Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate

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Hen egg-white lysozyme (HEWL) was the first enzyme to have its three-dimensional structure determined by X-ray diffraction techniques[1]. A catalytic mechanism, featuring a long-lived oxocarbenium-ion intermediate, was proposed on the basis of model-building studies[2]. The 'Phillips' mechanism is widely held as the paradigm for the catalytic mechanism of β-glycosidases that cleave glycosidic linkages with net retention of configuration of the anomeric centre. Studies with other retaining β-glycosidases, however, provide strong evidence pointing to a common mechanism for these enzymes that involves a covalent glycosyl-enzyme intermediate, as previously postulated[3]. Here we show, in three different cases using electrospray ionization mass spectrometry, a catalytically competent covalent glycosyl-enzyme intermediate during the catalytic cycle of HEWL. We also show the three-dimensional structure of this intermediate as determined by X-ray diffraction. We formulate a general catalytic mechanism for all retaining β-glycosidases that includes substrate distortion, formation of a covalent intermediate, and the electrophilic migration of C1 along the reaction coordinate.

The natural substrate of HEWL is the peptidoglycan cell wall of Gram-positive bacteria. It is composed of crosslinked oligosaccharides consisting of alternating 2-acetamido-2-deoxy-glucopyranoside (NAG) and 2-acetamido-2-deoxy-3-*O*-lactyl-glucopyranoside (NAM) residues. In the textbook mechanism proposed by Phillips (Fig. 1, path B), the enzyme cleaves the substrate by first binding the polysaccharide in a cleft six saccharide units long (A–F or −4 to +2). The substrate was proposed to bind in a distorted conformation in which a NAM unit in the −1 (D) subsite adopts a half-chair conformation, thereby forming a kink in the oligosaccharide between sites −1 and +1 (D and E). A catalytic acid/base, Glu 35, was suggested to protonate the glycosidic oxygen while Asp 52 stabilizes the developing oxocarbenium ion intermediate through electrostatic interactions. It was also proposed that Asp 52 acts to shield the α-face of the glycosyl oxocarbenium ion, such that it can only be intercepted by water from the β-face to yield the hydrolysed product with retained stereochemistry at C1. It was later demonstrated that the intermediate could also be intercepted by another saccharide moiety that had diffused into the active site, producing a transglycosylation product[4].

The nature and purported stability of the ion-pair intermediate have always been points of contention. The lifetime of the glycosyl cation in water (10^{-12} s) is estimated to be just slightly longer than a bond vibration—much less than the time required for diffusion events[5]. Furthermore, there is no experimental evidence for a long-lived ion-pair intermediate at the active site of any glycosidase, including HEWL. Conversely, for every retaining β-glycosidase studied in detail to date[6], the intermediate has been shown to be a reactive, covalent acylal ester or, in the case of retaining β-hexosaminidases using neighbouring-group participation, a covalent oxazoline intermediate[7]. The most rigorous demonstration of the covalent nature of the intermediate has been obtained by measuring α-deuterium kinetic isotope effects (k_H/k_D, where k_H and k_D are rate constants for reactions of protium- and deuterium-labelled substrates) with substrates for which the breakdown of the intermediate is rate determining. In all of these cases, k_H/k_D has been found to be greater than unity[6,8]. Effects greater than unity, measured on the second step, indicate that the anomeric centre undergoes rehybridization from sp^3 in the intermediate to sp^2 in the transition state. These results are consistent only with the collapse of a tetrahedral covalent intermediate through an oxocarbenium ion-like transition state. A long-lived ion pair may not be reconciled with these observations.

In order to observe the covalent intermediate experimentally, a situation in which the rate of formation (k_2) of the intermediate is significantly greater than its rate of breakdown (k_3) must be created (Fig. 1). Considerable efforts have previously been made, with HEWL, to find such a condition yet in no case have these efforts proven successful[9,10]. This observation strongly suggests that, for HEWL, hydrolysis of the intermediate (k_3) occurs at a much greater rate than does its formation (k_2). We used a mutant enzyme in which the catalytic acid/base carboxylate residue had been replaced by the corresponding amide: the HEWL(E35Q) mutant enzyme. Mutation of the catalytic acid/base residue of HEWL and other β-retaining glycosidases has been shown to greatly reduce the rate of hydrolysis of unactivated glycosides by slowing both steps of the reaction[11,12]. Substrates bearing activated leaving groups do not require significant acid catalytic assistance and so react at near wild-type rates to yield the glycosyl-enzyme intermediate[12]. Hydrolysis of this intermediate, however, is slowed enormously as the attack of water no longer benefits from the base catalysis ordinarily provided by the enzyme. Indeed, on incubation of HEWL(E35Q) with

