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NMR evaluation of adipocyte fatty acid binding protein (aP2) with *R*- and *S*-ibuprofen

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Abstract—We have examined global chemical shift perturbations for aP2 ligand complexes and compared these with amide temperature coefficients. Hydrogen bond potential was monitored by amide chemical shift's temperature coefficient. Based on this information, we propose that the binding energy contribution can be spread out to multiple distant residues. For aP2, the ability of the receptor protein to change its hydrogen bond interactions in the β-strands to accommodate different ligand scaffolds seems to make this receptor difficult for structure based drug design. While stabilization energy differential on hydrogen bonds is likely to be small for individual residues, the accumulative effect on multiple hydrogen bonds may have a dramatic impact on ligand affinity.

1. Introduction

Structure based drug design is showing great promise and is playing an essential role in the development of new chemical entities.[1] However, de novo rational drug design still struggles with the ability to predict ligand affinity due to the influence of conformational and dynamic changes resulting from ligand binding. This is especially true when the structural perturbations are distal from the ligand-binding site. Experimental results would be of great help in the ability of computer aided drug design to make more accurate predictions. It has been shown that NMR spectroscopy has the unequaled power of detecting very weak interactions between target biomolecules and ligands and is a crucial spectroscopic method to monitor ligand binding and the influence of this binding on the structure and dynamics of remote regions in a protein.[2–6] In particular, the NMR chemical shift perturbation effect obtained from ligand to target interactions reflects changes in the chemical and structural environment on ligand binding and has been well exploited in drug design.[7–11] Knowledge of subtle conformational and dynamical changes at regions remote from the ligand-binding pocket upon inhibitor binding is important to define the basis of differential affinities within ligand–protein systems and may allow the development of unique strategies for drug discovery.

Based on the HSQC chemical shift perturbation method,[7] the binding interface of a protein can be mapped. An underlying assumption in the interpretation of data obtained by this approach is that the ligand-binding pocket and the general molecular structure is rigid and will not drastically change upon ligand binding. However, proteins are not always static and may make structural accommodations. There are many instances where this appears to be the case,[12,13] and the crystal structure or detailed NMR structure may detect the structural change and aid the understanding of the binding epitope. In some cases the ligand binding which may cause a local variation can propagate to remote residues so that the impact is minimized.

These small adjustments are detectable as NMR chemical shift perturbations. When this occurs, the binding pocket may not be well mapped as chemical shift changes are seen over a wide area of the protein and misjudgement of the ligand–target binding energies may occur and lead to non-additive and confusing structure activity relationships.

Adipocyte lipid-binding protein, also known as aP2 or FABP4, is an important contributor to the maintenance

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of systemic glucose metabolism and adipocyte biology.[14–19] A deficiency of aP2 protects against the development of hyperinsulinemia and insulin resistance in genetic and diet induced obesity in mice and therefore it has been postulated that aP2 represents a vital link in the pathogenesis of diabetes mellitus and obesity.[20] aP2 is a potential pharmaceutical target, as its absence decreased hypercholesterolemia-induced atherosclerosis and in aP2 knock-out mice it was shown that they were less diabetic compared with wild-type controls.[5]

Structurally, aP2 built from 10 anti parallel β-strands that are connected via tight turns or α-helices has been described as a β-clam shell with the ligand binding at an internal site.[21–28] Crystal structures of apo and natural and synthetic ligand bound aP2s have been reported.[21,24–29] These structures have played an important role in understanding the entry and exit of the ligand to the active site. NMR has played a critical part in the studies of these dynamic processes.

In principle chemical shifts and chemical shift perturbation differences can also be interpreted in structural terms.[30–33] While many empirical relationships exist to 'calculate' chemical shifts, the precise relationship between the chemical shift perturbation and structural change is not fully understood. The chemical shift is affected by several conformational parameters, including local backbone dihedral angles, side chain dihedral angles, and hydrogen bonding.[30,33–35] Specifically, the amide ^{1}H chemical shift is governed by the combined effects of local secondary structure, hydrogen bonding, and long-range ring-current interactions.[36] Heteronuclear shielding is affected by backbone dihedral angles of neighboring residues, the nature and conformation of the alkyl substituents of the immediately preceding residue, the side chain conformation of the residue, the effects of hydrogen bonding to ^{15}N, and the effects of longer-range electrostatic fields.[37] In this regard, the chemical shift perturbation at distal sites induced by the binding of *R*- and *S*-ibuprofen to aP2 was studied.

2. Results

A cursory look at the X-ray structures for the aP2 complexes in the PDB (1TOU, 1TOW, and 2HNX) shows little effect on protein structure for different ligands and suggests a rather rigid binding pocket and no changes in the peripheral backbone positions. However, NMR ^{1}H–^{15}N HSQC data fail to generate a clear ligand-binding pocket map by chemical shifts. Instead, chemical shift perturbation takes place for a large number of residues, suggesting subtle global 'structure' reorganization upon ligand binding.

