A low energy short hydrogen bond in very high resolution structures of protein receptor–phosphate complexes

A very short hydrogen bond between an Asp and a phosphate is established in two high resolution structures (0.98 and 1.05 Å). A mutant complex that changes the Asp to an Asn , which forms a normal hydrogen bond, has a similar free energy of binding to the wild type complex, suggesting that the contribution of the short hydrogen bond is not extraordinarily strong.

Sir — Short (2.45 Å) or low barrier hydrogen bonds (LBHBs), which possess strengths of as much as 30 kcal mol^{-1} for model compounds in the gas phase[1], have recently attracted considerable attention and controversy for their possible major role in enzyme catalysis[1–5]. Previously, the existence of short hydrogen bonds between enzyme/protein and ligands has not been unambiguously demonstrated. Difficulties in establishing these hydrogen bonds in biological systems stem from uncertainties in the physical properties by which they are distinguished and experimental limitations in the assessment of their bond strength. As proposed[1,2], the properties that distinguish low barrier hydrogen bonds from normal hydrogen bonds are: short length, very close matching of proton affinity in acceptor-donor pairs ($\Delta pK_a = 0$), and exclusion of location solvation effects. These requirements provided an attractive mechanism for enzyme catalysis by which intermediate and transition states may be highly stabilized in the active site. More recently, however, it has been shown with model systems that the strength of hydrogen bonds, formed under conditions conducive to LBHB formation, are not exceptionally sensitive to ΔpK_a or particularly $\Delta pK_a = 0$ (ref. 6). Given the uncertainty in the dielectric constants of local environments in proteins, this finding has simplified the task of identifying and assessing the importance of LBHBs in biological systems. Herein we present the first solid data from very high resolution refined X-ray structures of ligand-bound phosphate binding protein (PBP), an initial receptor for active transport, that allow an accurate determination of hydrogen bond distances. We definitively show the presence of a short hydrogen bond between the receptor and phosphate and present further evidence against an exceptional role for it in the stabilization of the complex.

Short hydrogen bonds (<2.5 Å distances) between proteins and analogues, inhibitors or ligands have been reported in X-ray structures[7–11]; however, the resolutions (none better than 1.5 Å) and the restrained least squares minimization techniques used in the structure refinement (such as those implemented in PROLSQ[12] and X-PLOR[13]) precluded accurate determination of hydrogen bond distances. The positional coordinate errors in these structures are estimated to be about 0.1 Å and are likely to be dependent on the thermal disorders of regions of the refined structures. For example, based on the structure of the wild type PBP with bound phosphate refined using PROLSQ or X-PLOR against the 1.7 Å diffraction data collected at 20 °C, we observed that one of the 12 hydrogen bonds to the ligand (Asp 56 OD2 to phosphate O4), formed in the extremely well-ordered, solvent-free and completely sequestered site, is short (~2.4 Å distance)[8,14]. This short hydrogen bond confers the exquisite specificity of the receptor and its associated transport system[8,14]. Very similar short hydrogen bonds were also observed in five different crystal structures of active mutant PBPs using data sets collected to 1.9, 1.7, or 1.5 Å resolution at a temperature of 4 °C or -160 °C[14,15]. One of these mutant receptors contained an Asp substitution for Thr 141 (T141D) in the binding site which introduced an additional carboxylate group forming a hydrogen bond with the phosphate O2[14].

The initial observations suggesting the presence of a short hydrogen bond provided the impetus for extending the dif-

Table 1 Diffraction data and refinement statistics

	Wild type PBP	T141D Mutant
Protein M_r excluding H atoms	34,420	34,440
Diffraction data		
Resolution range (Å)	19.45 – 0.98	28.14 – 1.05
R-merge, no σ cut-off.	0.053	0.049
Completeness (%)	79.8	71.7
Refinement		
Observation to parameter ratio	144638 : 28434	111912 : 28497
R_{cryst}	0.111	0.109
R_{free}	0.138	0.139
Averaged positional error (Å)		
Protein atoms and phosphate	0.015	0.013
Phosphate and eight hydrogen-bonding residues	0.007	0.007
R.m.s. difference Fourier synthesis (e/Å^3)	0.06	0.07
Number of residues with dual conformations	6	6

