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Physical Chemistry Chemical Physics (2017)

DOI: <https://doi.org/10.1039/C7CP04315E>

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DOI link to article:

<https://doi.org/10.1039/C7CP04315E>

Date deposited:

18/09/2017

Embargo release date:

07 September 2018



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Cooperative Hydrogen Bonds Form a Pseudocycle Stabilizing an Isolated Complex of Isocyanic Acid with Urea

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

The shapes of macromolecules and their complexes with small molecules are often determined by extended networks of hydrogen bonds. Here, for the first time, we provide a detailed description of a cooperative pair of hydrogen bonds to an individual molecule of urea. The structure and properties of a gas phase complex formed between urea and isocyanic acid are characterised through microwave spectroscopy and *ab initio* calculations at the CCSD(T)(F12*)/aug-cc-pVTZ level.

1. Introduction

Networks of hydrogen bonds play a decisive role in the three-dimensional structures adopted by macromolecules and the effect of subtle changes in interaction strengths and directionality can be profound, ranging from switching between thermodynamically-favored crystalline polymorphs to causing a protein to denature. Multiple hydrogen bonds contribute to the stability of complexes between enzymes and their cofactors, substrates or inhibitors. Spectroscopy provides a powerful means for the characterization of individual hydrogen-bonding interactions.¹ Hydrogen-bonded networks often include functional groups that act simultaneously as a hydrogen donor and acceptor and therefore exhibit strong cooperative or anti-cooperative effects: the strength of an individual bond is influenced by the polarization induced by neighboring bonds and vice versa, propagating throughout the network.² High-resolution microwave spectroscopy is a powerful tool for the exploration of the relationship between cooperative hydrogen bonding interactions, structure and function. Spectroscopic experiments on small gas-phase hydrogen bonded water clusters enabled the definitive characterization of the structures and their energy ordering, providing insight into competing cooperative hydrogen-bonding effects in liquid water.³

In this article, we report a structural characterization of hydrogen-bonding to a single molecule of urea. The gas-phase microwave spectra conclusively show that the N-H group of isocyanic acid complexes to urea via two hydrogen bonds where the N-H group is both a hydrogen bond donor and acceptor, thus forming a 'pseudocycle', a structural motif important in some drug-enzyme interactions.⁴ The lengths and angles of the identified hydrogen bonds confirm the presence of strong, cooperative, stabilizing interactions. The hydrogen bonding properties of urea are of prototypical importance in biology and crystal engineering.⁵

Urea is commonly used in laboratory experiments to induce denaturing of aqueous proteins and initiate the *in vitro* exploration of physical and chemical aspects of protein folding.⁶ The mechanism of denaturing, however, is the subject of significant debate and is hypothesised⁷ to result from; (i) a direct mechanism in which urea interacts with the protein resulting in unfolding and/or; (ii) an indirect mechanism in which urea disrupts hydrogen bonds between solvating water molecules that are stabilizing the native form of the protein. Many experimental and computational studies⁸ have been performed with the aim of elucidating the nature of hydrogen bonding between urea, water and protein backbone or side-chains and their effects on the thermodynamics of unfolding, but have not included consideration of the bonding motif identified in this work. An unpublished study previously reported the isolation of a complex of urea and water, also identifying a structure that contains a pair of hydrogen bonds to a single O-H group.⁹ The clear implication is that cooperative effects are important in the hydrogen bonding of urea to proteins and water and that the NH and CO groups of urea can simultaneously attach to a single protein site wherever a suitable donor-acceptor functional group is situated.

2. Experimental and theoretical methods

The instruments and methods employed for the microwave spectroscopy experiments have been described in detail previously.¹⁰ A backing pressure of 6 bar was employed during pulsed injection of an argon buffer gas into a vacuum chamber. The

