ESF-EMAR workshop "Paramagnetic Tagging in NMR"

May 17-18, 2010 - Sevilla, Spain

Scientific Report

Contents

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Summary

The ESF-EMAR workshop on Paramagnetic Tagging in NMR was held on 17 and 18 May, 2010 in Sevilla, Spain. Eleven speakers presented their vision on paramagnetic tags to an audience of 25 participants. Nine speakers came from six European countries (Denmark, France, Germany, Spain, Switzerland and The Netherlands), one speaker from the United States of America and one from Australia. The meeting also included two discussion sessions and ample time for informal discussions. For this purpose the two lunches and the workshop dinner were open to all participants.

The topic of the meeting was the use of paramagnetic tags in biological NMR. Paramagnetic molecules contain unpaired electrons, which exhibit very strong magnetic spins. The presence of electron spins can have large effects on nuclear magnetic resonance (NMR) signals. The effects can be used to derive information about molecular structure from NMR spectra. However, unpaired electrons are found only in metal ions and stable radicals and, thus, are absent in most proteins, nucleic acids and saccharides. To take advantage of the electron-nuclear interactions, paramagnetic centres need to be introduced in such samples. Over recent years, a variety of paramagnetic tags has been described, with a range of applications.

The aims of the meeting were to evaluate the currently available tags, get an overview of their applications and limitations and discuss future improvements and new applications. The speakers represented most leading groups in this area, providing a good overview of both the current applications and tags. During the discussion after each presentation and in the special sessions, the advantages and limitations of specific tags became clear. The list of requirements of ideal tags is long: small; rigid; single isomer (for metal complexes); strongly paramagnetic, able to cause relaxation, shifts and alignment; easy to produce and easy to attach to the molecule of interest. It is evident that no single tag can fulfil all requirements. The best tag needs to be selected on the basis of its suitability for a specific research question. Several specific areas of tag improvements were identified. The range of applications was shown to be very wide, including structure determination of macromolecules, docking of protein complexes, characterisation dynamics in proteins and nucleic acids, the study of small molecule-protein interactions and the visualisation of lowly populated states. However, also several limitations in the interpretation of the observed paramagnetic effects were discussed, like the consequences of insufficient averaging between states when studying minor protein conformations.

The workshop illustrated the wide range of possibilities of this new tool in bioNMR and has also been important to identify the limitations of the current tags and methods, providing a focus for the further improvements in paramagnetic tags.

Scientific contents of the workshop

Below an overview of the presentations is provided.

Prof. Michael Sattler described a study of protein domain interactions using spin labels and an external paramagnetic compound, Omniscan, which contains a Gd^{3+} ion and also causes relaxation effects. A comparison of the relaxation caused by Omniscan on the domains free and in complex yields an image of the surface area that is protected in the complex. This information, in combination with interdomain PREs from the spin labels, can be used as restraints in docking. Also the use of PRE of ^{13}C nuclei was discussed. This approach offers the possibility to obtain structural restraints for protein sidechains. Lastly, showed the possibilities of combining neutron or X-ray scattering data with PREs. These data are complementary in that the former tends to bias the open form of a complex and the latter the closed form.

Dr Daniel Häussinger described work on cadherin, a transmembrane glycoprotein involved in cell-cell adhesions. In cancer cells that are prone to cause metastasis, cadherin is often lost. The structure of the protein depends on the concentration of Ca^{2+} ions. To study domain-domain interactions in cadherin, first spin labels were used to obtain interdomain PREs. However, this approach did not yield good results because the distance range of the restraints from spin label PREs was too small. Then a new lanthanide tag was produced, called M8-Spy. It is based on DOTA with additional methyl groups in the cyclen ring and the Ln-coordinating arms. NMR spectra show that the probe is in a single isomer. It yields large pseudocontact shifts (PCS) when attached to a protein to a cysteine residue via a single arm, possibly involving secondary interactions with the protein surface. It was demonstrated that the temperature dependence of the PCS can be used conveniently for assignment purposes.

Dr Guido Pintacuda talked about a very new area of paramagnetic NMR, the analysis of paramagnetic effects in the solid state. There are several critical differences between solution and solid state NMR that influence the paramagnetic effects. Curie relaxation, caused by fluctuations in the dipole-dipole interaction between the nucleus and the time-averaged component of the electron spin, is absent in the solid state. PCS can be observed and also the anisotropy of the PCS, which averages out in solution, is accessible in solids and causes broad powder spectra. However, ultra-fast magic angle spinning (MAS) can alleviate this problem considerably. It was shown how paramagnetic effects can be applied to reconstruct molecular structures in crystals using solid state NMR. Also the application of MAS NMR to microcrystalline superoxide dismutase was discussed, showing that it yields data on the paramagnetic Cu(II) site that complement the solution NMR results.

