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The influence of solvation on short strong hydrogen bonds: a density functional theory study of the Asp-His interaction in subtilisins†

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The effect of a structural water molecule on the electronic nature of the His64-Asp32 hydrogen bond in subtilisins is examined by DFT calculations; the structural water is found to favor a short strong hydrogen bond in the catalytic triad in sharp contrast to some current beliefs.

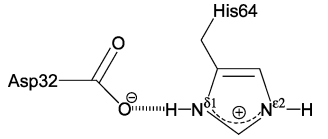
The possible involvement of low-barrier hydrogen bonds[1] (LBHBs) in enzyme catalysis has in recent years attracted much attention.[2–8] The original proposal[2–5] of the presence of special short strong hydrogen bonds (SSHBs) in enzymatic active sites was based on experimental findings of unusual physicochemical properties as *e.g.* highly deshielded ^{1}H NMR signals, low fractionation factors and short heteroatom separations.[1,6] It was estimated, that the hydrogen bond energy of LBHB could be as much as 24 kcal mol^{-1},[5] and that necessary requirements for formation of LBHB were nearly matching pK_as of the heteroatoms in the hydrogen bond, a non-polar environment and an equally shared proton between the heteroatoms.[2,4–6] The proposal set off an intense debate and arguments were presented against the importance of LBHBs in enzyme catalysis.[7,8] Warshel *et al*. argued that[7] "the stabilization of hydrogen bonds is largely electrostatic in condensed phases" whereas Bachovchin and co-workers,[8] based on careful NMR experiments on serine proteases, concluded that the proton is at least 85% located at the nitrogen atom and that the Asp-His hydrogen bond of subtilisin BPN′, Scheme 1, is accessible by solvent. Therefore, failing two of the criteria for LBHB formation, these were ruled out as playing a role in serine proteases and in enzyme reactions in general, even though the physicochemical properties of an LBHB are found in several enzymes.[7,8]

Recently, a very high resolution (0.78 Å) structure of *Bacillus lentus* subtilisin was published.[9] The hydrogen bond between Asp32 and His64 was analyzed and the hydrogen of interest was found to be "partially shared" between His64(Nδ1) and Asp32(Oδ2) with distances measuring 2.6 Å for the heteroatom separation and 1.2 and 1.5 Å for the N–H and O–H separations, respectively, with a slight deviation from a linear arrangement of the three atoms involved in the hydrogen bond.[9] The Asp32-His64 dyad was not termed a LBHB, as the structure showed "…it was not shielded from solvent and because the pK_as of His64(Nδ1) and Asp32(Oδ2) would appear to be unmatched."[9]

A hydrogen bond is defined as a LBHB (or SSHB) based on the shape of the potential energy surface – if the hydrogen atom lies in a double minimum potential well with zero-point vibrational energies similar to the barrier height, it is a LBHB.[1] This definition of the SSHB (LBHB) is used in the present study. In this paper we will examine the consequences of having an explicit water molecule close to the Asp-His dyad of subtilisins on the shape of the potential energy surface by high-level theory computations. Electron correlation effects must be included to model the hydrogen bond in question. Our recent studies on similar systems uses the DFT method.[10] For intermolecular hydrogen-bonded systems, it has been shown that DFT with the B3LYP hybrid functional[11] represents a very good method for structural optimizations.[12] Solvation effects are included by combining a continuum solvation method with the explicit water molecule. This approach presents a very accurate modeling for this hydrogen bond.[13] Surprisingly, the results reveal that the water molecule is necessary for formation of the SSHB, in contrast to earlier belief of a completely deshielded environment.

