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Evaluation of Solvent Accessibility Epitopes for Different Dehydrogenase Inhibitors

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Knowledge about the orientation of ligands or inhibitors bound to a protein is vital for the development of new drugs. It was recently shown that solvent accessibility epitopes for protein ligands can be mapped by transferring magnetization from water molecules to the ligand to derive the ligand orientation. This is based on the fact that NMR signals of ligands arising from magnetization transferred from solvent molecules via the protein have a different sign from those arising from direct magnetization transfer from bulk water. Herein we critically evaluate the applicability of solvent accessibility mapping to derive binding orientations for ligands of two dehydrogenases (AKR1C3 and HSD17β1) with very different binding pockets, including complexes in which the ligand is buried more deeply inside the protein. We also evaluate the possibility of using co-solvents, such as DMSO, for magnetization transfer.

Introduction

Structural knowledge has accelerated drug discovery by facilitating the design and optimization of new inhibitors in a more systematic manner by targeting specific protein–ligand interactions. While a complete structure determination of protein–ligand complexes by X-ray crystallography or NMR spectroscopy provides the most comprehensive information about the interaction, it is often too time consuming. Therefore, many other biophysical technologies such as fluorescence resonance energy transfer (FRET) and various NMR methods are employed to obtain more limited but specific structural information about the protein–ligand interaction. For proteins with a molecular weight <50 kDa, binding sites can usually be determined by monitoring chemical shift perturbations in NMR spectra obtained from isotopically labeled proteins.[1] Although this remains difficult for larger proteins, there is a range of NMR methods that can identify contact points of the ligand to the protein or the relative orientation of two ligands by observing the spectrum of the ligand. Most of these methods are based on the transfer of magnetization from the protein to the ligand[2–4] or between ligands.[5–7] WaterLOGSY has been employed for drug screening because it is one of the most sensitive NMR techniques used in the context of drug design although it has until recently not been used to map the ligand binding epitope. However, recent experiments show that a solvent accessibility epitope can be derived from such experiments which can be translated into the orientation of a ligand with respect to the protein or can be used to compare solvent accessibility epitopes for different ligands.[8] This experimental approach has been termed SALMON, Solvent Accessibility, Ligand binding and Mapping of ligand Orientation by NMR spectroscopy. This epitope information is to some degree complementary to that derived from the commonly used STD-NMR experiment[3,9] which maps ligand protons which directly contact the protein and should therefore not be solvent accessible. The mapping of the solvent-accessible ligand protons does not require isotopic labeling, works over a broad range of ligand affinities and is relatively sensitive allowing a fast qualitative assessment of ligand orientations. In addition, it allows the use of (co-)solvents such as DMSO as a starting point for the magnetization transfer. Herein we investigate the applicability of SALMON to measure solvent accessibilities of ligand protons for two different dehydrogenases, aldoketoreductase 1C type 3 (AKR1C3) and 17β-hydroxysteroid dehydrogenase type 1 (HSD17β1).

Results

Compared with the nitro reductase NQO2, which was recently studied to measure solvent accessibility,[8] the two hydroxysteroid dehydrogenases analyzed here have deeper binding pockets in which the ligands get buried. Because this limits the solvent accessibility of the ligand while increasing magnetization transfer from the protein the NOE mixing time was carefully adjusted to minimize spin diffusion across the ligand. Furthermore, STD experiments were measured for comparison and to evaluate the accuracy of the SALMON results. We also evaluate the use of DMSO rather than water for the magnetization transfer.

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AKR1C3

As a first example we studied binding of the non-steroidal anti-inflammatory indomethacin to aldoketoreductase 1C type 3 (AKR1C3).[10] AKR1C3 regulates both steroid and non-steroid signaling pathways. The 11-β-PGD_2 ketoreductase activity of AKR1C3 diverts PGD_2 towards 11-β-$PGF_2\alpha$ thereby diminishing the availability of 15,$\Delta^{12,14}PGJ_2$ to either activate PPARγ mediated signals for differentiation, apoptosis and inflammation or repress the constitutive expression of anti-apoptotic genes mediated by NF-κB activity. AKR1C3 is therefore an emerging target for the treatment of acute myeloid leukemia, Burkitt's lymphoma and chronic lymphocytic leukemia. For this study AKR1C3 was chosen because the structure of the indomethacin complex is known and can be used as a reference.