Prof. Chun Tang discussed an important issue in the analysis of PRE data used for the characterisation of minor states and dynamics. If a protein or protein complexes exists in two or more conformations and the minor state is studied by analyzing the effects of a relaxation probe on the NMR resonances of the major state, it is generally assumed that the exchange rate between both states is much faster than the PRE. However, this may not be generally true, because then the exchange rate needs to be very large in cases of strong PREs. The use of two probes with different paramagnetic strength at the same site, EDTA-Mn and MTSL, illustrated this point. The observed PREs did not scale according to the difference in the strength of the paramagnet, because the exchange rate between the conformers was not sufficient for complete averaging of both states.
It was also shown that a Cu(II) bound to two histidine residues and NTA provides a rigid probe with a K_d of about 4 μM.

Prof. Flemming Poulsen presented results on the use of spin labels to study unfolded proteins. The aim of the experiments was to identify regions with residual structure. The protein ACBP, which assumes a four helix bundle fold in the native state, is thought to exhibit residual helical structure in the acid-unfolded state. By introducing spin labels at multiple positions along the unfolded chain, distant regions that contact the spin label can be identified. This approach was compared with the analysis of chemical shifts changes upon introduction of Ala substitutions along the chain. Residual helical structure causes slight changes in the chemical shifts, so the changes in the shifts due to the Ala mutations report on increased or decreased residual helical structure in the unfolded state. A good agreement between the methods was observed, but the spin label approach is rather more complex and may not be the most efficient way to obtain the required data.

Prof. Martin Blackledge also reported on the study of unfolded proteins. The description of the ensemble of conformations of unfolded proteins is an ill-posed problem. The number of the degrees of freedom is much larger than provided by any experimental data set. The best approach is to combine PRE data with residual dipolar couplings (RDC) and chemical shifts. The best solution proposed by Blackledge comprizes the generation of a set of conformations selected on the basis of amino acid specific sampling, i.e. taking the neighbouring residues into account when evaluating possible conformation of a residue in the chain. Using a genetic algorithm and the experimental restraints ensembles can be generated that yield back-predicted data matching the experimental ones.

Prof. Miquel Pons reported on PRE work. Pathogenic factors are often localized on mobile genetic elements (plasmids etc.). The protein H-NS can silence horizontally acquired genes. PRE was used to demonstrate that the topology of the subunits in the dimer formed by the dimerisation domain is antiparallel. For the interpretation of the PRE data the spin label was represented by an MD generated ensemble of orientations. Also the interaction with partner protein Hha was studied, using mutagenesis, fluorescence anisotropy, chemical shift perturbations and intermolecular PRE. Surprizingly, PRE-based docking did not yield a good result. The reason for this is as yet unknown and may be due to conformational changes.

Prof. Gottfried Otting gave an overview of lanthanide tags that cause PCS. Many tags results in multiple peaks for nuclei in the protein, due to the presence of isomers of the tag. Lanthanide ions coordinate 8-9 ligands and the design of a tag in a single isomer is difficult. Recent small tags, such as 4MMDPA and several variants, were discussed. These are small and require additional coordination of the Ln ion by residues on the protein. Also the incorporation of 4MDPA-Bpa via an unnatural amino acid in a protein was discussed. A new probe that is not bound covalently to macromolecules is $[Ln(DPA)_3]^{3-}$. This compound tends to associate to proteins at specific sites and can yield pseudocontact shifts. It can also be used to generate scalable PREs by simple titration of the compound. Lastly, some W-band DEER spectra of two interacting Gd ions in different tags were reported.

Ms Katja Barthelmes acted as a substitute for Prof. Harald Schwalbe, who was unable to attend the workshop due to unforeseen circumstances. She presented work on lanthanide binding tags (LBT). These tags are extensions of the protein that bind lanthanides. Well-known are the LBTs at the N- or C-terminus of the protein, but new are the loop-LBTs, which are introduced by extending existing loops in protein with a sequence that can bind a Ln ion with nM affinity. It was demonstrated that a LBT can quench the fluorescence from nearby tryptophan residues and can be used to solve the phase problem in X-ray diffraction. For the generation of RDCs is its better to use a double LBT, binding two Ln ions, because it gives better alignment. When the LBT is introduced in a peptide that binds a protein, 'transferred RDCs', i.e. alignment of the protein, can be detected.