Table 2 Hydrogen bonds/dissociation constants of PBP–phosphate complexes

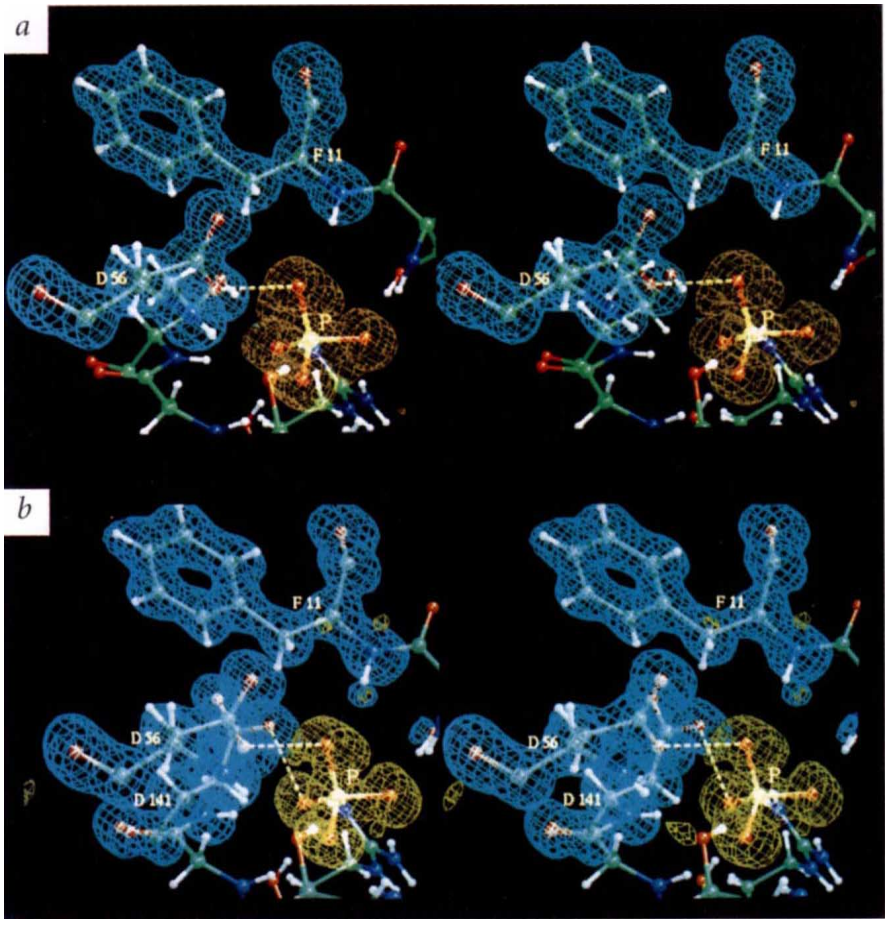
***a*, Hydrogen bonds between PBP receptor and phosphate**

Atom		Distance (Å)	
Phosphate	Residue	Wild type PBP	T141D Mutant
O1	Thr 10 N	2.765	2.805
	Thr 10 OG1	2.642	2.703
	Arg 135 NH2	2.848	2.864
O2	Arg 135 NH1	2.839	2.856
	Ser 139 OG	2.666	2.758
	Thr 141 N	2.866	2.906
	Thr 141 OG1	2.683	—
	Asp 141 OD1	—	2.591
O3	Ser 38 N	2.667	2.664
	Ser 38 OG	2.727	2.785
	Gly 140 N	2.729	2.753
O4	Phe 11 N	2.813	2.875
	Asp 56 OD2	2.432	2.435

***b*, Dissociation constants of PBP-phosphate complexes**

Protein	pH 4.5		pH 8.5	
	K_d (μM)	ΔG (kcal mol^{-1})	K_d (μM)	ΔG (kcal mol^{-1})
Wild type	3.8 (0.08)	-7.37	0.31 (0.009)	-8.85
D56N mutant	6.5 (0.08)	-7.05	0.36 (0.002)	-8.76

The estimated standard deviations of the distances, obtained rigorously from the full correlation matrix of the SHELXL refinement[16], vary from 0.006–0.009 Å for the wild type PBP and from 0.006 to 0.008 Å for the T141D mutant PBP.



fraction data to resolutions close to 1 Å in order to undertake refinements of both phosphate-bound wild type and T141D mutant receptor structures using full matrix least squares methods as implemented in SHELXL[16]. This method affords the investigator numerous advantages[17]. For example, it allows incorporation of anisotropic temperature factors for the heavy atoms (C, N, O, and P) and a very accurate assessment of the positional errors. It also allows the visualization of hydrogen atoms.

