BioXAS 2000

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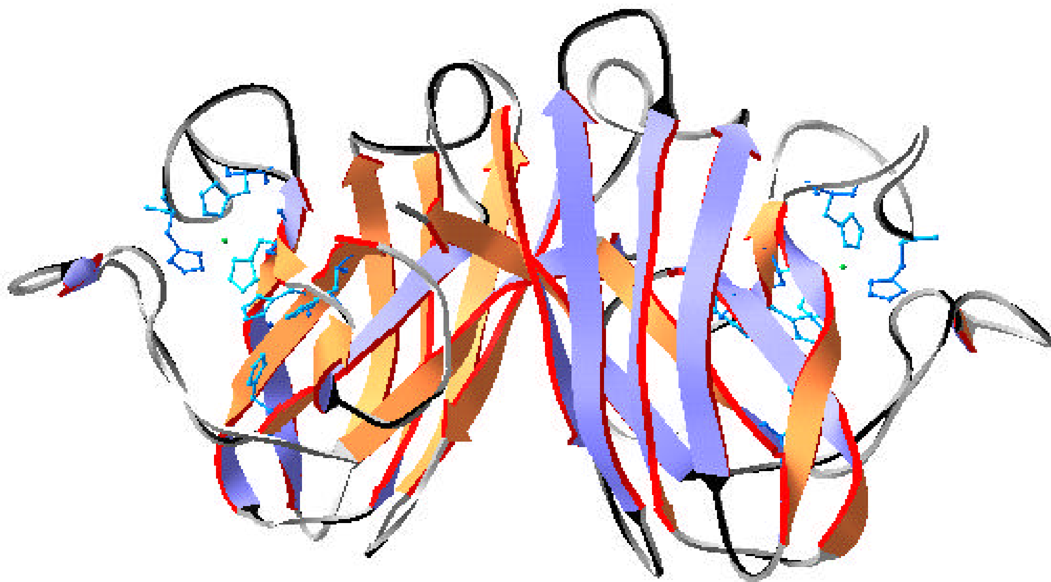
EUROPEAN WORKSHOP ON X-RAY ABSORPTION FOR BIOLOGY

LURE, Orsay - France - July 3-4 2000

Amphithéâtre Pierre Lehmann - bât. 200



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CONTENTS

- PROGRAMME
- ORAL COMMUNICATIONS
- POSTERS
- PARTICIPANTS

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MEN

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Roger FOURME	(France)
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José GOULON	(France – ESRF)
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Preface

X-ray Absorption Spectroscopy (XAS) has specific capabilities, which make it an important tool among available physical methods for structural biology and its interest has been identified from the early steps of the field. A number of articles have demonstrated that (i) it is possible to obtain a reliable local structure of the protein metal sites, (ii) XAS gives information on electronic structure and oxydation state, (iii) measurements can be performed on protein solution and powder as well as crystals, (iv) the protein metal site structure can be probed changing chemical or physical conditions.
Nevertheless, the development of the use of XAS for biomolecules - and in particular macromolecules - has been relatively slow. Due to the complexity of biomolecules and the high dilution of active species, BioXAS pushes the technique to its limits, from experimental aspects (data collection, signal extraction) to theoretical analysis. BioXAS experiments can be performed only with synchrotron radiation (SR) and require a concerted action of users and staff members of SR facilities; this is not the case of other spectroscopies where it is possible to develop the technique in smaller laboratories. Moreover, in most SR facilities, there are few XAS beam lines dedicated to biology and BioXAS proposals, that are time-consuming, compete for beam time with materials science and chemistry. Another, perhaps more subtle reason, is the shadowing effect of crystallography, which dominates applications of SR in structural biology. Yet, it seems likely that the most challenging forthcoming projects in structural biology will require a multi-technique approach combining protein crystallography, SAXS, BioXAS, time-resolved fluorescence and IR spectroscopy. Clearly, a better balance should be restored between investments in these various techniques.
These considerations, and the fact that there is no country in the EC with a critical mass of scientists concerned by BioXAS developments, gave us the motivation for the organization of this workshop, which was attended by 65 participants.
BioXAS 2000 follows the meeting organised by ESRF at Grenoble in February 1999. Together with the Programme Committee, we chose, in contrast to the Grenoble meeting, the option of a workshop with a global covering of the various aspects of BioXAS domain. In addition to two sessions presenting new or recent results, the workshop was structured in

topics on specific aspects of biological applications: recording conditions required to obtain information of biological relevance, methods and instrumentation for biological samples, specific problems of data analysis with biological samples, advances in data analysis programs. Finally, a practical session on computers was organised.

The programme allows to focus on some limiting factors to BioXAS developments, this being a necessary condition to make progresses in the field. Clearly all the issues cannot be solved during a workshop, nevertheless addressing specific points may contribute to their solution. We hope that this joint effort of both participants and organizers will continue in the future.

The implication of BioXAS community on issues like XANES simulations or sample preservation from X-ray damage will be useful also for non-biological applications.

Without attempting to make a summary of the meeting, let us underline some points which we feel are important. The potential of third generation SR sources for higher signal-to-noise ratio, extended k-range and time-dependent studies was demonstrated. The importance of sample control was a recurrent theme, including photodegradation (its importance and efficient ways to reduce it) and control of the electrochemical state. In methods, the development and bright future of microtechniques combining XANES and other spectroscopies at the micronic or sub-micronic scale were emphasized. The growing importance and capabilities of XANES was underlined by several speakers. We took note also of the derivation of three-dimensional information from multiple scattering and combined studies of anions and cations, the combination of XAS and protein crystallography refinements and progress in error treatment.

At the end of the workshop, the question of how to foster a coordinated action at the european level in order to develop BioXAS was indeed a major concern. There were several suggestions. The necessity of facilitating exchanges was immediately evident; in this context, dedicated web sites at various SR facilities should be created or improved, in order to give access to e.g. coordinates of users, lists of their proposals, publications and data analysis programs. It was decided to continue workshops; after Grenoble and Orsay, the following site next year might be in Italy (probably at Siena).

Finally, it is our pleasure to thank all those who contributed to the success of this meeting. We acknowledge the involvement of the European Programme Committee members in the preparation of the workshop. Chantal Jucha, as scientific secretary, and Dominique Michalowicz, as LURE webmaster, did a superb job. We are grateful to Alain Michalowicz who particularly contributed to the organization of the computer session, with the help of Daniel Dagneaux, Andrea Cognigni, Emmanuel Curis and Ioannis Nicolis.

Isabella Ascone, Roger Fourme and Simone Bénazeth.

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Programme

July 3, 2000

10:30 **ASCONE Isabella**
Introduction

Topic 1

New and/or recently published BioXAS results
Chairman: MARASSI Roberto

10:45 **PENNER-HAHN James - University of Michigan - U.S.A.**
Zn catalyzed alkyl-transfer reactions: a new class of biological Zn sites.

11:20 **GNIDA Manuel - Medical University of Lübeck - GERMANY**
Active site structure of CO dehydrogenase of *Oligotropha carboxidovorans*. Structural implications of different activities.

11:45 **MANGANI Stefano - Università di Siena - ITALY**
XAS characterization of the Atx1 protein from the prokaryote *Bacillus subtilis.*

12:10 **MURPHY Loretta - Daresbury Laboratory CLRC - U. K.**
The Role of XAFS In Understanding The Catalytic Cycle Of Cu, Zn SOD

12:35-14:00 **Lunch**

Topic 2

Primary data acquisition. Recording conditions required to obtain information of biological relevance (e.g. quality of spectra, stability of biological samples upon irradiation, electrochemical stabilization of redox proteins, control of sample chemical and/or physical conditions).
Chairman: FEITERS Martinus C.

14:00 **GOULON José - European Synchrotron Radiation Facility - FRANCE**
From Difference analyses to Dichroism: new experimental challenges to extract more information from X-ray Absorption spectra

14:35 **CHAMPLOY Frédéric - Université Paris XII - FRANCE**
Photoreduction of B12 samples caused by X-ray radiation.

15:00 **MARASSI Roberto - Università di Camerino - ITALY**
Hydroxocobalamin, a vitamin B12 model studied by XAS as function of pH under electrochemical control.

15:25 **SALVATO Benedetto - Università di Padova - ITALY**
Protection from X-ray damage by solid matrix embedding of proteins.

15:50-16:20 **Coffee break and posters**

Topic 3

Methods and instrumentation for biological samples.
Chairman: PENNER-HAHN James

16:20 **SIMIONOVICI Alexandre - European Synchrotron Radiation Facility - FRANCE**
State of the art and prospects for X-ray microtechniques: XAS and other spectroscopies.

16:55 **BERTRAND Loïc - Laboratoire de Recherche des Musées de France - FRANCE**
XAS study of metal fixation in archaelological keratinised biomaterials.

17:15 **SOLE Armando - European Synchrotron Radiation Facility - FRANCE**
ID26, an ultra-diluted sample beam line; equipment for biology.

17:35 **COGNIGNI Andrea - LURE - FRANCE**
Electrochemical cells dedicated to biological samples.

17:55-19:00 **Posters**

19:00 **Buffet dinner**

July 4, 2000

Topic 4

Specific problems of data analysis with biological samples (e.g. effect on XAS spectra of one ligand distance variation, complementarity of X-ray diffraction and XAS data, precision in measurements, statistical error treatment...)
Chairman: DENT Andy

09:00 **BENFATTO Maurizio - Laboratori Nazionali Frascati INFN - ITALY**
Recent developments in XANES analysis for biology.

09:35 **MICHALOWICZ Alain - Université Paris XII - FRANCE**
Is it possible to get accurate Co-axial ligands bond lengths in Vitamin B12 and related macrocyclic model compounds by fitting EXAFS spectra ?

10:00 **PURANS Juris - University of Latvia - LATVIA**
Very precise local structure from EXAFS (picoscopy): reinvestigation of local structure of hydrated Ln^{3+} ions.

