting species and of the inherent metal-protein interactions has not yet been possible because of the nearly complete lack of crystallographic information and the intrinsic difficulties in performing exhaustive NMR or mass spectrometry analyses of such complicated systems.

In view of the above arguments, we thought that an X-ray absorption spectroscopy (XAS) approach might provide additional and complementary information on those systems and thus help clarifying some unsolved issues of their chemistry. Biological applications of XAS (BioXAS) have

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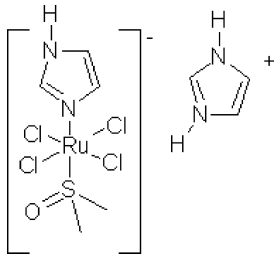

Figure 1. Schematic drawing of ruthenium(III) complex NAMI-A [transRuCl$_4$Me$_2$SO(Im)].

been widely used for the structural and electronic characterization of small protein portions and for the detailed description of the local environment of specific atomic probes.[6] In addition, in a few cases, XAS was successfully applied to follow the fate of metallodrugs within biological systems. For instance Hambley et al., relying on Pt X-ray Near Edge Absorption Spectroscopy (XANES) measurements, could monitor activation by reduction of anticancer Platinum(IV) compounds inside cells.[7,8]

Commonly, most BioXAS experiments are performed at energies higher than 5 keV, the typical threshold above which the K-edge absorption of elements having an atomic number greater than 22 occurs. Lower energy measurements present additional experimental difficulties and require specific setups.[9,10]

We report here a XANES investigation of NAMI-A [*trans*-RuCl$_4$(Im)(DMSO)][ImH] (where Im is imidazole and DMSO is dimethylsulfoxide) and of its 1:1 adduct with bovine serum albumin (NAMI-A/BSA).

NAMI-A is a very promising ruthenium(III) metallodrug.[11,12] While being weakly cytotoxic and not very effective against primary tumors, NAMI-A manifests outstanding antimetastatic activity and has recently entered phase II clinical trials. The chemical structure of NAMI-A is shown in Figure 1; the complex is octahedral with the RuCl$_4$ part being configured in a plane and the imidazole and DMSO ligands being axially arranged. Owing to the presence of different kinds of ligands, that may behave as potential leaving groups, and to its pronounced tendency to undergo reduction, NAMI-A shows a rather complicated solution chemistry, either alone or in its reactions with biomolecules.

Notably, a number of biophysical studies were specifically dedicated to the analysis of its solution chemistry,[13−17] and of its reactions with relevant biomolecular targets such as DNA and selected proteins.[13,18,19]

Moreover, a few recent investigations tried to reproduce the solution behavior of NAMI-A from a theoretical point of view mostly relying on density-functional theory (DFT) analysis.[20−22]

As shown above NAMI-A contains one ruthenium, one sulfur, and four chlorine atoms that may be independently monitored through XAS and may serve as useful probes. The XAS measurements that are reported in the present study were performed taking advantage of two recently constructed X-ray absorption beamlines: DXR-1 for soft X-rays at DAPHNE (Frascati, Italy) and SAMBA for hard X-ray at SOLEIL (Saint-Aubin, France).

With the present paper we aim at stressing the effectiveness of a XANES-based approach to characterize metallodrug−protein interactions. Assaying independently chlorine, ruthenium, and sulfur atoms of the metallodrug alone and of its BSA adduct has resulted in the unambiguous determination of the ruthenium oxidation state and of its interactions with chlorine ligands.

Experimental Section

Sample Preparation. NAMI-A was prepared according to reported procedures.[23,24] Model ruthenium(III) complex tris-acetylacetonate Ru(III), Ru(AcAc)$_3$, and bovine serum albumin were purchased from Sigma Chemical company. Samples were prepared in phosphate buffer (10 mM, pH 7.4) with a protein concentration of 1 mM and metal-to-protein ratios of 1:1. The reaction mixture was incubated for 24 h at 37 °C. The resulting sample was extensively ultrafiltered by using Centricon YM-3 (Amicon Bioseparations, Millipore Corporation) to remove the unbound metal complex. The upper fractions were separated and reconstituted with the buffer to a final volume of 1 mL. In all cases extensive metal binding to the protein was demonstrated (>80%) through ICP OES (inductively coupled plasma optical emission spectroscopy) analysis of the different fractions.