Dr Peter Keizers discussed two-armed lanthanide probes that require attachment to the protein via two cysteine residues. It was demonstrated that double-armed attachment yields a tag that is rigid relative to the protein, producing strong alignment and large PCS. Several applications for the study of protein-protein complexes were reported. The orientation of the proteins in the 152 kDa complex of nitrite reductase and pseudoazurin was determined using PREs generated with these tags. Also the structure of the complex of adrenodoxin reductase and adrenodoxin, a 64 kDa complex, was determined using both PCS and PRE. This was used as a showcase for paramagnetic NMR methods, because the low solubility of the reductase and the FeS cluster in adrenodoxin make it impossible to determine the structure of the complex with standard NMR methods. Lastly, the application of the tag in the study of the highly dynamic complex of cytochrome *c* and adrenodoxin was mentioned. Alignment of the complex via the cytochrome *c* does not result in any alignment of the adrenodoxin, indicating that the complex must be highly dynamic, with adrenodoxin sampling many different orientations around the cytochrome.

Prof. Christian Griesinger reported on tags that are derived from an EDTA skeleton. The probes have well-defined chirality, to avoid double peaks in the NMR spectra. A new tag with three nitrogen and six carboxy groups was reported, resulting in better Ln loading of the tag. Several applications were discussed, such as the study of domain motion in the Ca binding protein calmodulin, but also a tag for DNA and even a disaccharide (lactose). The DNA tag is attached to an unnatural base using click chemistry and yields large PCS.

Assessment of the results and impact on the field

The workshop brought together many leaders in the field. Getting an overview of all the applications of the paramagnetic tags helps to create a community feeling. This sense of common interest was clearly felt, which is important for propagation of the developed methodology among the many other NMR groups in the world. The workshop has initiated new collaborations between the participating groups, which will also strengthen the field. Lastly, the discussion on the limitations and problems with current tags leads to a focus on the most important issues to solve in the coming years in order to expand the possibilities still further.

On the basis of the presentations and the discussions sessions the following conclusions can be drawn.

1. Paramagnetic tags have found an amazingly wide range of applications that is still expanding. The areas comprize: protein folding, protein structure determination, structure determination of complexes of proteins with other proteins, DNA, RNA and ligands, structures of nucleic acids and sugars and macromolecular dynamics including both ensembles and exited states.
2. Many tags have been developed with widely varying properties, from very small to bulky, from mobile to rigid, from weakly to strongly paramagnetic. The right tags needs to be selected for the question at hand. It is the responsibility of the leaders in the field to keep providing reviews to give an overview of the tags and their possibilities and limitations.
3. Two specific problems with tags can be identified.
    a. Rigid probes are usually large and bulky and it appears as if none of the existing tags shows no mobility at all. Rigidity is essential to obtain large and unambivalent paramagnetic effects.
    b. Most tags, but not all, require attachment via exposed Cys residues. The engineering of Cys residues on protein surface and the removal of endogenous Cys residues can affect the stability and solubility of the protein in an unpredictable way. The use of unnatural amino acids may be a promising way to address this issue.
4. More development is required for the interpretation of the results of PREs, especially for the characterisation of ensembles and lowly populated states. The visualisation of ensembles is inherently an inverse problem, because the data represent some average of the measured parameter. The rate of exchange between the individual states in relation to the experienced PRE must be considered carefully.
5. An area with great potential for further development of paramagnetic NMR is solid state NMR, especially in combination with very fast MAS.

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Dear speaker/participant,

The aims of the workshop are to discuss current and future applications of paramagnetic tags in bio-NMR and, following from that, how paramagnetic tags can be improved to meet the (new) requirements.
Below you will find the programme and the list of participants. Talks are 30 min + 5 min for discussion. Also plenty of time for informal exchange of information will be available. Speakers, please involve the younger participants, all from the participating groups, in your discussions.

Conference room and accommodation of the workshop are the same as for the FEBS meeting 'Understanding Transient Interactions in Biology', which follows immediately after our workshop. The information below has been taken from the FEBS meeting website. For more information about the venue, please check: http://www.transient2010.ciccartuja.es.

I wish to thank Dr Irene Díaz-Moreno and her team for the local organisation and Ms Ana del Valle of the travel agent Viajes el Corte Inglés, s.a. division congresos for her help with the logistics. The sponsors, indicated at the top with their logos, are gratefully acknowledged for making this workshop possible.

Marcellus Ubbink

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cicCartuja

Venue address and location

Both the Hotel and the Workshop venue are located at the Cartuja Science and Technology Campus.

To get there from the Seville (*S. Pablo*) airport, you have the following two options:

1. BY TAXI: The taxi ride takes 20 min to the Campus (ca. €20) .
2. BY BUS: There is a Bus Service from the Airport to Seville Centre, stopping at *Sª Justa* Rail Station (Fare €5-10). There you can either take a taxi or connect the **C2** Public Bus Service to get to Workshop Venue and Hotel.