10:25 **CURIS Emmanuel - LURE - FRANCE**
A minimal hypotheses model for determination, propagation and usage of statistical errors in XAS.

10:50-11:10 **coffee break**

Topic 5

New and/or recently published BioXAS results
Chairman: MEYER-KLAUCKE Wolfram

11:10	**FEITERS Martinus C. - University of Nijmegen - THE NETHERLANDS** Differences in the Cu environments of *Helix pomatia* (vineyard snail) and *Limulus polyphemus* (horseshoe crab) hemocyanins detected by EXAFS.
11:35	**SABATUCCI Annalaura - Università di Camerino - ITALY** Structural properties of the binuclear ative site of mollusc and arthropod oxy- and deoxy-hemocyanins. Evidences from EXAFS and XANES spectroscopy.
12:00	**HEINZ Uwe - University of the Saarland - GERMANY** EXAFS investigations of Zinc and Cadmium beta-Lactamases.
12:25-14:00	**Lunch**

Chairperson: MURPHY Loretta

14:00	**HASNAIN S. Samar - University of Manchester - U.K.** Why EXAFS is necessary for metalloproteins studies? Some Recent Results
14:35	**MORANTE Silvia – INFM - Università Tor Vergata - ITALY** Resolving the structure of Tetanus Neurotoxin by X-ray Absorption Spectroscopy
15:00	**NICOLIS Ioannis – Université Paris V - FRANCE** XAS characterization and XRF monitoring, applied on an arsenic based antitumor drug

Topic 6

EXAFS and XANES data analysis programs: latest versions. Speakers should indicate specific performance in the treatment of data from biological samples e.g.: amino acid identification, interfaces between protein data bank/XAS programs and protein data bank/simulations, potentials. What is existing and what should be developed.
Chairman: BUBACCO Luigi

15:25	**PROVOST Karine GPMD - Université Paris XII- FRANCE** CRYSTALFF, a bridge between crystallography, molecular modelling and EXAFS multiple scattering calculation with FEFF.
15:45	**DENT Andy- Daresbury Laboratory CLRC - U.K.** EXCURV98 for Biological Systems.
16:05	**DI CICCO Andrea - Università di Camerino - ITALY** GNXAS program: advances in multiple scattering EXAFS data analysis.
16:25	**FOURME Roger** Conclusions

Practical session on computers

BioXAS 2000 workshop includes a practical session on computer for demonstration of several programs for the XAS data analysis. The demonstration of each program is planned for 1.5 hours so that each interested participant can discover two programs. Each demonstration should include the full treatment of a test case, from experimental data to a structural model. At the end of the first session, the participant will have two possibilities: either to discuss at an advanced level with the program specialist, or to follow another demonstration on a different program.

Participants involved: a) XAS non-specialists: first contact with XAS analysis; b) XAS specialists who would like to discover another software or to get information on updated versions; c) XAS specialists who have specific questions about a particular software.

16:40 **MICHALOWICZ Alain**
Introduction to the practical session

16:55 – 17:10 **Coffee break**

Five programs are available:

GNXAS, (DI CICCO Andrea and COGNIGNI Andrea), **EXCURV** (DENT Andy, MURPHY Loretta**), LASE** (CURIS Emmanuel and NICOLIS Ioannis)**, CRYSTALFF** and coupling with **FEFF** (PROVOST Karine and MICHALOWICZ Alain)**, XMAN** (BENFATTO Maurizio).

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ORAL PRESENTATIONS

Zn catalysed alkyl-transfer reactions: A new class of biological Zn sites

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Zinc is the most common trace element and is the only transition metal known to be required for at least one enzyme in each of the major classes of enzymatic activities. A common paradigm divides Zn sites in enzymes into "structural" and "catalytic" site. The former are typically thiolate ligated and have no direct involvement in catalysis, while the latter generally have carboxylate+imidazole ligation, together with one or more bound solvent molecules. In recent years, it has become clear that there exists a third class of Zn sites in which the Zn is thiolate ligated but plays a key role in catalysis, promoting the alkylation of thiolate substrates. Characterization of Zn(II) in biological systems is challenging since Zn(II), as a d10 ion, is "silent" to most traditional spectroscopies. This presentation will describe recent advances in understanding the structure and function of Zn-catalyzed alkyl-transferases using EXAFS and XANES spectroscopy.

Active site's structure of CO Dehydrogenase of *Oligotropha carboxidovorans* – Structural implications of different activities

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CO dehydrogenase (CODH) of *Oligotropha carboxidovorans* is a molybdo iron-sulfur flavoprotein that catalyzes the oxidation of CO to CO_2. It is the key enzyme in the aerobic utilization of CO by carboxidotrophic bacteria. Interestingly, CODH occurs with different activities which seems not to be consistently related to the bacteria's growth. In order to understand this phenomenon, CODH batches with three different activities have been investigated using X-ray absorption spectroscopy (XAS): the highest activity was 23 units, one sample exhibited 16 units activity, and finally one sample with 4 units activity represented the form of CODH most often obtained.
Analysis of the fine structure of the molybdenum-K-edge (EXAFS) leads to the following structural model for the 23 units sample: molybdenum is coordinated by two oxygens, three sulfur atoms (two of them have been interpreted as the dithiolene-sulfurs of a molybdopterin-cytosine-dinucleotide (MCD)), and another backscattering contribution at 3.8 Å, which is most likely due to a sulfur at that position. Assuming that the 23 units sample represents the pure and 100% active enzyme, the CODH samples with 4 and 16 units activity should contain 17% and 70% active CODH, respectively.
Difference spectra analysis allowed to extract the inactive part from the spectrum of the 4 units sample. This yields a structural model for the inactive species which is characterized by three changes compared to the active form. Each of these changes might correspond to a

malfunction of the enzyme: (i) The number of oxo-groups is significantly reduced and partially replaced by a sulfur contribution. (A reduced oxygen content reduces the ability to transfer oxygen to the substrate.) (ii) The dithiolene-sulfurs of the MCD have been found to be asymmetric. (It can be doubted that the MCD which is involved in electron transfer binds correctly to the molybdenum.) (iii) Finally, the sulfur contribution present in the active form at 3.8 Å is missing. (This sulfur may play a role in the substrate's binding to the active site or the fixation of the product in the transition complex. Its removal may inactivate the enzyme.) Finally, we have reproduced the experimental EXAFS spectrum of the 16 units sample by adding up 30% of the amplitude of the active species' model and 70% of the amplitude of the model obtained for the inactive part. Thus, the assumption of purity for the 23 units sample and of the ratios of active to inactive species for the 4 and 16 units enzymes is justified and excludes the possibility that 16 and 23 units CODH are mutants of the enzyme with 4 units activity.

XAS characterization of the Atx1 protein from the prokaryote *Bacillus subtilis*

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[3] EMBL-Hamburg Outstation c/o DESY, Notkestrasse 85, Hamburg, Germany.

Copper is necessary for living organisms and the intracellular trafficking of this metal to copper-dependent proteins is fundamental for a normal cellular metabolism (1).
Only a small number of proteins involved in these processes has been individuated. It has been also found that mutations on some of them produce not efficient proteins leading to lethal diseases.
The majority of the copper chaperonins individuated up to now shows a conserved motif CXXC which can constitute the metal binding site (2). However up to now no X-ray or NMR structural study of a copper-bound protein has been reported and therefore the coordination mode of copper has been inferred only on the basis of data on complexes with other metal ions. An EXAFS study is available on the Cu(I) adduct of the Atx1 protein from yeast (which transfers Cu to the membrane-bound Ccc2 protein, an P-type ATPase) but the coordination sphere of Cu is not yet completely understood (3). We have performed EXAFS studies on the Atx1 protein from *Bacillus subtilis*, a prokariotic organism, whose protein has about 30% homology with the yeast (eukaryotic) protein.
The experiments have been carried both on frozen solutions and on lyophilized samples in presence of different reducing agents as well as in presence of glutathione. The results show that some of the reducing agents used for the experiment are interacting with the metal center being bound to the copper first coordination sphere. A model of the copper coordination in *B. subtilis* Atx1 protein will be presented as well as the model of its complex with glutathione and with the reductant. The more general question of careful experimental planning and analysis of XAS data on biological samples will be also addressed.

1) Harrison MD, Jones CE, Dameron CT : Copper chaperones: function, structure and copper-binding properties. J Biol Inorg Chem. 1999 Apr;4(2):145-53.

2) Srinivasan C, Posewitz MC, George GN, Winge DR: Characterization of the copper chaperone Cox17 of Saccharomyces cerevisiae. Biochemistry. 1998 May 19;37(20):7572-7.
3) Pufahl RA, Singer CP, Peariso KL, Lin SJ, Schmidt PJ, Fahrni CJ, Culotta VC, Penner-Hahn JE, O'Halloran TV: Metal ion chaperone function of the soluble Cu(I) receptor Atx1. Science. 1997 Oct 31;278(5339):853-6.

