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A. Balerna, M. Belli, E. Bernieri, A. Bianconi, E. Burattini, M. Matzeu, F. Mazzei, S. Mobilio, C.R. Natoli, G. Onori, L. Palladino, A. Reale, E. Rongoni and A. Scafati:

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CHARACTERIZATION OF METAL SITES IN NUCLEIC ACIDS BY HIGH-RESOLUTION X-RAY SPECTROSCOPY *

M.Belli (a), A.Balerna (b), E.Bernieri (c), A.Bianconi (d), E.Burattini (e), M.Matzeu (a), F.Mazzei (a), S.Mobilio (c), C.R.Natoli (c), G.Onori (f), L.Palladino (c), A.Reale (b), E.Rongoni (a), A.Scafati (a).

(a) Istituto Superiore di Sanità and INFN - Sezione Sanità, Roma, Italy; (b) Università dell'Aquila and INFN - LNF, Frascati, Italy; (c) INFN - LNF, Frascati, Italy; (d) Università "La Sapienza" Roma, Italy; (e) CNR and INFN - LNF, Frascati, Italy; (f) Università di Perugia, Italy.

Summary - High-resolution X-ray spectroscopy with synchrotron radiation has been used at the Frascati Synchrotron Radiation Facility as a tool for studying the interaction of metal ions with biological structures. This paper reports the results obtained on some metal-nucleotide and metal-nucleic acid complexes in aqueous solution. The binding sites of Mn(II) in complexes with ATP and tRNA have been analysed by EXAFS and XANES spectroscopy and important information about the site geometry and the nature of neighboring atoms has been obtained. In Cu-DNA complex, the Cu K-edge XANES has been used as a probe for detecting the "in situ" reduction of Cu(II) to Cu(I) caused by chemical reductants and UV radiation.

Introduction

Metal ions play an important role in the living cell. Alteration in their amount or nature may have toxic, mutagenic and carcinogenic consequences. Understanding these effects requires the knowledge of metal ion-biological structure interaction(1).

High-resolution X-ray spectroscopy with synchrotron radiation has become an important tool in this research field. It is especially precious for investigating samples in aqueous solution where the usual X-ray diffraction techniques cannot be used.

The absorption spectrum of a metal compound in correspondence of inner-shell excitations (usually the K- or L-edges) is conventionally separated into the XANES (X-ray Absorption Near Edge Structure) and the EXAFS (Extended X-ray Absorption

Fine Structure) regions. The XANES region is determined by the multiple scattering processes of the excited photoelectron on the neighbor atoms so that information about the local geometry on the metal site and its effective charge can be obtained. The EXAFS region analysis can be used to obtain the distances from the absorbing (metal) atom to its neighbours, together with information about their nature.

Most of the experiments performed up to now on biological molecules have been addressed to metalloproteins, and only few have been devoted to metal-nucleic acid or metal-nucleotide complexes. This is rather surprising since metal ions are required for almost all the processes in which nucleic acids are involved, and the possible role of these ions together with the mechanism of their interaction are subjected to intensive investigation by many other techniques.

The few studies on nucleic acids and nucleotides performed by X-ray spectroscopy which appeared, to our knowledge, in the literature are reported in ref. 1-4, 9, 17.

This paper reports a brief account of the experiments which have been carried out at the Frascati Synchrotron Radiation facility, in the frame of a cooperation programme engaging the Istituto Superiore di Sanità, the Laboratori Nazionali di Frascati and the Universities of Rome, l'Aquila, Perugia. They include EXAFS and XANES analysis of Mn K-edge to investigate the metal site of Mn-ATP and Mn-tRNA complexes and XANES analysis of Cu K-edge to probe "in situ" reduction of Cu(II) in Cu-DNA complexes.

Mn-ATP complexes

The adenosine 5'-triphosphate (ATP) and the related di- and monophosphates (ADP and AMP) are fundamental to many cellular processes. ATP acts as an activator in several enzymatic reactions, and requires for its function the presence of divalent metal ions which can give specific conformations to the molecule. The solution structure of the metal-ATP complex is therefore of great importance for its intrinsic biological relevance as well as for its value as a model for metal-nucleic acid binding. Among the various metal ions, the paramagnetic Mn(II) has been the most investigated one, since it can be studied by magnetic resonance techniques.