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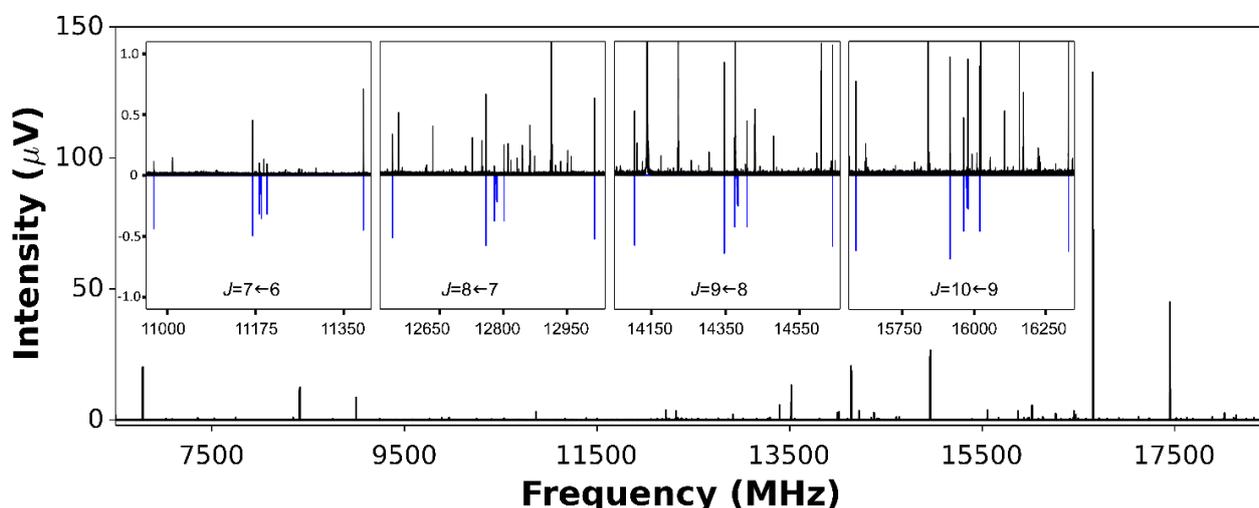


Figure 1. (Main panel) The broadband rotational spectrum recorded during the described experiment. Transitions of many small molecules are seen in addition to those of the $(\text{H}_2\text{N})_2\text{CO}\cdots\text{HNCO}$ complex. (Inset panels) The experimental spectrum (3.9M averages) (black) is displayed above a simulation (blue) of the spectrum of $(\text{H}_2\text{N})_2\text{CO}\cdots\text{HNCO}$ that uses the fitted values of spectroscopic constants.

generation of molecular complexes was achieved by laser-vaporising material from a solid target such that it becomes entrained in the buffer prior to supersonic expansion of the gas sample. The frequency doubled output of a Nd:YAG laser (532 nm, 20 mJ pulse^{-1} , 10 ns duration) is focussed on the target which is continually rotated and translated to ensure fresh surface material is exposed to each laser pulse. Target rods were prepared using a 1:1 molar ratio of urea and copper, using a table top press and an evacuable pellet die (Specac). The molar ratio of urea and copper was maintained during the experiments involving synthetically enriched ^{13}C , ^{18}O , ^{15}N and deuterium containing urea.

An arbitrary waveform generator (AWG) is used to supply a chirped microwave pulse that sweeps from 0.5–12 GHz over a duration of 1 μs . This chirped pulse passes through a 12.2 GHz low-pass filter and is mixed against the filtered output of a phase-locked dielectric resonant oscillator (PDRO) that supplies a 19.00 GHz signal. The AWG and PDRO are each phase-locked to a 10 MHz frequency accurate to 1 part in 10^{11} . The output after mixing passes through a 7–18.5 GHz bandpass filter and is amplified prior to irradiation of the molecular sample. The data presented herein were acquired using a 300W travelling wave tube amplifier. The microwave frequency sweep is introduced perpendicular to the propagation direction of the expanding gas via a microwave horn. The molecular emission signal (free induction decay, or FID, of the polarisation) is detected by a second horn antenna and amplified by a low noise amplifier. The FID is mixed down against the 19.00 GHz reference signal supplied by the PDRO and then passes through a 12.5 GHz low-pass filtered before detection. Each FID is measured over a 20 μs period following

each chirped polarisation pulse. A fast digital oscilloscope is phase-locked to the same 10 MHz external frequency reference used by the PDRO and AWG and used to digitise the molecular FID. The above sequence of microwave polarisation pulse, detection and recording of FID is repeated eight times following each individual nozzle (i.e. gas sample introduction) pulse to maximise the duty cycle of the spectrometer. In order to record the spectrum of urea-HNCO during this study, 3.9 million FIDs (acquired from 487k valve pulses) were co-added in the time domain. FID's are averaged in the time domain before Fourier transforming to yield the frequency domain spectrum spanning from 7 to 18.5 GHz. After application of a Kaiser-Bessel window function, all observed transitions have a full width at half maximum (FWHM) of 150 kHz.

