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Specific Methyl Group Protonation for the Measurement of Pharmacophore-Specific Interligand NOE Interactions

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In the recent decades NMR spectroscopy has emerged as a powerful tool in the drug-discovery field. Several NMR-based methods have proved beneficial for the optimization of low-molecular-weight lead structures. Among those, techniques relying on the nuclear Overhauser effect (NOE) can provide information on pharmacophores at atomic resolution. The transferred-NOEs (tr-NOEs)[1] allow access to the bioactive conformation of ligands and can also be applied as a screening tool to identify the interaction of a small molecule with a macromolecular receptor. Recently, we have reported on the measurement of protein-mediated interligand NOEs (INPHARMA), which are observed for a mixture of two ligands binding competitively and weakly to the same binding pocket of a common target.[2–4] These interligand NOEs do not result from a direct magnetization transfer between the protons of the two ligands, since the small compounds, being competitive binders, never occupy the protein binding pocket at the same time. Rather, a spin diffusion process, mediated by the protons of the receptor-binding pocket, leads to cross-peaks between the two ligands. We proposed and demonstrated that these interligand NOEs can be used to determine the relative binding mode of two drug leads or, in favorable cases, even their absolute binding pose (INPHARMA approach).[3]

The principle underlying the methodology relies on the dependence of the INPHARMA NOEs on the protein environment and more specifically on the distances between the protons of each ligand and the protons of the receptor. Consequently, the size of the INPHARMA NOEs depends on the binding mode of each ligand to the receptor and a quantitative interpretation of such effects can be used to derive the binding modes. However, extensive spin diffusion among the receptor protons reduces the specificity of the INPHARMA NOE signals, and the pharmacophore signature has to be retrieved by theoretically simulating the effect of spin diffusion. Thus, the determination of the ligands' binding modes relies on computationally intensive calculations based on the full-relaxation matrix approach in presence of chemical exchange,[5] including the protons of the ligands and all protons of the receptor within a reasonable distance from the binding pocket typically 10 Å.[1,3]

In the daily workflow of drug discovery, it is desirable to extract information from the INPHARMA NOEs without need for the demanding full-relaxation matrix calculations. We reasoned that a clearer fingerprint of the binding modes of the two ligands to the receptor would be obtained from the values of the INPHARMA NOEs if spin diffusion inside the protein could be either switched off or considerably reduced. An efficient way to attenuate intramolecular spin-diffusion is to reduce the proton density in the receptor, for example, by deuteration. In the past few years, a robust protocol has been developed for bacterial expression of proteins, in which methyl groups are selectively protonated in a highly deuterated background.[6] Here we employ this method to investigate the effects on protein-mediated interligand NOEs of depleting the background protein proton density while maintaining protonated side chains of specific amino-acids types.[6] Furthermore, we explore the attractive possibility of employing the INPHARMA method in combination with selectively protonated receptors to extract structural information on the relative binding mode of

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two competitive ligands without need for the time-consuming full-relaxation matrix calculations.

The system under investigation consists of the catalytic subunit of cAMP-dependent protein kinase A—a monomeric protein of 353 amino acids. The protein kinase A (PKA) is a ubiquitous enzyme of known crystal structure,[7–9] which regulates a large variety of cellular processes including ion flux, cell death and gene transcription. The selected ligands were the core hinge binding fragments of two known ATP competitive kinase inhibitors L_A and L_B, whose binding site has been previously identified (Figure 1).