XAS Experiments at DAPHNE. XAS spectra of powder samples (namely, BSA, NAMI-A, NAMI-A/BSA adduct, Ru(AcAc)$_3$, NiSO$_4$, and native sulfur) were recorded at DRX-1 soft X-ray

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beamline at DAFNE, a 0.51 GeV storage ring (Laboratori Nazionali Frascati, Italy). DRX-1[25] is 6-pole wiggler beam line, equipped with a Toyama double-crystal monochromator based on the "boomerang" mechanism that ensures a fixed-exit beam. Crystals used were InSb(111) which are suitable for scanning from 1800 eV to about 3100 eV. XAS experiments were performed at sulfur and chlorine K-edges respectively at 2472 and 2822 eV, and at the Ru L_3-edge at 2838 eV with an electron current ranging between 1.5 and 0.7 A. In such conditions the beam intensity allowed to record good quality spectra in transmission mode with an energy resolution of about 1.5 eV and acquisition times between 2 and 3 s per energy point. In data acquisition, constant energy steps, smaller than the energy resolution (0.2 eV), were always used. To monitor the incoming and transmitted X-ray beams, two ion chambers filled with N_2 gas were used.

The X-ray energy scale was calibrated using the native sulfur (S_8) sample, having (0) oxidation state and the $NiSO_4$ spectrum that clearly shows an edge energy shift of about 10 eV, because of the valence state changing. The inflection point of the S_8 K-edge was set at 2472.0 eV.

XAS Experiments at SOLEIL. XAS spectra of NAMI-A, NAMI-A/BSA adduct, and $Ru(AcAc)_3$ where performed at the SAMBA beamline at the Synchrotron SOLEIL Facility (St Aubin, France). SOLEIL is a 2.75 GeV machine which was running during the experiment at 150−200 mA. SAMBA is a bending magnet beamline which covers the 4−40 keV energy range. Spectra were recorded at the Ru K-edge with 0.5 energy steps. To calibrate the energy scale, the sample and the Ru foil reference spectra (22117 eV) have been measured simultaneously. The signal from the protein sample was detected by fluorescence using a seven element solid state detector (CANBERRA-Eurisys). The NAMI-A and $Ru(AcAc)_3$ spectra were recorded in transmission mode.

Data Analysis. The edge energy position for each tested sample varies over several electronvolts. To superimpose such different spectra, a specific normalization procedure has been used. Sulfur K-edge spectra were baseline subtracted using a linear fit in the pre-edge region 2458−2469 eV.

The 2487−2507 eV energy region was fitted with a linear polynomial and then assigned the value 1 to the intersection point between the linear function and the rising part of the spectrum. Spectra were scaled accordingly. A similar procedure has been used for Cl and Ru XANES spectra.

Sequences of BSA (Swiss-Prot entry P02769, entry name: ALBU_BOVIN) and human serum albumin (1AO6 in PDB) have been compared using freeware LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html).

Results and Discussion

Measurements at Ru L_3- and K-edges. NAMI-A presents a relatively high redox potential for the ruthenium(III)/(II) couple in comparison to other anticancer ruthenium(III) compounds.[16,26−28] Accordingly, it is known to undergo easy reduction *in vivo* to the corresponding ruthenium(II) species by the action of biologically occurring reductants (such as ascorbic acid and glutathione). Thus, it is likely that upon reaction with BSA, the ruthenium(III) center of NAMI-A may undergo reduction. For this reason, we monitored the chemical state of ruthenium in the free complex and in its protein adduct by measuring the Ru L_3-edge XANES spectra. In Figure 2a the L_3 spectra of NAMI-A and NAMI-A/BSA are shown. The Ru L_3-edge XANES spectrum of the model ruthenium(III) complex $Ru(AcAc)_3$ was also recorded for comparison purposes.

