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Enzyme Inhibition

A New Potent Inhibitor of Glycogen Phosphorylase Reveals the Basicity of the Catalytic Site

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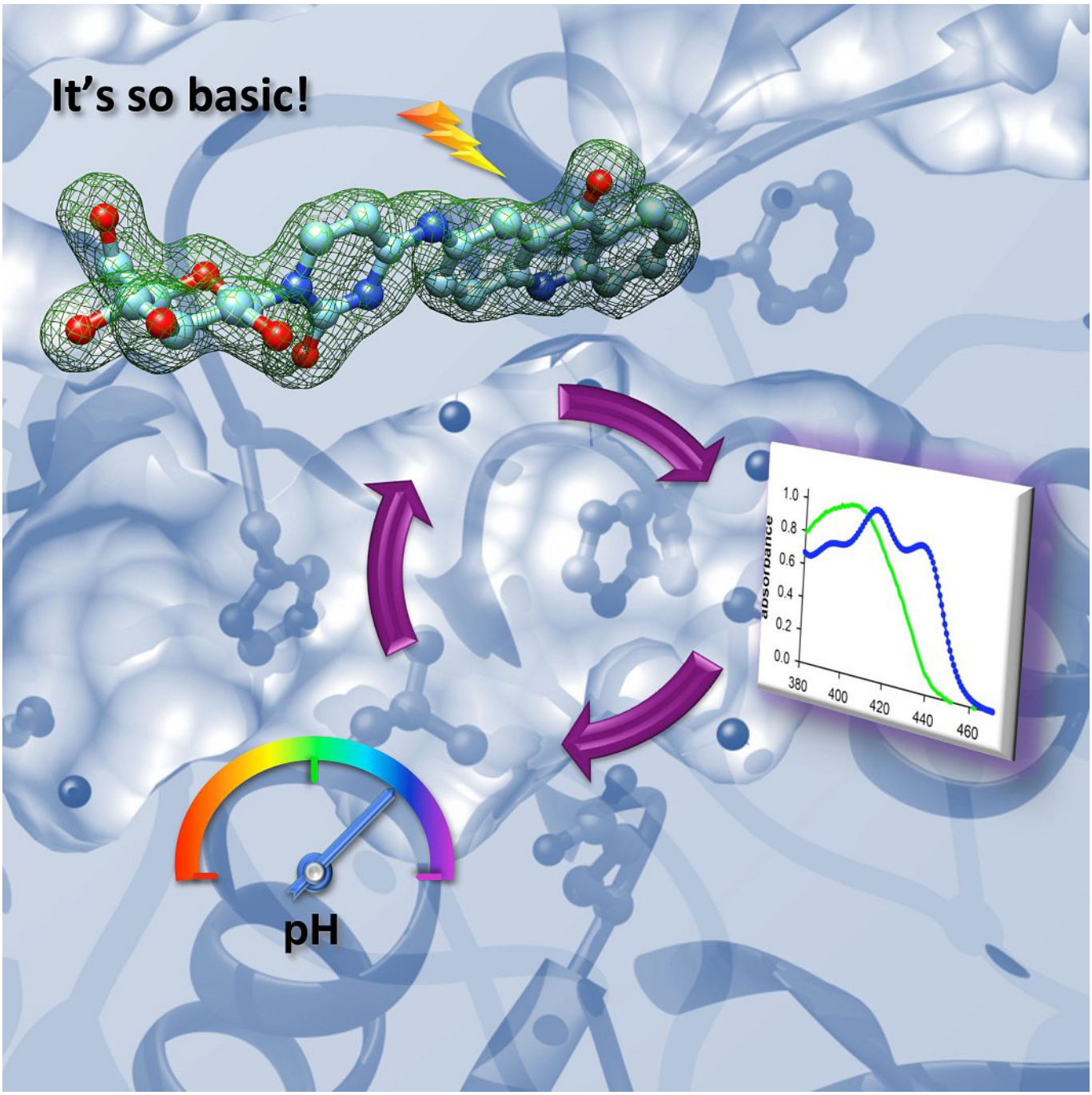
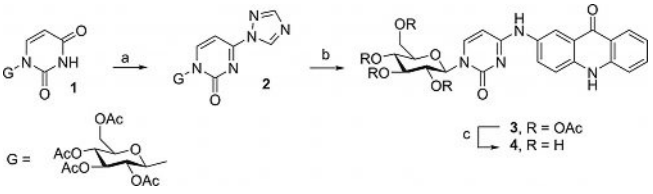