Table 1 summarizes the diffraction data collected at a synchrotron facility to 0.98 Å and 1.05 Å resolutions from crystals of the wild type and the T141D mutant that were grown at pH 4.5[8,14]. The table further indicates that both structures are extremely well-refined. The final model, especially the phosphate-loaded binding site, has excellent geometry and exceptionally well-defined positional and thermal parameters. Whereas the averaged positional error of all protein atoms is 0.015 Å, that of the atoms of the bound phosphate and eight hydrogen bonding residues is 0.007 Å. A detailed description of the refinement process and the very high resolution structures, including unusual clustering of ordered water molecules near the protein surface, will be published in a separate paper.

The presence of a short hydrogen bond, between OD2 of Asp 56 and O4 of the phosphate, is unambiguously established in both very high resolution refined structures (Fig. 1; Table 2*a*). The electron densities contoured at 3.5σ level of the two oxygen atoms of the short hydrogen bond in both structures are nearly touching (Fig. 1), a feature not seen for the other hydrogen bonds. The measured bond lengths in the wild type and T141D mutant receptor structures are 2.432 ± 0.007 Å and 2.435 ± 0.007 Å, respectively. This and 11 other normal hydrogen bond distances in the receptor–phosphate interaction are,

Fig. 1 Stereo view of the difference (F_o - F_c) electron density maps with superimposed models of the phosphate and binding site residues in ***a***, the 0.98 Å resolution wild type structure and ***b***, the 1.05 Å resolution T141D PBP receptor structures. The difference density maps were contoured at 3.5σ level with a grid spacing of 0.3 Å; phosphate is shown in gold and the residues in cyan. The map in (*a*) was calculated with atoms of the phosphate, Phe 11 and Asp 56 omitted from the model. The map in (*b*) was calculated as in (*a*) with Asp 141 additionally ommitted. Figures were generated with the MIDAS program[25].