Calculations were performed at the CCSD(T)(F12*) level of theory, that is, explicitly correlated coupled-cluster singles, doubles and perturbative triples¹¹ with the (F12*) approximation of Hättig, Tew and Köhn.¹² Only valence electrons were correlated and calculations were performed using the aug-cc-pVDZ or aug-cc-pVTZ basis sets.¹³ The exponent of the correlation factor was 1 a_0 ,¹⁴ the F12 method 2B was used,¹⁵ and the details of the auxiliary basis sets were that the aug-cc-pVTZ Coulomb fitting basis¹⁶ was used as CABS¹⁷; and the aug-cc-pwCVQZ Coulomb fitting basis¹⁸ and the aug-cc-pVTZ JK fitting basis were used for density fitting the correlation and Fock integrals, respectively. Structures of HNCO, urea and the lowest energy complex were optimised at the CCSD(T)(F12*) level of theory, first using the aug-cc-pVDZ basis and then the aug-cc-pVTZ basis set. At the optimised CCSD(T)(F12*)/aug-cc-pVTZ structures of HNCO, urea and the hydrogen-bonded complex, harmonic

frequencies were evaluated at the CCSD(T)(F12*)/aug-cc-pVDZ level of theory. The force constant matrix was computed numerically using a five point formula. Residual basis set incompleteness errors led to an imaginary frequency for the out-of-plane intermolecular vibration in the complex. To refine the harmonic frequencies, CCSD(T)(F12*)/aug-cc-pVTZ calculations were therefore performed: a 5-point formula was used for each mode individually, involving displacements along the normal mode vector from the CCSD(T)(F12*)/aug-cc-pVDZ Hessian. The harmonic wavenumbers are reported in Supplementary Data Table 6 together with their vibrational characteristics.

To synthesise the ^{18}O -urea, chlorotrimethylsilane (1.5 g, 1.08 mL, 13.8 mmol) was added dropwise to $[^{18}\text{O}]\text{H}_2\text{O}$ (2.0 mL, 100 mmol) in dry dioxane (12 mL), cooled in ice and stirred under nitrogen. Cyanamide (1.20 g, 28.6 mmol) was added in portions and the mixture was heated at reflux for 80 min. After cooling to room temperature, methanol (10 mL) was added followed by sodium carbonate (1.0 g), and the mixture was stirred for 30 min. The resulting solid was removed by filtration and the filtrate was evaporated to give a white solid that was dried in vacuo. The solid was extracted with boiling acetone (3×15 mL), the extracts were combined and the solvent was removed. The remaining white solid was recrystallised from ethanol-diethyl ether (1:1, v/v) to yield large white crystals of the labelled urea (1.32 g, 74%): ^1H NMR (300 MHz, d^6 -DMSO) δ 5.43 (bs, 4H, 2 \times NH_2); ^{13}C NMR (175 MHz, D_2O) δ 162.45 (C^{18}O); ESI m/z 61.0 (^{16}O - MH^+ , 6%), 62.9 (^{18}O - MH^+ , 100%)

3. Results and discussion

3.1 Spectral assignment and analysis

The most intense transitions in the observed spectrum assign to the isolated urea monomer but many other species¹⁹ were also identified (Figure 1) from their known microwave spectra. Disintegration of the urea precursor prompts the formation of CH_3CN , HC_3N , H_2CO and NH_3 within the expanding gas pulse. The experiment also revealed the spectrum of a molecule or complex that has not previously been characterized and which; (i) is a near-prolate asymmetric rotor; (ii) has a significant dipole moment component on its a inertial axis; (iii) is planar, or very nearly planar; and (iv) is somewhat of larger mass than the urea monomer based on the increments between its rotational transitions.