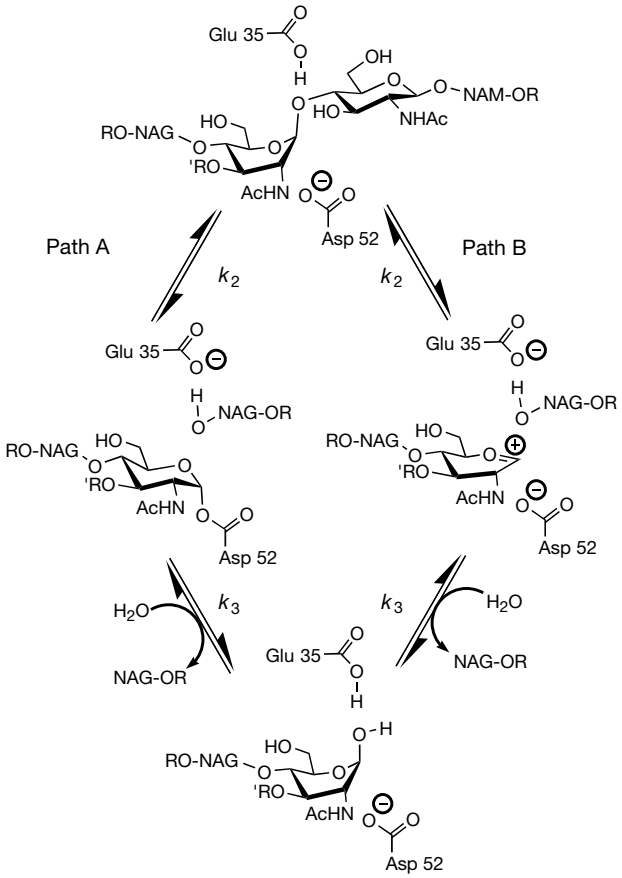

Figure 1 Two possible catalytic mechanisms for HEWL. Path A; the Koshland mechanism; path B; the Phillips mechanism. R, oligosaccharide chain; R′, peptidyl side chain.

chitobiosyl fluoride (NAG_2F)[13] we observed, using electrospray ionization mass spectrometry (ESI-MS), a mixture of the free enzyme HEWL(E35Q) and a high steady-state population (Fig. 2b) of a covalent intermediate (relative molecular mass (M_r) 14,719) with a mass increase (405) revealing the attachment of a chitobiosyl moiety (M_r 407) to HEWL. This intermediate turned over too rapidly for X-ray analysis, so a complementary strategy was sought.

To reduce the rate of the second step in a different approach, we incorporated fluorine in place of the 2-acetamido group of chitobiose, while keeping a good leaving group at the anomeric centre. Previous studies, designed to rule out a possible mechanism involving anchimeric assistance from the 2-acetamido group, demonstrated that substrates bearing hydrogen and hydroxyl substitutions at this centre are hydrolysed at similar rates to that of the parent compound bearing the acetamido group, and thus reveal that the amide functionality is not critical for catalysis[14,15]. An electronegative fluorine substituent at the 2 position will, however, inductively destabilize the oxocarbenium ion-like transition states leading to the formation and hydrolysis of the intermediate, slowing both steps. When a good leaving group is also incorporated to accelerate the first step, the net result is that the breakdown of the intermediate (k_3) is slowed to a greater extent than its formation (k_2), which leads to accumulation of the intermediate. Incubation of wild-type HEWL with 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride (NAG2FGlcF; synthesis to be described elsewhere) again yielded a significant steady-state population of the covalent glycosyl-enzyme intermediate as detected by ESI-MS (Fig. 2c). The mass difference between the wild-type enzyme (M_r 14,316) and the intermediate (M_r 14,683) is 367 (368 expected), demonstrating the accumulation of the 2-fluorochitobiosyl enzyme intermediate. This intermediate was formed some 126-fold slower than with NAG_2F (k_{cat}/K_m = 9.6 $\pm$ 0.1 $M^{-1}\,min^{-1}$ and k_{cat}/K_m = 1,200 $M^{-1}\,min^{-1}$, respectively, where k_{cat} is the rate constant when both substrates are at saturating concentrations and K_m is the Michaelis constant) but still turned over too rapidly ($k_3 > 96\,min^{-1}$) for X-ray crystallographic studies.

