Letter

Deuterium Isotope Effects on Carbonyl Carbon Chemical Shifts of BPTI. Hydrogen Bonding and Structure Determination in Proteins

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Hansen, P. E. and Tüchsen, E., 1989. Deuterium Isotope Effects on Carbonyl Carbon Chemical Shifts of BPTI. Hydrogen Bonding and Structure Determination in Proteins. – Acta Chem. Scand. 43: 710–712.

Protein engineering calls for fast methods for the determination of structural changes caused by site-directed mutagenesis, or to ensure that correct folding of the engineered proteins has occurred. Comparison of deuterium isotope effects on ^{13}C chemical shifts may be a useful method. It has an appreciable advantage over comparison of chemical shifts in being a differential method (the chemical shifts of the deuterio species is subtracted from that of the protonated one), thus avoiding difficulties with solvent effects, aromatic ring-current effects, etc. Moreover, the two-bond deuterium isotope effect has been shown to be a good gauge of hydrogen-bonding,[1-3] an essential determinant for protein folding and dynamics.

In this study BPTI (basic pancreatic trypsin inhibitor) was chosen as a model for several reasons. Firstly, it has the best characterized structure of any protein, including a solution conformation[4] and proton positions in the crystalline state.[5] Secondly, the majority of the carbonyl NMR resonances have recently been assigned.[6] Thirdly, specific hydrogen isotope labeling can be achieved for the amide groups due to detailed knowledge of the exchange rates.[7-9]

Isotope effects from the amide proton are propagated through the covalent bonds to neighboring carbon atoms. In the carbonyl region of the ^{13}C NMR spectrum, effects over two bonds are always observed, while the isotope effects over three bonds from the N–H to the carbonyl carbon of the same residue is seen in some cases and not in others. It is the aim of the present communication to describe how both two- and three-bond isotope effects can be used to determine hydrogen-bond patterns and hence structures of proteins and to gain new knowledge on the atomic level about the hydrogen-bond potential of main-chain hydrogen bonds in proteins.

Results and discussion

Deuterium isotope effects on the BPTI carbonyl carbon chemical shifts are obtained either by observing effects of deuterium exchange at specific amide NH positions (described in detail in Ref. 7) or from the resonance isotope splittings of samples in exchange equilibrium in H_2O/D_2O mixtures. With the former procedure we can measure both the isotope effects over two and three bonds, $^2\Delta CO(ND)$ and $^3\Delta CO(ND)$, respectively. With the latter procedure three-bond isotope effects can not always be measured separately.

The two-bond isotope effects fall in a fairly narrow range, 0.06–0.09 ppm. The lower limit is similar to the two-bond isotope effects found in many simple amides.[10] However, in phenols[2] and aromatic amines[11] with intramolecular hydrogen bonds, the $^2\Delta CO(ND)$ values are enhanced and are correlated to the H-bond mediated downfield change of the donor-proton chemical shift. Similar data have recently been obtained for *o*-acetamido azo compounds[12] which are better model substances for protein backbone amide groups. Using these data along with data from acetoacetylglycine [N-(carboxymethyl)-3-oxobutanoic amide], (δ_H = 8.37 ppm), a correlation eqn. (1) could be suggested for the H-bond potential *E*,

$$E = -11.7 \times \ln {}^2\Delta CO(ND) + 49.2 \text{ (kcal mol}^{-1}\text{)} \quad (1)$$

where the isotope effect is in ppb.

With reference to this eqn. (1) most of the hydrogen bonds in BPTI should have enthalpies of about −2 kcal mol^{-1}, and none of them should exceed −3.5 kcal mol^{-1}. The small size of these numbers may be surprising, particularly for hydrogen bonds in the stable BPTI β-sheet. Some understanding of the β-sheet stability may be derived from the following.

We were at first astonished by the flickering occurrence of three-bond isotope effects. Their origin could be determined unambiguously in H/D-exchange experiments involving small sets of amide groups, selected by an isotope-labeling procedure,[6] and the experiments clearly ruled out isotope effects from donor to acceptor groups of inter-chain hydrogen bonds. Three-bond isotope effects have been detected for the backbone carbonyls of the following resid-

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ues, Arg-20, Phe-22, Leu-29, Gln-31, Phe-33, Asn-44 and Phe-45. All except Asn-44 are part of the antiparallel β-sheet.

On examining the crystal structure, a striking correlation was found between $^3\Delta CO(ND)$ and short distances between H and O of backbone H–N–C^α–CO atom strands (Fig. 1). Large $^3\Delta CO(ND)$ values are clearly associated with residues with unusually short H··O distances. Fortunately, we can calculate these distances from the highly refined neutron diffraction structure of Wlodaver *et al.*[5] The limit for detection of $^3\Delta CO(ND)$ seems to be an O··H distance of 2.6 Å. This distance coincides with the van der Waals distance between an O and an H atom. There are only two additional residues with similarly short H··O distances, Cys-38 and Ser-47. Their carbonyl resonances are both unidentified. For Tyr-21 with an H··O distance of 2.75 Å there is no detectable three-bond isotope effect. The resolution of our spectra would in most experiments allow detection of effects down to about 0.01 ppm. Smaller three-bond isotope effects than those observed here and reported for the peptide viomycin dissolved in $CDCl_3$.[13] They would suggest H··O distances in the range 2.6–2.7 Å.