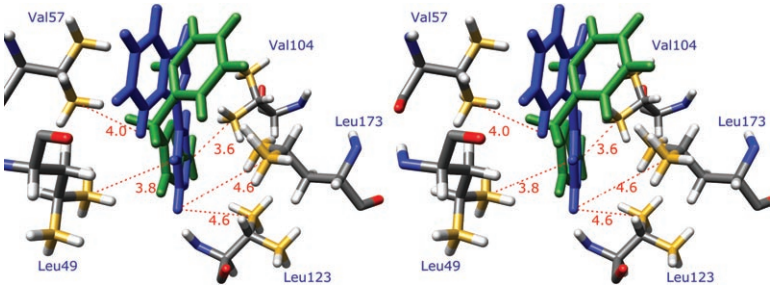

Figure 2. Overlap of the crystal structures of the PKA/L_A (3DNE.pdb) and PKA/L_B (3DND.pdb) complexes (stereo view). The Val and Leu amino acids are represented with sticks and the methyl groups are highlighted in gold. L_A is in green and L_B is in blue.

The catalytic unit of PKA was expressed in three different forms: i) fully protonated (FP), ii) protonated at specific amino acid side chains (SP), or iii) perdeuterated (PD). For the preparation of the specifically protonated protein, certain α-ketoacids can serve as precursors for a number of methyl-bearing amino acids for proteins over-expressed in minimal media.[10] In our case, among amino acids containing an aliphatic side chain, only valine and leucine are part of the binding pocket (Figure 2); therefore we chose α-ketoisovaleric acid as the precursor molecule for the production of deuterated proteins with protonation restricted to the Leuδ/Valγ positions. Typically, precursors with the desired labeling patterns were added to D_2O-based growth medium approximately 1 hour prior to induction of protein over-expression, with expression times kept reasonably short (3–4 h in our case) to maximize the incorporation levels.[11,12]

Three samples were prepared containing 450 μM of L_A and 150 μM of L_B. The 1:3 ratio in the concentration of the two ligands has been chosen to partially compensate for the different affinity of L_A and L_B for PKA ($K_{i,LA}/K_{i,LB} \cong 0.3$). The protein concentration was 25 μM for samples (ii, SP) and (iii, PD) (containing selectively protonated and perdeuterated protein, respectively), and 45 μM for sample (i, FP) (containing fully protonated protein). NOESY spectra were acquired on a 900 MHz spectrometer with a mixing time $\tau_m = 600$ ms (Figure 1a). The exact protein concentrations were determined by fitting the intensity of intraligand tr-NOEs. The absence of NOEs in the sample containing the perdeuterated protein confirms that the interligand NOEs do not originate from aggregation of the ligands or from simultaneous binding to neighboring binding pockets of the protein, but are indeed mediated by the protein protons.

Interligand NOEs are observed for samples containing either fully or specifically protonated protein (Figure 1), thus confirming that selective protonation of the Leuδ/Valγ positions in a perdeuterated background allows the measurement of protein mediated interligand NOEs. Figure 2 shows the binding pocket of PKA, in which L_A and L_B occupy alternately the same space. The short distances ($d < 5$ Å) of the methyl groups of Leu49, Val57, Val104, Val123 and Leu173 from both ligands ensure an efficient transfer of magnetization between the two ligands in the Leuδ/Valγ selectively protonated sample.