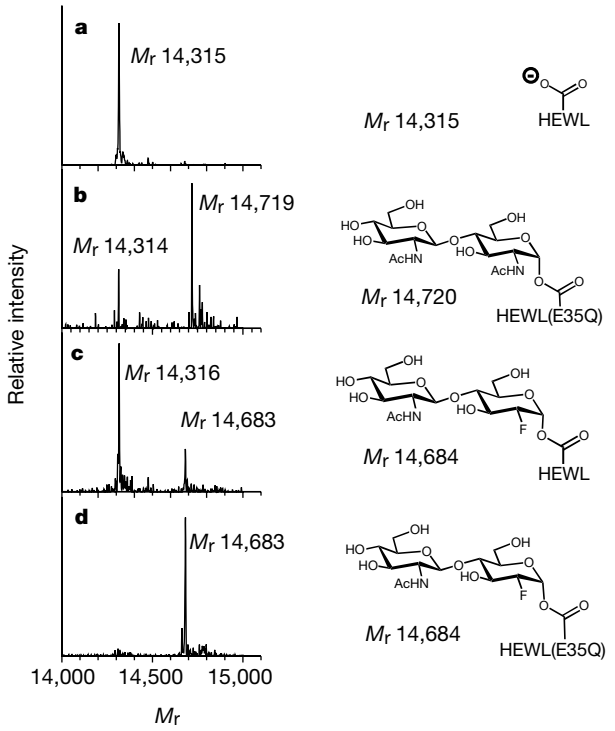

Figure 2 Transform of the ESI-MS mass spectra of HEWL and HEWL complexes. **a**, Wild-type HEWL. **b**, HEWL(E35Q) incubated with NAG2F. **c**, Wild-type HEWL incubated with NAG2FGlcF. **d**, HEWL(E35Q) incubated with NAG2FGlcF. Structures of the species corresponding to each peak observed in the mass spectra are shown to the right, with their expected relative molecular mass.

By combining these two completely independent approaches, we characterized the covalent glycosyl-enzyme intermediate formed on lysozyme by X-ray crystallography. On incubation of HEWL(E35Q) with NAG2FGlcF, the stoichiometric accumulation of a covalent intermediate could be observed by ESI-MS (Fig. 2d). The second-order rate constant governing the formation of the intermediate (k_i/K_i = 0.20 $\pm$ 0.05 $M^{-1}\,min^{-1}$) is only 50-fold less than that observed for the wild-type enzyme interacting with the same substrate. Hydrolysis of the covalent intermediate, however, was slowed by at least 5.3×10^5-fold ($k_3 < (1.8 \pm 0.6) \times 10^{-4}\,min^{-1}$) with the result that the covalent glycosyl-enzyme intermediate was stable for several days (half-life $t_{1/2}$ = 64 h) at 25 °C. The HEWL(E35Q) regenerated on such turnover could once again be labelled by incubating it with NAG2FGlcF (data not shown), indicating that the formation and breakdown of the intermediate leaves the enzyme unchanged and catalytically competent.

Diffraction data collected from crystals of the HEWL(E35Q) covalent glycosyl-enzyme formed by reaction with NAG2FGlcF reveal a complex in which the covalent linkage (~1.4 Å) between C1 and $O^{\delta 2}$ of Asp 52 is unambiguous (Fig. 3a). The covalent acylal linkage is formed on the *syn* lone pair of the catalytic nucleophile as has been observed for all retaining β-glycosidases studied to date[16–18]. One objection to the possibility of a covalent intermediate, based on the X-ray crystal structure of a NAM-NAG-NAM trisaccharide bound to the −3 to −1 (B–D) subsites of HEWL (MGM-HEWL), was that Asp52 was oriented such that only an *anti* lone pair was correctly disposed for nucleophilic attack at C1 of the −1 pyranose ring and that reorientation to align the *syn* lone pair would require major conformational change of the β-strands[19]. Such conformational change is not necessary and all that is observed is a rotation of 45° around χ_1 of Asp 52 such that the *syn* lone pair can engage the C1 atom to form the intermediate while leaving $O^{\delta 1}$ in an unchanged position (Fig. 3b). In the covalent complex observed here, the −1 pyranose ring adopts an undistorted $^4C_1$ chair conformation, as seen in the structures of all other hexopyranose covalent intermediates so far observed (Fig. 4a)[16–18].