In the waterLOGSY spectrum of indomethacin depicted in Figure 1B the signal of the aromatic protons 14 and 18 is remarkably negative while all the other protons have a positive sign when a small amount of AKR is present (10 μM AKR; 1 mM indomethacin). The sign of the signals in waterLOGSY spectra depends primarily on the affinity of the ligand to the protein, which is in the low-micromolar range[10] and on the off-rate (k_{off}); ligands binding to the protein show an opposite sign from those which do not interact with the protein.[11] Small molecules that do not bind the protein with high affinity show the positive NOE (cross-relaxation rate $\sigma > 0$) typical for fast tumbling molecules arising from a direct magnetization transfer from bulk water molecules. For ligands interacting with the protein, the negative NOE of the protein is transferred to the ligand, resulting in positive signals if the rate of ligand release is fast on an NMR time scale and an excess of ligand is used. However, solvent-accessible protons in a protein-bound ligand can maintain a negative sign when spin diffusion across the ligand is low.

For AKR1C3 this effect is in excellent agreement with the solvent accessibility map calculated from the X-ray structure[10] depicted in Figure 2 which shows that the aromatic protons 14/18 and the methyl group 11 are solvent exposed. For H14/18 a negative signal is observed (Figure 1B) arising from a dominance of direct magnetization transfer from bulk solvent, whereas the methyl group 11 shows only a decreased intensity. This can be attributed to a balance between magnetization arising from the protein and direct solvent accessibility. H15/17 are covered by a loop of the protein and are therefore better protected against direct interaction with bulk solvent. The observed effect depends on the choice of sample temperature and NOE mixing time. The negative signal for H14/18 was observed for temperatures of 37–40°C and a mixing time of 125 ms where spin diffusion is small. When DMSO is used as the source of magnetization transfer the spectrum depicted in Figure 1C is observed. As for transfer from water, protons 14 and 18 appear negative, whereas the signal of the methyl group 11 is positive and shows a large intensity. Buildup curves are shown in Supporting Information figures S3 and S4.