The Role of XAFS In Understanding The Catalytic Cycle Of Cu, Zn SOD

L. M. Murphy

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Recent articles in the press have highlighted superoxide dismutases (SOD) possible use as an anti-ageing drug. Indeed, this enzyme forms part of the cellular defence aginst oxygen radicals which are implicated in the ageing process. SOD is already in use as a treatment of hyperoxia related lung damage in premature babies and also for lung and heart muscles damaged following coronary heart attacks. Point mutations in human SOD have been linked with 20% of cases of familial amyotrophic lateral sclerosis (FALS) – a motor neuron disease.
SODs are relatively well characterised proteins whose function is believed to be the removal of the toxic superoxide radical. The majority of Cu,Zn SODs are homodimers. At the heart of bovine copper, zinc SOD (BSOD) lies a unique bimetallic active site where copper and zinc are bridged by a histidine residue (His-61). This feature is conserved in all structurally characterised Cu,Zn SODs.
XAFS and crystallography have played an important role in understanding the structural features associated with SOD's function. The presence of a three co-ordinate reduced copper ion is a key intermediate in the generally accepted enzymic mechanism(1,2). However a recent crystallographic study finds a five co-ordinate copper in the reduced form of the enzyme(3).
We have chosen to use both XAFS and high resolution PX in order to re-examine the question of whether three co-ordinate reduced copper is a viable intermediate during the Cu,ZnSOD catalytic cycle.
The results demonstrate the value of using a multi-technique approach to define the structure of metalloenzymes. XAFS clearly shows that reduced BSOD has a three co-ordinate copper site. Interestingly our high resolution crystal structure of $P2_12_12_1$ BSOD has one subunit with five co-ordinate copper and the other with three coordinate within the same molecule. Crystal packing forces are implicated as being responsible for the subunit asymmetry seen in the $P2_12_12_1$ form (4,5).
This multidisciplinary approach allows us to support three co-ordinate copper (I) as the key intermediate in the catalytic cycle.

(1) Tainer, J. *et al*. Nature, 1983, **306,** 284-287
(2) Blackburn, N. *et al.* Biochem J., 1984, **219**, 985-990.
(3) Rypniewski, W. *et al.* J. Mol. Biol., 1995, **251,** 282-296.
(4) Murphy, L. *et al*. Structure, 1997, **5**, 371-379.
(5) Hough, M. A and Hasnain, S.S, J. Mol. Biol.1999, **287,** 579-592.

From Difference Analyses to Dichroism: Can one extract more information from XAS ?

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The ESRF beamlines ID12A and ID26 are sophisticated beamlines which were technically optimized to fully exploit the remarkable optical properties of undulator sources in the X-ray range. Recording undistorted EXAFS spectra with such narrow-band sources is not a trivial task and required us to develop the so-called «Gap-scan » technique in close collaboration with the ESRF Machine Division. To illustrate the full reliability of the method, we will produce EXAFS spectra of metalloporphyrins recorded over ca. 2000 eV and also the Sulfur K-edge FT EXAFS spectrum of dl-cystine in which the signatures of the S…C and S…S distances at 1.82 and 2.04 Å are, for the first time, perfectly resolved. On the other hand, the high Signal-to-Noise ratio of the XANES spectra makes it now possible to deconvolve the spectra with appropriate numerical methods in order to get rid of the residual instrumental broadening and of the core-hole lifetime: such super-high resolution spectra are of considerable interest to study the electronic structure of the absorbing element.

Unfortunately, there are still a number of cases where it remains difficult to unravel the desired information from experimental EXAFS spectra. For instance, destructive interferences between unresolved scattering paths can spoil the information content of EXAFS and make standard numerical analysis methods ill-conditioned. At third generation synchrotron radiation sources where the level of sensitivity and reproducibility of the spectra can be very high, it is attractive to look for *small differences* in whole series of spectra. This led us to refine *Difference Analyses* of EXAFS spectra[1]: this approach made it possible to simplify the analyses and to disentangle the combined effects of an axial displacement of the metal out of the porphyrin cavity and those of a distortion (*e.g.* the *ruffling* or saddle-shape deformation) of the porphyrin core. Selected examples will be produced to illustrate the interesting potentiality of this method[1,2].

The polarization of the incident X-rays can indeed be exploited to extract more information: Differences mesured in excitation spectra recorded with orthogonal polarizations are termed *Linear* or *Circular Dichroism.* X-ray Magnetic Circular Dichroism was detected on applying a high magnetic field at low temperature on a paramagnetic complex (DPA)[Gd(OH)]$_2$ involving a cofacial bisporphyrinato ligand. High resolution X-ray Fluorescence emission spectra of the K_β lines of iron also revealed the existence of intense spin polarized satellites in high spin complexes[1]: again, *difference analyses* revealed subtle information regarding the electronic structure of μ-oxo- and μ-nitrido- bisporphyrinato iron complexes.

(6) Goulon, J. ; Goulon-Ginet, C. and Gotte, V. in *The Porphyrin Handbook* ; Kadish, K.M. ; Smith, K.M. and Guilard, R. Eds; Academic Press, San Diego, CA, 2000 ; Vol. 7, Chapt. 49, pp. 79-166

(7) Gotte, V. ; Goulon, J. ; Goulon-Ginet, C. ; Rogalev, A. ; Natoli, C.R. ; Périer, K. ; Barbe, J.M. ; Guilard, R. *J. Phys. Chem. B,* **2000**, *104*, 1927-1938

Photoreduction of B_{12} samples caused by X-ray radiations

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Crystal structures of several proteins with a B_{12} cofactor show abnormally long axial bonds between the cofactor's cobalt atom and its "lower" ligand, which is typically a protein-derived imidazole from a histidine residue. XAS (X-rays absorption spectroscopy) experiments were carried out with the following cofactor derivatives to examine the question whether the bond elongation might be due to an X-ray induced reduction of the cofactor's cobalt center: aquocobalamin, cyanocobalamin, methylcobalamin, 5'-desoxyadenosylcobalamin and cob(II)alamin. Each cofactor was investigated at 100K in a water/glycerol or water/trehalose glass, both as unbound free species and bound to the protein components of the enzyme glutamate mutase. XAS data were collected for each sample around the cobalt absorption edge before and after exhaustive (10 minutes) irradiation with X-rays of an energy of 7.76 keV. While XAS spectra for cob(II)alamin, methyl- and 5'-desoxyadenosylcobalamin presented the same evidences (within experimental error) before and after irradiation, both in the free and protein-bound state, the spectra of samples with aquo- and cyanocobalamin changed substantially upon irradiation. The spectra of the irradiated samples resembled each other and were similar - but not identical - to the spectrum of the reduced cob(II)alamin. The implications of these observations for the interpretation of the "long" axial Co-N bonds observed crystallographically in B_{12} proteins are discussed.

In situ X-ray absorption spectroelectrochemical study of hydroxocobalamin

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An in situ X-ray absorption spectroscopy (XAS) spectroelectrochemical study of aquocobalamin (system B_{12a}-B_{12r}-B_{12s}) has been carried out in acqueous solution buffered at different pH values. To the best of our knowledge, this is the first structural study of aquocobalamin at room temperature under controlled oxidation condition . Most of the

previous work was in fact performed using a frozen sample chemically treated to produce the species. The spectroelectrochemically approaches offers several advantages: (1) the reduction products may be studied without poisoning the system with chemical reductive reagents and (2) any possible variations of the oxidation state owing to the electrons produced by incident beam is avoided as the electrode , under potentiostatic control , acts as a scavenger . The spectroelectrochemical approach , together with more careful data analysis , has led to an improved interpretation of the XAS data . These conditions were no met in previous work were the oxidation states was not controlled and multiple scattering contributions were no taken into account . The general shape of XAS spectra of the different species is not greatly affected by pH. A sognature for the base-off square-planar coordination has been evidenced for the Co(II) compound at basic pH. A new signature for Co(I), indicating square planar coordination , has been identified on the experimental spectra and simulated in theoretical X-ray absorption near-edge structure (XANES) studies . The flexibility of the electrochemical approach that permits to unambiguously establish the formal oxidation state, has led to very reliable values for energy shift and peak intensity variation. The experimental XANES and extended X-ray absorption fine structure (EXAFS) spectra with a very good signal-to-noise ratiohave been processed using the GNXAS package that take into account multiple scattering contributions. EXAFS and XANES indipendent analysis result in the same structural model. The reduction from Co(III) to Co(II) produces the most significant structural changes : the cobalt coordination number decreases from six to five , and the edge position shift by 2.4±0.3eV . In addition, the XANES spectra are strongly modified . The reduction from Co(II) to Co(I) produces mainly electronic effects with no apparent change in the coordination number.

Reference

M. Giorgetti, I. Ascone, M. Berrettoni, P. Conti, S. Zamponi, R. Marassi ; JBIC (2000) 5: 156-166

Solid matrix embedded proteins: protection from X-ray damages

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Biological materials are very sensitive to X-ray irradiation owing to the fact that they are forced to have water as solvent
The application of synchrotron radiation techniques are also limited by the low concentration often available for biological materials. On these basis, we developed a new method based on the use of cryoprotectants like sucrose, ammonium quaternary salts and aminoacids for

obtaining solid samples of proteins embedded into the cryoprotectant matrix. The proteins we used are several hemocyanins (high molecular weight oxygen carrier copper proteins present in some invertebrates) and human hemoglobin. We applied different techniques to demonstrate that the procedure allows to preserve the protein conformation as well as the oxygenation state as that in the liquid state. These materials are also very highly concentrated, the protein representing about the 25% of the total weight.
XAS experiments on these proteins demonstrate the very high quality of these samples as far as the stability against X-ray damages is concerned as well as a high signal-to-noise ratio. Accordingly, we propose this procedure as a general tool for preparation of biological specimens for XAS experiments

Reference

I. Ascone, A. Sabatucci, L. Bubacco , P. Di Muro, B. Salvato; E.B.J. (2000) in press

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State of the art and prospects for X-ray microtechniques:
XAS and other spectroscopies

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Hard X-ray imaging plays an increasing role in micro-analysis, allowing non-destructive measurements on solid/liquid samples. While recent experiments on third generation synchrotrons center on high-energy phase-contrast imaging, it appeared essential to use established quantitative probes such as X-ray fluorescence and absorption spectroscopy in order to obtain 2D and 3D information regarding parameters such as elemental distribution, concentration, oxidation state, site geometry and even atomic structure.
While general XAS techniques have largely proved their usefulness and precision in chemical analysis, the extension of these techniques to the micron scale is fairly complicated. The focusing optics at third generation synchrotrons offers the high flux, high resolution parameters necessary for XAS. However, the stability requirements on these focusing elements is quite significant and the high sensitivity on the sample micro-heterogeneity is a factor of difficulty.
Frequently XAS, which gives a partial answer of the chemistry of samples, needs to be complemented by associated spectroscopies, such as fluorescence and tomography in order to probe the structure at the micron scale. We will describe measurements done on the ID22 beamline of the ESRF using samples from the fields of Mineralogy, Environmental and Life Sciences.
These combined techniques are currently under development and will become tomorrow’s reliable probes into the internal structure of materials and biological objects, in a fast and precise way. They are the only non-destructive analytical procedures capable of investigating

the structure of objects with micron-size resolution and with fully quantitative elemental sensitivity for elements of Z>20.

