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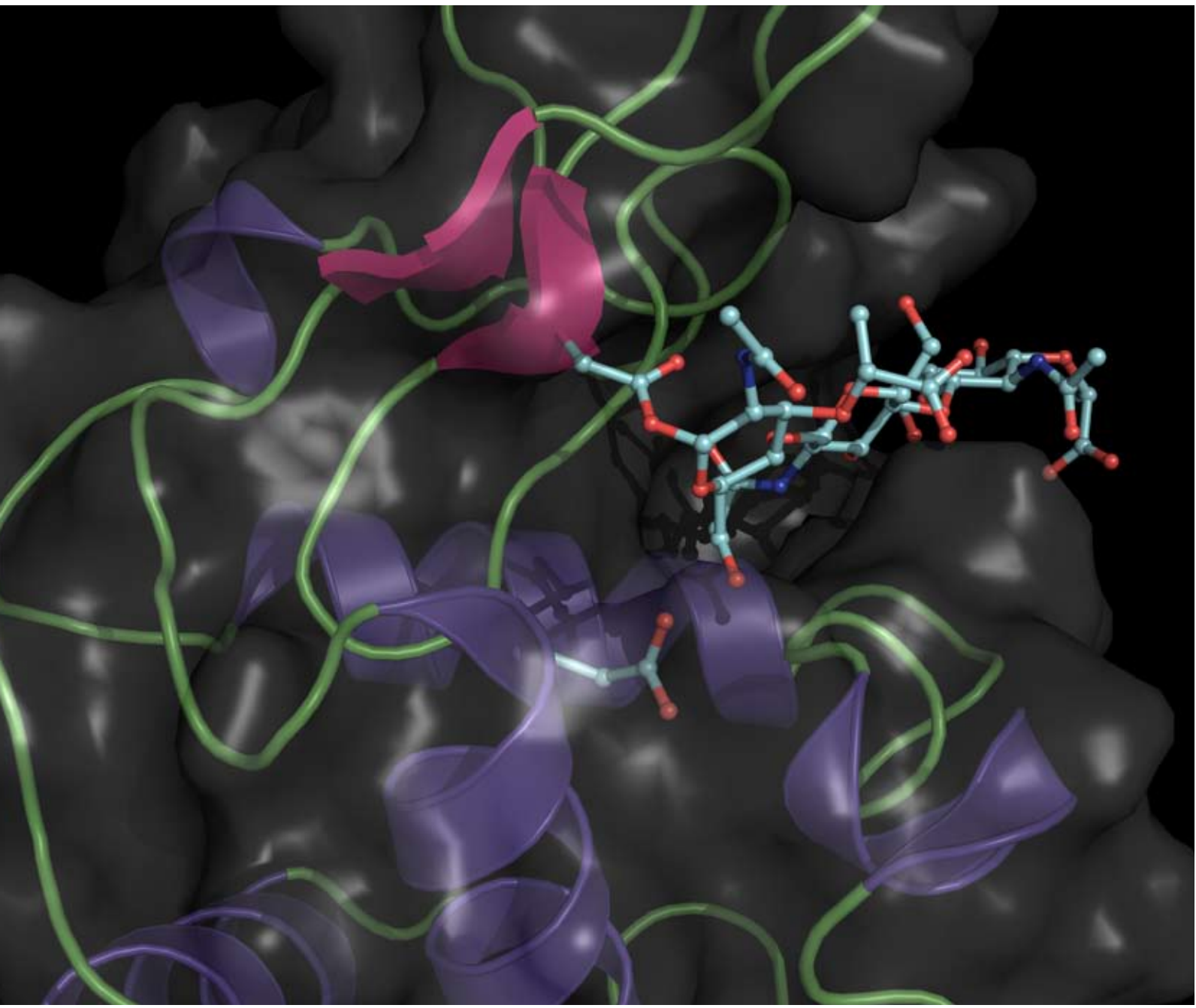

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QM/MM simulations predict a covalent intermediate in the hen egg white lysozyme reaction with its natural substrate†

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Quantum mechanics/molecular mechanics (QM/MM) molecular dynamics simulations indicate that the reaction of native HEWL with its natural substrate involves a covalent intermediate, in contrast to the 'textbook' mechanism for this seminal enzyme.