A survey of high-resolution structures (< 2.0 Å) of subtilisins from the protein data bank[14] revealed that a water molecule hydrogen bonding to Asp32 is indeed a common structural feature. Eight such structures are displayed in Fig. 1. To model the Asp32-His64 dyad of the subtilisins, coordinates were extracted from the high-resolution structure of *Bacillus lentus* subtilisin.[9] The model was truncated to contain an aspartate ion, a methylimidazolium ion and the water molecule after addition of hydrogens. Upon energy minimization the water molecule moves slightly to a position where it still hydrogen bonds to Asp32(Oδ1) but also interacts with His64(Cε1) making an O···H–C hydrogen bond. In the enzyme, His64(Cε1) is found to hydrogen bond to the backbone carbonyl of Ser125 with a heteroatom separation of 3.16 Å.[9] The water molecule thus serves two enzymatic functions; being a hydrogen bond donor to Asp32 and a hydrogen bond acceptor towards His64(Cε1). To model the interesting hydrogen bond between His64 and Asp32, a large basis set,[10a,b] was used in all density functional theory calculations combined with a continuum solvation model[15] (see ESI†).

In order to evaluate the shape of the potential energy surface, two isomers are considered. Chemical structures of the ion–ion complex, **1**, denoted N-side, and the neutral complex **2**, termed O-side, are displayed in Scheme 2, together with the structure of the transition state, **3**, TS, for the hydrogen transfer reaction. Important numbers are found in Table 1 for the three isomers, all of which are all fully optimized at the B3LYP(COSMO)/6-31++G (d,pd) level of theory. In **1** and **2**, the N–H and O–H bonds show considerable elongation compared to non-hydrogen bonded structures. The computed numbers reveal that the O-side isomer is preferred by only 1.45 kcal mol^{-1} relatively to the N-side isomer. The internal



Scheme 1 The Asp32-His64 dyad of subtilisins with a protonated His64 that is required for formation of the short hydrogen bond.

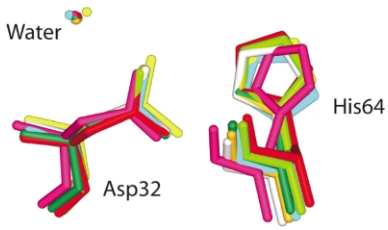
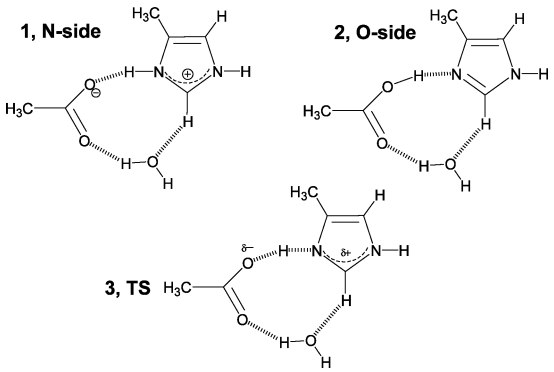


Fig. 1 Overlay of eight high-resolution structures (< 2.0 Å) of various subtilisins. The structural water molecule is found at the same volume of space in all structures. Pdb-codes are: 1DUI (cyan), 1GNS (white), 1SUP (magenta), 1GCI (dark green), 1CSE (red), 1SVN (orange), 1YJA (yellow) and 2SIC (light green).

† Electronic supplementary information (ESI) available: Computational methods and coordinates for the fully optimised structures of **1**, **2** and **3** at the B3LYP(COSMO)/6-31++G(d,pd) level of theory. See http://www.rsc.org/suppdata/cc/b3/b314228k/

DOI: 10.1039/b314228k



Scheme 2 Chemical structures of the O-side, N-side and TS-isomers.

barrier for hydrogen exchange is 0.15 kcal mol^{-1} when moving from N-side to O-side. Upon inclusion of the zero-point vibrational energies, the barrier vanishes, and the TS-structure becomes the lowest energy structure. The TS has an almost equally shared proton and a heteroatom separation of 2.48 Å, Fig. 2. This is slightly shorter than the experimentally reported number, 2.6 Å,[9] however one has to recall that an error of approximately ±0.1–0.2 Å is found in the experimental structure.[16] Interestingly, the computed structural parameters for **3** are very similar to the experimental numbers found for the low-temperature structures of a model compound.[17] In the TS, the hydrogen is partially transferred, however it resides closest to the nitrogen, in agreement with the NMR-results.[8]