XAS study of metal fixation in archaeological keratinised biomaterial

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Hairs are nowadays commonly used in medicine and forensics to get information on intoxications, alimentation habits or oligo-elements deficiencies . Making the distinction between metabolically fixed and exogenous metallic ions would open the way to a more rigorous analysis of trace elements content in archaeological keratinised materials.

Animal and human hairs are seldom preserved in an archaeological context. Nevertheless, specific environments can lead to their preservation in two main cases : *as is* under "severe" conditions (deserts, bogs, extreme dryness as in mummification processes) or embedded in the corrosion layer of metal objects (bronze, iron, etc...). A great number of samples has been gathered at the Laboratoire de Recherche des Musées de France. They mostly come from fabrics used to pack weapons, vessels or ornaments in a funeral context, one to three thousand years ago.

In a first step, we aim at understanding how these remains could be preserved thanks to their contact with metal objects. It is therefore necessary to analyze the first kinetic stages of metal ions fixation (as, for instance, copper and iron) in the fiber texture. XAS studies enables us to distinguish the different fixation sites.

A first set of samples has been analyzed by XAS (at copper and/or zinc K thresholds) :
hairs from an Egyptian mummy of the late period
hairs and fabrics coming from Naintré (Gallo-Roman site, IVth century AD). This sepulture has the specificity to contain two bodies in an hermetically confined lead coffin, without any other metallic object. Nevertheless, a high content of copper, from unknown origin, has been measured in these samples.

cashmere coming from Lattes site (Vth century BC).
contemporaneous human and animal hairs
contemporaneous human hairs after immersion in Cu^{2+} enriched solutions

Our first results are :
three well defined peaks can be distinguished in the Fourier transform of archaeological spectra

the first neighbors of copper and zinc absorbers consist of oxygen and/or nitrogen atoms, but not sulfur atoms in archaeological as in model hairs
copper environment seems identical in Naintré human hairs and Naintré wool
copper site is slightly different in Naintré and copper enriched samples
These data have been analyzed with Lase Software, developed at Lure.
XAS sensitivity to structural modifications enables us to probe small local changes which have occurred in the long term. This method can therefore help us to connect the history of particular biological samples to the cultural history of societies (in situations as diverse as mummification, lead poisoning or chemical treatment of fabrics).

ID26, an ultra-diluted sample beam line; equipment for biology

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The ESRF beamline ID26 is an undulator beamline dedicated to absorption spectroscopy on very dilute systems. Copper concentrations of a couple of ppm's have been successfully measured at this beamline. Nevertheless, the biological applications of this beamline are not just limited to low absorber concentrations. Its choice of components (from beamline components to detectors) allow us to carry out a wide range of experiments in which other factors are determinant: small sample volume, stroboscopic measurements, slow kinetics experiments, temperature studies... The beamline and some examples of user experiments will be presented.

Electrochemical cell dedicated to biological samples

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Charge transfer reaction between redox proteins and electrodes surfaces allow to understand functionality of this protein in the biological system.
Electrochemical approach over XAS study of biological samples let to avoid photoreduction problem of protein under measure. Electrochemical control allow to fix and maintain a particular oxidation state , and electrodes behave as scavenger in the solution removing free radicals .
In order to study metalloproteins, which change the metal oxidation states during the catalytic cycle, we have developed an electrochemical cell for in situ XAS measurement on biological solution samples. EXAFS and XANES spectra in fluorescence mode may be measured using small volumes of sample (total volume is 0.4 ml), this allows studies of proteins which are not

available in large amount. We have investigated the reduction of Microperoxidase (from Fe(III) to Fe(II)). We have then determined the correct energy-shift , by 1 eV ± 0.3 eV upon oxidation, of XANES.
Moreover , to approach study of small quantities hydrated protein : a new "quasi-solid state" spectroelectrochemical cell for in-situ XAS experiments was developed and tested using microperoxidase as reference material. The cell substantially improves conventional thin layer cells used for solution XAS spectroelectrochemistry in terms of assembling time and, more important, equilibration of the redox system under study with the applied potential. Spectra can be, in fact, recorded simultaneously during a slow scan rate cyclic voltammetric-scan thus permitting correlation of the spectra and the electrochemical curve. Other advantages are the possibility to use very small quantities of material also with first generation rings. With high intensity sources having focussed beams a further decrease of the specimen weight can be easily obtained.

Reference

A. Cognigni, I. Ascone, S. Zamponi, R. Marassi ; J.S.R. (2000) Submitted

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Recent developments in XANES analysis for biology

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X-Ray Absorption Near Edge Structure (XANES) spectroscopy is a powerful method to study the local structure around an absorbing site of various type of matter. Analysis of the spectrum can give both electronic and structural information on the site around the absorbing atom. The fit of the XANES part of the spectrum, i.e. from the rising edge up to few hundreds of eV, in terms of the geometrical structure has long been an aim of the users of this technique, especially in the cases of limited k-range of the experimental data where a standard EXAFS analysis cannot be easily done. Moreover the XANES data is more sensitive of the EXAFS signal to the structural details of the absorbing site, so that, in principle a complete recovery of the geometrical structure around the absorbing atom can be achieved from the measured spectrum.
However, the quantitative analysis of the XANES spectra present some difficulties mainly linked to the theoretical approximations in the treatment of the potential and the need of heavy time-consuming algorithms to calculate the absorbing cross-section in the framework of full multiple scattering approach. For these reasons, the "XANES analysis" is so far considered a "qualitative" technique, used as an help for standard EXAFS studies or more advanced investigations like the ones based on the develop of the absorbing cross section in term of contributions related to the correlation functions of order higher than two. Few attempts have been done to study the theoretical sensitivity of XANES to structural parameters, at least in low-symmetry structures like biological systems, in particular protein active sites.

In this paper we present a software package, named MXAN, to calculate the absorbing cross section with the aim to obtain a reliable fit of the experimental data in term of well defined set of structural parameters.
The method is based on the comparison between experimental data and many theoretical calculations performed by varying selected structural parameters starting from a putative structure, i.e. from a well defined initial geometrical configuration around the absorber. The calculation of XANES spectra related to the hundreds of different geometrical configurations needed to obtain the best fit of the experimental data is done in a reasonable time and the optimization in the space of parameters is achieved by a Monte Carlo search and the overall minimization algorithm is based on the MINUIT package. The solution is found independent from the minimization strategy. The x-ray photoabsorption cross sections are calculated using multiple-scattering theory, with a complex Hedin-Lunqvist energy-dependent potential to describe the exchange correlation interaction. No limitations in the energy range and polarization conditions are present.
The zinc site of the protein superoxide dismutase (SOD), obtained by x-ray diffraction data, has been chosen as an example. The experimental Zn K-edge XANES has been fitted in the space of the first shell coordination parameters following the behaviour of the chi-square as function of the local distortions from its crystallographic structure. The undistorted structure is recovered whatever the minimization pathway is. Strengths and limitations of the method in its application to real systems are also discussed.

Is it possible to get accurate Co-axial ligands bond lengths in Vitamin B12 and related macrocyclic model compounds by fitting EXAFS spectra ?

A. Michalowicz

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Very precise local structure from EXAFS (picoscopy): reinvestigation of local structure of hydrated Ln^{3+} ions

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The microscopic mechanisms of the water exchange reaction between the hydration shells of Ln^{3+} aqua ions play an important role in the development of novel diagnostic agents in Magnetic Resonance Imaging (MRI) in medicine. Nevertheless, the previous EXAFS results on Ln^{3+} aqua ions in solutions are an example of the controversary.
New high accuracy EXAFS measurements have been done on Ln^{3+} (La, Ce, … Lu) aqua ions to check the ultimate limits of the cumulant analysis. For the first time the *local structure have been established* for the first shell of Ln^{3+} aqua ions with picometer accuracy (10-3 Å).

The measurements have been carried out at the Ln L_3-edges with an energy reproducibility of 0.1 eV. The remarkable improvement of experimental data quality and analysis procedures has allowed to measure the local Ln-O expansion. For the first time, the asymmetry of Ln-O RDF (third cumulant) has also been experimentally observed.
The second shell XAFS signal have been interpreted within the multiple-scattering (MS) approach taking into account the first and second coordination hydration shells around Ln^{3+} ions. The MS contributions were calculated for the three different models of nine/eight water molecules coordinated around Ln^{3+} ions: (i) tricapped trigonal prism; (ii) bicapped trigonal prism; (iii) square antiprism.

A minimal hypotheses model for determination, propagation and usage of statistical errors in XAS.

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The statistical analysis of EXAFS spectra is required for all structural applications, and especially for diluted samples and fluorescence recordings, as is quite often done for bioinorganic systems. Since such experiments lead to an important number of spectra, it may be convenient to do this analysis at the very first step.
The usual models are based on strong hypothesis, such as the constance of the error bars with the energy, and provide no simple way to propagate the errors across the treatment, so the statistical analysis must be made at the end of the analysis procedure.
We develop a new model that allows us to propose a method to do that, with a very reduced set of hypotheses easily satisfied. The errors are estimated point by point by the classical statistical estimators; one can propagate them across all the treatment, including Fourier filtering (which also gives error bars for each point of the FT, and of the filtered spectrum).
The resulting errors are used in the classical chi-square estimator; we confirmed that this estimator is effectively a maximum likelihood estimator. Since the EXAFS equation is not linear, the chi-square law followed by the residual is questionnable. Hence, we use a Monte-Carlo procedure to obtain the statistical informations about the fitted parameters (average value, standard deviation, distribution function, correlations). This procedure also allows us to use some other estimators for the parameters, like the classical unweighted least-square estimator, and despite that include the uncertainties to estimate the parameters confidence intervals.