Hen egg white lysozyme (HEWL) was the first enzyme structure solved by X-ray crystallography, and the first for which a mechanism was proposed based on structural data.[1] Consequently, it is one of the most influential enzymes in the field of biochemistry. It hydrolyses a component of the polysaccharide cell wall in Gram-positive bacteria, cleaving the β(1–4) glycosidic bond between *N*-acetyl-muramic acid (NAM) and *N*-acetyl-D-glucosamine (NAG). The enzyme binds the polysaccharide in a cleft six saccharide units long, traditionally designated sites (or subsites) A to F (or in more systematic nomenclature −4 to +2).[2] In the original mechanism of Phillips *et al.*,[1] the D (−1) site NAM was proposed to be distorted from the chair conformation when bound to the enzyme.[3,4] A dissociative (S_N1-type) mechanism was suggested, in which glutamic acid 35 donates a proton to the glycosidic oxygen atom between rings D and E (−1 and +1). This leads to cleavage of the glycosidic bond and, according to the original mechanism, an ionic (oxocarbenium) intermediate results.[5] The positive charge of this proposed intermediate was suggested to be stabilized electrostatically by the negative charge of the carboxylate group of the neighbouring aspartate 52 side-chain (Fig. 1).

Evidence from crystallography and electrospray ionization mass spectrometry[6,7] indicates that reaction proceeds *via* a covalent intermediate. However, these techniques require either a (less active, *e.g.* E35Q) mutant HEWL or an unnatural (*e.g.* fluorinated) substrate. In contrast to the earlier proposal, this is an S_N2-type reaction with a covalent intermediate formed between the D site NAM and Asp52. This mechanism, originally proposed by Koshland (Fig. 1), is widely accepted for most retaining β-glycosidases other than HEWL.[8,9]

Here, we have applied combined quantum mechanics/molecular mechanics (QM/MM) molecular dynamics simulations, with high-level energy corrections, to investigate the mechanism of native (wild-type) HEWL with its natural substrate. The reaction was studied by free energy calculations based on QM/MM umbrella sampling molecular dynamics simulations, which allow for flexibility of the substrate and the HEWL active site as well as for explicit solvation of the enzyme. Previous calculations on small models did not include the protein and were inconclusive.[10] Here we include the essential effects of the protein (the whole protein is included in our model),[5] and the water environment, and allow for fluctuations in the enzyme structure.

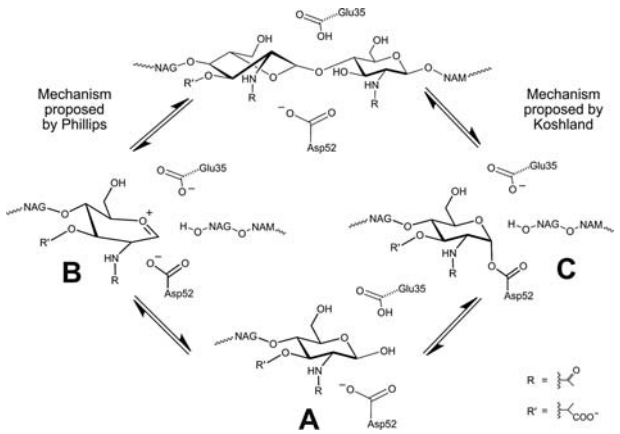

Fig. 1 Catalytic mechanisms for HEWL, proposed by Phillips[1] (*via* an oxocarbenium ion intermediate **B**) and Koshland (*via* covalent intermediate **C**). QM/MM results here indicate that HEWL follows the Koshland mechanism.[8] The transition state found here resembles the oxocarbenium ion.