The results are indicative of a short strong hydrogen bond (SSHB) of the low-barrier type, as the vibration frequencies are comparable to the internal hydrogen transfer barrier, thereby showing that the presence of a water molecule in close proximity to a potential SSHB does *not* prohibit its formation, in sharp contrast with what has previously been thought to be the case.[2,4–6] When either the explicit water molecule or the continuum is removed from the simulations, the N-side isomer no longer represents a minimum structure at the B3LYP(COSMO)/6-31++G(d,pd) level of theory. This observation shows that an explicit water molecule is a required structural element for formation of a SSHB in this model of subtilisin, probably because it stabilizes the system through hydrogen bonding to Asp32(Oδ1) and His64(Cε1), both of which are within interacting distance of Ser125(O) in the crystal structure. Asp32(Oδ1) and His64(Cε1) are hydrogen bonded to one another without the water molecule in the model.

In summary, the calculations in the present paper reveal that a mixed discrete continuum solvation model[13] is required to reproduce the experimental features of the His-Asp dyad in the catalytic triad of subtilisins. The results reveal that the water molecule hydrogen bonding to Asp32 in the crystal structure is essential for formation of a short strong hydrogen bond. When not present, the potential energy curve has just one minimum corresponding to **2**. This finding suggests that the hitherto belief of the necessity of having SSHB totally solvent screened is *not* important, and can thus *not* be used arguing against the formation of LBHB. Experimental reports for a strong HB in aqueous solution of pK_a-matched dicarboxylic acids have recently appeared in the literature,[18] supporting the findings in this paper. Recently, QM/MM studies of other serine proteases also show similar short N–O separations, when electron correlation is included.[19] The results may indicate that a partial proton transfer can be involved in the catalytic mechanism, as has been proposed in other enzymatic reactions.[20] Further studies are under way to gain deeper understanding of the role (electronic, energetic) of the explicit water molecule on the nature of the hydrogen bond.

Table 1 Optimized geometrical parameters and electronic energies (in Hartrees) calculated at the B3LYP/6-31++G(d,pd) level of theory using COSMO-solvation.[15] E_{elec} is the electronic energy in solution. The sum of the zero-point vibrational energies is added to E_{elec} in the last column (1 Hartree = 627.5 kcal mol^{-1})

	N–H/Å	O–H/Å	N···O/Å	N–H–O/°	E_{elec}/Hartree	(E_{elec} + E_{ZPV})/ Hartree
1	1.124	1.416	2.534	171.9	−571.141665	−570.957249
2	1.583	1.042	2.614	168.8	−571.143970	−570.959280
3	1.227	1.263	2.480	169.9	−571.141432	−570.960042

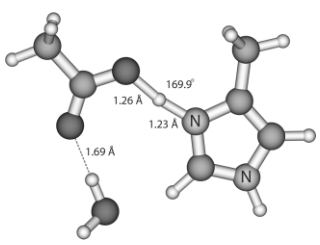


Fig. 2 Structure of the transition state, **3**, B3LYP(COSMO)/6-31++G(d,pd); important bond distances (Å) and angles (°) are shown. Oxygen atoms are displayed in dark gray, carbons in light gray.