Abstract: The design and synthesis of a glucose-based acridone derivative (GLAC), a potent inhibitor of glycogen phosphorylase (GP) are described. GLAC is the first inhibitor of glycogen phosphorylase, the electronic absorption properties of which are clearly distinguishable from those of the enzyme. This allows probing subtle interactions in the catalytic site. The GLAC absorption spectra, associated with X-ray crystallography and quantum chemistry calculations, reveal that part of the catalytic site of GP behaves as a highly basic environment in which GLAC exists as a bis-anion. This is explained by water-bridged hydrogen-bonding interactions with specific catalytic site residues.

Type 2 diabetes mellitus is among the globally prevailing diseases that constitute a major threat to society. Therefore, efforts have been directed towards developing satisfactory models for prevention as well as intervention of the disease.[1] Glycogen phosphorylase (GP) is a validated target for the development of new antidiabetic agents employing the structure-based drug design (SBDD) approach.[2,3] Previous studies performed on the T state, inactive, rabbit muscle GP (GPMM), a widely used target for the development of GP inhibitors, have shown that the chemical nature of a candidate drug plays a crucial role in its activity upon binding at the active site of the enzyme. With the aim of advancing our understanding of ligand-bonding interactions with the catalytic site residues, GPMM has been probed with a broad library of glucose derivatives.[2–5] A key point for the design of efficient drugs is the characterization of the interactions governing its binding to the enzyme, which can be advantageously investigated by optical spectroscopy.[6]

Here, we present a glucose-based acridone derivative (GLAC), which is shown to be among the most potent GPMM inhibitors. Its novelty resides in the fact that its optical spectra contain characteristic absorption bands, readily distinguishable from those of the enzyme, providing an additional tool for studying subtle interactions in the active site. In combination with X-ray crystallography and quantum chemistry calculations (TD-DFT), the optical spectra reveal that proton transfer to water molecules, present in the active site, stabilizes a highly basic bis-anion and possibly strengthens its binding to the enzyme.

The design of a catalytic site inhibitor was based on C-4 modification of β-D-glucopyranosyluracil, a relatively efficient, (micromolar) inhibitor of GPMM that we have reported.[5] According to previous crystallographic studies, addition of aromatic amines at the 4-position of the pyrimidine ring could orient these aromatic substituents towards the β-channel of GPMM, a $15\times10\times7.5$ Å^3 volume within the catalytic site of GPMM, isolated from the bulk solvent by the 280s loop.[5] Due to its dimensions and the residues sculpting this β-channel, it has been known to have an affinity for flat aromatic molecules. Little is known, though, about which aromatic functional groups, deep in the β-channel, can further increase the binding affinity. To probe these interactions in this region of the catalytic site, we chose to utilize an acridone moiety. The structure of acridone was selected as a possible chromophore as well as fluorophore[7] and compound **4**, N^1-(β-D-glucopyranosyl)-N^4-[2-acridin-9(10*H*)-onyl]-cytosine (GLAC), was prepared following the synthesis described in Scheme 1.



Scheme 1. Synthesis of GLAC. a) Triazole, $POCl_3$, Et_3N, CH_3CN, 0 °C, 95 %.[8] b) 2-aminoacridone, DMSO, microwave (60 W), 114 °C, 54 min, 90 %. c) 7 N NH_3, MeOH, RT, 95 %.

We based our synthesis on a modification of a recently developed methodology for introducing aliphatic and aromatic amines at the 4-position of β-D-glucopyranosyluracil (**1**).[8] Specifically, **1** was converted to 4-triazolyl derivative **2**.[9] Substitution of **2** by 2-aminoacridone, commercially available or prepared in-house (see the Supporting Information, Section 1), under microwave irradiation, in DMSO, provided the protected adduct **3** in 90 % yield.[10] Final deprotection furnished the desired product **4** in 95 % yield.