Differences in the Cu environments of *Helix pomatia* (vineyard snail) and *Limulus polyphemus* (horseshoe crab) hemocyanins detected by EXAFS

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Hemocyanins, the oxygen transport proteins from arthropods and molluscs, are large assemblies of protein subunits. The β_c fraction of *Helix pomatia* (vineyard snail) hemocyanin can be treated by limited proteolysis to give well-defined domains each containing a single dinuclear copper unit, which is the oxygen binding site [1]. Comparison of the domains by UV-vis and Raman spectroscopy reveals subtle differences [2]; the d and g domains are blue (λ_{max} 570 nm) whereas the h domain is purple (λ_{max} 550 nm).
The Cu EXAFS of the oxygenated d and h domains shows a large (approx. 2-fold) reduction in the amplitude of the major peak in the Fourier transfrom upon deoxygenation (dithionite reduction), implying that each Cu ion in the dinuclear copper site must interact with both atoms of the bound dioxygen. Comparison of the oxy spectra of the blue domain d and the purple domain h reveals that the differences are negligible, whereas a slight shift to a higher average distance is observed for deoxygenated domain h as compared to deoxygenated domain d.
The amplitude of the major peak in the Fourier transform of the Cu EXAFS is slightly lower in the spectra for oxygenated *Helix pomatia* hemocyanin domains than in that of *Limulus polyphemus* (horseshoe crab) subunit II hemocyanin measured in the absence of the allosteric effector, Cl^-. Preliminary simulations of the EXAFS, featuring multiple scattering in both the imidazole and Cu_2:O_2 units [3], show that in *Limulus* each Cu ion is coordinated by 3 imidazoles, in line with the crystal structure [4], whereas one of the Cu ions in *Helix* probably has only 2 imidazole ligands. This may be related to the fact that one imidazole ligand per dinuclear copper site of *Helix* is covalently linked to a cysteine by a thioether bridge [5].
The authors acknowledge support from EEC-TRM.

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Sructural properties of the binuclear active site of mollusc and arthropod oxy- and deoxy-hemocyanins. Evidences from X-ray absorption spectroscopy

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An investigation on the active site structure of hemocyanins (Hcs), oxygen transport proteins present in some species of arthropods and molluscs has been performed.
The structural differences between the active site of oxy- and deoxy-hemocyanins from different species of both arthropods and molluscs have been investigated using X-ray absorption spectroscopy both in the XANES and in the first shell EXAFS region. The XANES spectra of oxy-hemocyanins of the different species are remarkably similar, consistent with a very strongly conserved coordination geometry of the copper active site. In contrast, small but significant differences are observed between the deoxy-forms of arthropodan and molluscan proteins. In particular, the XANES spectra arthropodan deoxy-hemocyanins show a more planar geometry of the copper-ligands.
The first shell analysis of the EXAFS signal is consistent with the presence of n = 3 $N^{\varepsilon 2}$ imidazole nitrogens of the ligand histidines at an average distance of 1.93±0.03 Å from each copper ion in all deoxy-hemocyanins investigated. This result does not support the X-ray structure of *P. interruptus* deoxy-Hc, which involves four imidazole nitrogens to form a coplanar system together with the two copper ions, while two more imidazole nitrogens in axial position are located at a longer distance. Rather, the EXAFS data are in better agreement with the structure of *Limulus* deoxy-Hc where all nitrogens are at the same distance (2.03 Å) within 0.2 Å. The copper-to-ligand distances found by EXAFS are, however, smaller than those determined by X-ray crystallography.
Binding of dioxygen results for all hemocyanins in the increase of the number of first shell back-scattering atoms to n = 5 with average distances of 1.93±0.03 Å. Alternatively, by separating the contribution of $N^{\varepsilon 2}$ imidazole nitrogens and of peroxide O-atoms, n = 3 N ligands at 1.98±0.03Å and n = 2 O ligands at 1.87±0.03Å are found. These results are in agreement with the crystallographic results on *O. doefleini* and *L. polyphemus* oxy-hemocyanins although, as found in the case of deoxy-hemocyanins, the distances found by EXAFS are smaller.

EXAFS Investigations of Zinc and Cadmium beta-Lactamases

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Zinc-beta-lactamases are bacterial resistance factors with a rapidly increasing endemic potential. Development of suitable inhibitors deserves a thorough understanding of structure-function-relationships of the metal binding sites. These are binuclear as judged from their primary structure in comparison to the available crystal structures for some representatives, whereas full activity often is observed in the mononuclear species. In order to achieve correlations between metal localisation, activity and possible catalytic mechanisms, we have

characterised by EXAFS spectroscopy the metallo-beta-lactamases from A. hydrophila AE 036 and from B. cereus 569/H/9 containing one or two zinc or cadmium ions. In addition, we measured enzyme species in the presence of substrates or inhibitors. The data are discussed in the light of possible mechanistic implications.

Why EXAFS is necessary for metalloproteins studies? Some Recent Results

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Metalloproteins are an important class of proteins and perform a variety of fundamental biological processes and make up 30% of all known proteins. They exploit the redox & ligand chemistry of biological metals to perform a wide variety of chemical reactions. In order to understand how these metalloproteins utilise the chemistry of metals to perform a particular function, it is imperative to know the three dimensional structure of these proteins, in general, and of the metal site, in particular, to a very high resolution. Perhaps nowhere in the determination of molecular structure is precision more at a premium than in the case of metalloproteins.

Small changes at the metal centre and its ligands can be amplified by the protein to perform complex biological processes. This is most beautifully illustrated in the case of Haem proteins where small changes at the Fe results in large changes elsewhere which control the allosteric mechanism.

Despite tremendous efforts, only a few metalloproteins' crystallographic structures are known at atomic resolution i.e. <1.2Å. XAFS, which probes the metal centre and is capable of providing sub-atomic resolution (<0.2Å) has additional role to play in acting as control for the oxidation state of the metal centres in a protein structure determination. Some recent results will be presented to highlight the complementarity of the two techniques.

Resolving the structure of Tetanus Neurotoxin by X-ray Absorption Spectroscopy

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The biological problem at the origin of this work is the investigation of the molecular basis of Tetanus pathogenesis. This knowledge represents a necessary step in the quest for new, more effective, therapeutic strategies. The first step in this direction is to arrive at the identification

of the amino acidic residues primarily bound to Zn in the toxin active site. Since up to now all attempts to crystallize TeNT have failed, there exist no structural data available that can be exploited to this end. A clearcut and complete identification of the amino acids coordinated to the metal active site, was recently achieved by a careful analysis of the large set of XAS data collected in the last four years both at the Brookhaven National Laboratories (USA) [1] and at the ESRF facility in Grenoble [2] on Astacin, Thermolysin, Alkaline Protease and TeNT samples. The results beautifully prove that Zn is tetra-coordinated to two Histidines, a water molecule and a Tyrosine as a fourth ligand [2] and it has been obtained by carefully taking into account in the theoretical analysis of the data contributions coming from multiple scattering events. The data analysis has been performed by using the theoretical approach developed in [3] that has been implemented in the freely available package, gnXAS [4]. We would like to conclude by stressing that a part from the biological relevance of this work, which allowed to resolve the coordination mode of the active site of TeNT, its success is methodologically of great relevance as it opens new ways of structural investigations in cases in which no X-ray crystallography is available or possible.

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An Arsenic containing leukemia treatment: XAS structural analysis and XRF monitoring in hair

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Acute promyelocytic leukemias are treated by chemotherapy, nevertheless 25 to 40% of patients have a relapse after the first treatment. For these cases a new drug is tested, injectable solubilised arsenic trioxyde. Two different techniques based on X-ray absorption were applied to the study of this treatment.
In order to characterise the arsenic form in the injectable solution, we recorded XAS spectra of the drug and of a series of reference arsenic compounds, both in solid and solution form. XANES spectra indicate the arsenic being in valence (III) and fitting of the exafs spectra yields a coordination to three O atoms, as in an arsenious acid solution.
In the second part of the study, we recorded X-ray fluorescence spectra of hairs of patients having received the arsenic treatment. Recording spectra on successive points of single hairs

allowed us to monitor the arsenic content in patientÕs hairs for a period up to 5 months (taking into account a growth rate of 1Êmm/3Êdays). The spectra were recorded for at least 3 hairs per patient in order to average fluctuations. The results show a steep rise in arsenic content with the beginning of therapy and a fall to normal levels in a month after the end of the treatment.

CRYSTALFF – from crystallography to EXAFS multiple scattering calculation (FEFF)

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CRYSTALFF is an alternative utility to ATOMS containing most of the features of this standard program for converting crystallographic data to FEFF input. In addition it offers an interface with molecular modeling programs via the PDB format and new coordination sphere analysis options, in order to facilitate the comparison of multiple scattering calculations on similar complexes. Some examples of studied facilitated by CRYSTALFF will be given.

EXCURV98 for Biological Systems

Dent A. J.

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EXCURV98 is the latest version of the Daresbury Laboratory EXAFS analysis program. It uses rapid curved wave theory or Rehr-Albers theory to generate, a theoretical EXAFS function which can be compared with experiment. To analyse a structure a cluster of atoms is constructed with single and multiple scattering interactions included as required. Of particular relevance to biological samples are the ligand database, the protein database and constrained and restrained refinement which will be discussed.

GNXAS program: advances in multiple scattering EXAFS data analysis

Di Cicco A.