The primary reactive sites for metals in ATP complexes are the negatively charged oxygen atoms of the phosphate groups, and the nitrogen donor atoms of the adenine. Although a model in which the metal binds both to the phosphates and to the adenine ring was proposed by Szent-Gyorgyi in 1957, some important features of the model are being discussed since that year(5). In fact, the flexibility of phosphate chain gives to the molecule the capability of adapting to a variety of shapes, depending on the environment. Major controversial points are whether the

metal is bound to the ring directly or via a water molecule and whether 1:1 or 1:2 metal-nucleotide complexes are formed. Binding of Mn to all three phosphates was found by NMR of P-31 nuclei. Since the interaction of Mn also with the adenine ring has been observed by PMR techniques, a model was proposed (6) in which Mn(II) is octahedrally coordinated to form a macrochelate with the ATP molecule, where the α, β, γ phosphates and three water molecules are in the first coordination sphere and interaction with N(7) of the adenine ring is mediated by the H-bond of a water molecule (Fig.1). On the other hand, further NMR and ESR studies have indicated that the metal can bind directly to the N(7) nitrogen (7,8). This apparent discrepancy could be explained by the occurrence of an equilibrium among different complexes, depending on pH and on metal and ATP concentrations.

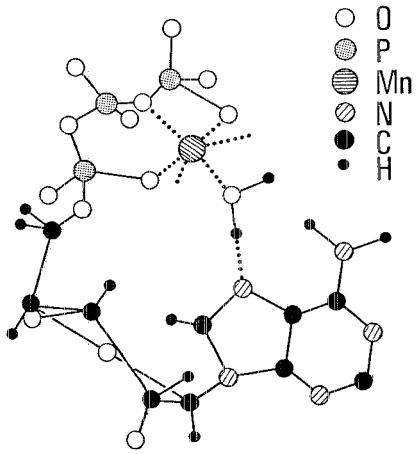

Fig.1 A possible structure for the Mn(II)-ATP complex, where the metal is octahedrally coordinated to six oxygen atoms beloging to the three phosphates and to three water molecules. Two of them have been omitted for simplicity (adapted from ref.6).

We performed an experiment where samples in solution at several pH and Mn to ATP concentration ratios have been examined by X-ray spectroscopy in order to look for different site structure favoured by different experimental conditions. Both EXAFS and XANES measurements were carried out on the different samples.

Mn-ATP complexes were prepared by adding $Mn(ClO_4)_2$ to concentrated (10% w/w) aqueous solutions of ATP. The pH of each solution was adjusted to the desired value before addition of Mn perchlorate. Three different samples were prepared at a Mn:ATP molar ratio of 1:1 (pH=3), 1:2 (pH=7), 1:10 (pH=9). The Mn-ATP solutions were put in special teflon cells with Kapton windows. The cells were 2-10 mm thick, depending on the Mn concentration in the sample, so as to obtain the better contrast at the K-edge. A 0.5 M solution of $Mn(ClO_4)_2$ contained in a 1-mm thick cell was also analyzed for comparison.

Inorganic Mn compounds (MnO, MnO_2 and MnP) were used as model compounds, mainly for determining the phase factor used in EXAFS analysis. This was performed by the method described in ref.(9).

Fig.2a reports Fourier transforms of the EXAFS spectra of Mn-ATP samples in solution. They show two distinct peaks (that at R < 1Å is an artifact due to the analitycal procedure), and the back-Fourier analysis is consistent with the assumption that the first peak is due to oxygen atoms and the second one to phosphorus atoms. The distances measured by this analysis, together with the relevant coordination numbers, are reported in Tab.1.