Our initial studies on aP2 were based on the interaction of 30 very diverse ligands and the ^{1}H–^{15}N HSQC data are shown in Figure 1. Ligand titrations were performed to insure that the aP2 complex chemical shifts did not depend on ligand concentration, that is, the protein is fully bound. Since in many studies ligands have limited solubility, the addition of DMSO is often used to solubilize the ligand. In our case, the addition of DMSO did not induce chemical shift changes in the HSQC data of the apo protein indicating the shifts observed are due only to the ligand added. The strategy for the assignment of the chemical shift perturbations of the aP2 complexes was that there would be the minimal deviation between each position of the free and the complexed peak in the ^{1}H–^{15}N HSQC spectra. This is a reasonable assumption and does not cause ambiguity except in the highly crowded regions of the spectrum.[38,39] Over 95% of the backbone assignments for aP2/ligand complexes could be assigned by this method. Those resonances in crowded parts of the spectrum or where uncertainty might occur were checked with TOCSY[40] data.

The chemical shift perturbation maps for a series of ligands binding to aP2 are shown in Figure 1. While the spectra resemble each other in terms of peak distribution, it is obvious that a larger number of amide peaks are affected by ligand binding than would be expected based on a model where only regions adjacent to the bound inhibitor would be perturbed. Furthermore, no obvious pattern of shifting was obtained. X-ray studies on many of these ligands showed them to be bound in analogous regions of the active site. (unpublished results) So in order to better understand the molecular recognition events in aP2, it was decided to backtrack from the set of diverse ligands to a more concise data set based on two molecules that differed at only a single center, *R*- and *S*-ibuprofen. This relatively small modification in the molecular structure of the ligand (albeit enantiomers that might induce changes in protein conformation) should more readily allow for the interpretation of the measured NMR parameters in a systematic manner. The chemical shift data for apo, and *R*- and *S*-ibuprofen bound proteins are given in Supplementary Table 1.

Figure 2 shows the chemical shift perturbation patterns observed for the two ligands, *R*- and *S*-ibuprofen. As was seen for the larger and more diverse data set of compounds, the chemical shift perturbations observed are spread throughout the protein structure in a manner similar to that seen in Figure 1. This result suggests that the structure of the local perturbation caused by the ligand plays a minor role in the fact that there are distal chemical shift perturbations.

In order to gain further insight into the mechanism of the chemical shift perturbations we measured the temperature dependence of the chemical shift for the amide protons and nitrogens. The temperature coefficients were obtained by measuring the chemical shift position on varying the temperature from 283 to 317 K. The values for the temperature coefficient ranged from −11 to −3.5 ppb/K (the full data set can be found in Supplementary Table 2). The shifts were seen to be monotonic and linear over the temperature range and representative temperature coefficient plots for residues 73V and 97W are shown in Figure 3.

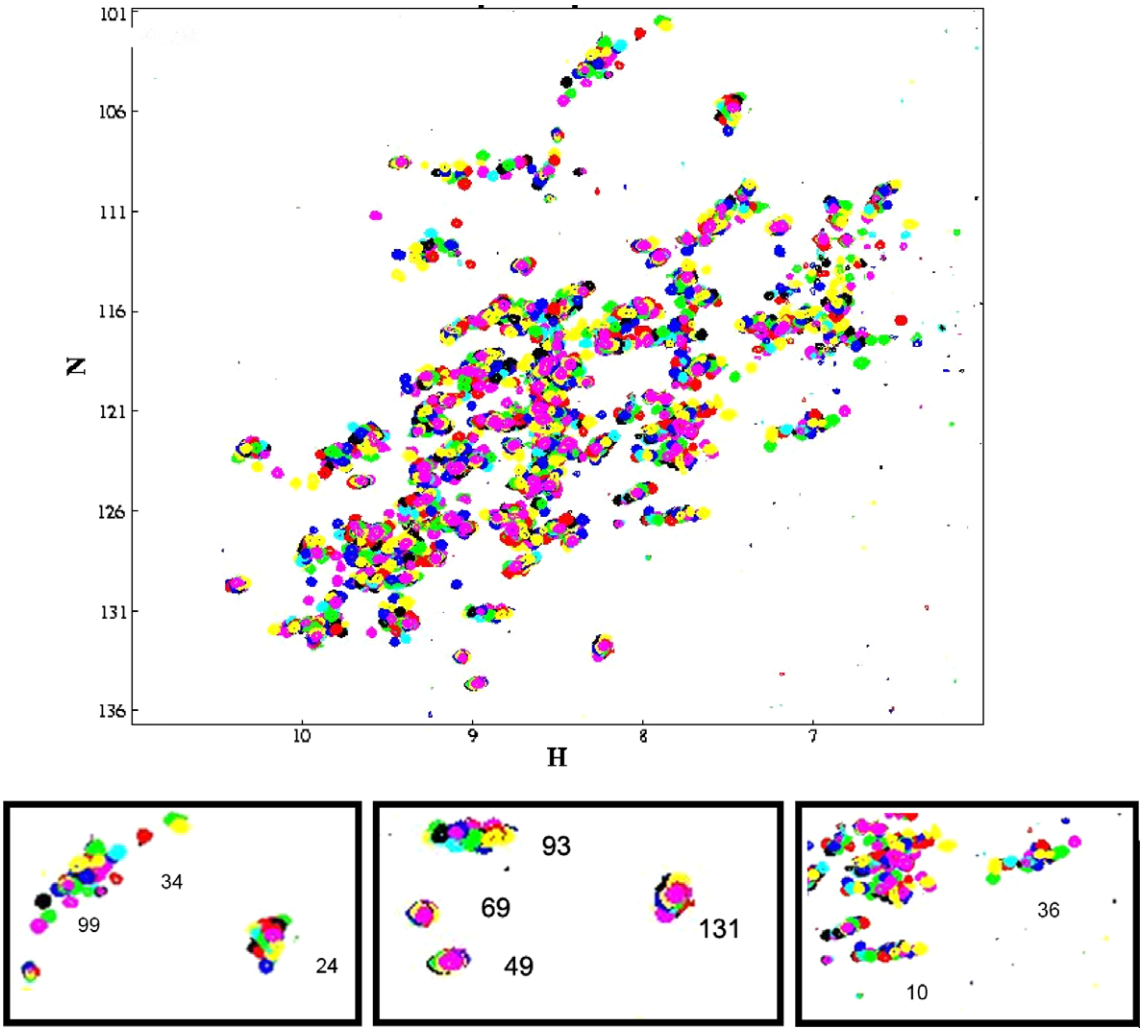
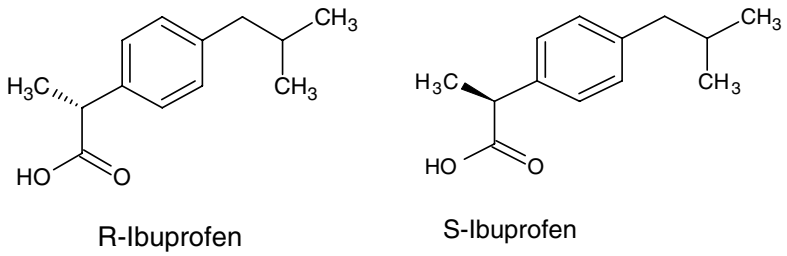