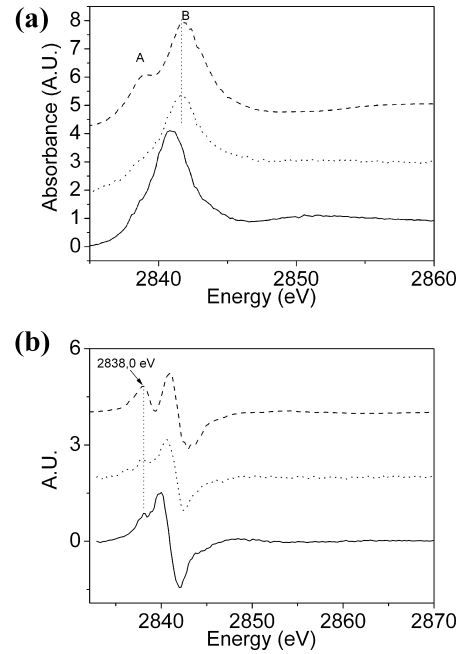

Figure 2. (a) Normalized spectra at the ruthenium L_3-edge of NAMI-A (solid line), NAMI/BSA adduct (dotted line), and $Ru(AcAc)_3$ (dashed line) with an arbitrary vertical shift. (b) First derivatives of spectra in Figure 2a. NAMI-A (solid line), NAMI/BSA adduct (dotted line), and $Ru(AcAc)_3$ (dashed line) with an arbitrary vertical shift.

The L-edges arise from atomic-like electric dipole transitions (change of angular momentum $\Delta l \pm 1$) from the $2p^{3/2}$ (L_3) core orbitals to unoccupied orbitals of both s and d symmetry.

Crystal-field theory can be used to interpret the above XAS spectra in line with previous observations.[29] Ruthenium(III) has a $4d^5$ configuration; in the presence of an octahedral crystal field the 4d orbitals transform into t_{2g} and e_g orbitals, separated in energy by the octahedral crystal field splitting energy (called 10Dq). A splitting of the L_3-edge into two bands was already observed in octahedral ruthenium(III) compounds similar to $Ru(AcAc)_3$, such as $[Ru^{III}(NH_3)_6]^{3+}$ [29] and the photogenerated $[Ru^{III}(bpy)_2bpy^-]^{2+}$ (bpy = 2,2′-bipyridine).[30] In agreement with the above arguments, the experimental L_3 spectrum of $Ru(AcAc)_3$ presents two bands denoted A and B, which should correspond to the $2p^{3/2} \rightarrow 4d(t_{2g})$ and $2p^{3/2} \rightarrow 4d(e_g)$ transitions, respectively. The two bands are separated by 3 ± 0.3 eV. The experimental Ru L_3-edge spectra of NAMI-A and of the NAMI-A/BSA

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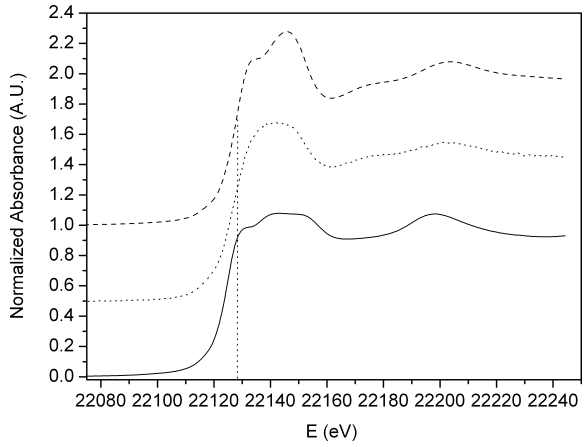

Figure 3. Normalized spectra at ruthenium K-edge of NAMI-A (solid line), NAMI/BSA adduct (dotted line) and $Ru(AcAc)_3$ (dashed line) with an arbitrary vertical shift.

adduct are dominated by a broad feature (Figure 2a). The first derivatives of the three spectra indicate that the inflection point of the A-band in $Ru(AcAc)_3$ (2838.0 ± 0.3 eV) is at the same energy as in NAMI-A and NAMI-A/BSA (Figure 2b). The B-band energy positions in $Ru(AcAc)_3$ (maximum at 2841.8 ± 0.3 eV) and in the NAMI-A/BSA adduct (2841.6 ± 0.3 eV) are consistent with the B band position of the oxidized form in photogenerated $[Ru^{III}(bpy)_2bpy^-]^{2+}$ (2841.4 ± 0.1 eV)[30] while they are shifted by +0.8 eV compared to NAMI-A.