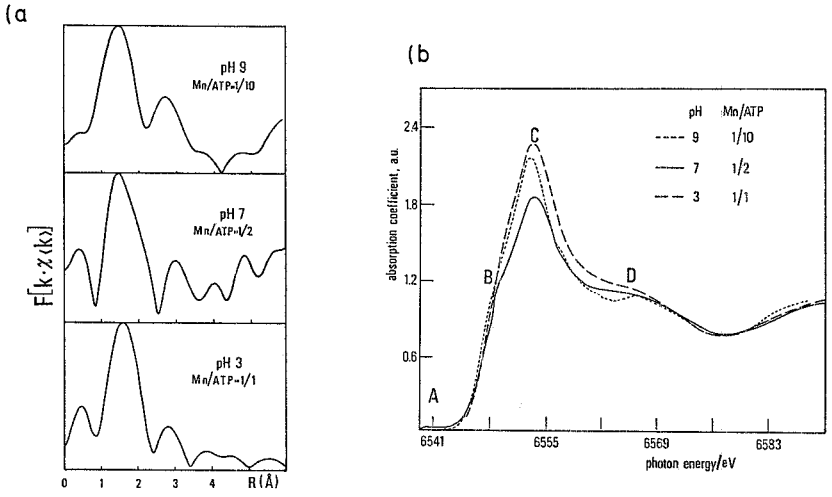

Fig.2. a): Fourier transforms of the EXAFS spectra of Mn-ATP samples in solution. Interatomic distances can be derived by taking into account the phase shift. b): XANES spectra of Mn-ATP samples in solution.

Table 1

	1 st shell		2 nd shell	
	Distance (Å)	Coord. n.	Distance (Å)	Coord. n.
Mn-ATP (pH 9)	2.15 ± 0.05	7 ± 1	3.30 ± 0.05	7 ± 1
Mn-ATP (pH 7)	2.16 ± 0.05	4 ± 1	3.40 ± 0.05	4 ± 1
Mn-ATP (pH 3)	2.15 ± 0.05	5 ± 1	3.40 ± 0.05	2 ± 1
$Mn(ClO_4)$,aq.sol.	2.15 ± 0.05	5 ± 1		

The results indicate that the oxygens of the first shell are at the same distance (2.15 Å) as that for the aqueous ion, and that this distance is independent of pH. The Mn-P distance of 3.3 - 3.4 Å is in agreement with that found in Mn-AMP complex in solution (10). From these data a Mn-O-P bonding angle of 126° can be calculated.

Evaluation of the coordination numbers, though affected by large errors, indicates that the number of phosphorus atoms increases with pH, which is consistent with the observation that at low pH only the β and γ phosphates are capable of entering the first coordination shell because of the protonation at the α group. The results also are consistent with the formation of a 1:2 complex at pH 9. Edge analysis (Fig.2b) supports this hypothesis, since the sample at pH 9 shows a more resolved peak D, which is mainly related to the second shell. Since the orientation of the phosphate groups appears unchanged, an increase in the number of these groups at pH 9 can be deduced. Indeed, the formation of 1:2 complexes is also favoured by the low Mn: ATP ratio used.

The XANES analysis can give other significant information about the metal site. The very low oscillator strenght associated with the small pre-edge feature A is an indication that the Mn ion is in a centro-symmetric position. A detailed analysis (9) of this feature suggests that only oxygen atoms should be present in the first coordination shell of Mn-ATP complexes.

In conclusion, our data show that:

1) The Mn metal ion is in a centro-symmetric position, octahedrally coordinated to the oxygen ligands; 2) oxygens from phosphate groups are present in the first shell, and phosphorus atoms in the second one; 3) the first and second shell distances appear the same in all the samples, being equal to (2.15 $\pm$ 0.05) Å and (3.40 $\pm$ 0.05) Å respectively; 4) the presence of a nitrogen atom of the adenine ring in the first coordination shell seems to be excluded at the conditions we used, so that possible interaction of Mn with the adenine ring should be mediated by water molecules; 5) a $Mn(ATP)_2$ complex is probably formed in solution at pH=9 at room temperature; 6) the geometry of the Mn binding site and therefore the structure of the complex is dependent on pH and on the Mn:ATP ratio in aqueous solutions, and this must be considered when comparing experiments performed under different conditions; equilibrium among different conformations should also be taken into account.

Mn-tRNA complex.

Transfer RNA (tRNA) is a small nucleic acid molecule which takes part in the protein synthesis. The metal ions specifically bound to tRNA contribute to determine the structure of the molecule and its ability to interact with the other

molecules involved in protein synthesis.

It is generally assumed that Mn binding to nucleic acids involves different groups (phosphates and bases) and that this may be related to the presence of different sites for the metal (12). Indeed, two distinct structure modifications in tRNA were evidentiated by UV spectroscopy upon increasing the Mn:nucleotide molar ratio (r) in tRNA solutions (13).