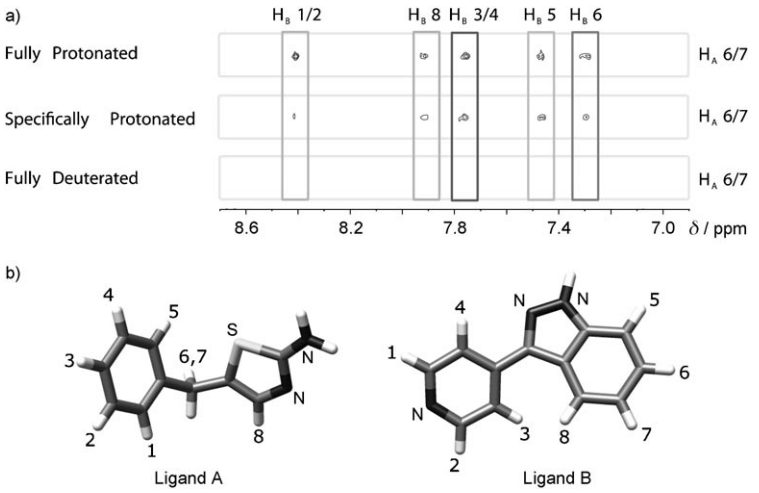

Figure 1. NOESY spectra for the measurement of interligand NOEs. a) Slices from 2D NOESY spectra at 3.72 ppm with a mixing time of 600 ms. Protein samples were expressed in a fully protonated form (upper slice); with the methyl group side chains of Leu and Val specifically protonated (middle slice); or fully deuterated (lower slice). b) Chemical structures of the ligands L_A and L_B with proton numbering.

A comparison of the theoretical values of the INPHARMA NOEs expected for a sample containing L_A (450 μM), L_B (150 μM) and either fully or selectively protonated PKA (25 μM) shows that a much lower intensity is expected for the interligand NOEs in the presence of selectively protonated protein, which is in agreement with the paucity of receptor protons available for the transfer (Figure 3). The data set in the presence of the selectively pro-

tonated protein has been calculated assuming a typical protonation efficiency of 80%, which, for the five Val and Leu amino acids present in the binding pocket, results in 16 species with population $p > 0.5\%$ (1 species with $\boldsymbol{p}=32\%$, 10 species with $\boldsymbol{p}=8.2\%$ and 5 species with $p=2\%$). All species with $p < 0.5\%$ were neglected in the calculations. The two theoretical data sets correlate reasonably well, with the exception of two large outliers: the interligand NOE between H_8 of L_A and $H_{1,2}$ of L_B and the NOE between $H_{1,3,5}$ of L_A and H_5 of L_B (Figure 3). The ratio of the interligand

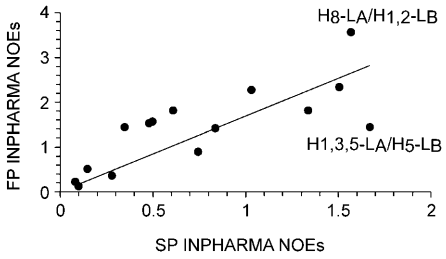

Figure 3. Correlation of the theoretical INPHARMA NOEs expected for a sample containing fully protonated PKA (FP) versus those expected using selective protonation (SP) of the Leuδ/Valγ positions. The NOEs are not normalized and are in arbitrary units.

NOEs between H_8-L_A and $H_{1,2}$-L_B for the SP vs the FP sample is lower than average; this reflects the depletion of the protons of Phe327, which are the closest to both proton H_8-L_A and protons $H_{1,2}$-L_B and therefore are most responsible for the corresponding protein-mediated exchange of magnetization between them (Figure S1). On the other hand, the same ratio for the NOE between $H_{1,3,5}$-L_A and H_5-L_B is higher than average, reflecting the elimination of additional spin-diffusion pathways inside the protein. The principal mediators in the transfer of magnetization between the $H_{1,3,5}$-L_A protons and the H_5-L_B proton are the methyl groups of Val57, which are not depleted in the selectively protonated protein. In general, it is expected that the ratio of the interligand NOE peaks observed for the SP sample versus those observed for the FP sample is high for the ligand protons close to Leuδ/Valγ methyl groups, due to the absence of dissipating spin-diffusion pathways, while it is lower for the ligand protons that are distant from the protonated methyl groups. This should result in a specific fingerprint of the binding mode of the ligands.