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Reliable determination of the local structure in molecules and crystals can be obtained using advanced data-analysis methods (GnXAS) taking proper account of multiple-scattering terms and atomic background shapes in the x-ray absorption spectra. Methodology and results on simple molecular and crystalline systems will be presented. Recent applications including multiple-edge structural refinement of short-range n-body atomic configurations (n up to 4) in molecular complexes will be briefly discussed.

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POSTERS

Structural Changes in Bacterial Cytochrome-c Oxidase induced by Chemical Reductionand a structural Investigation of a Model Complex for the reduced CuA Site

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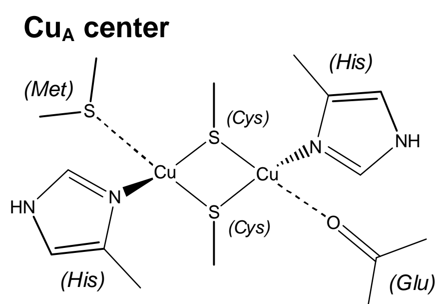
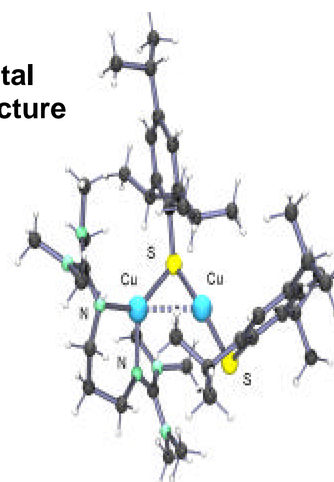
Cytochrom-c oxidase is an integral membrane protein that catalyses the terminal reaction in aerobic respiration, the reduction of oxygen to water. The redox energy for this reaction step is converted into a proton gradient across the plasmatic or mitochondrial membrane which is utilized for the biosynthesis of ATP. The enzyme contains several metal centers of which the homodinuclear Cu_A site acts as the primary electron acceptor. EXAFS investigations of the Cu_A site in the $Cu^{+1.5}/Cu^{+1.5}$ state provided the first definitive proof for cysteine ligands in bridging positions. [1] An X-ray structure of the ba_3-oxidase from *Thermus thermophilus* with improved resolution has recently been published and confirms all our results. [2]
EXAFS investigations of the fully reduced ba_3-oxidase have been used to characterize Cu_A in its reduced form (Cu^{+1}/Cu^{+1}). This provide the first insights into structural changes induced by the uptake of one electron.
The dinuclear complex $[Cu_2(STip)_2btmgp]$ (**1**) is a chemical model for Cu_A with respect to the number and the definition of its ligand donor functions. In the crystalline state, both Cu atoms are in different ligand environments (see figure). In contrast to this result, two thiolate bridges are observed when **1** is dissolved which is one of the characteristic properties of the central Cu_2S_2 core of Cu_A. [3]





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Heme-iron coordination complex in carp hemoglobinstudied by XANES and Resonance Raman spectroscopies

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As unliganded hemoglobin exhibits a low ligand (O_2, CO) affinity, it adopts a thermodynamically stable T conformation. Liganded hemoglobin with its high affinity adopts an R conformation. The cooperative ligand binding properties of hemoglobin are interpreted in terms of a reversible transition between the T and R states, and an evolution of the T-R conformers repartition with the fraction of ligand binding.

Studies on carp hemoglobin showed a very large influence of the pH on the affinity and on the cooperative transition of the hemoprotein [1]. At low and high pH, the protein does not exhibit a cooperative fixation of ligand. It has been suggested that the transition between the T and R states does not occur [2]: in the absence or in the presence of ligand, the protein would be blocked in either the T or R state, at low or high pH, respectively.

XANES is a powerful tool to detect subtle differences in the electronic repartition and structural properties of the iron coordination complex. In complement, Resonance Raman (RR) spectroscopy provides a particularly sensitive probe of heme properties because a number of vibrational bands have been correlated to the spin and position of the iron atom. These give the means to investigate whether changes in the protein conformation can induce changes in the strains on the heme.

To test the effect of the protein T-R switch subsequent to the pH change on the heme, it is interesting to study the heme-iron coordination complex properties of the unliganded and liganded species of carp hemoglobin by changing the pH.

Results of the XANES and RR studies on carp deoxy- and carbon monoxide (CO) hemoglobin species are reported here at several pH values (i.e. 5.8, 7 and 8). These data indicate that there is no correlation - subsequent to the pH change - between the affinity of carp hemoglobin for CO, the T and R protein states and the structure of the heme-iron coordination complex.

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Metal binding site of Hepatitis C virus NS3 protease studied by XAS

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We study by X-ray absorption spectroscopy (EXAFS and XANES) the NS3 protease in solution, at Zn K-edge.
This protein, cristallized at IRBM laboratory, is a proteolytic enzyme whose activity is necessary for hepatitis C virus replication. Two important aspects for the protease's activity are: i) Zn binding, required for the stability of the protein structural conformation (1) ; ii) the NS4A peptide presence, needed to increase the cleavage efficency of NS3.
XANES and EXAFS spectra at different pH, in presence and in absence of NS4A peptide, have been acquired at D21 LURE beam line. EXAFS analysis of NS3 protease in solution, in the same crystal buffer conditions, reveals differences in the distance of Zn with respect to its coordinated atoms (3 S of Cys and 1 O of a water molecule). Moreover, XANES spectra show interesting structural variations with and without NS4A.

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Purification and spectroscopic characterisation of purple acid phosphatase from Sweet potatoes (Ipomoea batatas)

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Purple acid phosphatases catalyze the hydrolyses of phophoric acids and -anhydrides. So far only one plant purple acid phosphatase from red kidney beans has been characterized by spectroscopic methods including a X-ray structure analysis. We report on the purification and spectroscopic characterisation of a new plant purple acid phosphatase from sweet potatoes. The metal content has been determined. EXAFS spectroscopy has been used to determine the first coordination sphere as well as the metal metal distance. A structure for the active site will be discussed.

Synchrotron induced X-Ray microfluorescence on single-cells

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Recent improvements in Synchrotron X-ray sources (third generation) and in X-ray focusing elements have been realized. This result in delivering highly collimated quasi-monochromatic x-ray beam with tunable energy and highly focused beam with a (sub) micrometer diameter. Elemental mapping of single-cells was obtained for the first time by synchrotron induced X-ray fluorescence in the hard X-ray range with high spatial resolution. Pink beam and compound refractive lenses were used resulting in an incident flux of around 10^{12} photon/s and a micrometer beam size. Taking into account the properties of synchrotron radiation, experiment confirms that in our conditions, high energy, high intensity x-rays are well suited for microanalyses of sensitive biological specimen (freeze-dry cells). Results show that the synchrotron microprobe set-up at ESRF allows high accuracy in trace element measurements for cell treated with pharmacological doses of anticancer drug. Micro-SXRF on single-cells is at its starting point and is expected to become a powerful non-destructive method, highly complementary to Particle Induced X-ray Emission (PIXE) and other types of micro-analytical methods. Moreover, SXRF will be shortly improved toward fully quantitative analysis and will be used in conjunction with X-ray absorption spectroscopy (micro-XANES) and phase-contrast x-ray micro-imaging. Finally, microanalysis of living cells is an exciting perspective that could be reached using micro-SXRF.

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Carboplatin and oxaliplatin decomposition in chloride and acidic solution, monitored by XAS

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Carboplatin and oxaliplatin are second generation antitumoral drugs belonging to the cis-platinum structural family. The transformation of carboplatin in presence of chloride ions into cis-platinum has been previously suggested from HPLC experiments, nevertheless product degradation in the HPLC column is not excluded. Confirm this transformation is of importance as in practical hospital use the chloride ions might be brought either by bag container or through multicomponent chemiotherapy.
XAS is particularly well suited for such a study, because it enables to follow the evolution of the Pt coordination sphere. We have first checked the EXAFS features to be strongly different between these complexes in solid state, related to the substitution in the first shell coordination of two oxygen atoms (carboplatin and oxaliplatin) by two chloride atoms (cis-platinum). In a second series of experiment, we have added to carboplatin solutions, chloride ions at various concentrations and pH, and recorded solution EXAFS spectra in different time lapses after chloride addition. Analysing these data, we have concluded to fast (a few-hours) cis-platinum formation especially at low pH and to slower (days order) reaction at neutral pH. So the main factor in the carboplatin degradation is pH.
We applied recently this approach to new antitumour complexes such as oxaliplatin. Solutions show a similar decomposition, with formation of cis-platinum like complexes. Furthermore, structural features of oxaliplatin EXAFS spectra allows a more accurate study of the acidic solutions evolution.

Localisation of zinc in doped hydroxyapatite by studying X ray absorption with a synchrotron radiation

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Hydroxyapatite (HAP) is generally used as coating biomaterial in orthopaedic surgery. We have studied its biological behaviour after implantation in ovine femur[1]. So as to enhance the resorption of this biomaterial, a doping by metal elements has been made.

We have shown that zinc has a prominent role in the kinetic resorption of HAP[2]. In this work, we are looking for clarifying the role of this metallic element expected to increase the solubility of HAP in biological environment, which is not the case for Mn in HAP. It is interesting to determine the position occupied by zinc in the crystal lattice in order to put out if its action is due to a disorder introduced by geometrical position in the lattice. This phenomenon added to zinc biological role can explain the ossification kinetic of the composite. We have performed EXAFS experiments by photoexciting zinc atoms. This spectroscopy gives structural information about the local order of the absorbing atoms and is well adapted to our research.

We use EXAFS experimental station of LURE (Orsay, France). Despite the low zinc concentration, we get good quality fluorescence mode spectra. These spectra show medium range order of the material that is consistent with its crystalline form. To perform the analysis, we compare the result obtained with another models that we can met (as β tricalcium phosphate) and we create theoretical models (zinc in substitution of calcium) in order to reproduce as well as possible the experimental spectrum. Furthermore, to get the uncertainties on the fit procedure, we used a Monte Carlo simulation method which gives the values of each parameter with the errors. After this study, only two models are coherent with experimental spectrum, zinc in substitution of calcium in site I and zinc in the interstice between the two hydroxydes. In order to put out the final localisation of zinc in the structure of apatite, we must perform other experiments with higher concentration in zinc. Perhaps, the variation of this can explain the unusual edge structure of the absorption spectrum and make the difference between the last two hypothesis.