Figure 1. $^{1}H–^{15}N$ HSQC Data for 30 diverse ligands binding to aP2. Insets show detailed chemical shift perturbations and assignments.

3. Discussion

The $^{1}H–^{15}N$-correlation spectrum of a protein and its specific pattern can be regarded as a fingerprint of its structure. In proteins, amide NH chemical shift perturbations serve as direct probes by which evaluation of both local and proximal interactions can be evaluated. These perturbations can be caused by chemical shielding upon ligand binding, side chain movements or altered hydrogen bond geometries. Therefore, the perturbations of ^{15}N and HN chemical shifts of a protein upon complexation with a ligand are a qualitative tool for mapping of residues involved in binding sites and/or identifying conformational rearrangements. However, chemical shift effects induced by ligand binding are difficult to interpret directly in structural terms. A comparative analysis for different ligands allows for a better understanding of the effects. This is especially true if one wants to investigate chemical shift changes at distal protein regions. For example the chemical shift perturbation comparison between the apo state and *R*- or *S*-ibuprofen bound protein shows large differences in the helices (i.e., large difference in structure). In the same comparison for R versus S bound forms this effect is substantially mitigated suggesting the structures are more similar (Supplementary data).

When the chemical shift perturbation differences for the amide protons induced by *R*- and *S*-ibuprofen are plotted onto the X-ray structure of aP2 an interesting pattern is revealed. As observed in Figure 4, chemical shift variations between the two ligands are seen extensively throughout the backbone and seem to occur at regular intervals. For the β sheets similar trends are seen for apo versus *R*- or *S*-ibuprofen. See Supplementary Figure 1. It appears that the chemical shift information is communicated from one β-strand to another, in a 'molecular wave' pattern. The chemical shift perturbation results shown here are not unlike that reported for wild-type IFABP where it was found that the mutation of a single residues propagated throughout the molecule and this effect can be measured by the $^{1}H–^{15}N$ HSQC NMR spectrum.[41] Based on this observation, it is tempting to propose that the ligand induces adjustments in hydrogen bonds across the β-sheet structure to overcome what may be unfavorable entropic contributions to binding. This is similar to that observed for SH2 proteins where strong evidence was provided that