The starting models for the present simulations were entirely rebuilt and refined from the high-resolution crystal structure of trisaccharide 2-acetamido-2-deoxy-D-muramic acid-β(1→4)-2-acetamido-2-deoxy-D-glucose-β(1→4)-2-acetamido-2-deoxy-D-muramic acid (NAM-NAG-NAM), bound to sites B, C, and D (−3, −2, and −1) in the active site of HEWL.[3,11] The D site NAM sugar ring of the product of catalysis is distorted from the chair form when bound in the active site.[3,11] The whole enzyme was included in all simulations. Our calculations are run backwards from the product **A**, simulating the second half of the reaction: the crystal structure which was used for the simulations is of the (trisaccharide) product of the reaction. The simulations used a stochastic boundary approach.[12,13] The protein was solvated by superimposing a 25 Å radius sphere of TIP3P water molecules and equilibrated by a procedure similar to ref. 13.

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The QM region (*i.e.*, the entire D site NAM (NAMD) and side-chains of Glu35 and Asp52, 53 atoms in total) was treated with the PM3 semi-empirical method. In order to describe the three bonds crossing the QM/MM boundary, 'link atoms' were placed between O4 of the D Site NAM and C1 of the C site NAG, and between the Cβ and Cγ of the Glu35 and Asp52 side-chains.[14] The charge on the QM system was −2. All other atoms (6642) were treated using the CHARMM22 (all-hydrogen) MM force field.[15] Simulations were carried out with a modified version of CHARMM version 27b2.[16]

To model the proton transfer from Glu35 to the glycosidic oxygen of the D site NAM (O1), a first reaction coordinate was defined as: r_1 = *d*(Glu35 Oε1–Glu35 Hε1) − *d*(Glu35 Hε1–NAMD O1). The second reaction coordinate was defined as: r_2 = *d*(NAMD O1–NAMD C1), for the glycosidic bond being broken (water molecule leaving). Simulations were also performed with a reaction coordinate defined as: r_3 = *d*(NAMD C1–Asp52 Oδ2) to model the conversion of the covalent intermediate to the ionic form. More than 1 ns of sampling was performed (20 ps at every 0.1 Å along each reaction coordinate), using a 1 fs timestep. The reaction coordinate statistics of the various simulations were combined by means of the weighted histogram analysis method.[13,17]

High level quantum chemical energy corrections[18] were applied to obtain reliable free energy profiles (to overcome limitations of the PM3 method; see Supplementary Information†). Representative small models of important species in the reaction were optimized in the gas phase at various levels of theory, and their energies compared. The energies quoted below are corrected at the B3LYP/6-311 + G(2d) level; where the MP2/6-311 + G(2d) result is significantly different, it is quoted in parentheses.

Protonation of the glycosidic oxygen (O1) is unfavourable by ∼5 kcal mol^{-1}, with a barrier of $\Delta^{\ddagger}G$ ∼16 kcal mol^{-1} (Fig. 2). The proton transfer is complete by r_1 = 0.4 Å. There is a subsequent barrier to breaking the glycosidic bond of approximately another 12 kcal mol^{-1} (∼17 kcal mol^{-1}) relative to the (protonated NAMD sugar) minimum between **A** and **B**. The highest energy point, **B**, resembles an oxocarbenium ion; as shown by positive Mulliken charges on the C1 (+0.30 *e*) and the O5 ring oxygen (+0.02 *e*), and its planar conformation (∼^4E). The average value of ω (the C5O5–C1C2 dihedral angle of NAMD recorded every 0.5 ps during r_2) for the transition state, **B**, was 0.9 ± 3.1°; the oxocarbenium ion character of the transition state is in agreement with experimental kinetic isotope effects.[19] In the TS, the Asp52 carboxylate oxygen is on average 0.6 Å closer to the sugar ring than in **A** (average C1–Oδ2 distances 2.9 ± 0.1 Å and 3.5 ± 0.1 Å, respectively): Asp52 stabilizes the positive charge of the transition state.