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Determination of metal-metal distances. Significance and accuracy

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Nature utilizes a variety of metal clusters as catalytic centers. Some of them make use of two or more metals in the catalytic cluster. The variation of metal-metal distances plays the key-role in several processes like charge transfer and weakening of bonds. X-ray absorption spectroscopy can determine these metal-metal distances in several states (crystal, solution or amorphous). But sometimes backscattering from light elements hides the metal-metal contribution to the fine structure.
Here we point out significance and accuracy of metal-metal distances in a model system. Measurements on these trimers [LFeNiFeL] were taken at a variety of temperatures and at two different absorption edges. Therefore a number of different refinement protocols could be applied to the data. These protocols will be discussed focussing on the significance and accuracy of the metal-metal distances extracted from the data.
Three different approaches were compared: i) a single edge refinement for each metal site, ii) simultaneous Ni and Fe-edge refinements for each temperature, and iii) a fit using additionally the Debye model for the thermal vibrations in a simultaneous fit of all temperatures and the two edges. Model iii) results in an improvement of the accuracy that suggests being the most appropriate model in cases where the overlap of the light elements with the metal-metal distances is considerable.

EXAFS Picoscopy:
Local structure of bio-inorganic molecules Gd(DOTA) and Gd(DTPA)

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Key words: Gd(DOTA), Gd(DTPA), MRI, EXAFS, Picoscopy.

The microscopic mechanisms of the water exchange reaction between the hydration shells of Gd^{3+} aqua ions play an important role in the development of novel diagnostic agents in Magnetic Resonance Imaging (MRI) in medicine.
High accuracy EXAFS measurements have been done on Gd(DOTA), Gd(DTPA) and Gd^{3+} aqua ions to check the ultimate limits of the cumulant analysis. For the first time the *local structure have been established* for the first shell of Gd^{3+} aqua ions with picometer accuracy (10-2 Å). The measurements have been carried out at the Gd L_3-edges with an energy reproducibility of 0.1 eV. The remarkable improvement of experimental data quality and analysis procedures has allowed to measure the local Gd-O expansion. The second shell XAFS signal have been interpreted within the multiple-scattering (MS) approach taking into account the first and second coordination hydration shells around Gd^{3+} ions.
In these solutions the gadolinium ions in the complex $Gd(DOTA)^-$ are bonded to the four carboxylate oxygens : R(Gd-O_{av}) 2.38 Å, Debye -Waller (DW) factor 0.006 $Å^2$, to the four nitrogens : R(Gd - N_{av}) 2.65 Å, DW factor 0.006 $Å^2$ and to one water molecule : R(Gd -O_w) 2.46 Å, DW factor 0.012 $Å^2$. Concerning the complex $Gd(DTPA)^{2-}$ the gadolinium ions are bonded to the five carbonyl oxygens : R(Gd -O_{av}) 2.39 Å, DW factor 0.007 $Å^2$, to the three nitrogens : R(Gd -N_{av}) 2.64 Å, DW factor 0.006 $Å^2$ and to one water molecule : R(Gd -O_w) 2.47 Å, DW factor 0.018 $Å^2$.

EXAFS investigation of uranium (VI) complexes formed at *Acidithiobacillus ferrooxidans* types

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Uranium (VI) complexes formation at surfaces of *Acidithiobacillus ferrooxidans* types was studied using uranium L_{III}–edge Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy. In all samples the uranium is coordinated by two axial oxygen atoms (Oax) at distance of 1.77-1.78 Å. The distance between uranium and the equatorial oxygen atoms

(Oeq) is 2.35 Å. To within the experimental error, there are no differences in the U-O bond distances between samples from the 3 types of *A. ferrooxidans.* The coordination number for Oeq is 5-6. The fit to the EXAFS data of samples measured as wet pastes gave the same results as for dried samples. No significant structural differences were observed for the uranium complexes formed by the eco-types of *A. ferrooxidans.* However, the EXAFS spectra are indicating formation of uranium complexes which are different from those formed by Bacilli /1/.

/1/ C. Hennig et al., EXAFS investigation of uranium(VI) complexes formed at *Bacillus cereus* and *Bacillus sphaericus* surfaces, Radiochim. Acta (submitted)

Structural study of cobalt(III) complexes related to nitrile hydratase metallic site by EXAFS spectroscopy

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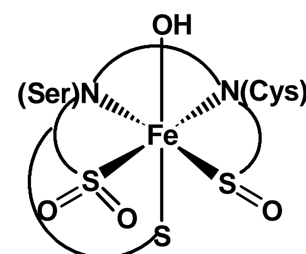
Nitrile hydratases (NHases) are non-heme iron or non-corrinoid cobalt containing enzymes which catalyze the hydration of nitriles to amides. Recent X-ray and ENDOR (Electron Nuclear Double Resonance) studies of NHases indicated that the iron is bound to two nitrogens from peptidic bonds, three sulfurs from one cystein-thiolate and two post-translationally modified cystein-sulfinic (RSO_2^-) and cystein-sulfenic (RSO^-) moieties, and one hydroxide group in the active form.[1,2,3] The EXAFS data suggest that the iron and cobalt sites are similar.[4]



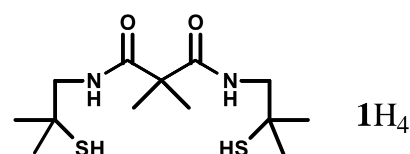
metallic site of active iron containing NHase.

To mimic the metallic site of NHase, one tetradentate N_2S_2 ligand $\mathbf{1}H_4$ containing aliphatic thiols and aliphatic amides was synthesized. The corresponding dithiolato, di-*N*-carboxamido square-planar cobalt(III) complex [Co**1**]⁻ **2** was characterized by X-ray diffraction.

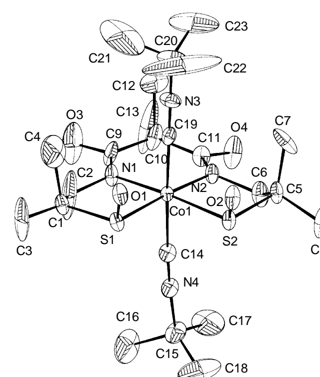
[Co**1**]⁻ gives with isocyanide or cyanide ligands hexacoordinated cobalt(III) complexes.

[Co**1**]⁻ could be oxidized by air or H_2O_2 in the presence of axial ligands to afford *S*-sulfinato or *S*-sulfenato cobalt(III) complexes, like the structurally characterized di-*S*-sulfenato, diisocyanido, di-*N*-carboxamido complex **3**. These results suggest that the cysteine post-translational modifications in NHases could be due to the axial thiolate and hydroxide ligands, respectively present as endogenous and exogenous moieties.

The EXAFS study of the non oxidized and the oxygenated complexes will be presented and the structural data will be discussed.



1H_4



[Co^{III} **1-*N*₂*SOSO*** (*t*BuNC)$_2$]⁻ **3**

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Use of Difference of EXAFS spectra in order to get information on the axial ligand in glyoxims and cobalamins

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It has been shown that it is impossible to get some reliable information on the axial ligand in glyoxims and cobalamins when working on EXAFS filtered spectra. The best approach is then to model the whole spectra, including multiple scattering (1), which may imply long calculations. When we just want to compare complexes differing only from one ligand, an alternate approach is to use difference of spectra.
We used this method on glyoxims and cobalamins. The different comparison show than when the structures of the conserved ligands are little changed, it is possible to get a very good idea of the structural parameters for the axial ligand. But this technique may be very sensitive to structural modifications of the conserved ligands. We applied this technique to the study of sulfur ligand binding to vitamin B12.

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XAFS Study of Gadolinium and Samarium Bisporphyrinate Complexes

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We present for the first time X-ray absorption spectroscopy study of bisporphyrinate Ln^{3+} complexes: $Gd(oep)_2$, GdH(oep)(tpp), $GdH(tpp)_2$ and SmH(oep)(tpp), $Sm(oep)_2$, $SmH(tpp)_2$. The XAFS spectra were recorded on the LURE-DCI storage ring (Orsay-France) in transmission mode on the crystalline samples at the Gd and Sm L_3 edges.
The protonated and nonprotonated bisporphyrinate complexes have different XAFS features. Applying one shell and two shell XAFS analysis procedures we reconstructed the Ln^{3+} local environment. The gadolinium ion (at 80 K) is bonded : (i) for $Gd(oep)_2$ to eight nitrogen atoms at R(Gd -N) 2.51 Å, Debye -Waller (DW) factor 0.004 $Å^2$; (ii) for GdH(oep)(tpp) to seven nitrogens R(Gd -N) 2.50 Å, DW factor 0.005 $Å^2$ plus one nitrogen at long distance; (iii) for $GdH(tpp)_2$ to six nitrogens R(Gd -N) 2.51 Å, DW factor 0.006 $Å^2$ plus two nitrogen at long distance. The Sm bisporphyrinate complexes show similar behaviour. The samarium ion (at RT) is bonded: (i) for $Sm(oep)_2$ to eight nitrogen atoms at R(Sm -N) 2.53 Å, DW factor 0.006 $Å^2$; (ii) for SmH(oep)(tpp) to seven nitrogens at R(Sm -N) 2.53 Å, DW factor 0.006 $Å^2$ plus one nitrogen at long distance; (iii) for $SmH(tpp)_2$ to six nitrogens R(Sm -N) 2.53 Å, DW factor 0.006 $Å^2$ plus two nitrogen at long distance. For $Ln(oep)_2$ complexes the increase Ln-N distance in the series $Gd^{3+} < Eu^{3+} < Sm^{3+}$ reflects an increase in the ionic radii, that are in good agreement with previously published XRD data (Bucler et al) on $Eu(oep)_2$. But the protonated LnH(oep)(tpp) and $LnH(tpp)_2$ complexes gives the systematic shorter distances about 0.02 Å between the data of XAFS and XRD technique. The difference is attributed to the asymmetry of the distribution of distances Ln -N.