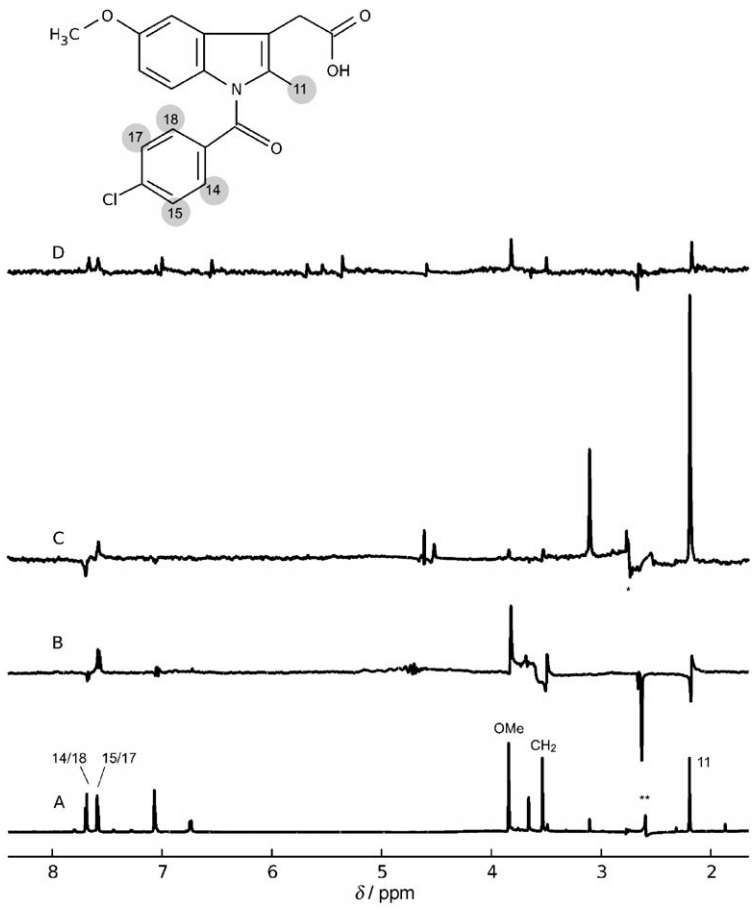

Figure 1. NMR spectra of indomethacin with a small amount of AKR1C3 present: A) 1D spectrum, B) waterLOGSY spectrum with magnetization transfer from water, C) waterLOGSY spectrum with magnetization transfer from DMSO, D) STD spectrum. * Residual signal of DMSO after dual band excitation sculpting[22] solvent suppression; assignments were taken from reference [25].

These results were compared with saturation transfer difference (STD) spectra. In the STD spectrum (Figure 1D) the two doublets for protons 14/18 and 15/17 show a similar intensity, whereas the signal for the methyl group 11 is comparably large. This lends itself to an interpretation where the methyl group shows much better contact to the protein than the protons 14/18 and 15/17. However, the solvent accessibility analysis suggests that protons 14/17 are solvent exposed, while the protons 15/18 are more shielded from solvent by a loop of the protein. This apparent contradiction is easily resolved by considering the experimental conditions of both experiments: the waterLOGSY spectrum is recorded with a small mixing time and is therefore more sensitive to small differences in solvent accessibility whereas the STD experiment requires a long mixing time causing spin diffusion over the ligand. In fact, the waterLOGSY spectrum recorded with a long mixing time is similar to the STD spectrum. Moreover, the solvent accessibility of the methyl group 11 is only observed for SALMON using water excitation but not for DMSO excitation. This must either be the consequence of a DMSO mol-

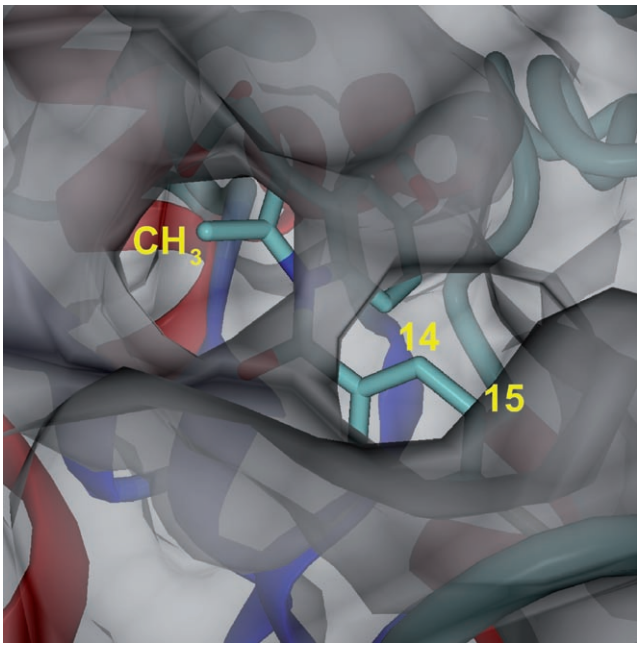

Figure 2. Solvent accessibility surface of indomethacin in AKR1C3 calculated from the crystal structure of the complex[10] showing a large pocket for the aromatic proton 14 and a smaller pocket for the methyl group 11. The solvent accessibility surface was calculated using the molecular surface tools by Michael Sanner assuming a solvent radius of 1.5 Å.