The higher specificity of the pharmacophore signature observable for the SP sample with respect to that of the FP sample prompted us to investigate the possibility of interpreting the INPHARMA NOEs in a semiquantitative way, namely without considering the effect of spin diffusion inside the protein. For this purpose we defined the parameter *D*, as an indicator of the distances of each ligand proton to the PKA protons:

$$D_{AB} = \sum_{\text{Leu,Val}} \sum_{i=1,2} d(H_A H_{\text{Met}_i})^{-6} \cdot \sum_{i=1,2} d(H_B H_{\text{Met}_i})^{-6}$$

where $d(H_{A/B}H_{Met})$ is the distance between proton $H_{A/B}$ of $L_{A/B}$ and the carbon of one of the two methyl groups of a particular valine or leucine amino acid. Whenever two or more ligand protons have a degenerate chemical shift the sum extends over all degenerate protons and the resulting indicator D_{AB} is divided by the number of degenerate protons. Only distances $d < 5$ Å are considered in the calculations. Our goal is to demonstrate that the interligand NOEs values correlate well with the indicator D_{AB} for each H_A–H_B NOE and that the quality of the fitting can be used to distinguish between different binding modes of the two ligands. In Figure 4 we plot the experimental INPHARMA NOEs between L_A and L_B versus the indicator *D* for five pairs of PKA/L_A and PKA/L_B complexes. The interligand NOEs were measured for the mixture of L_A, L_B and the selectively protonated PKA (SP) and were normalized with respect to the diagonal peak in ω_1. The pair of docking modes in panel a) corresponds to the crystal structures of both complexes and represents the "correct" docking poses. The pairs in panels b)–d) contain one wrong binding mode each and have been generated by rotating L_A or L_B by 180° around the *z* or *y* axis, as indicated in the figure. The difference in the quality of the correlation is striking, with the correct pair a) exhibiting $R=0.83$ for the linear correlation of the INPHARMA NOEs with the crude distance indicator *D*, while the incorrect pairs show very poor correlations ($R < 0.5$). From this result we conclude that a semiquantitative interpretation of the INPHARMA NOEs measured in presence of selectively protonated protein might be sufficient to discriminate between docking modes. Such semiquantitative interpretation could replace the lengthy full-relaxation matrix interpretation necessary when the INPHARMA NOEs are measured in the presence of fully protonated protein. Work is in progress in our laboratory to thoroughly characterize the discriminatory power of the correlation graphs of Figure 4 and to define the minimum change in the ligands orientations that can be differentiated with this approach. In addition, the specificity of the pharmacophore signature could be further improved by enlarging the SP INPHARMA NOE data-set. This can be achieved by means of multiple complementary schemes of selective protonation, targeting for example either the methyl groups or the aromatic side-chains.[13]

The possibility of discriminating between binding poses by correlation of the INPHARMA NOEs with the distance indicator *D*, or a similar function, opens the way to easy implementation of the INPHARMA NOEs in structure calculation programs. While simulation of spin diffusion at each step of structure calculation and comparison of theoretical and experimental NOEs is a computationally demanding task, due to the large size of the matrices involved, the correlation between the INPHARMA NOEs and a distance indicator could be easily translated into an energy term. In this way, the INPHARMA NOEs could be used to actively drive the docking protocol towards the correct binding poses of the two ligands. Our laboratory is actively exploring

