EUROPEAN ORGANISATION FOR NUCLEAR RESEARCH

CERN-INTC-2006-xxx
INTC-P-xxx
xx May 2006

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

Pb(II) and Hg(II) binding to *de novo* designed proteins studied by ^{204m}Pb- and ^{199m}Hg-Perturbed Angular Correlation of γ-rays (PAC) spectroscopy: Clues to heavy metal toxicity

L. Hemmingsen[1], Th. Agne[2], T. Butz[2], S.K. Das[3], S.-B. Ruy[2], V. L. Pecoraro[4], J.G. Correia[5]

Spokesman: Th. Agne

Contactman: J.G. Correia

Abstract

De novo design of proteins combined with PAC spectroscopy offers a unique and powerfull approach to the study of fundamental chemistry of heavy metal - protein interactions, and thus of the mechanisms underlying heavy metal toxicity. In this project we focus on Pb(II) and Hg(II) binding to designed three stranded coiled coil proteins with one or two binding sites, mimicking a variety of naturally occurring thiolate rich metal ion binding sites in proteins. The ^{204m}Pb- and ^{199m}Hg-PAC experiments will complement data already recorded with EXAFS, NMR, UV-Vis and CD spectroscopies.

[1] Department of Natural Sciences, The Royal Veterinary and Agricultural University, 1871 Frederiksberg C, Denmark.
[2] Fakultät für Physik und Geowissenschaften, Universität Leipzig, 04103 Leipzig, Germany.
[3] Babha Atomic Research Centre, Kolkata, Indien.
[4] Department of Chemistry, University of Michigan, USA
[5] ITN, Sacavem, Portugal and CERN, Geneva.

I. Introduction

Heavy metals such as mercury, lead and cadmium are among the elements that have so far shown only poisonous effects on living organisms [1]. The toxic effects are generally assumed to be related to binding of the heavy metal ions to proteins, and in particular to the thiolate groups found at many naturally occurring metal ion binding sites in such biomolecules. For example, anemia resulting from lead exposure is thought to be a result of Pb(II) binding to δ-aminolevulinic acid dehydratase, an enzyme in the heme biosynthetic pathway, with an active site Zn(II) coordinated by three cysteine ligands. Despite this and many other demonstrations of the widespread clinical relevance of Pb(II) toxicity, only very limited fundamental chemistry describing the interaction of Pb(II) with protein associated thiolates is known, and similarly the molecular basis of Cd(II) and Hg(II) toxicity is not fully uncovered yet.
Over the past decade *de novo* design of proteins has emerged as a powerful tool for the study of protein folding, function, and in this context interaction with metal ions [2,5]. The TRI and Coil-Ser series of *de novo* designed three-stranded coiled coil proteins are known to bind heavy metals in thiolate rich sites, see Figure 1. These sites provide similar coordination environments to proteins such as δ-aminolevulinic acid dehydratase and the metalloregulatory mercury binding protein MerR [1,4-6].

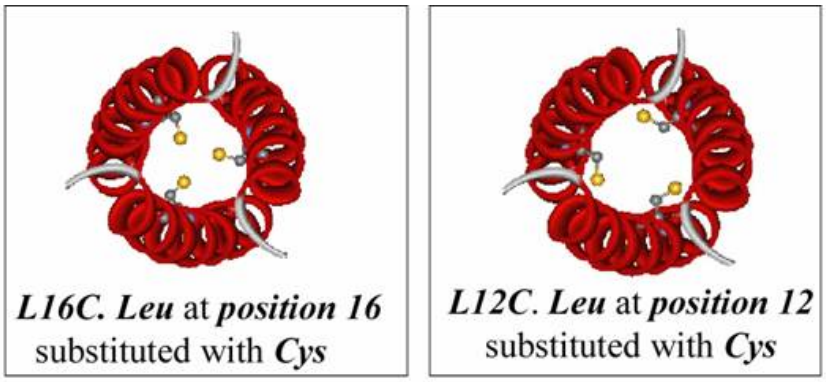

Figure 1 *Top view of a de novo designed three-stranded coiled coil protein with a heavy metal ion binding site consisting of three cysteine residues at the center of an otherwise hydrophibic core. Left: A so-called "a"-site where Leu is replaced by Cys at position 16 of the amino acid sequence (L16C) of each of the three strands; Right: A so-called "d"-site where Leu is replaced by Cys at position 12 of the amino acid sequence (L12C). Notice the structural differences between these two binding sites.*

