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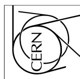

EUROPEAN ORGANISATION FOR NUCLEAR RESEARCH

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Proposal to the ISOLDE and Neutron Time-of-Flight Experiments Committee

Ag(I), Pb(II) and Hg(II) binding to biomolecules studied by Perturbed Angular Correlation of γ-rays (PAC) spectroscopy: Function and toxicity of metal ions in biological systems

L. Hemmingsen[1], Peter W. Thulstrup[1], V. L. Pecoraro[2], R. Sigel[3], E. Freisinger[3], Jens Mueller[4], K. Johnston[5]

Spokesman: L. Hemmingsen

Contactman: K. Johnston

Abstract

Metal ions display diverse functions in biological systems and are essential components in both protein structure and function, and in control of biochemical reaction paths and signaling. Similarly, metal ions may be used to control structure and function of synthetic biomolecules, and thus be a tool in the design of molecules with a desired function. In this project we address a variety of questions concerning both the function of metal ions in natural systems, in synthetic biomolecules, and the toxic effect of some metal ions. All projects involve other experimental techniques such as NMR, EXAFS, UV-Vis, fluorescence, and CD spectroscopies providing complementary data, as well as interpretation of the experimental data by quantum mechanical calculations of spectroscopic properties.

[1] University of Copenhagen, IGM, LIFE, Denmark.
[2] Department of Chemistry, University of Michigan, USA
[3] Institute of Inorganic Chemistry, University of Zurich, Switzerland
[4] Department of Inorganic and Analytical Chemistry, Westfälische Wilhelms-Universität Münster, Germany
[5] ISOLDE, CERN

I. Binding of Ag(I), Hg(II), and Pb(II) to metallothioneins, RNA, DNA, and model systems

The clustering and specific placing of metal ions closely in space is a reoccurring feature, in Nature as well as in Nanotechnology. Metallothioneins (MTs) are cystein-rich proteins that can bind and accumulate high amounts of both, essential metal ions such as Zn(II) and Cu(I) as well as toxic heavy metals like Hg(II), Cd(II), and Pb(II). Plant MTs have been discovered several decades ago, but knowledge about their structures and properties is still very scarce, although these metalloproteins perform crucial functions in all stages of development and life of a plant.[1] One of the proposed functions is an important role in Zn(II) and Cu(I) homeostasis. In this respect, Ag(I) can be used as an (almost) isostructural replacement for Cu(I) ions to probe their coordination environment. In addition, we intend to use Pb(II) both, as a mimic for Zn(II) and as one of those metal ions that might be detoxified by plant MTs. Today's knowledge on such clusters in plant MTs is basically absent, and hence unprecedented insights will be gained from these PAC experiments.

Nucleic acids find increasing applications in nanotechnology: DNA and RNA can serve as scaffolds to align metal ions in a specific way and hence to create electronic tools, e.g. molecular wires or magnets.[2] The knowledge on such structures is very scarce, but is crucial to develop and optimize such nanotools. Here we investigate the alignment of Ag(I) and Hg(II) ions within natural and modified RNA and DNA duplexes, e.g. stacked uracil-Hg-uracil base pairs,[2,3] or Ag(I) ions trapped between two modified imidazol nucleotides.[2,4] In both cases, i.e. with MTs and nucleic acids, PAC is the method of choice to elucidate the metal clusters and their neighborhood, because it allows for studies of binding ranging from extremely low amounts of metal ion up to stoichiometric amounts (adding non-radioactive metal ions). Thus PAC spectroscopy holds the capacity to follow the formation of metal ion clusters and their effect on the structural organization of the biomolecules, from the initial stages with very few metal ions present to the complete 3D architecture of the cluster.