After the hemiacetal C–O bond breaks, the covalent intermediate, **C**, is formed spontaneously (at r_2 = ∼2.3 Å). The +0.3 *e* positive charge on C1 at r_2 = 2.0 Å is reduced to −0.1 *e* as the bond is formed between the D site NAM C1 and Asp52 Oδ2 at r_2 = 2.3 Å. The total barrier (from **A** to **B**) to forming the intermediate is ∼18 kcal mol^{-1} (∼23 kcal mol^{-1}) (Fig. 2), which compares well with the experimental value of 17 kcal mol^{-1} (derived from the rate constant for the overall hydrolysis reaction in the forward direction).[20] Although the reverse reaction is modelled here, the barrier is probably fairly similar to that of the forward reaction: water is a comparable leaving group to the NAG-NAM (E–F sites) disaccharide, with a relatively small basicity difference (1–2 pK_a units).

Prior to proton transfer (at r_1 = 0.4 Å) there is an average of 0.95 ± 0.05 hydrogen bonds between Glu35 Hε1 and the D site NAM O1. There is also an average of 0.98 ± 0.02 hydrogen bonds between the D site NAM HN2 of the acetyl side chain and Asp52 Oδ1. However, after the covalent intermediate is formed (*i.e.* after around r_2 = 2.3 Å) this latter hydrogen bond is not observed. The separation between Asp52 Oδ2 and Glu35 Oε1 decreases from ∼5.8 Å in **B** to ∼5.1 Å in **C**. Hydrogen bonding between Val109 NH and the D site NAM O6 increases (from ∼0 to ∼1) as a result of protonating the sugar. This hydrogen bond persists throughout the r_2 reaction path, including for the intermediate (**C**). A hydrogen bond was present throughout between Asp52 Oδ1 and Asn46 Hδ21, but that between Asp52 Oδ1 and Asn59 Hδ21 is lost when the covalent intermediate is formed (r_2 = 2.3 Å). The large free energy gain from forming the covalent intermediate easily outweighs the loss of this hydrogen bond.

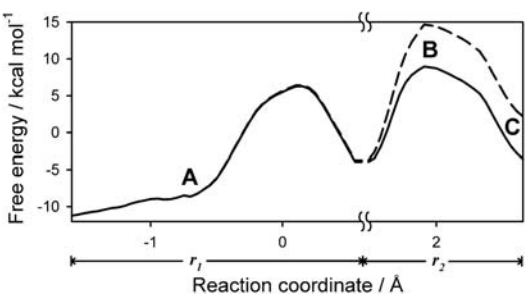

Fig. 2 Free energy profiles for the proton transfer from Glu35 to the D site NAM O1 (r_1) and subsequent breaking of the D site NAM C1–O1 bond in HEWL (r_2) at B3LYP/6-311 + G(2d)–PM3/CHARMM22 (solid line) and MP2/6-311 + G(2d)–PM3/CHARMM22 (dashed line) levels.

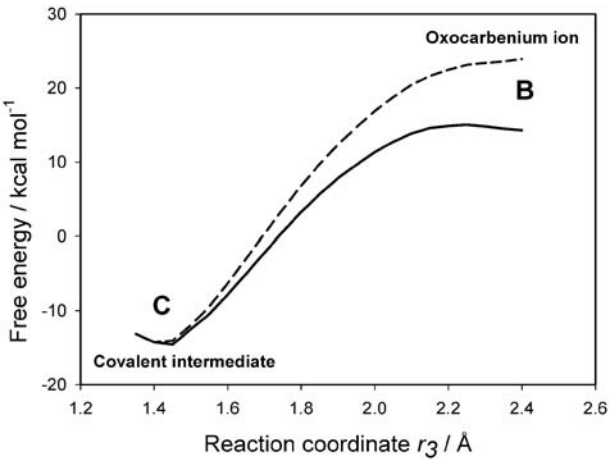

Fig. 3 QM/MM free energy profiles for the breaking of the bond between the D site NAM C1 and Asp52 Oδ2 (*i.e.* for conversion of **C** (left hand side) to **B** (right hand side)): B3LYP/6-311 + G(2d)–PM3/CHARM22 (solid line), MP2/6-311 + G(2d)–PM3/CHARM22 (dashed line). Reaction coordinate: r_3 = *d*(NAMD C1–Asp52 Oδ2)