Another argument put forward against the possibility of a covalent intermediate in the HEWL reaction[19] is based on the near van der Waals distance (3.2 Å) between the C1 atom of the distorted −1 (D) pyranose ring and Asp 52 observed in the MGM-HEWL product complex (Fig. 3b). These two groups cannot be expected to be significantly closer in a noncovalent complex, and in order to form the intermediate, in which the C1–$O^{\delta 2}$ distance is approximately 1.45 Å, C1 and $O^{\delta 2}$ have moved 1.75 Å closer. Much of this motion occurs at C1, arising from relaxation of the distorted pyranose ring to the chair conformation, leaving the sugar–enzyme contacts involving O6 and O4 almost unchanged (Fig. 3b). A conformational rearrangement of Asn 46 is necessary to prevent steric clashes between the 2-fluoro substituent and the enzyme. Although Asn 46 is disordered in the structure obtained here, it is entirely possible, with substrates in which a 2-acetamido group assumes the place of the 2-fluoro substituent, that both Asn 46 and Asp 52 may hydrogen bond to the nitrogen of the acetamido group. The ESI-MS results (see above) clearly indicate that the presence of the 2-acetamido group does not preclude formation of a covalent intermediate.

A detailed comparison of the structure determined here with that of the MGM-HEWL complex is complicated by the mobility observed in the MGM complex, manifested as high-temperature factors, that may be a consequence of it being a product complex. Fortunately, HEWL has an active-site architecture very similar to that of almost all other retaining β-glycosidases, wherein the two key carboxyl groups are positioned ~5 Å away from each other, and

this suggests a common mechanism. Therefore, detailed insights into how HEWL accomplishes catalysis may be gained by examining the conformations of Michaelis and intermediate complexes of several β-retaining glycosidases.

Several such Michaelis complexes have now been observed using X-ray crystallography and in all of these cases the reactive pyranose adopts a skew-boat or distorted envelope conformation (Fig. 4b, c)[18,20,21]. C1 assumes a position above the plane of the sugar ring approximately 3 Å away from the catalytic nucleophile with the leaving group held in a pseudo-axial position by the enzyme. A comparison of representative Michaelis complexes with the covalent HEWL glycosyl enzyme structure determined in this study reveals that C1 undergoes the greatest change in position (Fig. 4b, c), whereas both the nucleophile and leaving group remain relatively fixed in space throughout the catalytic cycle. The mechanism can thus be described as an electrophilic migration of C1 from above the ring plane to below the ring plane where it easily bridges the 1.6–1.8 Å gap required to form a covalent intermediate. Additionally, the