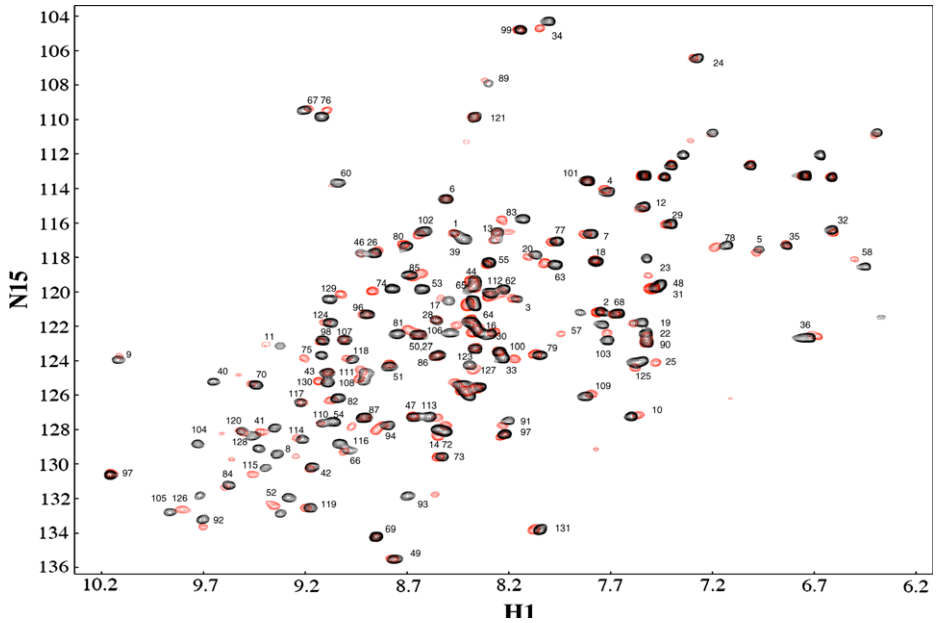


Figure 2. Chemical shift perturbation patterns observed for *R* (red) and *S* (black) ibuprofen. HSQC assignments are represented by residue numbers.

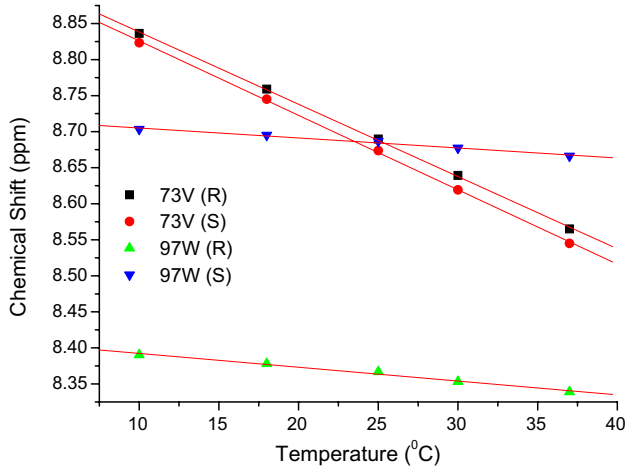


Figure 3. Temperature dependence plot for typical NH protons in aP2.

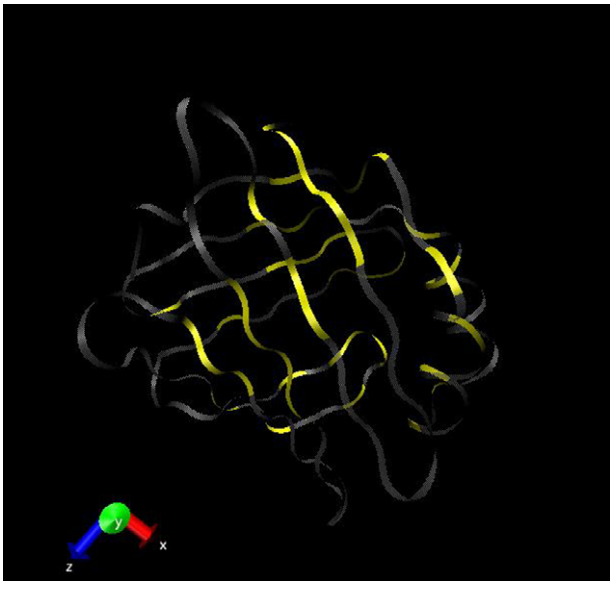


Figure 4. Representation of the chemical shift changes, plotted in yellow ribbon, between *R*- and *S*-ibuprofen binding mapped on the structure of aP2.

the observed dynamics in the β-sheet involved correlated motions between hydrogen bonded peptide planes and alternating exposure of amino acid side chains along the strands. [42] These correlated dynamics across the β-sheet were stabilized by the hydrophobic core.

The chemical shift perturbation changes appear to be much more sensitive than coordinate displacements seen in the crystal structures. In general, crystal structure comparisons are limited by the atomic coordinate precision, typically on the order of 0.1–0.3 angstroms for a good structure and by the fact that many new structures are solved by molecular replacement strategies.[43,44]

Often, it is not possible to distinguish perturbations caused by direct contacts across the intramolecular interface from perturbations caused by a conformational change associated with binding.[45–47] While open to interpretation, the present results suggest that the more intense and more broadly distributed perturbations are a reflection of changes in the intramolecular hydrogen bonds beyond the binding interface.

The cumulative chemical shift perturbation for remote residues is at least as large as for the residues in direct contact (within 5 Å from ligand), suggesting the driving force ($-\Delta G$) for aP2 binding to a ligand is distributed over a number of distant residues. A similar observation of chemical shift perturbations was reported for FABP upon the mutation of peripheral amino acids;[41] however, our finding here suggests that structural perturbation can propagate much further along the protein sequence. Although one can strive to find strengthening of protein hydrogen bonds for higher affinity ligands, by looking at donor (N) and acceptor (O) distance or the N…H…O angle, caution has to be taken not to overinterpret the distance measurement from an X-ray crystal structure. There are also alternative approaches to evaluate protein hydrogen bonding, such as direct measurement of coupling between ^{15}N and ^{13}C nuclei[48] and this is currently being pursued.