The capacity of GLAC to act as an inhibitor of GPMM was determined by kinetic studies, following previously described protocols.[11] A measured K_i value of 71 nM indicated that GLAC is a potent inhibitor of T state GPMM. Full kinetics of GLAC inhibition of GPMM are reported in Figure S1 in the Supporting Information. This low value places GLAC among the strongest catalytic site inhibitors of GPMM reported to date.[12,13]

The binding mode of GLAC to GPMM was explored by X-ray protein crystallography with synchrotron radiation and data were collected at 1.78 Å at the EMBL-Hamburg Unit, PETRA III (see the Supporting Information, Section 2, Table S1). The crystal structure of the GPMM:GLAC complex was determined by employing standard protocols for refinement.[14] The results confirmed tight binding along the catalytic channel forming an

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increased network of both hydrogen bond (18) and van der Waals interactions (159). The most profound changes were observed in the solvent structure that was perturbed to accommodate the bulkier substituent. In particular, at the β-subsite, Asn282 is hydrogen-bonded to Glu287 through a network of water molecules (Figure 1, Tables S2, S3), which are displaced and induce a cascade of conformational changes in the side chains of the 280s loop residues.

GLAC may exist in a number of tautomeric/ionic forms whose relative stability has been studied by theoretical calcula-

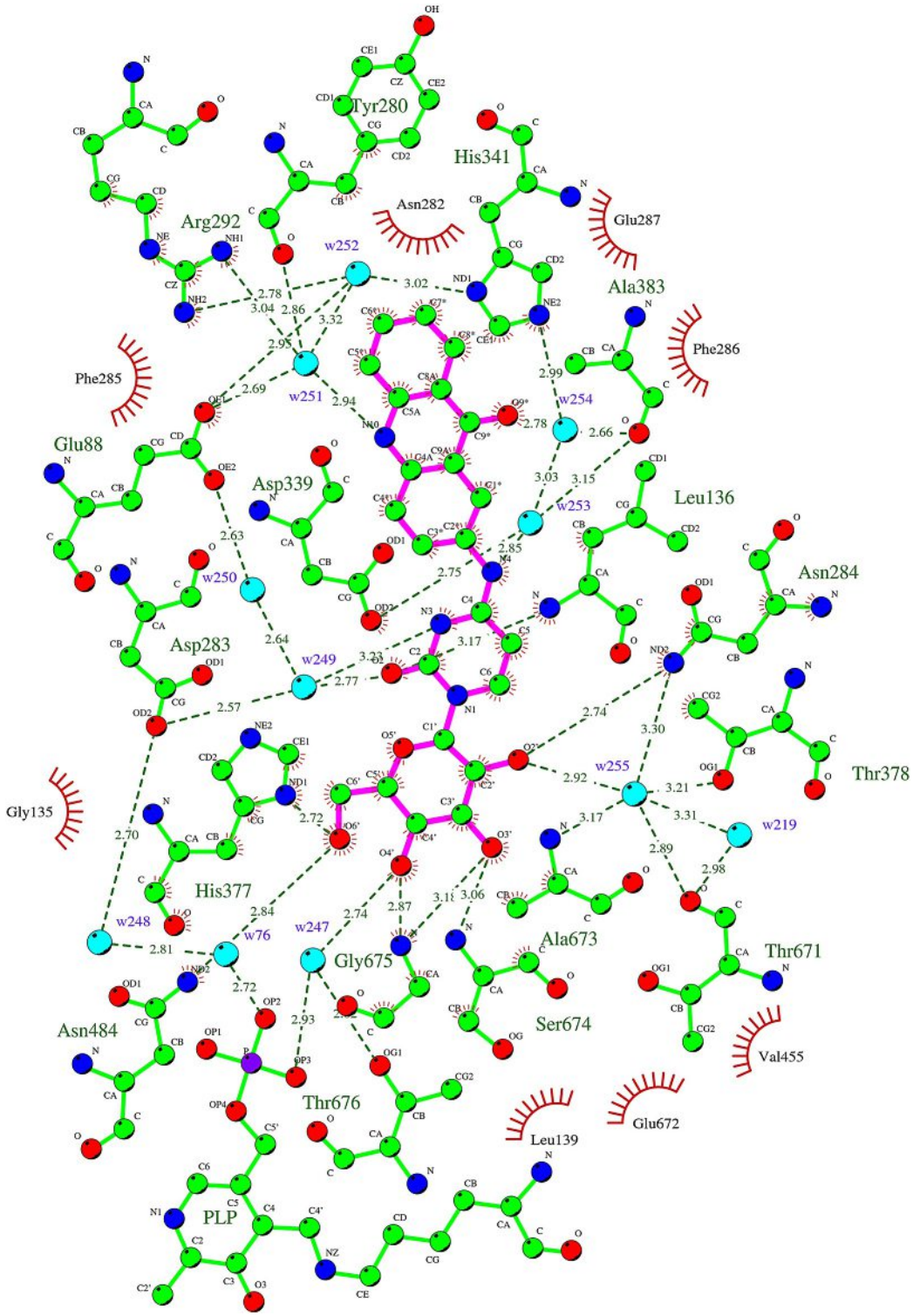

Figure 1. LIGPLOT diagram of GLAC interacting with the active-site residues lying in the vicinity. The ligand bonds are shown in magenta, whereas bonds of the residues lining the site are shown in green. Hydrogen bonds are shown as dark green dashed lines with distances indicated in Å. Additional residues forming van der Walls interactions with GLAC are represented by red semicircles with radiating spokes.