We used the X-ray spectroscopy to characterize the Mn binding sites in tRNA and to try revealing possible differences in site structure related to changes in the ratio r.

Concentrated solutions of tRNA from E.coli were made in 10 mM tris-HCl (pH=7) buffer. Concentrated $MnCl_2$ solution was then added to obtain the desired r value, which was not more than 0.5, to keep the unbound Mn at very low levels. The Mn concentration, at these conditions, was in the mM range.

The XANES spectrum obtained at r = 0.5 in shown in Fig.3a . Its shape is very similar to that found for the Mn-ATP complex at neutral pH. This indicates that the Mn sites (or their majority) have an octahedral simmetry, with the metal ion surrounded by six oxygens.

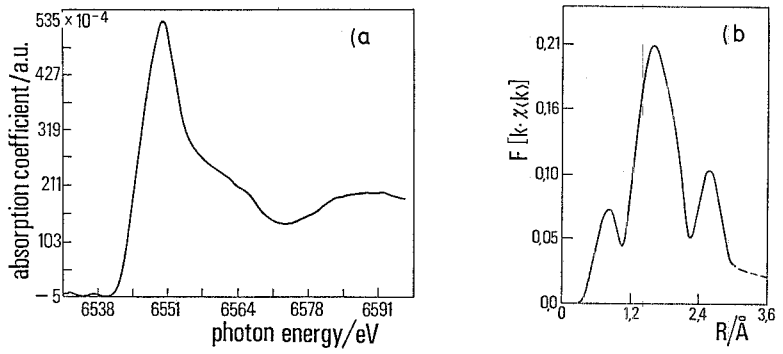

Fig.3. a): XANES spectrum of Mn-tRNA complex in solution at r = 0.5. b):Fourier transform of EXAFS spectrum of the same sample.

The radial distribution function deduced by the EXAFS analysis (Fig.3b) gave for the first shell a distance of (2.15 $\pm$ 0.05) Å and its back-Fourier transform is consistent with the presence of oxygen atoms, in agreement with the Mn-ATP results. The peak at R < 1 Å is an artifact and the structures at R > 3 Å are covered by the noise. Analysis of the second peak yielded no clearcut result, perhaps because of site heterogeneity in the second shell. Indeed, a single class of sites is expected only at very low r values. However, XANES analysis failed in revealing any changes in the nature of the binding sites on lowering the ratio r. This may be ascribed to

the poor sensitivity of the method, due to the very low signal to noise ratio at the Mn concentration used. It is expected that significant improvement in the experimental methodology, such as those offered by fluorescence techniques, will be able to settle this issue in the future.

- UV-induced reduction of Cu(II) in DNA complex.

Cu(II) ions have a strong ability to bind DNA where they are chelated by the purine bases. It has been shown that these ions can be reduced "in situ" by reducting agents. Since copper reduction and oxidation may affect the local stability of the double stranded DNA, it has been suggested that this process is involved in the control of the DNA function as a primer (14).

There is an indirect evidence from optical spectroscopy that irradiation of DNA-Cu(II) complex with UV light at 310 nm causes photoreduction of some of the Cu(II) to Cu(I) sites (15). Any biochemical changes produced by this wavelength may be important in connection with biological damage caused by solar radiation.

We attempted to use XANES analysis at the Cu K-edge to probe the "in situ" reduction of Cu(II) to Cu(I). We found that the percentage of reduced copper sites can be measured via the intensity of a localized atomic-like excitation characteristic of the Cu(I) ion.

The DNA-Cu(II) complex was prepared from calf thymus DNA at a Cu:nucleotide molar ratio of 0.25. UV irradiation was performed at 310 $\pm$ 10 nm with fluences up to 750 kJ/m^2. Chemical reduction by $NaBH_4$ has been performed for comparison. Xanes spectra have been recorded at the Frascati wiggler beam line using the transmission technique. The stability of the DNA-Cu(II) complex under the X-ray beam used for obtaining XANES spectra has been checked by repeated measurements and by an independent study performed at LURE with a dispersive apparatus which allows time-dependent investigations (16).