The amino acid sequence and thereby the structure of the *de novo* designed proteins can be controlled and manipulated, and thus they form an ideal system to study heavy metal – protein interactions and to investigate how changes in the amino acid sequence affects fundamental properties such as metal ion selectivity and affinity.

In PAC spectroscopy, like for example Mössbauer-, NMR,- and ESR-spectroscopy, the hyperfine interaction of a nucleus with extranuclear fields is recorded. In biological applications of PAC spectroscopy usually the nuclear quadrupole interaction (NQI)

between the nuclear quadrupole moment and the electric field gradient (EFG) from the surrounding charge distribution is measured. This provides unique information on the structure and dynamics at the binding site of the PAC probe [6,7].

Recently ^{204m}Pb was added to the limited list of PAC isotopes applied in biology, in an effort by the group from Leipzig, where expertise in biological application of ^{199m}Hg PAC has also been accumulated over the past 5-10 years. Cadmium binding to the de novo designed proteins has already been studied by ^{111m}Cd-PAC spectroscopy as well as EXAFS, NMR, UV-Vis and CD spectroscopy in a collaboration between the groups in Copenhagen and Michigan. I turned out, for example, to be possible to control the coordination number to be either 3 or 4 depending on the design of the protein [5]. Another central finding illustrating the strength of PAC spectroscopy was that one signal was observed in ^{113}Cd NMR spectroscopy, but, surprisingly, two NQIs were unambiguously present in the ^{111m}Cd-PAC spectra. A subsequent temperature profile study with PAC spectroscopy revealed that the two structures are in dynamic exchange on the nanosecond timescale [4,8], and thus in fast exchange on the NMR time scale. Such dynamics is most likely an integral part of the function of many metal ion binding proteins.

In this project we wish to extend these studies to two other relevant heavy metals: Lead and mercury. In this context PAC spectroscopy is a particularly well suited technique, as the two isotopes (^{204m}Pb and ^{199m}Hg) decay by isomeric transitions, and experiments are not complicated by the problems related to element transformation occurring for all other useful PAC isotopes. The projects described in this proposal is focused on the fundamental chemistry of heavy metal – protein interactions. On a longer term perspective we aim at characterizing heavy metal binding proteins and toxicity *in vivo*, an as yet largely unexploited possibility where PAC spectroscopy offers unique advantages. We are currently addressing this problem with ^{111m}Cd PAC spectroscopy [9].

II. Status of research and proposed experiments

^{204m}Pb-PAC: Status of research and proposed experiments

Previously, Pb(II) binding to the TRI peptides has been studied by UV-Visible (UV-Vis), Circular Dichroism (CD) spectroscopies and EXAFS [3]. These techniques are useful in determining that Pb(II) is coordinated by cysteine ligands and to establish the average Pb-S bond length. PAC spectroscopy is an excellent complementary tool to these techniques, as the PAC signal is very sensitive to the angular positioning of the metal ion ligands.
In particular, we are interested in differences in Pb(II) affinity to peptides with cysteines in *a* and *d* positions of the amino acid sequence. Previous work with Cd(II) and Hg(II) showed that peptides with cysteines in the *a* position has a higher binding affinity than proteins with cysteine in the *d* position, as well as a lower pK_a of metal binding [4]. Pb(II) displays the opposite trend, with Pb(II) binding stronger to peptides with cysteine in the *d* position than peptides with cysteine at the *a* position. Similarly, differences in the CD spectra are observed, where a Pb(II)-protein with cysteines at the *a* positions displays a more intense signal than proteins with cysteines in the *d* position, see Figure 2. We have hypothesized that this is due to a structural differences at the metal binding sites. PAC spectroscopy will be a highly useful technique to determine whether there is indeed a structural difference between the two binding sites, and possibly to uncover in more detail the nature of this structural difference.