tions. All possible tautomers were optimized in implicit water (PCM/PBE0/6–311 + G**//PCM/ PBE0/6–311G**).[15,16] The lowest energy neutral (**a**, **b**, **c**), mono-anionic (**d**, **e**), bis-anionic (**f**) and mono-cationic (**g**) forms are shown in Figure 2. Calculations predicted the energy of **c** to be 15.2 kcal mol^{-1} higher than that of **a**, whereas the energy of tautomer **b** was found to be only 3.8 kcal mol^{-1} higher. On the other hand, the monoanionic form **d** was calculated to be 4.3 kcal mol^{-1} more stable than its tautomeric form **e** (see the Supporting Information, Section 3, Table S5, Figure S5). The acridone chromophore in the minimized energy structures **b** and **e** is not coplanar with the pyrimidinone ring, but lies on a slightly inclined plane (Figure S6), similar to what is observed in the crystal structure (Figure S2). On the contrary, in the lowest energy cationic structure **g**, the acridone is almost perpendicular to the pyrimidinone (Figure S6).

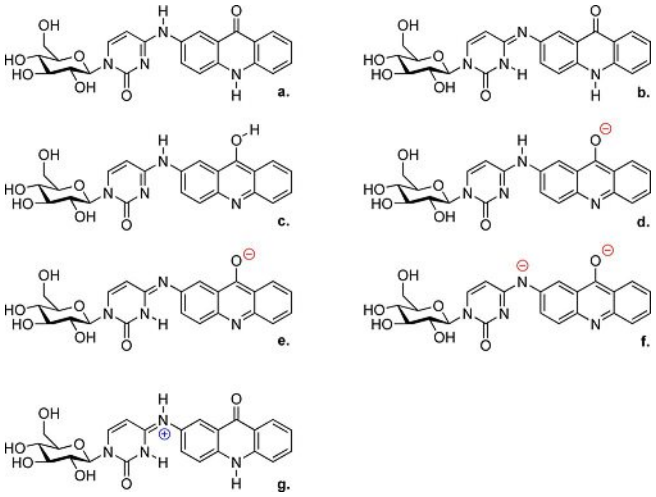

Figure 2. Seven possible ionic and/or tautomeric forms of GLAC (**a**–**g**).

The equilibrium between neutral and cationic/anionic forms of GLAC is affected by pH, and it is well known that in biological assemblies, the local pH at a given site may differ from the bulk pH value. Well-documented examples are the anomalously low pK_a of Tyr57 in ketosteroid isomerase,[17a] and the basicity of a catalytic glutamate in hydrolytic aldehyde dehydrogenases.[17b] To get an insight into this aspect, we subsequently focused on the optical spectra, which are sensitive to such ionic changes.