Additional features of residues showing three-bond isotope effects are (1) that the amide proton resonances are shifted to low field, (2) that the carbonyl carbon resonances are shifted to high field,[14] and (3) that the C=O double-bond is shorter on the average (Fig. 1). An elongation of the C=O bond might have been expected for these residues, due to the simultaneous inter-strand hydrogen bonds accepted by the carbonyl O. Apparently, the existence of the 5-membered ring leads to an increased bond order of the C=O bond. The nature of the interaction could be an orbital overlap between the H orbital and the rear lobe of the sp^2 orbitals of the carbon.

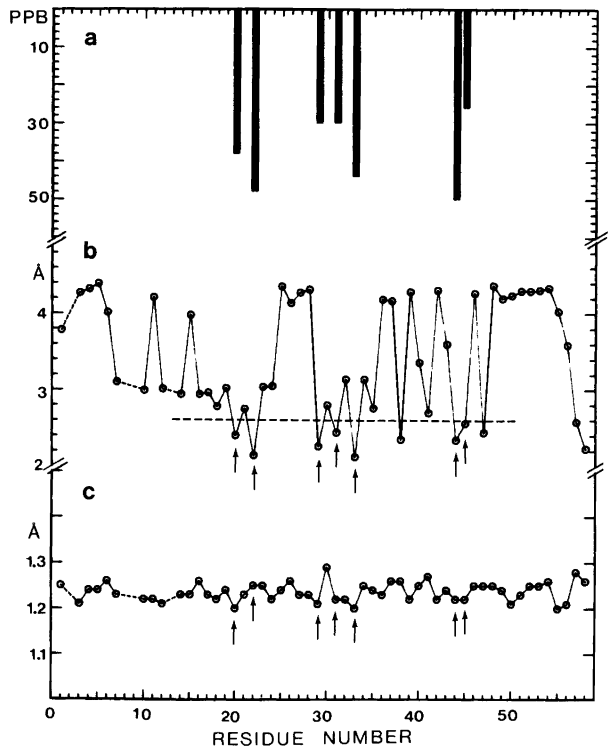

Fig. 1. (a) The size of three-bond isotope effects observed in BPTI (in parts per billion); (b) The distance between the peptide H and carbonyl O (following peptide group) for each residue; (c) peptide carbonyl bond lengths. The distances are calculated from the neutron-diffraction structure of A. Wlodaver *et al.*

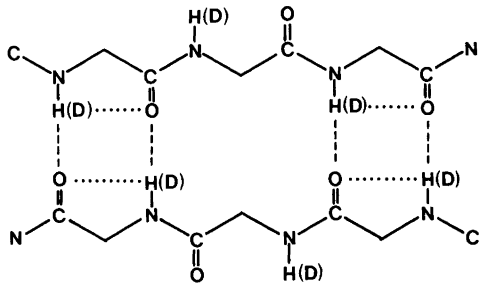

Fig. 2. The typical location of the intra-residue hydrogen bonds (dotted lines) that result in three-bond isotope effects. Broken lines indicate the inter-strand hydrogen bonds. D indicates the positions of deuterium.

The short H··O distances for these residues are clearly within the range of H-bonds of appreciable energy, in accordance with the sizable three-bond isotope effects. The finding suggests a principle for the H-bonding in antiparallel β-sheet, as outlined in Fig. 2, in which the 10-membered H-bonded rings are cross-linked with extra (and perhaps weaker) hydrogen bonds, forming two 5-membered rings. Ramachandran *et al.*[15] many years ago suggested a potential function for inter-residue hydrogen bonds like these with a maximum stabilizing energy of 2.5 kcal mol^{-1}.

The occurrence of three-bond isotope effects is clearly related to residues with main-chain dihedral angles typical for the antiparallel β-sheet structure. Pairs of three-bond isotope effects would be diagnostic for an antiparallel β-sheet such as that shown in Fig. 2. Residues with large three-bond isotope effects, however, are not necessarily incorporated in any extended β-structure. Asn-44 is a good example, displaying the largest three-bond isotope effect observed in the present study. This residue is incorporated in a sharp turn consisting of Asn-44, Asn-43 and Arg-42.

We imagine that the three-bond isotope effects can be used to gauge sensitively tiny structural changes in proteins. These could originate from a variety of sources such as changes in solution conditions, ligand binding, chemical modification or residue replacements brought about by genetic engineering.

Acknowledgment. We are indebted to Novo Industries, Copenhagen, for a generous gift of BPTI.

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Received February 6, 1988.