EUROPEAN ORGANIZATION FOR NUCLEAR RESEARCH

Clarification Letter
to the ISOLDE and Neutron Time-of-Flight Experiments Committee
concerning proposal INTC-2014-063, P-427

Cu(I), Ag(I), Cd(II), Hg(II), and Pb(II) binding to biomolecules studied by Perturbed Angular Correlation of γ-rays (PAC) spectroscopy

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Abstract

Metal ions display diverse functions in biological systems and are essential components in both protein and nucleic acid structure and function, and in control of biochemical reaction paths and signalling. 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.
The isotopes to be employed in the proposal are the following: ^{111m}Cd, ^{111}Ag, ^{199m}Hg, ^{204m}Pb, 61Cu, ^{68m}Cu.

Requested shifts: 22 shifts, (split into 4 major runs over 2 years; 2 test runs to be accommodated when possible)

In the following we address the issues raised by the INTC:

"… two specific questions need to be clarified: (i) how do PAC measurements complement the information gained using the other techniques mentioned in the proposal - more specifically the proposers should indicate how the PAC studies fit in with the other techniques and provide unique insights to enable greater understanding, as opposed to solely providing overlapping information to support and confirm insights gained from the other techniques; (ii) how the proposers plan to address risk factors associated with the complexity (or even lack of) PAC signals given the rather complex systems proposed for study."

We acknowledge that both points are important and relevant. The focus in the following clarification is on describing in which way the PAC experiments are unique and how risk poor / lack of signal is handled. In a short appendix (appendix 1), the other spectroscopic techniques used in this work are described very briefly.

(i) The PAC measurements are unique in at least three respects:

a. The nuclear quadrupole interaction displays a different dependence on the metal ion coordination number, type of coordinating atom, metal-ligand distance and ligand-metal-ligand angle than the other more conventional techniques applied in this work. Thus, for the elucidation of the structure(s) present in the sample, the PAC data are truly complementary to the other spectroscopic data, such as NMR, EXAFS, UV and visible absorption of light, etc. An example from previous work is the identification of two co-existing Cd^{2+} coordination geometries in a synthetic protein [Matzapetakis et al. J. Am. Chem. Soc. **2002**, 124, 8042], where the assignment of the CdS_3 and CdS_3O metal site structures relied on the PAC data, and in fact was initially interpreted incorrectly using the NMR data. In addition, the electric field gradient is an electronic ground state property which is relatively easily calculated using available program packages for electronic structure calculations, providing a means to systematically interpret the experimental data (this is also possible for calculations of shielding tensors (NMR data), but it is more complex as excited states must be included). It is expected that similar complementarity of the PAC and other spectroscopic data will be important for the interpretation of the data in the current proposal.

b. PAC spectroscopy is much more sensitive than the other spectroscopic techniques applied in this work, allowing for experimental conditions at very low concentration of the biomolecule to be explored, or very low loading of the available biomolecular metal ion binding sites. An example from previous work where this was essential, was investigation of metal ion binding to so-called zinc finger proteins, which regulate gene expression, and this regulation is achieved by Zn^{2+} binding to the protein inducing a structural change of the entire biomolecule. In this work it was demonstrated that one metal site structure was present at very low metal to zinc finger stoichiometry, but another structure was present when the zinc finger was fully loaded with the metal ion, with implications for the regulatory function of the protein [Heinz et al. Chem. Eur. J. **2009**, 15, 7350].

In this proposal the high sensitivity of PAC spectroscopy is explicitly used, for example, in the study of Ag^{+} binding to nucleic acids. In this subproject, two

Ag^+ ion binding sites are designed in the biomolecule, and we aim to characterize the structure with one metal ion bound as well as with two metal ions bound to the biomolecule. This is practically impossible with other spectroscopic techniques, because there is pronounced cooperativity of the binding of the two metal ions, i.e. once the first metal ion is bound, the binding of the second metal ion is highly favoured. Only at very low metal ion concentration it is expected to be possible to observe the species with one metal ion bound, and these experimental conditions can be realized with PAC spectroscopy but not with the other techniques used in this proposal.

c. PAC spectroscopy is sensitive to dynamics on the nanosecond time scale (dictated by the lifetime of the intermediate nuclear level). Nanosecond metal site dynamics is very difficult to explore with other spectroscopic techniques at equilibrium conditions, and at the same time it is a time scale which is important in the function of catalytic metal sites as well as in transport of metal ions in biology. In this respect PAC and NMR spectroscopy are also complementary, as NMR spectroscopy (in the way it is applied in this work) is sensitive to dynamics on the millisecond time scale. An example is the metal site in a synthetic protein [Stachura et al., in preparation], where only one resonance is observed in NMR spectroscopy but two NQIs are observed in PAC spectroscopy, indicating that the two species are in fast exchange on the NMR time scale, but in slow exchange on the PAC time scale. Carrying out a temperature series, it is demonstrated that the nanosecond exchange dynamics may be characterized using PAC spectroscopy.
In the current proposal, a similar study is planned on the naturally occurring Cu^+ transporter HAH1, where PAC data and NMR data display the same complementarity, i.e one resonance in NMR spectroscopy and two or more NQIs in PAC spectroscopy at room temperature. This will extend the metal site exchange dynamics studies to real systems and functional dynamics at biomolecular metal sites.