ecule bound to the protein in close proximity to the indomethacin ligand or of the small size of the cavity in the solvent accessibility surface which allows water but not DMSO to access the methyl group of the ligand. The STD spectrum shows that the magnetization transfer via the protein is very efficient for methyl group 11 in H_2O/D_2O/DMSO 80:10:10, but shows decreased intensity in $H_2O/D_2O/[D_6]$DMSO 80:10:10 (not shown). This result is consistent with the assumption of a bound DMSO molecule near the methyl group. The methoxy signal of indomethacin (at 3.84 ppm) as well as the CH_2 signal (3.53 ppm) and the methyl 11 (2.19 ppm) seem to be subject to artifacts in all waterLOGSY spectra and showed significant changes in intensity for different solvent compositions in STD spectra. This could be due to the vicinity of very polar groups (the methoxy or carboxyl group) and is probably related to slow exchange in indomethacin initiated by the interaction with the protein (c.f. Supporting Information figures S4 and S5).

The example of the AKR1C3–indomethacin complex with a known structure is useful to evaluate the accuracy of the SALMON and STD analysis and demonstrates the complementarity of SALMON and STD but can also yield additional information on ligand binding owing to the lower mixing times in the waterLOGSY experiment. The ligand orientation suggested by the SALMON analysis is in good agreement with the solvent accessibility calculated for the structure of the complex.

HSD17β1

The SALMON approach has been further explored for complexes of the 17β-hydroxysteroid dehydrogenase type 1 (HSD17β1). HSD17β1 is responsible for high levels of estradiol in malignant breast cells[12–14] stimulating the growth of the tumor and is therefore an important drug target for the treatment of breast cancer.[12–14] HSD17β1 is a homodimer with a molecular weight of 70 kDa and is, like many other dehydrogenases, not readily amenable to NMR spectroscopy, especially as the protein aggregates at higher concentrations. To obtain soluble protein we used a construct with an N-terminal protein G (B1 domain; GB1)[15] which formed soluble aggregates and showed activity in assays measuring the reduction of estrone, even at high concentrations of DMSO (up to 40%). The addition of DMSO was necessary because typical substrates of HSD17β1 (estrone, estradiol) and many inhibitors are not sufficiently water soluble to obtain NMR spectra. Compared with AKR1C3, the binding pocket of HSD17β1 for the substrates estrone and estradiol is deeply buried inside the protein and therefore less solvent exposed.

For HSD17β1 signals in waterLOGSY spectra for the flavonoid inhibitors apigenin and luteolin with micromolar IC_{50} values[16,17] were never negative, but intensities were in some cases significantly decreased relative to one-dimensional spectra. Furthermore, intensity patterns were different for magnetization transfer from water versus DMSO. The waterLOGSY signal intensity depends primarily on the interaction between the solvent and the protein. This was studied by comparing magnetization transfer between different solvent compositions: a) from H_2O in H_2O/DMSO/D_2O (50:40:10), b) from DMSO in H_2O/DMSO/D_2O (50:40:10), and c) from DMSO in D_2O/DMSO (60:40). The results for these transfers are summarized in Table 1. For luteolin as an inhibitor a significant difference between the protons in the two ring systems was observed. Apigenin yields the same epitope although somewhat less pronounced. The transfer from DMSO depends significantly on the presence of H_2O; in pure D_2O (c) we observe a more pronounced epitope for both, luteolin and apigenin (Table 1). This may be attributed to spin-diffusion via OH groups or to changes in the hydrogen-bonding network in the solvation shell of the protein. As a consequence, the interaction of DMSO with the protein surface can be more efficient in D_2O where weaker hydrogen bonds between D_2O and protein hydrogen atoms are formed.[18] The use of D_2O will also prevent against loss of magnetization via exchangeable protons of the protein to bulk H_2O. The crystal structure shows a number of water molecules close to indomethacin and one DMSO molecule close to the ligand binding site (c.f. Supporting Information figures S6 and S7). Interestingly H3 of luteolin but not apigenin becomes significantly more solvent accessible in (c) relative to (b) and (a).

The similarity of the epitope between luteolin and apigenin (Figure 3) suggests that the two inhibitors bind HSD17β1 in the same orientation as would be expected. However, the epitope observed for estradiol is different from that for apigenin and luteolin if the A and B rings of the steroid are identified

Table 1. Relative signal intensities[a] in waterLOGSY spectra of flavonoid inhibitors and of estradiol in the presence of HSD17β1.