In total, spectra were recorded and spectroscopic parameters determined for twelve distinct isotopologues with single isotopic substitutions achieved at every atom within HNCO, and the C and O atoms of urea, allowing for an accurate and quantitative determination of the molecular geometry. These experiments confirmed that the complex contains at least two non-equivalent atoms of each of carbon, oxygen and nitrogen. Observed transition frequencies were fitted to model parameters in Watson's S -reduced Hamiltonian²⁰ for a near-prolate asymmetric rotor using Western's PGOPHER program.²¹ The identified transitions were exclusively a -type. Components of the dipole moment aligned with the a , b and c

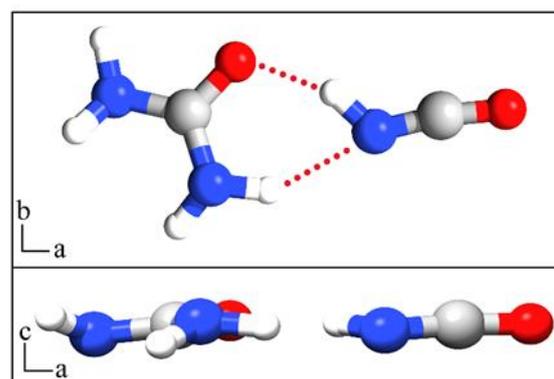


Figure 2. (Top panel) The geometry of $(\text{H}_2\text{N})_2\text{CO}\cdots\text{HNCO}$ with hydrogen bonds indicated by dotted rod lines. (Bottom panel) A side perspective of the same complex.

inertial axes of the complex were calculated at the CCSD(T)(F12*)/aug-cc-pVTZ level to be 5.84, 1.05 and 0.01 D respectively. b -type transitions were apparently insufficiently intense to be observed at the signal/noise of the present measurements. Full details of all fits and evaluated parameters are included in Supplementary Tables 1 and 2. During accompanying ab initio calculations, the HNCO molecule was placed at various hydrogen bonding positions around urea and the geometry was optimized at the MP2/aug-cc-pVTZ level of theory. At each MP2 structure, single point CCSD(T)(F12*)/aug-cc-pVTZ energies were computed. Three low-energy bonding motifs were identified with the lowest energy of these structures being that displayed in Figure 2. The results of the computations for higher energy conformers are provided in Figure 1 of the Supplementary Data. The experimentally-determined (B_0) rotational constants are compared with (B_e) results determined through a full geometry optimization at the CCSD(T)(F12*)/aug-cc-pVTZ level²² in Supplementary Table 2. The experimental observations and ab initio calculations confirm that urea coordinates to isocyanic acid via two hydrogen bonds to form a complex of the geometry shown in Figure 2. This result provides the basis for a determination of bond lengths, angles and atomic coordinates.

Table 1. Fitted values of structural parameters.

	exp		calc ^a
	r_s	r_0	r_e
$r(\text{O}_B\cdots\text{H}_A) / \text{\AA}$	1.820(6) ^b	1.866(11)	1.773
$r(\text{N}_A\cdots\text{O}_B) / \text{\AA}$	2.762(8)	2.809(9) ^c	2.790
$r(\text{N}_A\cdots\text{H}_B) / \text{\AA}$	-	2.261(13) ^c	2.306
$\angle(\text{N}_A\text{H}_A\cdots\text{O}_B) / ^\circ$	151.2(23)	156.8(6)	160.4
$\angle(\text{C}_B\text{O}_B\cdots\text{H}_A) / ^\circ$	113.9(27)	112.5(8)	112.6
$\angle(\text{N}_B\text{H}_B\cdots\text{N}_A) / ^\circ$	-	138.4(78) ^c	137.2

^a CCSD(T)(F12*)/aug-cc-pVTZ level of theory. ^b Numbers in parentheses are one standard deviation in units of the last significant figure. ^c Deduced from fitted values of other parameters.

3.2 Molecular Geometry

The isotopic substitution method (denoted as r_s) of Kraitchmann²³ provides a method by which the positional coordinates of an individual atom can be determined free from assumptions about the positions of all other atoms. An alternative method (denoted as r_0) fits internal coordinates to the vibrational ground state moments of inertia but requires some initial assumptions about the respective geometries of the urea and HNCO sub-units.²⁴ Bond lengths and angles that are internal to each of urea and isocyanic acid were fixed to values appropriate to the r_0/r_s geometries of the isolated monomer sub-units when fitting the r_0 geometry.²⁵ It was not possible to perform the H/D isotopic exchange with selectivity in the number and position of substituted hydrogen atoms and therefore not possible to unambiguously determine the position and orientation of the hydrogen atoms of urea within the $(\text{NH}_2)_2\text{CO}\cdots\text{HNCO}$ complex. The $\angle(\text{H}-\text{N}-\text{H})$ angles within urea, and the dihedral angles that define the orientations of the various N-H bonds, were fixed equal to the results of the ab initio calculation for the equilibrium (r_e) geometry when fitting the r_0 geometry of the complex. Coordinates of atoms determined by the r_0 and r_s methods are shown in Supplementary Table 3 alongside ab initio calculated results. The determined values of internal coordinates are shown in Table 1. Subscripts "A" and "B" respectively denote atoms internal to isocyanic acid and urea. The H_B and N_B atoms referred to by Table 1 are those which are nearest to the isocyanic acid sub-unit in the model geometry.