In-Situ XAFS Study of Dehydration Process on Ce doped Diosmectite

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We present in-situ X-ray absorption spectroscopy study of the structural transformations of interlamellar Ce^{3+} ions complexes in the montmorillonite clay at different temperatures (RT-245 °C) presenting various dehydration state of clay. Using X-ray absorption fine structure (XAFS) analysis procedures and ab initio multiple scattering calculations of XAFS spectra at the Ce L_3-edge, we reconstructed the Ce^{3+} local environment and compared it with existing structural models. The valence of the Ce^{3+} ions in montmorillonite clay has been determined by X-ray absorption near-edge spectroscopy (XANES).

From RT measurements of Ce^{3+} doped montmorillonite clay, we found that the local environment and complex dynamics around the interlammellar cerium aqua ion complexes were conserved, and the complex structure agreed well with the known structural data of Ce^{3+} aqua ion complexes in the water solutions : cerium ions are bonded to the nine water oxygen atoms with the average distance R(Ce-O_{av}) 2.54 Å and Debye-Waller (DW) factor 0.013 $Å^2$.
In the first temperature range (RT - 60°C) of dehydration, thanks to the dependence of the XAFS and XANES signals in the doped montmorillonite clay, we observed a small transformation of interlamellar Ce^{3+} aqua ion complexes: the water coordination number decrease from nine to about eight, structural disorder increase about two time (DW factor increase up to 0.02 $Å^2$) and the electronic structure shows progressive decrease of covalency character of the bonds in the complex.
In the second temperature range (80 - 135 °C), thanks to strong dependence of the XAFS and XANES signals, we observed a structural transformation of interlamellar Ce^{3+} aqua ion complexes into intermediate type complexes of Ce^{3+} ions. Ce^{3+} ions directly interact with the surface oxygen anions of the silicate sheets. The water coordination number decrease from eight to about five, structural disorder increase about three time (DW factor increase up to 0.0 35 $Å^2$) and the electronic structure shows progressive decrease of covalency character of the bonds in the complex. Therefore, we suggest that the intermediate type complexes of Ce^{3+} ions have mixed type ligands : water oxygen and surface oxygen of the silicate sheet. The dehydration process up to 110 °C is reversible and the characteristic XAFS spectra of Ce^{3+} aqua ions appears after the rehydration of the clay.
Finally in the temperature range 160 - 245 °C the montmorillonite clay completely loose interlameller water molecules and Ce^{3+} ions form bonds with oxygen anions of the silicate sheets.

E.X.A.F.S. analysis for copper complexation by aminoacids : treatment of menkes disease

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Menkes disease is a lethal genetic disorder resulting into a copper transport malfunction. This dysfunction can be attributed by the alteration of membranous copper transporting P-type ATPase, inducing skeletal abnormalities and neurodegenerative disorders. Pathological signs can be explained by the deficiency of the functional copper enzymes. In fact, in this disease;the intestinal absorption of copper is altered, inducing a deficiency of this element in plasma and the tissues, in particularly in the brain. Furthermore, it should be noted that an abnormal copper sequestration into cells of the peripheral tissues resulting from the impaired activity of the same ATPase.
The increase of the copper availability has been achieved therapeutically by different approaches. Oral and parenteral administrations of copper salts being inefficient, the copper-

histidine complex, administered subcutaneously, has been suggested as this complex is present in human serum, with success for few patients. In addition, the results are promising only if the medication begins very early.
Our purpose is the elaboration of a new treatment of Menkes disease and one of possibilities is the administration of other copper-aminoacids complex. So, a preliminary physical-chemical study of these complexes must be investigated. XAFS spectroscopy at the K edge absorption exhibits characteristic changes upon coordination modifications for copper. We applied this method in the characterisation of the copper-aminoacids complexes. All spectra were recorded on the LURE-DCI storage ring (Orsay, France).
In the present study , the choice of the complexes has been copper-histidine (as model) and copper-threonine-histidine complexes (predominantly ternary complex present in human serum). We present the XAFS analysis of copper interactions with histidine and threonine in solution, clearly demonstrating complex formation between different aminoacids and copper. The resulting structural parameters are in good agreement with the solid state models. Theses results are confirmed by different physical-chemical techniques such UV spectrophotometry, Electron Spin Resonance.

Recent advances of our study on the azide binding in hemocyanins by XAS and the multiple scattering approach

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Our project "The oxidation of hemocyanin by X-ray absorption studies. Characterization of native and derivative forms", that has been approved by the ESRF scientific committee in Grenoble, is concerning the characterization of the active site of the met-Hc forms and of the azide-met-Hc adducts from *Octopus vulgaris* (mollusc) and from *Carcinus maenas* (arthropod) at different pHs.
The [Cu(II)Cu(II)] EPR silent met-Hc form is an interesting derivative in defining the structure-function correlation for Hc active site. The oxy-Hc form converts spontaneously, but very slowly, into met-derivative; however the conversion can be stimulated by various anion. The azide is a suitable exogenous ligand for probing the active-site environment since the UV and CD LMCT features of the azide-Cu(II) adduct can distinguish between terminal and bridging mode of the ligand.
Our aim is to get more detailed information on the coordination modes to the metal centres of Hc active site of these derivatives and the ultimate goal is to find definitive evidences (presence and nature of hypothetical exogenous ligands responsible) for the anti ferromagnetic coupling on the met-Hc forms.
As model compounds six dinuclear complexes of the $[Cu(II)_2(L)(X)_2]^{+n}$ $(ClO_4)_n$ families (with L=L-5,5, L-6,6 and X=OH^-, OH_2, N_3^-) has been considered. The two poly(benzimidazole) ligands have identical donor groups, L-5,5 is providing a 5-member ring, whereas L-6,6 form is providing a 6-terms chelate. The spectroscopic characteristics of these compounds have been proved to be essential for a better understanding of the structure of the

met-Hc active site [1]. The azide binding mode in the binuclear derivatives has been assigned to a μ-1,3 bridging coordination for L-6,6 and to a μ-1,1 for L-5,5. However, only the analogue metoxy derivative $[Cu(II)_2(L\text{-}5,5)(OH)_2](ClO_4)_2$ has a X-ray solved structure. Mononuclear model compounds of the family $[Cu(II)(L)(X)]^{+n}$ $(ClO_4)_n$ (with L= 2-BB and X=OH_2, N_3^-) have also been used, since the poly(benzimidazole) ligand 2-BB is a structural analogue of the tris(imidazole) array with different coordination numbers and stereochemistries. Diffraction data are available for the analogue $[Cu(2\text{-}BB)(MeOH)ClO_4]$ (ClO_4) and for the complex $[Cu(2\text{-}BB)(N_3)](ClO_4)$, which shows an end-on coordination mode for the azide ligand with two statistically equivalent N-N bonds [2].

The XAS fluorescence experiments, in the XANES and EXAFS approaches, have been carried out on the GILDA beamline of the ESRF facility in Grenoble.

The results on the met- and metazide-Hc forms from *Octopus vulgaris* at pH 7.5 and on the $[Cu(II)_2(L\text{-}5,5)(OH)_2](ClO_4)_2$ complex have been already presented (BioXAS, 1999 [3]).

Here we present in a first instance a comparative XAS study on the met and metazide derivatives of Hcs from both the mollusc *Octopus vulgaris* and the artropod *Carcinus maenas*. A further a comparison between dinuclear and mononuclear complexes of the poly(benzimidazole) ligands L-5,5 and L-6,6 and 2-BB, will be presented. XAS data analysis of the higher shells by the MS approach are in progress using the GNXAS package programs and the program CONTINUUM with the aim to obtain theoretical absorption coefficient in the XANES region.

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Differences in the Cu environments of Helix pomatia (vineyard snail) and Limulus polyphemus (horseshoe crab) hemocyanins detected by EXAFS

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Hemocyanins, the oxygen transport proteins from arthropods and molluscs, are large assemblies of protein subunits. The β_c fraction of *Helix pomatia* (vineyard snail) hemocyanin can be treated by limited proteolysis to give well-defined domains each containing a single dinuclear copper unit, which is the oxygen binding site [1]. Comparison

of the domains by UV-vis and Raman spectroscopy reveals subtle differences [2]; the d and g domains are blue (λ_{max} 570 nm) whereas the h domain is purple (λ_{max} 550 nm).
The Cu EXAFS of the oxygenated d and h domains shows a large (approx. 2-fold) reduction in the amplitude of the major peak in the Fourier transfrom upon deoxygenation (dithionite reduction), implying that each Cu ion in the dinuclear copper site must interact with both atoms of the bound dioxygen. Comparison of the oxy spectra of the blue domain d and the purple domain h reveals that the differences are negligible, whereas a slight shift to a higher average distance is observed for deoxygenated domain h as compared to deoxygenated domain d.
The amplitude of the major peak in the Fourier transform of the Cu EXAFS is slightly lower in the spectra for oxygenated *Helix pomatia* hemocyanin domains than in that of *Limulus polyphemus* (horseshoe crab) subunit II hemocyanin measured in the absence of the allosteric effector, Cl^-. Preliminary simulations of the EXAFS, featuring multiple scattering in both the imidazole and Cu_2:O_2 units [3], show that in *Limulus* each Cu ion is coordinated by 3 imidazoles, in line with the crystal structure [4], whereas one of the Cu ions in *Helix* probably has only 2 imidazole ligands. This may be related to the fact that one imidazole ligand per dinuclear copper site of *Helix* is covalently linked to a cysteine by a thioether bridge [5].
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