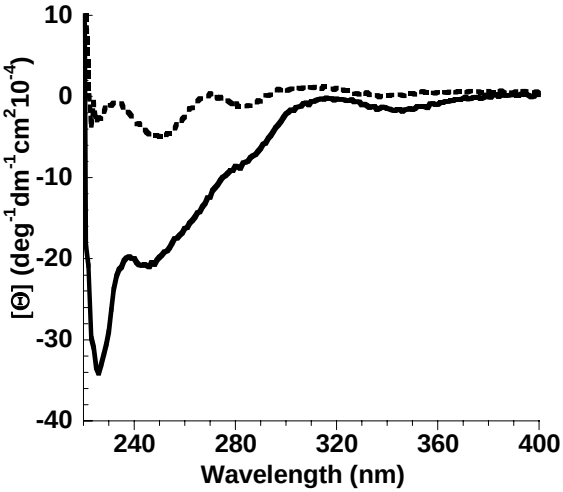

Figure 2: *CD Signals of Pb(L16C)$_3$, a protein with cysteines in the a position (solid line) and Pb(L12C)$_3$, a protein with cysteines in the d position (dashed line)*

Proteins having *two* metal binding sites separated by a leucine layer were designed that have site-selective metal binding. In the protein L9C/L19C (leucines number 9 and number 19 replaced by cysteines), with one *a* site and one *d* site were shown to bind Cd(II) specifically to the *a* site whereas Pb(II) has a preference for the *d* site. Investigation into the simultaneous Pb(II) and Cd(II) binding to this protein by ^{113}Cd NMR enable us to determine the number of Cd(II) species, however, we have to infer information indirectly about Pb(II). ^{111m}Cd-PAC experiments (carried out at the PAC-laboratory in Denmark) and ^{204m}Pb-PAC experiments would give us direct information about both the Cd(II) and Pb(II) speciation in solution. In addition, if it is possible to

distinguish Pb(II) binding to the *a* and *d* sites by PAC, we can determine directly to which binding site Pb(II) is coordinated.

^{199m}Hg-PAC: Status of research and proposed experiments

In an ongoing project CD, UV-Vis and ^{199}Hg NMR spectroscopies have been applied to different variants of the Coil-Ser (CS) proteins: A comparative study was carried out with Hg(II) binding to CS-L9C (cysteine in *a* position) and CS-L19C (cysteine in *d* position, respectively.
Addition of 2 equivalents of CSL9C to a solution of Hg(II) (10 μM) at pH 8.5 generates only minor UV-Vis spectral changes, whereas a strong ligand-to-metal-charge-transfer (LMCT) excitation is observed at 247 nm when the third equivalent of CS-L9C is added, see Figure 3. This may be interpreted as the formation of a linear complex $Hg(CS\text{-}L9C)_2$ when less than 2 equivalents of peptide is present and the formation of a trigonal complex $Hg(CSL9C)_3^-$ at higher stiochiometries. The same behavior is observed for the titration of CS-L19C (cysteine in *d* position) into a solution of Hg(II) (10 μM) at pH 9.5. In this case, however, a higher pH is necessary for the formation of the trigonal thiolato Hg(II)