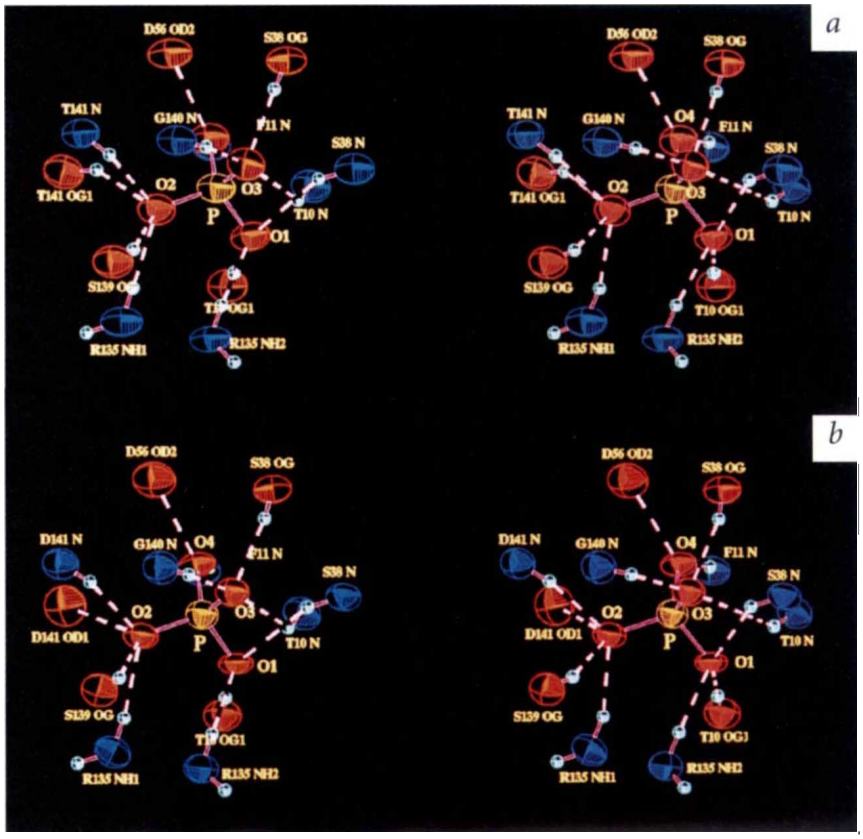


Fig. 2 Stereo drawing of the thermal ellipsoids of the atoms making hydrogen bonds between the phosphate and residues of the PBP receptor from ***a***, the 0.98 Å resolution wild-type receptor structure and ***b***, 1.05 Å resolution T141D mutant structure. The drawing was generated using ORTEP-III[26] with modifications provided by L. Zsolnai and G. Huttner.

thus far, the most accurately established for a protein–ligand interaction (Fig. 2; Table 2*a*). The thermal ellipsoids of the atoms involved in these hydrogen bonds are nearly isotropic, attesting to the exceptionally well-ordered and stable nature of the binding site and the low positional errors of the atoms. It is noteworthy that the distances of the hydrogen bonds, especially that of the short hydrogen bond, obtained in many structures at resolutions between 1.5 and 1.9 Å[8,14,15] are comparable to those established in the very high resolution structures.

The equilibrium dissociation constant (K_d) values of the wild type PBP-phosphate complex measured for this study at low ionic strength and pHs 4.5 or 8.5 are shown in Table 2*b*. The previously determined K_d values of the complex with the T141D mutant receptor are approximately equal to or no greater than two-fold of those of the complex with the wild type receptor in the range of pH 4.3–7 at which the mutant and native proteins are most active[14]. The lowest K_d (0.31 μM) corresponds to a difference in Gibbs free energy (ΔG) of -8.85 kcal mol^{-1} (Table 2*b*). As the ΔG is generally a complex effect of several competing factors, assessments of the individual energy contributions of each hydrogen bond observed between the protein and the phosphate cannot be made.

However, further structural and phosphate binding studies of an Asp 56 to Asn (D56N) mutant receptor indicate that the energy contribution of the short hydrogen bond is nowhere near the range (12–24 kcal mol^{-1})[1–4] assumed for LBHBs. The structure of the D56N mutant receptor complexed with phosphate refined to 1.9 Å indicated a normal hydrogen bond distance (2.6 Å) between the Asn 56 OD2 and the phosphate O4. (The other 11 hydrogen bonds to the phosphate are very similar to those observed at similar resolutions in several complexes with the wild type and other mutant proteins[8,14,15].) Despite the increase to a normal hydrogen bond distance, the phosphate binding energy is little changed ($\Delta\Delta G$ of 0.3 or 0.1 kcal mol^{-1}; Table 2*b*). This finding is inconsistent with the idea of a LBHB unless one proposes large entropic and/or enthalpic compensations for the loss of the short hydrogen bond as a result of the Asp to Asn mutation. The nearly identical native and mutant X-ray structures provide no support for compensations of 12-24 kcal mol^{-1}.

Similar observations have been obtained from studies of the binding of two similar potent inhibitors (affinities in the nanomolar range) to citrate synthase[11]. Structural analysis at ~1.7 Å resolution indicated the formation of a short hydrogen bond (<2.4 Å) with one inhibitor and a longer hydrogen bond (~2.5 Å) with the other in a partially exposed site with bound water molecules. However, despite the difference in hydrogen bond distances, the inhibition constants (K_i) of each inhibitor differs by only a factor of 20.

Our very high resolution structural data show definitively the formation of a very short hydrogen bond between a protein and its ligand. Despite meeting all proposed physical and structural criteria of a LBHB in the receptor–phosphate interaction, we find no evidence to indicate that the short hydrogen bond contributes exceptionally high energy. Thus a unique role for low barrier hydrogen bonds in biological interactions is, at present, without solid foundation.