^{1}H δ [ppm]	Atom[b]	(1)[c]	(2)[d]	(3)[e]	STD
Estradiol					
7.3	1	0.56	0.59	0.56	0.68
6.7	2	1.0	0.84	0.88	1.0
6.65	4	0.93	1.0	1.0	0.92
2.4	6	0.21	N/A	N/A	0.72
2.2	16	0.31	N/A	0.32	0.97
2.1	11	0.31	N/A	0.32	0.93
1.9	9	0.31	0.43	0.32	0.63
1.7	16, 15, 7	0.46	0.61	0.44	0.62
1.55	12	0.46	0.61	0.44	0.63
1.47	15	0.46	0.61	0.45	0.62
1.37	14	0.46	0.61	0.44	0.62
0.8	18	0.46	0.45	0.44	1.21
Luteolin					
7.62	2′	0.68	0.77	0.51	0.65
7.59	4′	0.72	0.70	0.57	0.54
7.1	3′	0.68	0.63	0.51	0.66
6.8	3	0.59	0.67	0.31	0.79
6.7	8	1.0	0.94	1.0	0.91
6.4	6	1.0	1.0	1.0	1.0
Apigenin					
8.05	2′	0.60	0.74	0.62	0.63
7.05	3′	0.62	0.73	0.67	0.85
6.7	3	0.59	0.78	0.61	1.03
6.6	8	1.0	1.0	1.0	0.97
6.4	6	1.0	0.94	0.94	1.0

[a] Intensities of signals in waterLOGSY spectra were compared with those in the 1D spectrum; therefore, low intensities represent high solvent accessibility. [b] Assignments as shown in Figure 3. [c] H_2O/DMSO/D_2O 50:40:10, magnetization transfer started from H_2O. [d] H_2O/DMSO/D_2O 50:40:10, magnetization transfer from DMSO. [e] DMSO/D_2O 40:60, magnetization transfer from DMSO.

with the benzo-γ-pyrone ring of the flavonoids. For estradiol the solvent accessibility is generally lower for the aromatic A ring than for the rest of the molecule (Table 1). This is in good agreement with the solvent-accessible surface calculated from the crystal structure of the HSD17β1–estradiol complex which shows the D ring at the solvent exposed end of a tunnel into the protein[19] (figure S1 in the Supporting Information). The result for apigenin is also supported by previous pharmacophore modeling which suggested the same orientation as derived from the SALMON analysis.[20] STD experiments provide complementary information. For most signals showing low solvent accessibility, intensities are high in STD spectra indicative of good contact to the protein. However, we also observe some differences between SALMON and STD. For estradiol protons 16 and 18 show remarkably high STD intensities although they seem to be solvent accessible. This reflects the fact that these protons experience polarization transfer from the protein in the STD experiment as the only contribution to signal intensity whereas the shorter mixing time in the waterLOGSY experiment shifts the balance towards magnetization transfer from the solvent. The STD results for luteolin and apigenin are largely consistent with the SALMON results except for proton 3, which shows a large solvent accessibility for luteolin but also an efficient magnetization transfer from the protein in the STD experiment with identical absolute values for the transfer in both compounds, luteolin and apigenin. It should be noted that all protons for which STD and SALMON showed different behavior are located at the edge of water accessible regions and therefore reflect the different balance between polarization transfer from the protein and solvent accessibility.