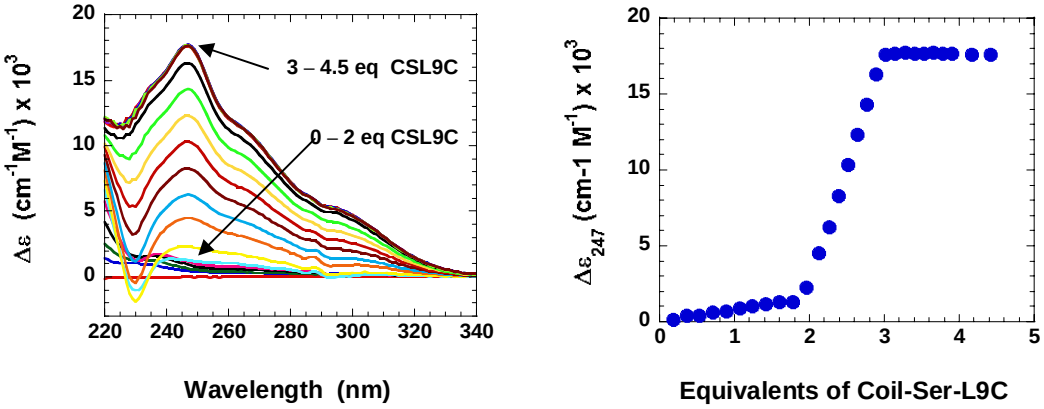

Figure 3. *UV-Vis titration of CSL9C into a solution of Hg(II) at pH 8.5 ([Hg(II)] = 10 μM).*

complex. It is noteworthy, that the CD spectra of the complexes $Hg(CSL9C)_3^-$ and $Hg(CSL19C)_3^-$ display opposite ellipticities (data not shown), indicating that structural differences occur between the two Hg(II)-protein complexes. In addition, ^{199}Hg NMR spectral data (Table 1) show that not only *a* and *d* sites generate different trigonal environments around Hg(II) but also different linear environments. This is fascinating since these binding sites are made off the same amino acids. PAC spectroscopic studies on these metal complexes will provide complementary spectroscopic information on the differences the *a* and *d* metal site structures, and should provide a sound basis for discrimination between 2- and 3-coordinated Hg(II) complexes. In addition, an unresolved question is whether the 3-coordinated site has regular planar trigonal structure or a T shaped structure. PAC spectroscopy should allow this to be clarified, as the prior

idealized structure has an asymmetry parameter of 0, whereas the latter has an asymmetry parameter of 1 – the two extreme values of this experimentally determined parameter.

Table 1. *^{199}Hg NMR chemical shift observed for the different Hg(II)-protein complexes*

peptide	Hg(II) Metal complex (3 mM, pH 9.5)	^{199}Hg chemical shift (ppm)
CS-L9C	$Hg(CSL9C)_3^-$	- 186
	$Hg(CSL9C)_2$	- 835
CS-L19C	$Hg(CSL19C)_3^-$	- 312
	$Hg(CSL19C)_2$	- 948

For all the peptides the following equilibrium appears to exist:

$$Hg(peptide)_3\ (= HgS_2SH) \quad \leftrightarrow \quad Hg(peptide)_3^-\ (= HgS_3) \quad + \quad H^+$$

With the data we have so far we can not determine if the protonated cysteine in the complex HgS_2SH is coordinated or not to the Hg(II). PAC spectroscopy is ideally suited to address this problem.

Proteins having *two* metal binding sites have also been synthesized for Hg(II) binding studies. In these, two layers of three cysteines are positioned next to each other (6 cysteines in total), providing a variety of possibilities for the coordination of Hg(II) including both 2-, 3-, and 4-coordinated sites, possibly with bridging thiolates when more than one equivalent of Hg(II) is added. PAC spectroscopy will be applied to study the coordination geometries appearing when up to 2 equivalents of Hg(II) is added.

Reference PAC data for relevant Hg(II) model compounds [10,11] and Hg detoxification enzymes [12,13] have been determined by the group in Leipzig, and will form a strong basis for the interpretation of the experimental data in this project.

III. 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 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 one of the hoods of the Solid State Physics Laboratory is required. There is also enough space for the cooling centrifuge and a cryobox (for storing the proteins).
Due to the rather short half-life of the isomeric TDPAC isotopes the collection point, the PAC-Cameras and the Solid State Physics Laboratory have to be as close as possible to each other, i.e. the best collection points for us would be close to the stairs in the experimental hall of ISOLDE.