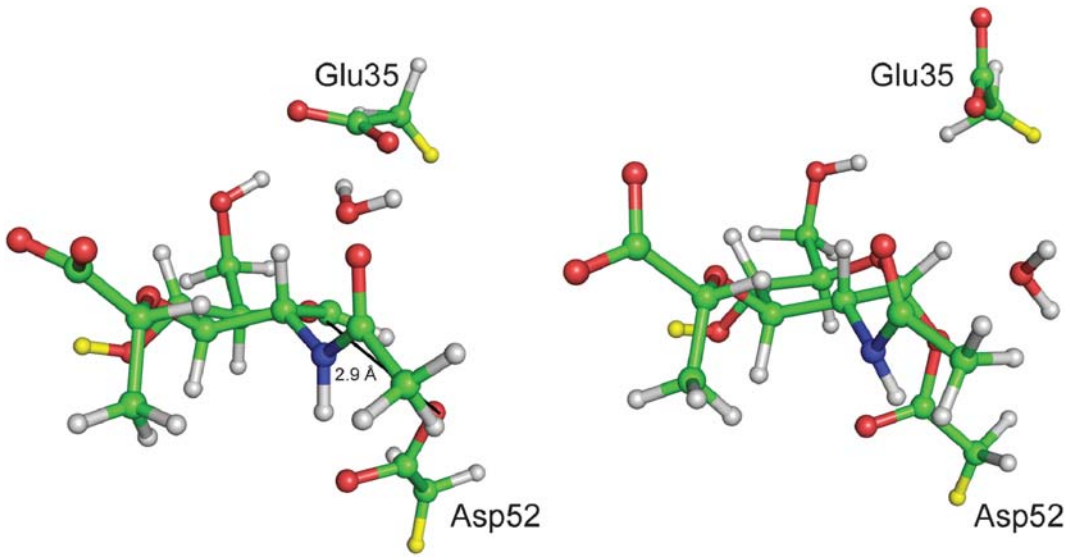

Fig. 4 Structures of the transition state **B** (r_2 = 2.0 Å), left hand side, and the covalent intermediate **C** (r_2 = 2.5 Å), right hand side. Representative snapshots from QM/MM (PM3/CHARMM22) umbrella sampling molecular dynamics simulations at the respective reaction coordinate values are shown. Only atoms in the QM region are shown, for clarity (*i.e.* the D site NAM sugar and the side-chains of Glu35 and Asp52). The distance between Asp52 Oδ2 and the D site NAM C1 decreases from ~2.9 Å in **B** (indicated in the figure) to ~1.4 Å in **C**. 'Link' atoms are shown in yellow.

Finally, we compared the relative stability of the ionic (**B**) and covalent (**C**) forms by simulating their interconversion (using reaction coordinate r_3). The free energy profile (Fig. 3) shows the covalent intermediate (at r_3 = 1.4 Å) to be significantly more stable than the oxocarbenium ion (at r_3 = ~2.4 Å), by ~30 kcal mol^{-1} (38 kcal mol^{-1}). This large difference in energy is likely to be well outside the error range of the calculations given the high levels of theory used for correction of the energies. We have also simulated the endocyclic protonation mechanism,[21] but find it to be prohibitively high in energy compared to the exocyclic protonation mechanism presented here.

We conclude that catalysis in HEWL proceeds *via* a covalent intermediate (Fig. 4). The conclusion is reinforced by the use of several levels of theory (*e.g.*, AM1/CHARMM22, see Supplementary Information†). This finding is contrary to long-standing theories and to the mechanism shown in most textbooks. The results here indicate that findings for a mutant HEWL with a fluorinated substrate[7] are indeed relevant for the wild-type enzyme with its natural substrate. This work is the first demonstration of formation of a covalent intermediate in wild-type hen egg white lysozyme.

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