The urea monomer adopts a planar geometry in the solid state²⁶ but is non-planar in the gas phase.^{25a} The latter is characterised by a pyramidal arrangement at each RNH_2 . One possible conformer of gas phase urea has C_s symmetry with the hydrogen atoms of both NH_2 groups positioned on the same side of the plane defined by the heavy atoms of urea but with a very low barrier to inversion at an NH_2 group to yield a second low-energy conformer of C_{2v} symmetry.^{25a} The uncertainties stated in Table 1, alongside the fitted values of internal coordinates, reflect the statistical uncertainties of the fits while the differing initial assumptions employed by the various methods are also responsible for broader differences. Some atomic coordinates have small magnitude and are imprecisely determined by either r_0 or r_s methods. Hydrogen atoms are not located experimentally with high precision as reflected in small inconsistencies in the determined values of $r(\text{O}_\text{B}\cdots\text{H}_\text{A})$. Zero-point vibrational effects are likely to be significant given that the ab initio calculations identify intramolecular vibrational modes of very low energy. Better agreement exists between the ab initio calculated, r_e , and experimentally-determined, r_0 , distances from the nitrogen atom of HNCO to the oxygen atom of urea, denoted by $r(\text{N}_\text{A}\cdots\text{O}_\text{B})$. Attempts to determine a greater number of structural coordinates were unsuccessful owing to sensitivities to the values of small coordinates. Complete results of all structural fits are provided alongside ab initio calculated geometries and vibrational frequencies in Supplementary Tables 3-6.

All methods of structure determination find that the distance between the oxygen atom on urea and the hydrogen atom of

isocyanic acid, $r(\text{O}_\text{B}\cdots\text{H}_\text{A})$, is between 1.77 and 1.87 Å, consistent with isocyanic acid acting as hydrogen bond donor to urea. The results obtained for $\angle(\text{N}_\text{A}\text{H}_\text{A}\cdots\text{O}_\text{B})$ span from 151° to 161°. Given that the lowest energy configuration of an individual, isolated hydrogen bond tends to linearity,²⁷ the deviation of this parameter from 180° suggests the presence of a secondary bonding interaction. An additional hydrogen bond is evidently formed between the nitrogen atom of isocyanic acid and a hydrogen atom on urea. The results obtained by the r_0 and r_e methods for this distance, $r(\text{N}_\text{A}\cdots\text{H}_\text{B})$, are 2.261(13) Å and 2.306 Å respectively. The values determined for $\angle(\text{C}_\text{B}\text{O}_\text{B}\cdots\text{H}_\text{A})$ are around 113° and thus slightly lower than would be expected for an isolated hydrogen bond which would tend to align with the axis of a non-bonding electron pair on the oxygen atom.²⁸

4. Conclusions

All quantified bond lengths and angles confirm the hypothesis that the complex contains two hydrogen bonds in the pseudocyclic arrangement shown in Figure 2. Various previous works have shown that the N-H group of isocyanic acid may simultaneously act as both a donor and acceptor of hydrogen bonds. For example, it was calculated ab initio that the lowest energy conformers of each of $\text{HNCO}\cdots(\text{NH}_3)_n$ and $\text{HNCO}\cdots(\text{H}_2\text{O})_n$ (where $n \geq 2$) involve geometries in which HNCO acts as both hydrogen bond donor and acceptor.²⁹ We believe the present work is the first to experimentally observe this dual role of the NH group of isocyanic acid. A preliminary study of an isolated complex of urea and water tentatively proposed a geometry that also contains a pair of hydrogen bonds.⁹ The geometry proposed for the $(\text{NH}_2)_2\text{CO}\cdots\text{H}_2\text{O}$ complex is strikingly similar to that identified here for $(\text{NH}_2)_2\text{CO}\cdots\text{HNCO}$ and identifies hydrogen bond distances of; (i) 1.85 Å between the oxygen of urea and a hydrogen of water; and (ii) 2.09 Å between a hydrogen of urea and the oxygen of water. The geometries of the hydrogen bonds in each of $(\text{NH}_2)_2\text{CO}\cdots\text{HNCO}$ and $(\text{NH}_2)_2\text{CO}\cdots\text{H}_2\text{O}$ thus strongly imply that the interactions within these complexes are strengthened by cooperative effects. In each complex, the presence of a pair of hydrogen bonds in a pseudocyclic arrangement enhances the polarization of neighboring bonds synergistically so as to increase the binding energy of the complex significantly beyond that which would result from a single hydrogen bond.