Considerable variations can exist between the thermal behavior of different backbone hydrogen bonds and these indicate regions of particular thermal lability or stability within the protein.[49] It has been suggested that a correlation between the chemical shift of the amide proton and the hydrogen bond length exists for proteins but that the thermal behavior of hydrogen exchange rates and H-bond 'strength' as determined by the H-bond couplings is not necessarily correlated.[50–52] However, we think that the overall perturbation pattern should at least be reflected by a global hydrogen bond network perturbation.[48] We measured the temperature coefficients for the amide chemical shifts between 280 and 308 K in both complexes to see if there were observable differences.

In Figure 5, when the temperature coefficient of *R*-ibuprofen is plotted against the *S*-ibuprofen data, a linear correlation with a slope of 1 is observed, indicating that there are not major differences in the protein's response to the two-ligand binding events. We feel that comparison of the temperature coefficients to apo ap2 is not warranted since we already know from dynamics data that the apo structure has a considerable amount of flexibility in solution.[38] This is also borne out by the larger chemical shift differences between apo and either R or S ligated protein. (Supplemental data) A plot of the temperature coefficient data against the amide ^{15}N chemical shift perturbation reveals an interesting trend as seen in Figure 6 for *R*-ibuprofen. (A similar trend is seen for *S*-ibuprofen as well.) The data seem to suggest that for many of the amide nitrogen chemical shifts, the absolute magnitude of the temperature coefficient is inversely correlated to the chemical shift perturbation as represented by the expanding arrows in Figure 6, that is, the greater

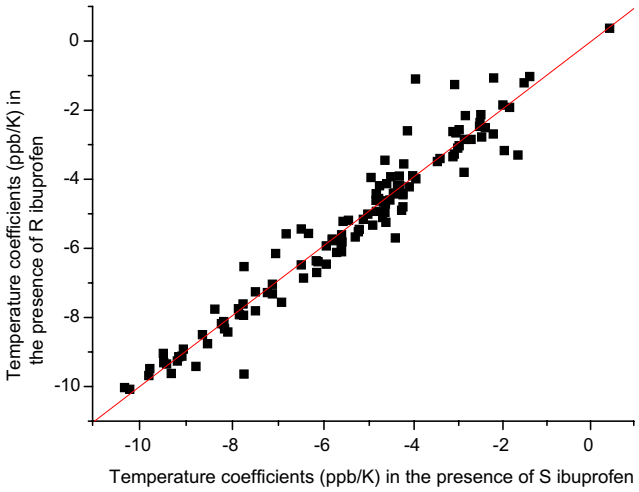


Figure 5. Correlation between the temperature coefficients of *R*- and *S*-ibuprofen binding aP2.

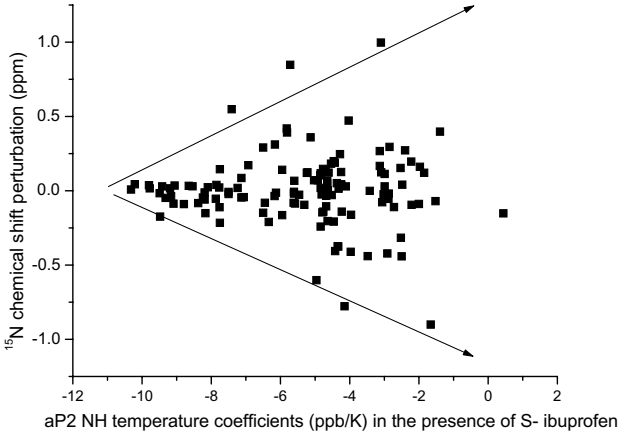


Figure 6. Correlation of the NH temperature coefficients versus the ^{15}N chemical shift perturbation.

the temperature coefficient (absolute values) the less the impact on the chemical shift perturbation.

This observation is intriguing in that it allows one to suggest that the residence time of the proton on the amide group affects the ability of the nitrogen chemical shift to be affected. In addition, it also suggests that this may be a necessary requirement for the transfer of 'information' across the β sheets. While this does not explain why the two ibuprofen enantiomers have differing chemical shift perturbation profiles, it does supply an avenue to explore in more detail. Also, it does suggest that, the interactions of hydrogen bonding effects may not extend uniformly throughout the protein and some may play a more important role than others. Some hydrogen bonds in the peripheral may actually have negative impact on binding. In fact, the transmission of chemical shift perturbation effects may be unidirectional, in that a perturbation at one site propagates to the other but not necessarily in the backward direction. While observations of chemical shift perturbation are very descriptive, it is still not clear as to why certain residues are selected to contribute to the transduction of

perturbations and why others are not involved. This will have to wait for more detailed studies using residual dipolar coupling measurements.

4. Conclusion

The question arises as to how or why long-range structure perturbation is achieved. One of the explanations could be that the ordered interior water molecules are disturbed.[25] We do not favor this explanation because X-ray structures show that most of the bound waters are conserved. Alternately, changes in the hydrogen bonding network of amino acid side chain and bound waters have been implicated.[53] Our result supports the hypothesis that such a hydrogen network is further extended to include backbone amides.