(ii) Addressing risk factors associated with the complexity (or even lack of) PAC signals given the rather complex systems in this proposal

It is indeed possible that some of the proposed experiments turn out to be too complex to lead to reliable results and interpretation. We aim to address this issue – as we have in previous beam times – by two different actions:

a. The biomolecular metal sites are characterized as well as possible using standard spectroscopic techniques before the PAC experiments are carried out. This allows for design of experimental conditions (concentrations of metal ion and biomolecule, temperature, pH, …) of the PAC experiments to maximize the likelihood of clear and useful results. Also, if the experiments involve an external biochemical collaborator, we usually have a person present and participating in the beam time from the collaborating group with detailed knowledge and experience in the specific sample preparation for the system of interest. This is probably the most important prerequisite for successful beam

time, and over the past 6 years where we have had beam time at ISOLDE, our track record is rather good (we think), although there have of course been cases that did not lead to useful results.

b. Prior to a given beam time we always prepare a list of experiments that is longer than what can be expected to be achieved. The list consists of several independent subprojects most of these are divided into 1) "Need to do", i.e. a minimal number of experiments that are necessary to address the most important scientific question, and 2) "Nice to do", i.e. additional experiments that are also very important. This gives flexibility during the beam time to re-prioritize between subprojects, if one subproject turns out to not to give useful results. In this manner we can optimize the exploitation of a given beam time.

c. Specifically, the experiments on ^{204m}Pb are expected to be challenging because of the complexity of the PAC spectra and their interpretation, arising from the fact that the intermediate nuclear level in the γ-γ cascade has I = 2. We have demonstrated that useful data can be obtained for a Pb^{2+} containing complex in the crystalline state [Vibenholt et al. Inorg. Chem. **2012**, 51,1992], but the line broadening observed in solution (due to slight differences in metal site structure from one molecule to the next and due to rotational diffusion) has so far obscured reliable interpretation. We therefore aim to study a biomolecular metal site which is known to be rigid, and to carry out the experiments at 77 K (using liquid nitrogen), following the initial experiments along this line carried out by prof. T. Butz and co-workers.

With the hope that the above sufficiently clarifies the issues raised by the INTC, and I am of course at your disposal if further clarification is required,

Respectfully,

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Appendix 1

The other experimental techniques which may be employed in this work comprise NMR, EXAFS, UV-Vis absorption, fluorescence and circular dichroism spectroscopy. In this appendix these standard techniques are very briefly described. Instruments required for these techniques are all present (except EXAFS, which requires beam time at a synchrotron facility) at the University of Copenhagen or collaborators.

UV-Vis absorption and circular dichroism spectroscopy:
Absorption spectroscopy in the ultraviolet and visible wavelength range probe electronic transitions of the biomolecules. For the systems investigated in this proposal, it is for example employed to determine concentration in solution of proteins, using a standard value for the given protein for the absorption at 280 nm, where certain groups ion the protein (the aromatic amino acid side chains) absorb UV light in a characteristic manner. It may also be employed to follow the emergence of absorption bands arising from electronic transitions directly originating from the metal site, and as such allow for determination of how many metal ions bind to the metal site(s) of interest, as well as of equilibrium constants and kinetics of the binding. There is some correlation between the absorption bands and the local metal site structure, but it is usually not possible to use the UV-Vis absorption spectra to determine the structure.
Circular dichroism is a variant of UV-Vis absorption spectroscopy, where the differential absorption of right and left circularly polarized light is recorded. This allows for characterization of the so-called secondary structure elements of biomolecules (for example helices in proteins), and is used, for example, to follow folding or similar structural reorganization of the entire biomolecule. Also absorption bands specifically originating from the metal site may be recorded.
There is some correlation between the absorption bands and the local metal site structure, but it is usually not possible to use the UV-Vis and CD spectra to determine the metal site structure for the systems explored in this work.

Fluorescence spectroscopy:
Fluorescence occurs from groups inherent in for example proteins, or the biomolecules may be labelled with fluorescing molecules. This fluorescence may change upon binding of metal ions, either directly or due to structural change of the protein induced by the binding of the metal ion. The technique thereby may allow for characterization of the metal ion binding equilibrium constant and kinetics, but not the metal site structure.

Nuclear Magnetic Resonance (NMR) spectroscopy:
NMR is the most versatile spectroscopic technique to explore structure and dynamics of biomolecules in solution. However, relatively large amounts of material is required, i.e. the sensitivity is poor. As indicated above, the resonance frequency does provide unique information on the metal site structure, but the interpretation is not trivial, and almost exclusively relies on empirical comparison with NMR spectra of model compounds for the metal ions explored in this work. Furthermore, the way we use NMR spectroscopy in this work, it is sensitive to dynamics on the millisecond time scale, and therefore complements the PAC

experiments in this respect. Thus, it has turned out that NMR and PAC spectroscopy are a strong combination when elucidating structure and dynamics at proteins metal sites.

Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy:
As the name indicates, it is an x-ray based technique, where the details of absorption of x-rays as a function of energy, for example at the K- or L-edge of a given element, reflect the local structure around that element. It is particularly sensitive to metal-ligand bond lengths, and thus complements PAC and NMR data well.