Methods

High resolution structural refinement of wild type PBP and T141D. Crystals of both the wild type and T141D mutant proteins grown at pH 4.5 at ~20 °C, using methods similar to published procedures[8,14], belong to space group $P2_12_12_1$. The unit cell dimensions of crystals of the wild-type PBP are a = 41.506 Å, b = 63.379 Å, and c = 122.373 Å, and the T141D mutant are a = 41.645 Å, b = 63.493 Å, and c = 123.788 Å. Each diffraction data set was collected from one crystal at -160 °C at the Stanford Synchrotron Radiation Laboratory (SSRL) beamline 7-1 (λ = 1.08 Å) and reduced with the program package DENZO-SCALEPACK[18]. The starting models used in the SHELXL refinements were obtained from corresponding structures previously refined to 1.7 Å for the wild-type PBP and 1.9 Å for the T141D mutant using XPLOR[13], which included a simulated annealing step[14]. The CHAIN molecular graphics program[19] was used for electron density fitting and examination of refined models. Isotropic *B*s of the hydrogens riding on the host atoms were restrained to be 1.5 times the equivalent isotropic *B*s of the C atom of methyl and O atom of hydroxyl groups and 1.2 times those of other host atoms[16]. In the SHELXL refinement procedure, no hydrogens were introduced to the model for the weakly ionizing groups of Asp, Glu and His residues[16]. This is also the case for the phosphate, thus, avoiding introducing a bias as to the location of the hydrogen involved in the hydrogen bonds between Asp 56 or mutant Asp

141 and the phosphate. Protein coordinates of both structures have been deposited in the Protein Data Bank (ID codes 1IXG and 1IXH).

Mutation, activity and structure analysis of D56N. The D56N mutant PBP was generated as described[14,15]. Phosphate-binding activity for the wild type and D56N mutant PBP was measured by the resin method designed for the sulphate-binding protein[20,21], adapted for the phosphate-binding protein[22], and further modified in our laboratory in order to attain the best activity at low ionic strength condition[15]. The K_ds obtained by this method agree well with those using different techniques[23,24]. Assays were performed at pH 8.5 or 4.5 where either dibasic or monobasic phosphate exists. PBP binds both forms of phosphate[8,14].

Activity assays for the study reported here (Table 2*b*) were conducted at room temperature using an incubation mixture of 0.1 g AG1X8 acetate resin, 82 μM ^{32}P (1.8×10^{13} c.p.m. mol^{-1}), 5 μM protein, and 50 mM Tris-acetate at pH 8.5 or 50 mM acetate at pH 4.5 in a 1.0 ml volume. Samples were allowed to incubate at room temperature for 15 min with intermittent mixing and were then centrifuged for two minutes in a microfuge at 12,000 r.p.m. Aliquots (80 μl) from the supernatant of each tube were counted for radioactivity. Blank samples, containing no protein, were treated identically and, the c.p.m. value measured represents unbound phosphate, [Pi_{free}], which under these conditions is 0.25 μM and 3.8 μM at pHs 8.5 and 4.5 respectively. The c.p.m. value of the incubation mixture with protein represents both [Pi_{free}] and Pi bound by protein [Pi-protein]. By subtracting [Pi_{free}] from this value, [Pi-protein] is obtained. Finally, the concentration of unbound protein, [$Protein_{free}$], is obtained by subtracting [Pi-protein] from the total protein. The K_d is then

$$\frac{[Pi_{free} * Protein_{free}]}{[Pi\text{–}protein]}$$

The average of three blanks is used in each experiment. Samples are run in triplicate to obtain an average K_d and an indication of error (standard deviation) (Table 2*b*). The K_d values[15] are lower than those determined at higher ionic strength in the study of the T141D mutant[14].

Crystals of the D56N mutant were grown at pH 4.5 and diffraction data to 1.9 Å was collected with laboratory equipment at 4 °C as described[14]. As the mutant crystals were isomorphous with the wild type crystals, the structure was solved by direct phasing with the 1.7 Å X-PLOR-refined wild type structure[14] with the Asp 56 side chain omitted. The structure was refined by X-PLOR[13] which included simulated annealing step. Asn 56 side chain was fitted to the density in the late stages of the refinement. The final *R*-factor is 0.200 against 22167 reflections (83.1% complete), and the R_{free} is 0.294. The r.m.s. deviations from ideal bond lengths and angles are 0.012 Å and 1.851°, respectively. Coordinates have been deposited in the Protein Data Bank (ID code 1IXI).

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