II. Binding of Ag(I), Hg(II), and Pb(II) to *de novo* designed proteins

For over a decade, our group has been interested in the biological chemistry of heavy metals such as mercury, lead, cadmium and arsenic [5-10]. Our research approach has been to design and synthesize small proteins that can mimic the structure of the metal binding sites found in regulatory proteins, metal chaperones and the targets of metal toxicity. Typically these proteins contain sulfur rich environments, so our first emphasis was on making solely cysteine thiolate containing peptides. We have been extraordinarily successful in our objectives having prepared the first aqueous models for merR, CadC and aminolevulinic acid dehydratase binding Hg(II), Cd(II) and Pb(II), respectively. The strength of this work has been directly dependent on the ability to carry out PAC studies of these peptides. PAC was the only technique to demonstrate that our original design for Cd(II) proteins was complicated by two species. Subsequently, PAC studies proved that

we had prepared the desired three and four coordinate cadmium structures. Based on these studies, we have now refined the use of the complementary technique Cd(II) NMR on biological samples. A similar achievement was made with Hg(II) where the PAC spectra have been correlated with Hg NMR in order to assess the extent of distortion in 2 and 3 coordinate homoleptic Hg(II) thiolates which are found in biology. Our future studies will focus on our Pb(II) peptides where Pb NMR has so far been unreliable. In addition, we are examining Hg(II) binding to Hah1, the human copper chaperone, in order to assess how this toxic metal may be transported within human cells. Finally, the studies with Ag(I) are particularly interesting as we have yet to characterize a monovalent cation in our peptide scaffold. Thus, the combination of different elements should allow us to make significant new advances in studying the basic chemistry of heavy metals in biology and further develop the field of de novo protein design.

III. Metal ion mediated protein folding and misfolding

The function of living cells depends on the correct function of thousands of proteins, whereas the function of the individual proteins usually depends on their correct folding into a well defined 3D structure. The protein folding process is surprisingly fast and still not very well understood. We have formulated the so-called "transient binding site hypothesis" which implies that metal ions, especially zinc ions, might be involved in the steering of cellular protein folding. Thus, we aim to challenge the paradigm that only the amino acid sequence is required for correct folding.
Since different metal ions have different preferences for binding sites in proteins heavy metals like cadmium, mercury and lead can lead to misfolding whenever a metal ion is able to stabilize a protein fold different from the native one by creating its preferred element-specific binding site. We have successfully combined Perturbed Angular Correlation of γ-Rays spectroscopy with freeze-quench techniques which allows us to follow time-dependent processes in metal ion binding to proteins. We plan to verify our "transient binding site hypothesis" with this method in combination with rapid kinetic measurements and supplementary spectroscopic techniques applied to model peptides and native metallo-proteins. The study of model peptides will deliver the basis for the understanding of time-resolved and metal-dependent folding processes of native proteins. We primarily aim at a better understanding of metal-dependent protein folding, but this may also lead to essential and novel basic understanding of metal ion homeostasis, and heavy metal toxicity.

IV. In vivo studies

We aim at characterizing heavy metal binding proteins and toxicity *in vivo*, an as yet largely unexploited possibility where PAC spectroscopy offers unique advantages [11]. The advantages of PAC spectroscopy for *in vivo* studies are that it is non-invasive, no external fields or similar perturbation of the system is necessary and highly specific information about the immediate surroundings of the PAC isotope is obtained. The tricky part, of course, is to get the PAC isotope into selected parts of an organism, for example proteins or cellular compartments.

In particular the fact that it is possible to probe high affinity sites using only the very small amount of radioactive PAC isotope and probe potentially weaker binding sites by adding non-radioactive heavy metal provides an exclusive opportunity: This may reveal if there is a unique scavenger of heavy metal ions, for example the metallothioneins (vide supra) or several such biomolecules, and if new binding sites appear as the capacity of these defense systems is exceeded can they be identified and correlated with the toxic effects of the heavy metal. In a pilot study carried out at ISOLDE in 2008, the roots and leaves of barley was exposed to Hg(II), and a surprisingly clear signal appeared for this rather complex biological system, see Figure 1.