The lowest in energy absorption band of GLAC is located in the 380–480 nm spectral region (Figure 3 A), in which the assay buffer and the enzyme do not absorb. In pH 7.0, the absorption spectrum peaks at 405 nm and contains two shoulders at approximately 385 and 420 nm. Going from neutral to pH 1.7, at which deprotonation is hindered, a relatively more intense peak appears at 388 nm, whereas the shoulder at 420 nm is no longer visible (Figure 3 A).

The absorption spectrum of a neutral solution (pH 6.8), containing both the inhibitor and the enzyme, at molar ratios (GLAC)/(GPMM) ≤ 1, is red-shifted (red spectrum in Figure 3 A) compared to that of free GLAC in the same pH. A ten-fold

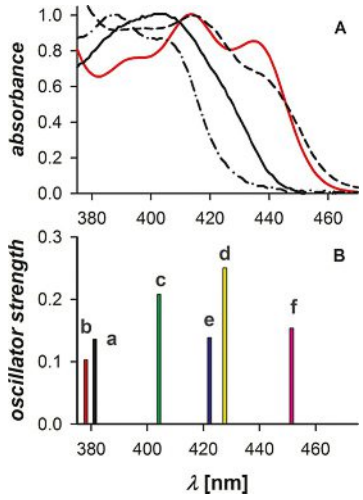

Figure 3. a) Normalized absorption spectra of free GLAC (black) in buffer at pH 1.7 (dash-dotted line), pH 7.0 (solid line) and pH 12.7 (dashed line). The red spectrum corresponds to a molar ratio (GLAC)/(GPMM) = 1 in assay buffer (pH 6.8, see the Supporting Information, Section 4). b) Lowest energy electronic transitions of GLAC calculated at the PCM/TD-PBE0/6–311 + G**//PCM/PBE0/6–311G** computational level considering implicit water; the letters **a** to **f** correspond to the forms shown in Figure 2.

change of the GPMM concentration, from 5×10^{-5} to 5×10^{-4} mol L^{-1}, did not lead to any observable modification of the spectrum. The inhibitor–enzyme spectrum exhibits three clearly distinguishable features: a shoulder around 395 nm and two peaks at 414 and 435 nm. Surprisingly, the same absorption features are present in the spectrum of free GLAC at pH 12.7.

To rationalize the spectral changes, we calculated the energy and oscillator strength of the lowest energy electronic transitions (LEET) contributing to the absorption spectra by TD-DFT at the PCM/TD-PBE0/6–311 + G**//PCM/PBE0-6–311G** computational level. The calculated transition energies and oscillator strengths of the low energy transitions $S_0 \rightarrow S_1$ are reported in Table S7. For comparison purposes, some models were optimized in implicit water but including three explicit water molecules forming hydrogen bonds with GLAC, as were found in the X-ray structure (Figures 1, S2, S3, Table S6). Inclusion of the three explicit water molecules, either "frozen" at their crystallographic positions or fully relaxed, in addition to implicit water, did not significantly change the results in most cases. The purpose of this test was to assess whether significant changes of the absorption spectra are due to explicit hydrogen bonds of GLAC with water molecules present in the active site. The exploration of the complex interactions undergone by the inhibitor when bound at the catalytic site requires a more sophisticated computational model (as in Figure 1 and Scheme S3) which will be addressed in future studies.

The above computational results were compared with those we obtained using two other density functionals, CAM-B3LYP and M06 (PCM/TD-M06/6-311 + G(d,p)//PCM/PBE0/6-311G** and PCM/TD-CAM-B3LYP/6-311 + G(d,p)//PCM/PBE0/6-311G**).[18] In all cases, the absorption is due to the same $\pi \rightarrow \pi^*$ transition centered on the acridone moiety (Table S7

and Figure S5). Furthermore, although M06 shows values close to the PBE0 results, CAM-B3LYP shows the well-known overestimation of the transition energies.[19]