IV. Beam Time Request

We ask for a total of 24 shifts within 3 years to be used as follows:

2006:	^{204m}Pb	(UC, LIS):	4 shifts
2007:	^{199m}Hg	(Pb Plasma Source):	4 shifts
	^{204m}Pb	(UC, LIS):	6 shifts
2008:	^{199m}Hg	(Pb Plasma Source):	4 shifts
	^{204m}Pb	(UC, LIS):	6 shifts

The number of shifts per year of the two isotopes corresponds to one beam time per isotope and year. We stress the fact that we do need the beam every two to three hours only. Therefore, we can share the isotope beam with other users.
In order to use the allotted beam time as efficiently as possible we have permanently installed PAC-Cameras at the Solid State Physics Laboratory at ISOLDE. If required, two other PAC-Cameras from Leipzig can be transported to ISOLDE.

References

1) Kaim, W.; Schwederski, B.; "Bioinorganic chemistry: Inorganic elements in the chemistry of life", John Wiley and Sons, 1991
2) Bryson, J.W.; Betz, S.F.; Lu, H.S.; Suich, D.J.; Zhou, H.X., O'Neil, K.T.; DeGrado, W.F.; "Protein design: A hierarchic approach" Science, 1995, 270: 935-941.
3) Matzapetakis, M. et al., Submitted to J. Biol. Inorg. Chem.
4) Matzapetakis, M.; Farrer, B. T.; Weng, T.-C.; Hemmingsen, L.; Penner-Hahn, J. E.; Pecoraro, V. L.; " Comparison of the Binding of Cadmium(II), Mercury(II), and Arsenic(III) to the de Novo Designed Peptides TRI L12C and TRI L16C" J. Am. Chem. Soc., 2002, 124: 8042-805.
5) Lee, K.-H.; Cabello, C.; L., Hemmingsen; Marsh, E.N.G.; Pecoraro, V.L.; "Using non-natural amino acids to control metal coordination number in three stranded coiled coils" Angew. Chem. Int. Ed., 2006, accepted for publication
6) Tröger, W.; Butz, T.; "Inorganic biochemistry with short-lived radioisotopes as nuclear probes", Hyperfine Int., 2001, 129: 511-527
7) Hemmingsen, L.; Sas, K.N.; Danielsen, E.; "Biological applications of perturbed angular correlation of γ-rays spectroscopy", Chem. Rev., 2004, 104: 4027-4061.
8) Hemmingsen, L.; Olsen, L.; Lee, K.-H.; Matzapetakis, M.; Pecoraro, V.L.; "Metal site dynamics on the nanosecond time scale in a de novo designed protein", manuscript in prep.
9) Sas, K.N.; Kovacs, L.; Zsiros, O.; Gombos, Z.; Garab, G.; Hemmingsen, L.; Danielsen, E.; "Fast cadmium inhibition of photosynthesis in cyanobacteria *in vivo* and *in vitro* studies using PAC spectroscopy" submitted to J. Biol. Inorg. Chem.
10) B.Ctortecka, W.Tröger, S.Mallion, T.Butz, R.Hoffmann and the ISOLDE-Collaboration "Hg-coordination studies of oligopeptides containing cysteine, histidine and tyrosine by199mHg-TDPAC." Hyperfine Interactions 120/121, 737, 1999.
11) T.Soldner, W.Tröger, T.Butz, P.Blaha, K.Schwarz and the ISOLDE-Collaboration "Measurement and Calculation of Electric Field Gradients in Hg-Mercaptides." Z. Naturforsch. 53a, 404, 1998.
12) W.Tröger, "Nuclear probes in life sciences", Hyperfine Interactions 120/121, 117, 1999.
13) C. Lippert, W. Tröger, and T. Butz, "TDPAC in Biophysics", in: "Proceedings of the XXX. Zakopane School on Physics", K. Tomala and E. A. Görlich (eds.), Jagiellonian Univ., Kraków, 1996.