In summary, we observed global chemical shift perturbations for the amide protons in the aP2 complexes. Hydrogen bonding potential was monitored by amide chemical shift's temperature coefficient and it was found that 'reorganization' of hydrogen bonds is found to spread out to distant residues. While stabilization energy differential on hydrogen bonds is likely to be small for individual residues, the accumulative effect on multiple hydrogen bonds can have dramatic impact on ligand affinity. According $\Delta G = -RT\ln K_i$, a hydrogen bond with energy contribution of 1–5 kcal/mol can change binding constant K_i by 10- to 100-fold or more. The fact that multiple hydrogen bonds can be readjusted distal to the protein active site advocates that this effect needs to be taken into account when considering SAR in the drug discovery process. In this particular case, the ability of the receptor protein to modify its hydrogen bond interactions in the β-strands to accommodate dramatically different ligand scaffolds might make this receptor difficult for structure based drug design.

The detection of these hydrogen bond effects via chemical shift perturbations and the correlation with NH bond temperature coefficients points out the high sensitivity of the NMR experiment to minor structural changes or adjustments. The question of whether these structural perturbations have a functional role remains to be answered. Never-the-less these will play an important role in the evaluation of SAR and the prediction of binding energies, since the system must be considered as a whole.

It has been shown that in solution the structure of aP2 is mobile especially at the portal region of the protein near residue Phe57.[54] X-ray structures of more than thirty structures of ligand–target complexes of human aP2 have been determined and an outstanding feature of these structures is how the ligands cluster in one region of the binding pocket and the large number of conserved water molecules.[55] It was shown that while there is no gross structural modification between apo and ligand bound aP2, crystal packing forces are different and there is an increase of the overall binding cavity.[23] For these reasons, we believe that comparisons between ligated states, $\Delta\Delta(A - B)$, make for better understanding of the differences in molecular recognition than do Δ comparisons (apo-A or apo-B).

It was shown that *R*- and *S*-ibuprofen bind in analogous positions in intestinal fatty acid binding protein.[56] The similarity in binding constants, while not conclusive, does suggest that there is little enantiomeric selection for these compounds and that there are no substantial protein backbone positional differences between the two ligands necessary to accommodate binding.

5. Experimental procedures

5.1. Protein expression and purification

The recombinant aP2 was prepared by subcloning the His-tagged cDNA into a pQE30 expression vector. The new plasmid was then transformed to *Escherichia coli* M15 cells, which were inoculated in 1L LB medium at 37 °C. Upon reaching OD_{600} of ~0.7, the cells were pelleted by a 10 min centrifugation at 5000*g* and washed. Subsequently, the pellets were re-suspended in 250 mL M9 minimal media[57] supplemented with 0.25 g ammonium chloride and 1 g glucose (either or both can be labeled with ^{15}N or ^{13}C if isotopically enriched protein is made).

The cells were induced with 0.5 mM IPTG after incubation in M9 for 1 h to allow the recovery of growth and clearance of unlabeled metabolites, and were harvested 4 h later by centrifugation at 4 °C. The cell pellet was re-suspended in a 50 mL pH8 buffer containing 50 mM phosphate, 300 mM NaCl, 5 mM BME, and then the homogenate was applied to Ni–NTA column (Bio-Rad) equilibrated with the same re-suspension buffer. The protein was eluted with 120 mM imidizole. The yield was 60 mg/L or better.

5.2. NMR sample preparation

All protein samples were prepared in buffers containing 50 mM NaPi, 50 mM NaCl, 2 mM DTT at pH 7.0 with 5% D_2O. Ligand concentrations were 10 times of protein unless otherwise stated. For assignment purpose, aP2 sample was labeled with ^{15}N and ^{13}C at a concentration of 0.6 mM. For temperature coefficient measurements, the protein concentration was 0.4 mM.

To minimize impacts introduced by ligand in DMSO, 50 mM pH 7.0 buffer was used, supplemented with 50 mM NaCl and 2 mM DTT. Ligand titration was also performed to make sure aP2 complex chemical shifts do not depend on ligand concentration. DMSO factor was also excluded as it alone does not induce any chemical shift change for the apo protein under the condition studied.

5.3. NMR data collection and processing

5.3.1. Chemical shift perturbation studies. All NMR experiments were carried out at 25 °C unless otherwise stated. Chemical shift perturbation studies were per-

formed on a Varian AS500 spectrometer (operating at 500.13 MHz ^{1}H frequency) equipped with a Varian 5 mm $^{1}H/^{13}C/^{15}N$ triple-resonance, triple-axis PFG probe. Each 2D ^{1}H–^{15}N HSQC experiment was recorded with 200 (t_1) × 2048 (t_2) complex points with spectral width of 2.0 and 9.0 KHz.

5.3.2. Sequential resonance assignment. ^{13}C and ^{15}N double labeled protein sample with *S*-ibuprofen was used to make the assignments of ^{15}N–^{1}H HSQC peaks. 3D HNCA[58] and HNCACB[59] spectra were collected on Bruker AVANCE 800 (800.27 MHz for protons) to get the information of $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ of the current and the preceding amino acid. CBCA(CO)NH[60] experiment was recorded on Bruker DMX600 (600.13 MHz for protons) to give the information of the $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ of the preceding amino acid.