Discussion

To evaluate the validity of the SALMON approach it is useful to estimate the required residence time of solvent molecules to generate a significant magnetization transfer from bulk water directly to the ligand versus transfer through the protein. While the cross-relaxation rate σ has positive values for small molecules it adopts negative values for larger molecules. Considering that the polarization transfer to the ligand arises from water interacting with the protein, water molecules must have a significant residence time on the protein. Depending on the size of the protein the water residence time must be between 0.1 and 1 ns to develop a negative NOE ($\sigma < 0$)[4,21] (Figure 4). The magnetization

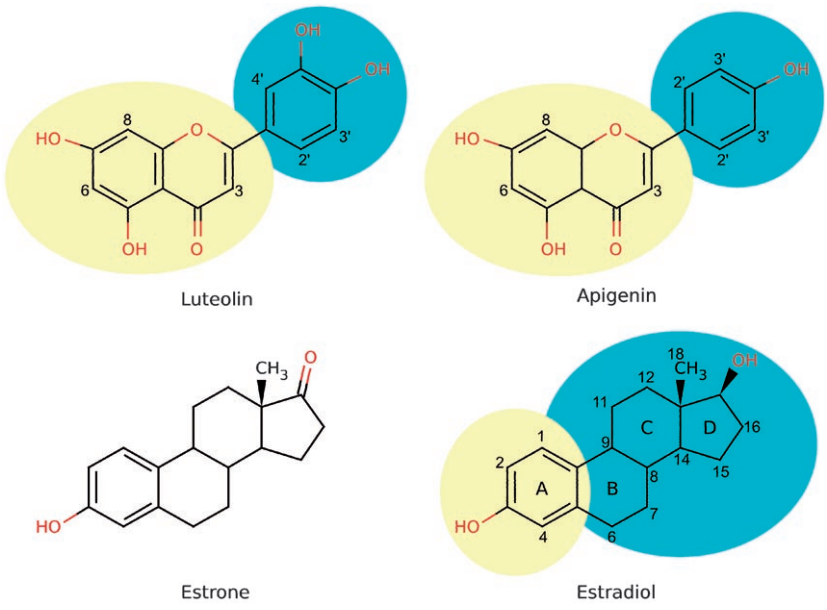

Figure 3. Structures of the flavonoids luteolin and apigenin and the hormones estradiol and estrone, showing binding epitopes for HSD17β1 observed in waterLOGSY spectra. Protons in the region marked in cyan showed higher solvent accessibility than protons in the region marked in yellow. See Table 1 for details.