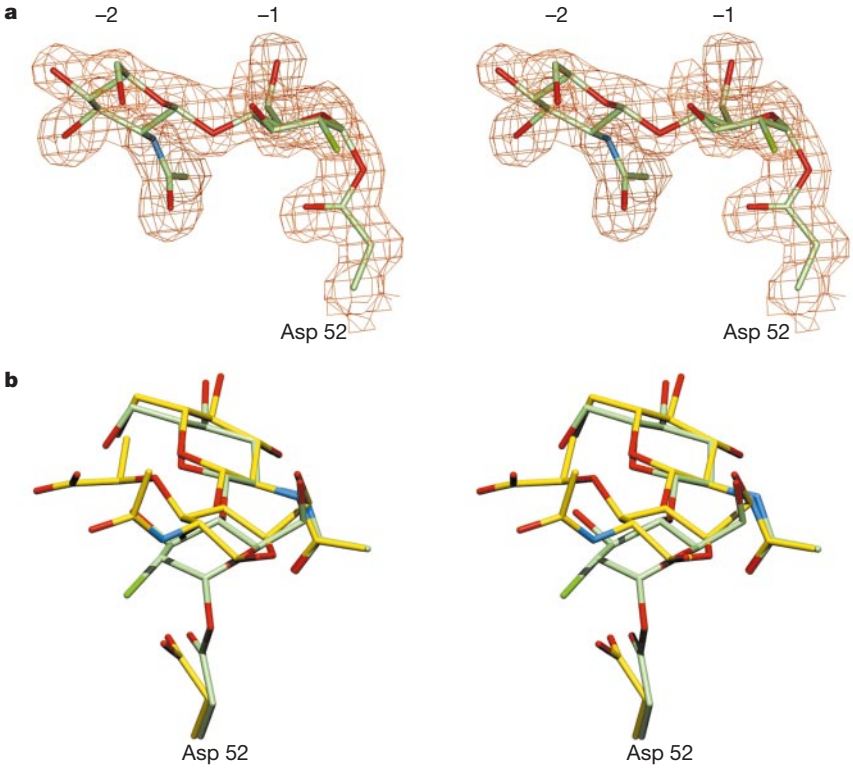

Figure 3 Structure of the covalent intermediate in the HEWL reaction. **a**, Maximum likelihood / σ_A-weighted $2F_O - F_C$ electron density for the covalent glycosyl-enzyme intermediate of HEWL, contoured at 0.4 electrons per Å^3. **b**, Overlap of the HEWL covalent intermediate and the product complex with MGM[19]. Formation of the intermediate is accompanied by an approximate 45° rotation around χ_1 of Asp 52.

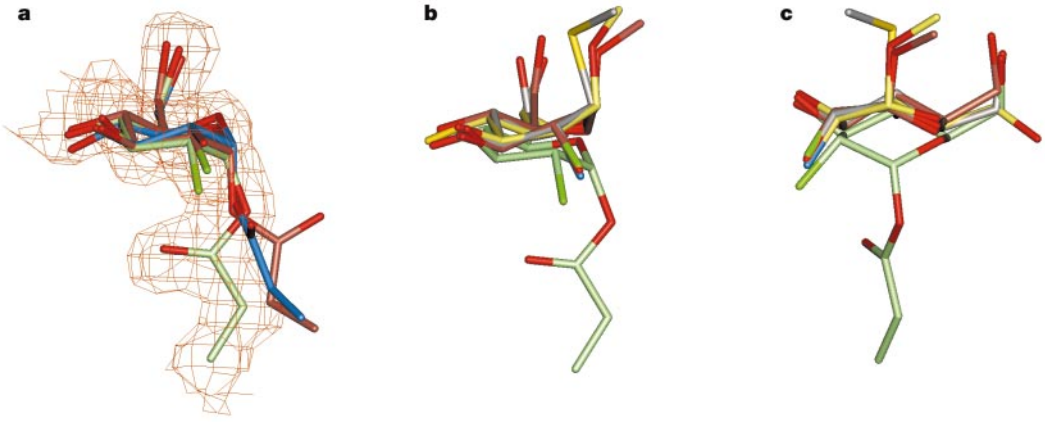

Figure 4 Comparison of hexopyranose covalent intermediates. **a**, 'Side' view of the overlap of three covalent glycosyl-enzyme intermediates: the HEWL intermediate described here (green), the cellobiosyl-enzyme intermediate from the *Cellulomonas fimi* Xyn10 (blue; ref. 17), and the 2-F cellobiosyl-enzyme intermediate from *Bacillus agaradhaerens* Cel5A (brown; ref. 18). For simplicity, only the –1 subsite sugars are shown. Electron density for the HEWL complex is shown contoured at 0.48 electrons per Å^3. **b**, **c**, 'Side' (**b**) and 'end' (**c**) view of three Michaelis complexes of retaining β-glycoside hydrolases (*Serratia marcesans* chitobiase[21] (yellow; the 2-acetamido group has been truncated at N2 for clarity), *Fusarium oxysporum* Cel7B (grey; ref. 20) *and B. agaradhaerens* Cel5A (brown; ref. 18)) together with the HEWL covalent glycosyl-enzyme intermediate (green). C1 has to migrate ~1.2 Å to form the covalent bond with the nucleophilic oxygen. Only the –1 subsite sugars are shown.

formation of the intermediate does not require any atoms of the −1 (D) pyranose ring, other than C1, to approach Asp 52. Indeed, as C1 migrates towards the nucleophile, the 2-substituent necessarily twists away from the β-sheet of the enzyme on relaxation of the ring to the chair conformation. The transition state must be reached as C1 assumes a position along this coordinate where it approaches coplanarity with the C2, O5 and C5 atoms of the sugar ring. Kinetic isotope effects measured on the first step of catalysis by HEWL[14,22] and other retaining glycosidases[6,8] reveal a highly dissociative, oxocarbenium ion-like transition state that is consistent with such an electrophilic migration mechanism[23].