The L_3-edge sensitivity to the electronic structure of the metal site gives information on its oxidation state. Upon decreasing the metal oxidation state, the edge moves toward lower energies.[30] Therefore, the Ru L_3-edge spectral changes, observed upon reaction of NAMI-A with BSA, indicate that the ruthenium center remains in the oxidation state +3.

To further substantiate this hypothesis, we have obtained additional XANES spectra of $Ru(AcAc)_3$, NAMI-A, and the NAMI-A/BSA adduct at the Ru K-edge (Figure 3.) The spectral behavior at the K-edge is similar to that observed at the L_3-edge. The K-edge positions of the NAMI-A/BSA adduct and of $Ru(AcAc)_3$ are almost the same (22127.8 ± 0.5 eV and 22128.5 ± 0.5 eV respectively). Again, the K-edge position for the NAMI-A/BSA adduct shows a blue shift compared to the NAMI-A K-edge but larger (+4 eV) than that observed at the L_3 edge. This shift is consistent with the replacement of Cl ligands upon NAMI-A interaction with BSA. In Ru(III) model systems, the K-edge energy position was found to vary significantly depending on the nature of ligands, for instance, the RuO_2 hydrate spectrum is shifted of +4 eV compared to that of the $RuCl_3$ hydrate.[31] An eventual Ru reduction would have produced a red shift as it was shown for $Ru^{III}Cl_2L$ and $Ru^{II}Cl_2L$ (L = 2,5,9,12-tetramethyl-2,5,9,12-tetraazatridecane).[32]

In conclusion, L- and K-edge spectra strongly suggest conservation of the +3 ruthenium oxidation state for the metallodrug−protein adduct. Notably, XAS data confirm previous suggestions based on spectroscopic data.[17] Indeed, it was reported that the intense charge transfer band of NAMI-A, at 400 nm, characteristic of the $[RuCl_4]^-$ chromophore, is still observed, although greatly shifted (346 nm), when NAMI-A reacts with albumin. In contrast, this absorption band virtually disappears when preparing the corresponding ruthenium(II) species by chemical reduction.

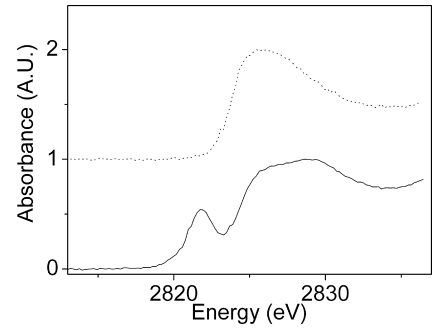

Figure 4. Normalized spectra at chlorine K-edge of NAMI-A (solid line) and NAMI/BSA adduct (dotted line) with an arbitrary vertical shift.

Measurements at Chlorine K edge. To further characterize the NAMI-A/BSA adduct the Cl XAS spectra of the same system were recorded. The Cl K-edge is expected to fall around 2824 eV because of the electric dipole-allowed 1s→np transitions which probe ligand−metal bonding interactions.[33,34] The Cl K-edge absorption spectra, for NAMI-A and the NAMI-A/BSA adduct, are deeply different (Figure 4); in particular, the pre-edge structure (at 2821.7 ± 0.3 eV) is not present in NAMI-A/BSA. An intense pre-edge transition in the Cl K-edge spectra was observed at 2820 eV for $CuCl_4^{2-}$ compounds with either a square planar (D_{4h}) or a distorted tetrahedral (D_{2d}) geometry.[33] This feature corresponds to a Cl 1s→Cu $3d_{x^2-y^2}$ transition that occurs when $3d_{x^2-y^2}$ orbitals contain a significant contribution of Cl p character, which must result from covalency.[33] A pre-edge transition, in the 2820−2823 eV range, was also observed in other complexes with chloride ligands bound to open-shell central atoms ($[MCl_4]^{n-}$).[35]

In all the ruthenium complexes, having the $4d^5$ electron configuration such as NAMI-A, the ligand field around the central ruthenium ion has nearly octahedral (O_h) symmetry and the $4d_{x^2-y^2}$ and $4d_{z^2}$ orbitals are unoccupied.[36] The pre-edge in NAMI-A could thus be due to a Cl 1s→Ru $4d_{x^2-y^2}$ transition. Further studies are required to determine the Cl 3p character of Ru $4d_{x^2-y^2}$ in NAMI-A and confirm this hypothesis. The absence of the pre-edge in the NAMI-A/BSA adduct might indicate a lower Cl p character of $4d_{x^2}-y^2$ orbitals because of the chloride dissociation. This is suggested also by the deep difference observed between the edge features of the two compounds (Figure 4): the NAMI-A maximum presents a double peak while the NAMI-A/BSA