5.3.3. Temperature coefficient measurement. HSQC experiments were run at 38, 30, 25, 18, and 10 °C to measure the temperature coefficient of each amino acid of aP2 in the presence of *R*- and *S*-ibuprofen, respectively, on Bruker 800. Temperature calibration was done with ethylene glycol. All data were processed on Mac OS X 10.4.10 with the software package NMRPipe,[61] and all proton chemical shifts are reported with respect to the H_2O or $^{1}H^{2}HO$ signal, which is taken as 4.72 ppm downfield from external TSP (0.00 ppm) at 25 °C.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.092.

References and notes

1. Gohlke, H.; Klebe, G. *Angew. Chem. Int. Ed.* **2002**, *41*, 2644.
2. van Dijk, A. D.; Kaptein, R.; Boelens, R.; Bonvin, A. M. *J. Biomol. NMR* **2006**, *34*, 237.
3. Schieborr, U.; Vogtherr, M.; Elshorst, B.; Betz, M.; Grimme, S.; Pescatore, B.; Langer, T.; Saxena, K.; Schwalbe, H. *Chembiochem* **2005**, *6*, 1891.
4. Revington, M.; Holder, T. M.; Zuiderweg, E. R. *J. Biol. Chem.* **2004**, *279*, 33958.
5. Alexander Dehner, J. F.; Klaus, Richter; Ioana, Schuster; Johannes, Buchner; Horst, Kessler *Chembiochem* **2003**, *4*, 870.
6. Song, J.; Markley, J. L. *J. Mol. Recognit.* **2001**, *14*, 166.
7. Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531.
8. Sun, C.; Song, D.; Davis-Taber, R. A.; Barrett, L. W.; Scott, V. E.; Richardson, P. L.; Pereda-Lopez, A.; Uchic, M. E.; Solomon, L. R.; Lake, M. R.; Walter, K. A.; Hajduk, P. J.; Olejniczak, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 7875.
9. Wyss, D. F.; McCoy, M. A.; Senior, M. M. *Curr. Opin. Drug Discov. Dev.* **2002**, *5*, 630.
10. Zartler, E. R.; Hanson, J.; Jones, B. E.; Kline, A. D.; Martin, G.; Mo, H.; Shapiro, M. J.; Wang, R.; Wu, H.; Yan, J. *J. Am. Chem. Soc.* **2003**, *125*, 10941.
11. Chen, A.; Shapiro, M. J. *J. Am. Chem. Soc.* **2000**, *122*, 414.
12. Vogtherr, M.; Fiebig, K. *EXS* **2003**, 183.
13. Zartler, E. R., Shapiro, M. J. *Current Pharmaceutical Design*, in press.
14. Baar, R. A.; Dingfelder, C. S.; Smith, L. A.; Bernlohr, D. A.; Wu, C.; Lange, A. J.; Parks, E. J. *Am. J. Physiol. Endocrinol. Metab.* **2005**, *288*, E187.
15. Sun, X.; Zemel, M. B. *J. Nutr.* **2004**, *134*, 3054.
16. Shaughnessy, S.; Smith, E. R.; Kodukula, S.; Storch, J.; Fried, S. K. *Diabetes* **2000**, *49*, 904.
17. Scheja, L.; Makowski, L.; Uysal, K. T.; Wiesbrock, S. M.; Shimshek, D. R.; Meyers, D. S.; Morgan, M.; Parker, R. A.; Hotamisligil, G. S. *Diabetes* **1999**, *48*, 1987.
18. Hotamisligil, G. S.; Johnson, R. S.; Distel, R. J.; Ellis, R.; Papaioannou, V. E.; Spiegelman, B. M. *Science* **1996**, *274*, 1377.
19. Kopecky, J.; Clarke, G.; Enerback, S.; Spiegelman, B.; Kozak, L. P. *J. Clin. Invest.* **1995**, *96*, 2914.
20. Makowski, L.; Brittingham, K. C.; Reynolds, J. M.; Suttles, J.; Hotamisligil, G. S. *J. Biol. Chem.* **2005**, *280*, 12888.
21. Ory, J.; Kane, C. D.; Simpson, M. A.; Banaszak, L. J.; Bernlohr, D. A. *J. Biol. Chem.* **1997**, *272*, 9793.
22. Ory, J. J.; Banaszak, L. J. *Biophys. J.* **1999**, *77*, 1107.
23. Ory, J. J.; Mazhary, A.; Kuang, H.; Davies, R. R.; Distefano, M. D.; Banaszak, L. J. *Protein Eng.* **1998**, *11*, 253.
24. LaLonde, J. M.; Bernlohr, D. A.; Banaszak, L. J. *Biochemistry* **1994**, *33*, 4885.
25. LaLonde, J. M.; Levenson, M. A.; Roe, J. J.; Bernlohr, D. A.; Banaszak, L. J. *J. Biol. Chem.* **1994**, *269*, 25339.
26. Xu, Z.; Bernlohr, D. A.; Banaszak, L. J. *J. Biol. Chem.* **1993**, *268*, 7874.
27. Xu, Z.; Bernlohr, D. A.; Banaszak, L. J. *Biochemistry* **1992**, *31*, 3484.
28. Xu, Z. H.; Buelt, M. K.; Banaszak, L. J.; Bernlohr, D. A. *J. Biol. Chem.* **1991**, *266*, 14367.
29. Marr, E.; Tardie, M.; Carty, M.; Brown Phillips, T.; Wang, I. K.; Soeller, W.; Qiu, X.; Karam, G. *Acta Crystallogr., Sect. F Struct. Biol. Cryst. Commun.* **2006**, *62*, 1058.
30. Le, H.; Oldfield, E. *J. Biomol. NMR* **1994**, *4*, 341.
31. Oldfield, E. *J. Biomol. NMR* **1995**, *5*, 217.
32. Wishart, D. S.; Sykes, B. D.; Richards, F. M. *Biochemistry* **1992**, *31*, 1647.
33. Xu, X. P.; Case, D. A. *J. Biomol. NMR* **2001**, *21*, 321.
34. Xu, X. P.; Case, D. A. *Biopolymers* **2002**, *65*, 408.
35. Schwarzinger, S.; Kroon, G. J.; Foss, T. R.; Chung, J.; Wright, P. E.; Dyson, H. J. *J. Am. Chem. Soc.* **2001**, *123*, 2970.
36. Doreleijers, J. F.; Nederveen, A. J.; Vranken, W.; Lin, J.; Bonvin, A. M. J. J.; Kaptein, R.; Markley, J. L.; Ulrich, E. L. *J. Biomol. NMR* **2005**, *32*, 1.
37. Wishart, D. S.; Case, D. A. *Methods Enzymol.* **2001**, *338*, 3.
38. Constantine, K. L. *Biochemistry* **1998**, *37*, 7965.
39. Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley and Sons: New York, 1986.
40. Hwang, T. L.; Shaka, A. J. *J. Magn. Reson.* **1998**, *135*, 280.
41. Rajabzadeh, M.; Kao, J.; Frieden, C. *Biochemistry* **2003**, *42*, 12192.
42. Bouvignies, G.; Bernado, P.; Meier, S.; Cho, K.; Grzesiek, S.; Bruschweiler, R.; Blackledge, M. *Proc. Natl. Acad. Sci. U.S.A* **2005**, *102*, 13885.
43. Roe, S. M.; Prodromou, C.; O'Brien, R.; Ladbury, J. E.; Piper, P. W.; Pearl, L. H. *J. Med. Chem.* **1999**, *42*, 260.