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Notes and references

1 F. Hibbert and J. Emsley, *Adv. Phys. Org. Chem.*, 1990, **26**, 255–379.
2 W. W. Cleland, *Biochemistry*, 1992, **31**, 317–319.
3 J. A. Gerlt and P. G. Gasmann, *J. Am. Chem. Soc.*, 1993, **115**, 11552–11568; J. A. Gerlt and P. G. Gasmann, *Biochemistry*, 1993, **32**, 11943–11952.
4 W. W. Cleland and M. M. Kreevoy, *Science*, 1994, **264**, 1887–1890.
5 P. A. Frey, S. A. Whitt and J. B. Tobin, *Science*, 1994, **264**, 1927–1930.
6 P. A. Frey, *Magn. Reson. Chem.*, 2001, **39**, S190–198.
7 A. Warshel, A. Papazyan, P. A. Kollman, W. W. Cleland, M. M. Kreevoy and P. A. Frey, *Science*, 1995, **269**, 102–106.
8 (*a*) E. L. Ash, J. L. Sudmeier, E. C. De Fabo and W. W. Bachovchin, *Science*, 1997, **278**, 1128–1132; (*b*) W. W. Bachovchin, *Magn. Reson. Chem.*, 2001, **39**, S199–S213.
9 P. Kuhn, M. Knapp, M. Soltis, G. Ganshaw, M. R. Thoene and R. Bott, *Biochemistry*, 1998, **37**, 13446–13452.
10 (*a*) J. Overgaard, B. Schiøtt, F. K. Larsen, A. J. Schultz, J. C. MacDonald and B. B. Iversen, *Angew. Chem., Int. Ed.*, 1999; (*b*) J. Overgaard, B. Schiøtt, F. K. Larsen and B. B. Iversen, *Chem. Eur. J.*, 2001, **7**, 3756–3767; (*c*) B. Schiøtt, B. B. Iversen, G. K. H. Madsen and T. C. Bruice, *J. Am. Chem. Soc.*, 1998, **120**, 12117–12124; (*d*) B. Schiøtt, B. B. Iversen, G. K. H. Madsen, F. K. Larsen and T. C. Bruice, *Proc. Natl. Acad. Sci.*, 1998, **95**, 12799–12802.
11 (*a*) A. D. Becke, *J. Chem. Phys.*, 1993, **98**, 5648–5652; (*b*) C. Lee, W. Yang and R. G. Parr, *Phys. Rev. B*, 1988, **37**, 785–789.
12 (*a*) M. Lozynski, D. Rusinska-Raszak and H.-G. Mack, *J. Phys. Chem. A*, 1998, **102**, 2899–2903; (*b*) J. J. Novoa and C. Sosa, *J. Phys. Chem.*, 1995, **99**, 15837–15845.
13 (*a*) D. Sicinski, P. Paneth and D. G. Thrular, *J. Phys. Chem. B*, 2002, **106**, 2708; (*b*) A. J. A. Aquino, D. Tunega, G. Haberhauer, M. H. Garzabek and H. Lischka, *J. Phys. Chem. A*, 2002, **106**, 1862–1871.
14 H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindalov and P. E. Bourne, *Nucl. Acid Res.*, 2000, **28**, 235.
15 (*a*) A. Klamt and G. Schüürmann, *J. Chem. Soc., Perkin Trans. 2*, 1993, 799–805; (*b*) A. Klamt, *J. Phys. Chem.*, 1995, **99**, 2224–2235; (*c*) A. Klamt, *J. Phys. Chem.*, 1996, **100**, 3349–3353.
16 A. S. Mildvan, M. A. Massish, T. K. Harris, G. T. Marks, D. H. T. Harrison, C. Viragh, P. M. Reddy and I. M. Kovach, *J. Mol. Struct.*, 2002, **615**, 163–175.
17 T. Steiner, I. Majerz and C. C. Wilson, *Angew. Chem., Int. Ed.*, 2001, **40**, 2651–2654.
18 (*a*) P. A. Frey and W. W. Cleland, *Bioorg. Chem.*, 1998, **26**, 175–192; (*b*) J. Lin and P. A. Frey, *J. Am. Chem. Soc.*, 2000, **122**, 11258–11259.
19 (*a*) C.-H. Hu, T. Brinck and K. Hult, *Int. J. Quantum Chem.*, 1998, **69**, 89–103; (*b*) W. M. Westler, F. Weinhold and J. L. Markley, *J. Am. Chem. Soc.*, 2002, **124**, 14373–14381; (*c*) P. A. Molina and J. H. Jensen, *J. Phys. Chem. B*, 2003, **107**, 6226–6233.
20 D. M. Smith, B. T. Golding and L. Radom, *J. Am. Chem. Soc.*, 1999, **121**, 1383–1384.