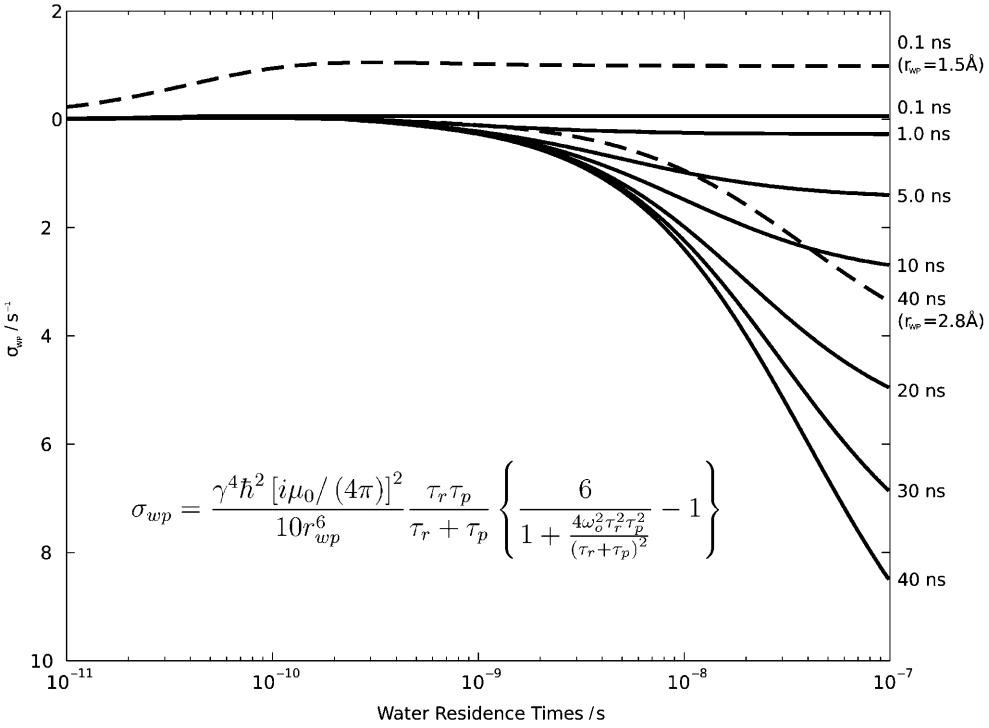

Figure 4. Simulation of magnetization transfer efficiency depending on water residence times τ_r, protein autocorrelation times τ_c, and water–ligand distances r_{WP}.[4,21] Protein correlation times τ_p were varied between 100 ps and 40 ns, and are shown on the right. The water–ligand distance was set to 2.4 Å[4] unless stated otherwise (dashed lines for 40 ns, 2.8 Å and 0.1 ns, 1.5 Å). γ is the gyromagnetic ratio of protons and μ_0 is the Bohr magneton.

transfer becomes more efficient with increasing protein size owing to faster spin diffusion and for closer proximity of the water molecules as shown in Figure 4. For short mixing times where spin diffusion is limited and for an excess of ligand, two components contribute to the NMR signal of a ligand: 1) a negative signal ($\sigma>0$) arising from the free ligand and 2) a positive signal ($\sigma<0$) arising from polarization originating from the protein. Differences within one ligand molecule may arise if some protons do not experience efficient polarization transfer from the protein or if bulk water molecules can access the bound ligand. The balance between the size of the polarization depends on the distance to water molecules which may be large in proteins but is likely to be smaller for the free ligand (Figure 4, dashed curve for $\tau_c=0.1$ ns and a distance of 1.5 Å). In the most extreme case this causes signals to remain negative for parts of the molecule while others change sign. In the presence of spin diffusion this effect may be less pronounced causing all signals to show a positive sign with decreased intensity for those signals which experience a residual contribution with $\sigma>0$ arising from the free ligand. It should be noted that the SALMON effect does not depend on the scaling of polarization transfer from the protein to different sites of the ligand but rather on the balance between polarization transfer from the protein versus from bulk solvent.

Exchangeable protons on the protein surface will facilitate magnetization transfer to the ligand via the protein. However, exchangeable protons on the ligand will cause positive signals (same sign as NOE with $\sigma<0$) in waterLOGSY spectra and neighboring protons may be affected by this as has recently been observed for HC protons next to NH protons in tryptophan side chains (unpublished results). In such cases a reference spectrum of the free ligand helps to deconvolute NOEs from exchange signals (see for example the NH_2 protons of CB1954).

Conclusions

The examples shown here demonstrate that the SALMON approach is suitable to identify solvent-accessible regions of ligands bound to proteins, even for ligands which are buried in the protein as long as a small part of the ligand remains solvent accessible. The results obtained from the SALMON analysis for the AKR1C3–indomethacin complex with known structure demonstrates that solvent accessibility can be translated into ligand orientations because only indomethacin oriented with the H14/18 and methyl 11 pointing away from the core of the protein is in agreement with the observed signal intensities. This was verified by calculating solvent accessibility surfaces from an existing crystal structure of the AKR1C3–indomethacin complex. The more polar methoxy group showed inconsistencies in signal intensities for both STD and SALMON.

Compared with AKR1C3, the ligand binding site for HSD17β1 is buried more deeply inside the protein. This binding mode highlights the limitations of the method; the effect is somewhat weaker for atom 14 relative to surrounding atoms because polarization transfer from the protein is low as shown by the STD spectrum. Nevertheless, the SALMON approach yields a solvent accessibility epitope which is in agreement with an existing structure for estradiol and which is consistent between ligands with similar flavonoid structure.

The use of magnetization transfer originating from DMSO also helped to identify relative binding orientations of estradiol and two flavonoid inhibitors (apigenin and luteolin). This approach takes advantage of the need to use co-solvents such as DMSO to dissolve inhibitors that are not readily soluble in water. Binding sites of different polarity may require different types of additives such as acetonitrile, DMF or even lipids depending on the protein–ligand complex under investigation. Co-solvent concentrations of 5% are sufficient for magnetization transfer from the co-solvent.