The dissociation energy, D_e , associated with separation of the $(\text{NH}_2)_2\text{CO}$ and HNCO monomer units was computed to be 41 kJ mol^{-1} at the CCSD(T)(F12*)/aug-cc-pVTZ level of theory using the counterpoise correction method. The counterpoise correction amounted to only 1 kJ mol^{-1} and we are therefore confident that the binding energy is tightly converged with respect to basis set. This dissociation energy is much greater than would be expected for a single hydrogen bond where values between 20 and 25 kJ mol^{-1} are expected.³⁰ The identified pseudocycle is formed of only six atoms implying that the pair of hydrogen bonds induces significant polarizations in neighboring bonds. The standard enthalpy of sublimation of urea³¹ is $\Delta_{\text{sub}}\text{H}^\circ = 95.5 \pm 0.3 \text{ kJ mol}^{-1}$. This quantity effectively represents the

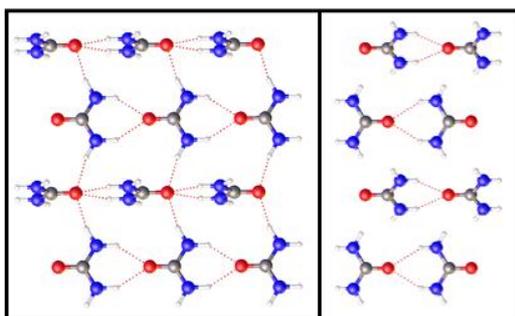


Figure 3. (Left panel) The structure of the most stable isomorph of crystalline urea under ambient conditions.^{26,32} Each oxygen atom participates in four hydrogen bonds. (Right panel) Application of high pressure (0.5 GPa) to solid urea yields an isomorph where each oxygen atom participates in only two hydrogen bonds.^{5b}

energy required to break all hydrogen bonds in the crystal structure of the urea isomorph that is most stable under ambient conditions as represented in Figure 3.³² Each carbonyl O atom acts as an acceptor of hydrogen bonds from four distinct N–H groups leading to a high degree of stability and structural order. The lengths of the two forms of N–H...O hydrogen bond are 2.00 Å and 2.06 Å respectively in this crystal. These are both slightly longer than the N–H...O distance of 1.820(6) Å (r_s geometry) identified in (NH₂)₂CO...HNCO during the present work. Evidently, the pair of hydrogen bonds in (NH₂)₂CO...HNCO confers almost half the stabilizing effect of the four hydrogen bonds present within solid urea.

Urea was amongst the first organic solids studied by X-ray crystallography and isomorphs of the pure solid and many co-crystals have been extensively studied. A very common structural motif in urea co-crystals involves a (nearly planar) pair of urea monomers that interconnect via a reciprocal pair of hydrogen bonds, occurring between the oxygen atom of each monomer and an NH group on the other. The energy calculated for the binding interaction³³ in this configuration is between 34 and 50 kJ mol⁻¹, similar in magnitude to that in (NH₂)₂CO...HNCO. Unfortunately, the geometry of this urea dimer does not possess a dipole moment precluding its detection by microwave spectroscopy. Evidently, the two hydrogen bonds in (NH₂)₂CO...HNCO are sufficient to generate a strong interaction while still leaving three hydrogen atoms of urea available to participate in other bonding. An important question is prompted by the observation of a single molecule of urea engaged in a cooperative hydrogen bond with HNCO. To what extent, and in which protein environments, will isolated urea molecules attach to proteins via multiple, cooperative interactions?

Conflict of Interest

There are no conflicts of interest to declare.

Acknowledgements

The authors thank the European Research Council for a postdoctoral fellowship awarded to C.M., a PhD studentship awarded to J.C.M., and for project funding (Grant No. CPFTMW-307000). A.C.L. thanks the University of Bristol for a Senior Research Fellowship. The Royal Society of Chemistry are thanked for an undergraduate research bursary awarded to L. L-B.

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