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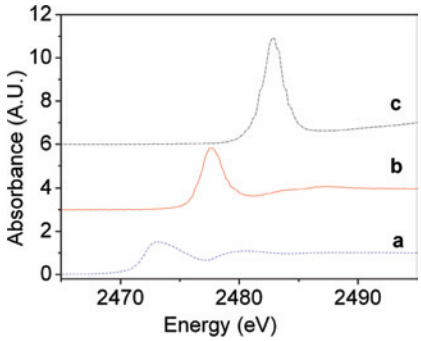

Figure 5. Normalized spectra at the sulfur K-edge of S_8 (a), NAMI-A (b), and $NiSO_4$ (c).

adduct shows just a single broad feature. The rising edge inflection points are 2824.4 ± 0.3 eV and 2823.9 ± 0.3 eV, respectively. Thus, all the described spectral features at the chlorine K-edge imply a profoundly different chemical environment for chlorine in the two systems.

Moreover, as suggested above, the observed shift between the Ru K and L_3 edge spectra of NAMI-A and of the NAMI-A/BSA adduct might be due to ligand substitution reactions taking place in the ruthenium(III) coordination sphere, in particular by replacement of chloride ligands. Notably, Messori et al. demonstrated, through analysis of spectrophotometric data, that release of two coordinated chlorides and their replacement by water molecules precede NAMI-A binding to BSA[17] in agreement with other experimental[13] and theoretical studies.[21]

Measurements at Sulfur K-edge. XANES spectra at the sulfur K-edge are dominated by dipole-allowed transitions ($\Delta l = \pm 1$) of the 1s electron to vacant p molecular orbitals. In principle, sulfur K-edge spectra can provide details on the electronic structure of sulfur itself allowing a rather precise insight on its chemical form. Sulfur K-edge spectra are particularly rich in information as sulfur is known to form stable compounds with nearly all elements, varying its oxidation state between −2 and +6. Moreover, sulfur K-edge spectra may be exploited to determine metal−ligand charge distribution.[37,35]

In Figure 5 (trace b) the sulfur K-edge spectrum of NAMI-A is shown. This spectrum is compared with those of two other characteristic inorganic sulfur species, namely αS_8 (trace a) and $NiSO_4$ (trace c). It is evident that the energy positions of the various edge maxima are shifted by several electronvolts in relation to the oxidation state of sulfur, in agreement with previous observations.[38] The maximum of absorption intensity for NAMI-A falls at 2477.5 eV, consistent with the presence of a sulfoxide sulfur.[38]

BSA includes a great number of sulfur containing amino acids, precisely 35 cysteines and 5 methionines; 34 out of 35 cysteines are engaged in the formation of 17 disulfide bonds whereas only one cysteine (Cys-34) is free. Accordingly, the sulfur K-edge absorption spectrum of serum albumin is expected to exhibit a main contribution from the 17 disulfide bridges and a minor contribution from the 5 methionines. Interestingly, the typical spectrum of a disulfide bridge presents a double peak around 2473.4 eV whereas aliphatic thiols show only one peak in the same energy

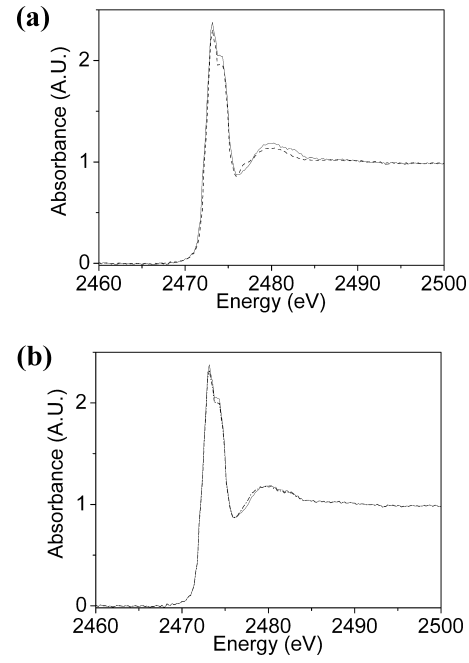

Figure 6. (a) Normalized spectra at the sulfur K-edge of BSA (solid line) and NAMI/BSA adduct (dashed line). (b) Linear combination (weighted with a 40:1 ratio) of experimental S K-edge spectra of BSA and NAMI-A (dash-dotted line) compared to the BSA spectrum (solid line).