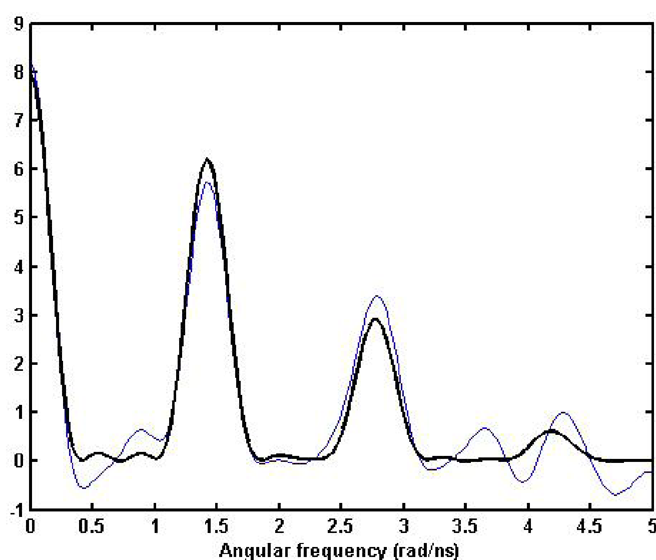

Figure 1 Proof of principle: ***In vivo*** ^{199m}***Hg-PAC spectroscopy may provide high quality data - Spectrum of only the radioactive Hg(II) bound to roots of barley***. *Blue: Fourier transformed PAC data; black: Fourier transform of fit to PAC data. Considering the quite complex biological system, the PAC spectrum is surprisingly simple, reflecting Hg(II) bound in a linear geometry most likely to two thiolates (cysteinates), based on similarity to reference data and quantum mechanical calculations. Spectrum recorded at ISOLDE/CERN September 2008.*

We wish to extend this project for example by increasing the concentration of the Hg(II) in the solution absorbed in the plants, to probe different binding sites as described above, as well as to investigate other systems in vivo such as bacteria and yeast, and binding of other heavy metal ions.

V. Experimental Methods

In 2002, two modern and very efficient 6-detector-TDPAC spectrometers, the so-called PAC Cameras, were installed permanently at the Solid State Physics Laboratory of the ISOLDE on-line isotope separator at CERN. This outstation of the Leipzig TDPAC

Laboratory is dedicated especially for TDPAC experiments in biochemistry and biology with rather short-lived isomeric TDPAC isotopes, like ^{111m}Cd, ^{199m}Hg, and ^{204m}Pb with half-lifes of less or about one hour. The synthesis and purification of the proteins will be done at the laboratory at the University of Michigan, and University of Zurich where outstanding expertise in this respect is available. The samples will be transported to ISOLDE on dry ice (~ -80 °C).

The proposed experiments require the implantation of ^{199m}Hg, and ^{204m}Pb for 40 to 70 minutes into ice at 100 K (in order to guarantee the vacuum in the beamline). In the last years we developed our own target holder, cooled by liquid nitrogen, at the collection point. Occasionally, we subsequently add inactive carrier (Hg(II)-, and Pb(II)-salts dissolved in adequate buffer solutions) to the molten ice to increase the metal concentrations from nanomolar to micromolar. After the incubation of the biomolecules with the ^{199m}Hg- and ^{204m}Pb-solution with or without carrier for 5 to 30 minutes the biomolecules are immobilized by precipitation/centrifugation, freezing, or adding sucrose. For the final preparation steps the new fume hood of the Solid State Physics Laboratory is required. There is also enough space for the cooling centrifuge, a refrigerator/freezer for protein samples, and other basic lab equipment, in the chemistry lab that has been (re-)establihed during the past 2-3 years.

VI. Beam Time Request

We ask for a total of 21 shifts within 2 years to be used as follows, all with min. $5 \cdot 10^7$ ions/s:

2009:	^{111}Ag (UCx,RILIS):	3 shifts
	^{199m}Hg (Pb Plasma Source):	5 shifts
	^{204m}Pb (UC, RILIS):	5 shifts
2010:	^{111}Ag (UCx,RILIS):	3 shifts
	^{199m}Hg (Pb Plasma Source):	5 shifts

The number of shifts per year of the two isotopes corresponds to one beam time per isotope and year.
In order to use the allotted beam time as efficiently as possible two PAC-Cameras at the Solid State Physics Laboratory at ISOLDE will be used in parallel for the ^{199m}Hg and ^{204m}Pb PAC experiments, whereas the ^{111}Ag (with a half life of 7.5 days) will be shipped to Copenhagen.

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