The address of the Workshop Venue (**www.ciccartuja.es**) is:

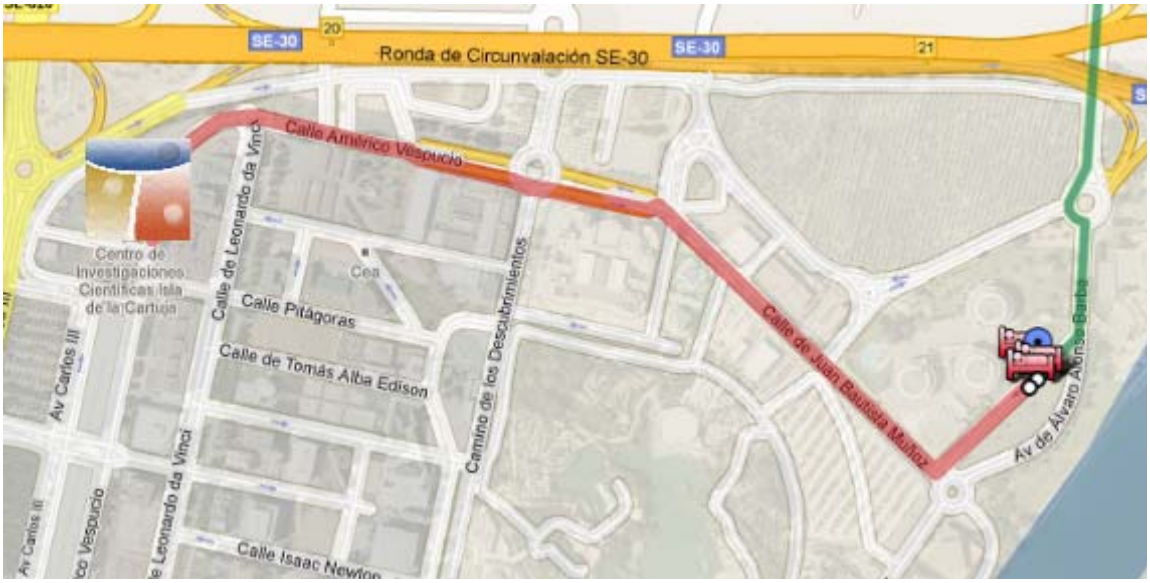
Centro de Investigaciones Científicas Isla de la Cartuja
Avda. Américo Vespucio, 49
41092 Sevilla
Phone: +34 954 489 500
Coordinates: 37.411542,-6.006785

The address of the Workshop Hotel (**www.barcelorenacimiento.com**) is:

Hotel Barceló Renacimiento – Isla de La Cartuja
Isla de la Cartuja s/n
41092 Sevilla
Phone: +34 954 462 222
Coordinates: 37.409997, -5.995436

The Hotel is only 15-20 minutes walking East from the Workshop Venue. Just check the red trail on the map in this page.

Seville, with ca. one million inhabitants, is the capital of Andalusia. Seville is rich in History and magnificent buildings and monuments. The city is well provided of an International Airport, a High-Speed Train line (AVE) and a renovated highways network.



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Programme for Monday, May 17, 2010
All presentations are 30 min. + 5 min. for questions

9:50 Welcome

Chair: Martin Blackledge

9:55 Michael Sattler (München, Germany)
Spin labeling and PREs for structural analysis of protein complexes in solution

10:30 Daniel Häussinger (Basel, Switzerland)
DOTA-M8 - an extremely rigid, high-affinity lanthanide chelating tag for PCS NMR spectroscopy

11:05 Coffee break

11:30 Guido Pintacuda (Lyon, France)
Paramagnetic shifts in solution and in the solid-state

12:05 Chun Tang (Columbia, MO, USA)
DiSPRE, tagging different paramagnetic probes in characterizing protein dynamics

12:40 Discussion I

13:10 Lunch

Chair: Guido Pintacuda

15:00 Flemming Poulsen (Copenhagen, Denmark)
The use of paramagnetic relaxation effects in comparing the unfolded state of a four helix bundle wild type and folding relevant single site mutations

15:35 Martin Blackledge (Grenoble, France)
NMR studies of local and long-range order in intrinsically flexible proteins

16:10 Tea Break

16:40 Miquel Pons (Barcelona, Spain)
PRE information and the topology of nucleoid associated protein complexes

17:15 Free time

20:00 Dinner

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Programme for Tuesday, May 18, 2010
All presentations are 30 min. + 5 min. for questions

Chair: Michael Sattler

9:30 Gottfried Otting (Canberra, Australia)
Paramagnetic tags for protein NMR

10:05 Katja Barthelmes on behalf of Harald Schwalbe (Frankfurt am Main, Germany)
Encodable LBTs in proteins and RNA

10:40 Coffee break

11:10 Peter Keizers (Leiden, The Netherlands)
Two-armed Ln-tags to study protein structure and dynamics

11: 45 Christian Griesinger (Göttingen, Germany)
Some exercises with paramagnetic tags: Dynamics and Complexes

12:20 Discussion II

13:00 Lunch & Departure

List of participants

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