44. Prodromou, C.; Roe, S. M.; O'Brien, R.; Ladbury, J. E.; Piper, P. W.; Pearl, L. H. *Cell* **1997**, *90*, 65.
45. Zhuravleva, A.; Korzhnev, D. M.; Nolde, S. B.; Kay, L. E.; Arseniev, A. S.; Billeter, M.; Orekhov, V. Y. *J. Mol. Biol.* **2007**, *367*, 1079.
46. Zuiderweg, E. R. P. *Biochemistry* **2002**, *41*, 1.
47. Landry, S. J. *Biochemistry* **2003**, *42*, 4926.
48. Cordier, F.; Wang, C.; Grzesiek, S.; Nicholson, L. K. *J. Mol. Biol.* **2000**, *304*, 497.
49. Cordier, F.; Grzesiek, S. *J. Mol. Biol.* **2002**, *317*, 739.
50. Cordier, F.; Grzesiek, S. *J. Am. Chem. Soc.* **1999**, *121*, 1601.
51. Grzesiek, S.; Cordier, F.; Dingley, A. J. *Methods Enzymol.* **2001**, *338*, 111.
52. Jaravine, V. A.; Cordier, F.; Grzesiek, S. *J. Biomol. NMR* **2004**, *29*, 309.
53. Lucke, C.; Fushman, D.; Ludwig, C.; Hamilton, J. A.; Sacchettini, J. C.; Ruterjans, H. *Mol. Cell Biochem.* **1999**, *192*, 109.
54. Simpson, M. A.; Bernlohr, D. A. *Biochemistry* **1998**, *37*, 10980.
55. van Dongen, M.; Uppenberg, J.; Svensson, S.; Lundback, T.; Akerud, T.; Wikstrom, M.; Schultz, J. *J. Am. Chem. Soc.* **2002**, *124*, 11874.
56. Velkov, T.; Chuang, S.; Wielens, J.; Sakellaris, H.; Charman, W. N.; Porter, C. J.; Scanlon, M. J. *J. Biol. Chem.* **2005**, *280*, 17769.
57. Marley, J.; Lu, M.; Bracken, C. *J. Biomol. NMR* **2001**, *20*, 71.
58. Kuboniwa, H.; Grzesiek, S.; Delaglio, F.; Bax, A. *J. Biomol. NMR* **1994**, *4*, 871.
59. Wittekind, M.; Mueller, L. *J. Magn. Reson.* **1993**, *B101*, 201.
60. Grzesiek, S.; Anglister, J.; Bax, A. *J. Magn. Reson.* **1993**, *B101*, 114.
61. Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277.