The information content of the SALMON analysis is to some degree complementary to that obtained from STD-NMR,[3] but

differs in places where a shorter mixing time can prevent against magnetization transfer from the protein. This study demonstrates that the SALMON approach can be applied to map protein ligand binding sites which bury large parts of the ligand as long as some fraction of the ligand remains solvent accessible. Identifying solvent-accessible parts of a ligand may help the design of new compounds even in the absence of crystal structures for individual protein–ligand complexes, although the existence of a protein structure will facilitate the interpretation of such data.

Experimental Section

AKR1C3 was expressed and purified as described before.[10] HSD17β1 was expressed with an N-terminal His_6-tagged protein G (B1 domain) (GB1).[14] GB1-HSD17β1 was expressed in BL21-DE3 cells by induction with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 16 h at 20 °C. Cells were resuspended in 50 mM sodium phosphate, 1 mM 1,4-dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 20% glycerol, pH 7.5 including protease inhibitors (complete, Roche) and subsequently lysed using a French press at 2.1×10^5 kPa. The lysate was spun in a Beckmann centrifuge, JA 25.50 rotor, for 1 h at 24000 rpm. The supernatant was applied to a 20-mL anion-exchange Q Sepharose HP column (Amersham). Extensive washing with 100 mL resuspension buffer was performed, followed by a slow elution using a gradient over 240 mL from 0 to 600 mM NaCl. GB1-HSD17β1 eluted at approximately 300 mM. Further purification was performed by size-exclusion chromatography using a Superdex 200 26/60 pg (Amersham). GB1-HSD17β1 eluted at an early stage suggesting a molecular size larger than expected for the dimer. Circular dichroism showed that the protein was properly folded. NMR and UV/Vis analyses show that the protein converts estrone into estradiol in the presence of 40% DMSO at pH 6.8.

Protein concentrations were 10–20 μM for AKR1C3 and 5 μM for HSD17β1. Ligand concentrations were 1 mM for indomethacin and 500 μM for luteolin, apigenin, and estradiol. The NMR data for HSD17β1 were acquired at 25 °C and pH 6.8 using 30 mM sodium phosphate buffer and 40% DMSO, whereas experiments for AKR1C3 were carried out at 40 °C at pH 8.1 using only 5% DMSO and a buffer containing 50 mM sodium phosphate and 300 mM NaCl.

All spectra were acquired on a Varian INOVA 800 MHz spectrometer equipped with a cryogenically cooled HCN probe. Spectra were typically recorded with 128 transients. Excitation sculpting[22] was used with polychromatic pulses for simultaneous suppression of water, DMSO and glycerol (the latter only in the case of HSD17β1). To decrease difference artifacts for SALMON we used a modified waterLOGSY pulse sequence which employs gradient encoding for the selection of NOE transfer pathways rather than difference spectra which are more prone to create artifacts.[21] The NOE mixing time was adjusted to obtain the most pronounced solvent accessibility epitope. WaterLOGSY spectra were phase corrected to show positive signals for resonances that experience the negative NOE of the protein. STD-NMR spectra were recorded using a train of 100 low flip angle Gaussian pulses (B_1 field strength was 65 Hz, using a pulse length of 22 ms) to saturate methyl groups in the protein at −2 ppm, while an irradiation frequency of +23.4 ppm was used for off-resonance irradiation. Excitation sculpting was used with polychromatic pulses for simultaneous suppression of water, DMSO and glycerol (the latter only in the case of HSD17β1).

Solvent-accessible surfaces were calculated using the molecular surface tools by Sanner.[23] Figure of solvent accessibility surfaces were generated using the DINO3D software (http://www.dino3-d.org). All spectra were analyzed using NMRlab.[24] Intensity ratios were calculated as ratios between the waterLOGSY signal and the signal from a normal ^{1}H spectrum, the highest value was set to 1.0.

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Keywords: epitope mapping · NMR spectroscopy · pharmacophore mapping · protein–ligand interactions

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