The hydrolysis of the covalent intermediate occurs by the near reverse of the first step, except that C1 migrates from its position below the ring plane to form a bond with a water molecule positioned in the location previously occupied by the substrate glycosidic oxygen. Water and other oxygen nucleophiles have been observed to occupy this position in several structures of covalent intermediates[4,16,18].

The crucially important role of Asp 52 as a nucleophile is underscored by the extremely low levels of activity that HEWL(D52N) mutants have on well defined substrates (<0.1%)[11]. The considerable residual activity (~5%) of the HEWL(D52A) mutant on complex cell-wall substrates has been shown to arise from chemical rescue wherein free carboxylate groups of the peptide moieties of the cell wall take the place of Asp 52 (ref. 24). Goose egg-white lysozyme (GEWL), which bears some similarity to HEWL, lacks an equivalent to Asp 52, and was long considered to provide some evidence pointing to a non-critical role for Asp 52 in HEWL. However, GEWL in fact uses a mechanism completely different from that of HEWL, which results in the net inversion of configuration at the anomeric centre[25].

The results of this study point to the nature of the intermediate in the HEWL-catalysed hydrolysis of glycosides. On the basis of these results, we conclude that HEWL, in common with all other retaining β-glycosidases, performs catalysis by the formation and subsequent breakdown of a covalent intermediate species and not by the formation of a long-lived ion pair. This revised mechanism unifies the substrate distortion proposed by Phillips, the covalent intermediate first postulated by Koshland, and the electrophilic migration of C1 along the reaction coordinate. It is consistent with both the anti-periplanar lone-pair hypothesis and the principle of least nuclear motion. Most importantly, this mechanism is supported by all experimental data including kinetic isotope effects, mass spectrometry, crystal structures and enzyme kinetics for all retaining β-glycosidases studied, to our knowledge. This suggests that all members of this very broad class of enzymes use the same fundamental mechanism. □

Methods

ESI-MS spectra were recorded on a PE-Sciex API III mass spectrometer equipped with an ion-spray ion source. Samples were introduced by reverse-phase HPLC (C_{18} column with MeCN, H_2O and TFA elution) directly interfaced with the mass spectrometer. Crystals of the HEWL covalent intermediate were grown by the hanging-droplet vapour-diffusion method over 4 d at 16 °C. The droplet, containing 2.5 μl of a solution of 7.5 mg ml^{-1} HEWL(E35Q), 20 mM NAG2FGlcF, 200 mM sodium acetate buffer at pH 5.0 and 2.5 μl of reservoir solution (200 mM sodium acetate, containing 4.0% NaCl (w/v), pH 4.5), was suspended over the reservoir solution. Crystals were transferred to cryprotectant solution that contained 25% glycerol in addition to the reservoir buffer before freezing. Data were collected on an RAXIS IIc image-plate detector using CuKα radiation and were processed using the HKL suite of programs[26]. All subsequent procedures used the CCP4 suite of programs unless otherwise stated[27]. Data are 99.4% complete to 1.64 Å with an R_{merge} of 0.054, a mean I/σ of 25 and a multiplicity of 5.2. All structures crystallized isomorphously with the $P4_32_12$ crystal form of HEWL with approximate cell dimensions $a = b = 78.63$, $c = 36.7$, $\alpha = \beta = \gamma = 90°$. The uncomplexed HEWL(E35Q) structure (not shown) was used as the starting model for refinement. Five per cent of the observations were flagged for cross-validation analysis and were 'released' through an extended simulated annealing run, to 3,000 K, using the program CNS[28]. The complex structure was refined using REFMAC[29]. The final model structure has an R_{cryst} of 0.15, an R_{free} of 0.20, and stereochemical deviations from target values of 0.013 Å for bonds and 1.5° for angles.

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