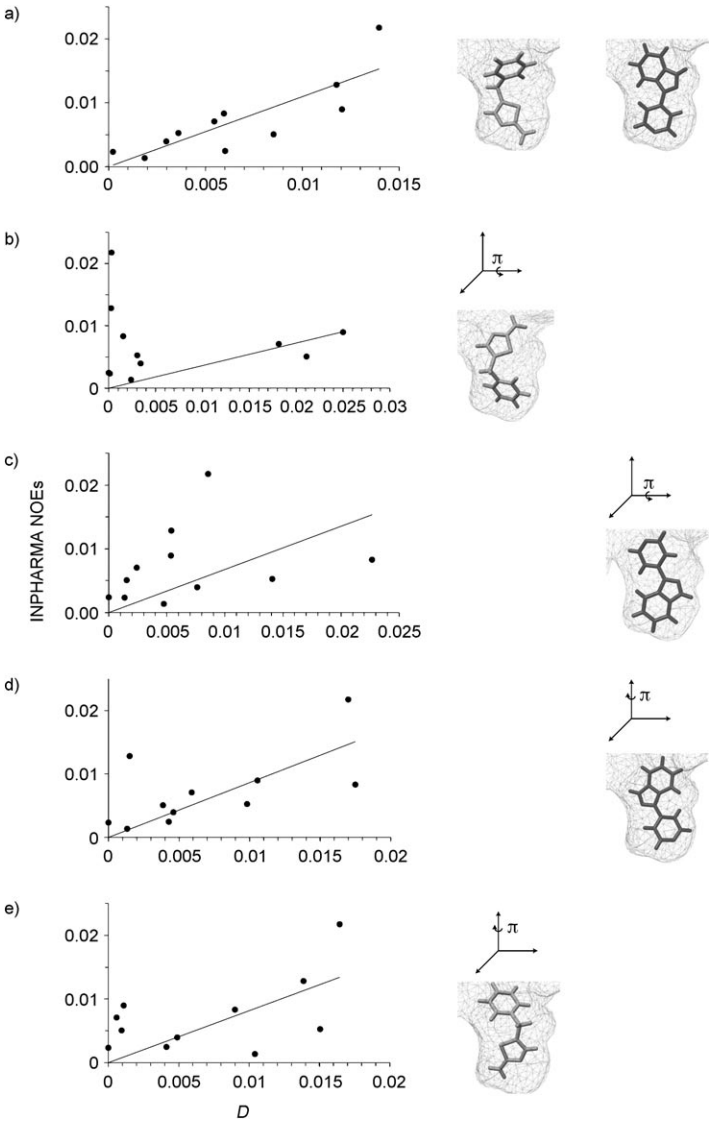

Figure 4. Correlation of the INPHARMA NOEs measured for a mixture of L_A, L_B and selectively protonated PKA (Leuδ/Valγ) with the distance indicator D calculated for five model-pairs of the PKA/L_A and PKA/L_B complexes. The first model-pair (panel a) corresponds to the crystal structures of the PKA/L_A and PKA/L_B complexes. Panels b)–d) present the correlations for model-pairs where one of the ligand has been rotated to an incorrect orientation in the binding pocket: b) L_A has been rotated by 180° around the y axis; c) L_B has been rotated by 180° around the y axis; d) L_B has been rotated by 180° around the z axis; d) L_A has been rotated by 180° around the z axis. The quality of the correlation is acceptable only for panel a) ($R=0.83$), while the next best correlation is seen in panel d) with $R=0.51$.

this intriguing and promising approach to the determination of receptor–ligand structures.

Experimental Section

Protein expression and purification for the NMR experiments: The catalytic subunit of Chinese hamster cAMP-dependent protein kinase A (PKA) was expressed and purified according to the published procedure.[14] In detail, the expression plasmid pETPKA, containing the coding sequence of the cAMP dependent protein kinase, was used for transformation of E. coli strain BL21 (DE3) competent cells. To produce the deuterated and specifically protonated protein samples, cells were initially grown on normal M9 media and then transferred to 10 mL expression cultures with increasing percentages of D_2O. The main culture in 100% D_2O was then inoculated with the adapted cells. Cells for the fully protonated protein sample were grown on LB medium. For the specifically protonated sample, the precursor with the desired labelling pattern (2-keto-3-methyl-butyrate) was added to the growth medium approximately one hour prior to induction. The protein, containing a histidine-tag, was purified on a Ni-NTA Fast-Flow column (Qiagen, Hilden Germany) following the manufacturer's recommendations. Cleavage of the His-tag was performed by adding 80 μL of Tev-protease (1 mg mL-1) and incubating overnight at room temperature. Further purification was achieved by anion-exchange chromatography with a SourceQ column. Finally, samples (10–15 mL) were dialyzed against 1 L NMR buffer (PBS-buffer, NaCl 150 mM).

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Keywords: drug discovery · NMR spectroscopy · pharmacophore mapping · selective protonation · specificity

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