region. The experimental XAS spectrum of human serum albumin (HSA) was reported earlier;[39] in line with expectations, the main absorption feature is split into two peaks, separated by about 1 eV.

BSA and HSA display 75.8% sequence homology and contain the same number of disulfide bridges. Thus, the S K-edge spectrum of BSA, shown in Figure 6a, is very similar to that of HSA, being dominated by the contribution of disulfide bridges. However, at variance with HSA, the two main peaks are not well resolved in the XAS spectrum of BSA because of the lower energy resolution of the setup (1.5 eV).

After recording the XAS spectra of NAMI-A and BSA, taken individually, we moved to analyze the XAS spectra of their 1:1 mixture after incubation and extensive ultrafiltration. Figure 6a compares the spectra of BSA and NAMI-A/BSA samples recorded at the sulfur K-edge. The sulfur single-atom signal from NAMI-A, in comparison to that of the 40 sulfur atoms belonging to the protein, contributes only marginally to the overall XAS spectrum of the drug/BSA adduct. Accordingly, addition of NAMI-A scarcely perturbs the environment of the sulfur atoms in the protein (Figure 6b). However, careful data analysis reveals that a weak new feature appears in the NAMI-A/BSA spectrum at 2476.7 eV (see Figure 6a), about 1 eV lower than the absorption maximum reported for NAMI-A.

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The above observations imply that S K-edge XANES, at least in principle, represents a useful tool to analyze metallodrug−protein interactions. In the case of BSA the value of this approach is greatly limited by the presence of several thiol groups; of course, more solid and selective information would be achieved in the case of proteins bearing only a few sulfur groups.

Sample Stability Assessment. We have investigated the possible occurrence of radiation damage for biological and inorganic samples exposed to X-rays.[9] In our experimental conditions, the photon fluxes were 3×10^8 photons s^{-1} (beam size 2 mm × 10 mm) and 10^{11} photons s^{-1} (beam size 1 mm × 4 mm) on DAPHNE (1A) and SOLEIL (200mA) beamlines, respectively. The effect of the X-ray damage on XAS spectra is the photoreduction of the metal with a consequent red shift. In our data, the ruthenium edge of NAMI-A/BSA adduct is blue-shifted compared to the NAMI-A edge and has about the same energy position of $Ru(AcAc)_3$. The samples' stability toward X-ray damage is further supported by the similar behavior of the spectra at both the K- and L_3-edges of ruthenium which was observed at two different facilities (SOLEIL and DAPHNE) using fresh sample preparations.

During the experiments, we have always minimized the X-ray exposure and performed a rapid scan of the XANES spectrum before the acquisition. Data analysis showed that no changes occurred during and at the end of the measurements.

Moreover, NAMI-A/BSA adducts were analyzed by visible absorption spectroscopy before and after XAS measurements: in all cases no significant spectral changes were detected (data not shown).

Conclusions

XAS methods may be conveniently exploited to elucidate unsolved issues of the reaction of metallodrugs with protein targets. Specifically, the interaction of NAMI-A with BSA was studied by XANES using Ru K- and L_3-edges, Cl K-edge, and S K-edge in such a way as to monitor the same binding process from *three chemically independent* points of view. Ruthenium K- and L_3-edges spectra documented unambiguously that the protein-bound ruthenium center remains in the oxidation state +3. Chlorine K-edge spectra evidenced changes in the chlorine chemical environment following NAMI-A binding to BSA in agreement with partial chloride release. The S K-edge spectrum of BSA is scarcely affected by addition of NAMI-A. Overall, new relevant chemical information has been gained on the present system; in addition, the effectiveness, the value, and also the limits of an experimental approach based on XAS for the characterization of metallodrug/protein interactions are well documented.

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