The XANES spectrum of the DNA-Cu(II) complex is reported in Fig. 4a where the pre-edge background has been subtracted and the absorption coefficient has been normalized to the high energy atomic absorption at about 100 eV above the edge. The main peak A can be identified as the multiple scattering resonance for excitations of the photoelectron of p-symmetry in the continuum, and P_2 is the characteristic pre-edge dipole-forbidden 1s$\longrightarrow$3d transition. A new feature P_1 appears at threshold after chemical reduction. We assign this transition to an atomic-like $1s^2\ 3d^{10}\ 4p^0 \longrightarrow 1s^1\ 3d^{10}\ 4p^1$ excitation characteristic of Cu(I) compounds. The intensity of the P_1 peak is therefore a direct probe of the number of reduced Cu sites, since the absorption of Cu(II) ions is small at the same energy. The P_1 feature also appears in the UV-irradiated complex although its smaller intensity indicates a partial reduction.To obtain the percentage of the reduced copper sites, this

spectrum has been fitted with a linear combination of the spectrum of the $NaBH_4$ (fully) reduced and that of the untreated DNA-Cu(II) complex (17). Fig. 4b shows the results of this procedure after subtraction of the atomic contribution to the transition, assumed as an arctan function. We found in this way that only 35% of the Cu sites have been reduced by UV radiation suggesting the presence of several classes of Cu sites in DNA.

When these findings are related to the results obtained by optical spectroscopy, the following picture comes out. The DNA-Cu(II) complex exhibits a small absorption band in the mid-UV, which is almost absent in DNA. This band, assigned to a charge transfer excitation, is responsible for absorption at 310 nm, which causes photoreduction of only one class of Cu(II) ions. The mechanism for such a reduction is probably a one-electron transfer from a ligand to the Cu(II) ion, leading to a Cu(I) ion and a radical. The resulting rearrangement of the Cu interactions may explain the change of the local stability of the DNA double helix.

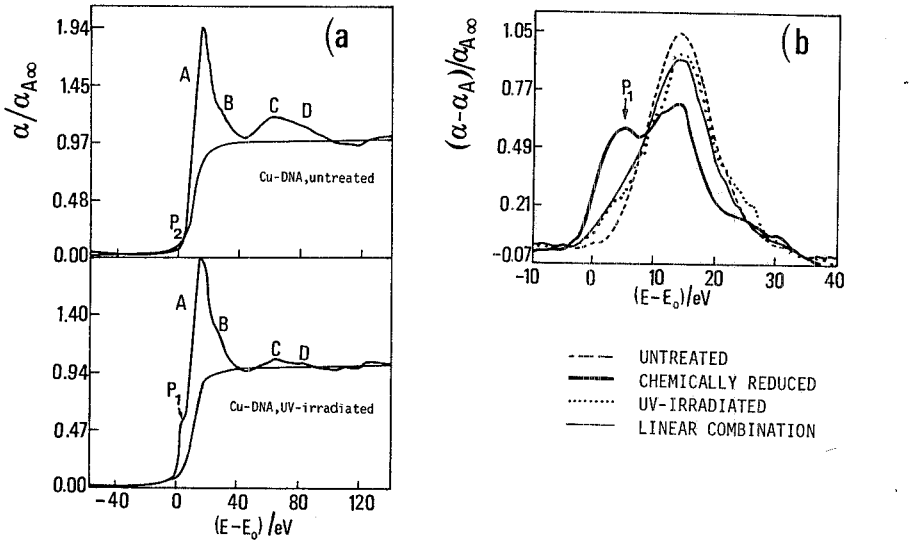

Fig.4. a): XANES spectrum of Cu-DNA complex at r = 0.25, before (upper panel) and after (lower panel) UV irradiation. Atomic contributions approximated by arctan curves are also shown. b): comparison of XANES spectra of Cu-DNA complex after subtraction with the arctan curves.

Conclusions

In spite of the small number of studies carried out up to now on the subject, the potentiality of high-resolution X-ray spectroscopy for investigating the nucleic acids structure in solution appears well established.

Some important developments in X-ray spectroscopy are expected to extend this capability, since very fast progress is being achieved both on the theoretical side (application of the multiple scattering description, use of more powerful methods in EXAFS data treatment), and on the experimental side (set-up of dedicated fluorescence instrumentation for dilute samples, development of dispersive methods for time-dependent studies).

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