The calculated $S_0 \rightarrow S_1$ electronic transition of cation **g** was found at 350 nm, whereas those of the forms **a**–**f** fall in the 380–460 nm spectral domain (Figure 3B), the same region occupied by the experimental spectra (Figure 3A). The exact position of the transition as well as the oscillator strength depend on the specific form of GLAC. Thus, it appears that the experimental spectra, displayed in Figure 3A, may be seen as an envelope of several transitions corresponding to various GLAC forms, depending on the pH, in equilibrium. An important point is that the lowest transition energies of the neutral forms (**a**, **b**, **c**) are located at shorter wavelengths than those of the anionic forms **d**, **e** and **f**. This theoretical finding is in perfect agreement with the experimental observation that the spectrum of GLAC at pH 1.7 is blue-shifted with respect to that at pH 7 (Figure 3A). Considering that the GLAC spectrum at pH 1.7 exhibits peaks up to 410 nm, with the strongest absorption band at 380 nm, this spectrum could be attributed to the neutral species **a**, **b** and/or **c**. Two pK_a values (2.7 and 12.2) were determined using, for better precision, shorter wavelength absorption bands (see the Supporting Information, Section 4). The observed acidity of GLAC is comparable to that of acridones substituted by electron-withdrawing groups at the 2-position.[20] No major spectral changes are observed from pH 3.9 up to 11. Therefore, we assume that around physiological pH, GLAC exists in solution mainly as a monoanion (**d**, **e**). The red shift of the inhibitor–enzyme complex at a molar ratio (GLAC)/(GPMM) = 1 (Figure 3A) could be explained by the stabilization of the bis-anionic form **f** within the catalytic site. This is in perfect agreement with the spectrum of GLAC at pH 12.7 and the calculated LEET of bis-anion **f**. The fact that the spectra of free GLAC at pH 12.7 and of the inhibitor–enzyme complex do not strictly overlap, could be due to different vibronic coupling exhibited by the chromophore in homogeneous solution and when bound in a highly heterogeneous environment with restricted degrees of freedom.

The origin of the observed double deprotonation may be traced down to the interactions of the four key GLAC atoms involved, namely N4, O2, N10 and O9*, with residues in the vicinity (Figure 1 and Scheme S3). The interactions of O2 affect the basicity of N4 since these two atoms are conjugated in the same manner that O9* is conjugated with N10. An extended network of the solvent structure in the crystal (Scheme S3) appears to connect Asp339 OE1, through w253 and w254 with both N4 and O9*. O2 and N10, in turn, form water-mediated interactions with Glu88 through water molecules w249, w250, and w251, respectively. Additionally, O2 and N10 interact through w249 with Asp283, a key residue that belongs to the 280s loop, that further interacts through a water bridge comprised of w248 and w76 with the phosphate group of PLP (Scheme S3). The latter water-mediated network of interactions was previously reported to be implicated in the stabilization of T-state GP.[21]

To get a better insight in the origin of the basicity of the catalytic site, the web server DEPTH[22–24] was used to calculate the predicted pK_a values of the ionizable residues involved in this network of interactions. We found pK_a values of 12.53, 11.14, and 11.93 for Asp339, Asp283, and Glu88 carboxylates, respectively. These residues may abstract GLAC N4 and N10 protons only if they exist, at least partially, as carboxylate anions, and this could be possible through the mediation of the phosphate group of PLP in the form of mono- or bis-anion,[21] as shown in Scheme S3. These interdependent interactions may explain the basic environment experienced by GLAC within the catalytic site and the prevalence of bis-anion **f**.

The potency of the only known inhibitor stronger than GLAC,[13] with a K_i of 31 nM, has been ascribed to hydrogen bonding with His377, in addition to Van der Waals interactions.[12] In the case of GLAC, we believe that the solvent structure and in particular, the water-bridged hydrogen bonding interactions formed with the catalytic site residues in the β-channel (Tables S2, S3) are responsible for the observed inhibition potency. Such interactions induce an environment similar to what free GLAC experiences in a highly basic pH (> 12.5). This affinity of the catalytic site of GPMM for anionic species may be exploited in the design of future inhibitors. The water-mediated interactions of the key residue Asp283 with PLP through conserved water molecules in the active site[21] are also maintained in GPMM:GLAC complex structure suggesting that ligands that utilize all the known features of the catalytic site, and have an appropriate pK_a, achieve the desired inhibition by stabilizing T-state GP.

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Conflict of interest

The authors declare no conflict of interest.

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