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Rational design, synthesis and evaluation of first generation inhibitors of the *Giardia lamblia* fructose-1,6-biphosphate aldolase $\overset{\leftrightarrow}{\sim}, \overset{\leftrightarrow}{\sim} \overset{\leftrightarrow}{\sim}$

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ABSTRACT

Inhibitors of the Giardia lamblia fructose 1,6-bisphosphate aldolase (GIFBPA), which transforms fructose 1,6bisphosphate (FBP) to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, were designed based on 3-hydroxy-2-pyridone and 1,2-dihydroxypyridine scaffolds that position two negatively charged tetrahedral groups for interaction with substrate phosphate binding residues, a hydrogen bond donor to the catalytic Asp83, and a Zn^{2+} binding group. The inhibition activities for the *Gl*FBPA catalyzed reaction of FBP of the prepared alkyl phosphonate/phosphate substituted 3-hydroxy-2-pyridinones and a dihydroxypyridine were determined. The 3-hydroxy-2-pyridone inhibitor **8** was found to bind to *Gl*FBPA with an affinity ($K_i = 14 \,\mu$ M) that is comparable to that of FBP ($K_m = 2 \mu M$) or its inert analog TBP ($K_i = 1 \mu M$). The X-ray structure of the *GI*FBPA-inhibitor **8** complex (2.3 Å) shows that **8** binds to the active site in the manner predicted by *in silico* docking with the exception of coordination with Zn^{2+} . The observed distances and orientation of the pyridone ring O=C-C-OH relative to Zn^{2+} are not consistent with a strong interaction. To determine if Zn^{2+} coordination occurs in the *GI*FBPA–inhibitor 8 complex in solution, EXAFS spectra were measured. A four coordinate geometry comprised of the three enzyme histidine ligands and an oxygen atom from the pyridone ring O=C-C-OH was indicated. Analysis of the Zn²⁺ coordination geometries in recently reported structures of class II FBPAs suggests that strong Zn²⁺ coordination is reserved for the enediolate-like transition state, accounting for minimal contribution of Zn²⁺ coordination to binding of 8 to GlFBPA.

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1. Introduction

Giardia lamblia, a waterborne human parasite, inflicts billions of people worldwide with a chronic infection (giardiasis) that leads to malnutrition, growth retardation in children, and sometimes death [1–3]. Because of its impact on impoverished countries, and the meager effort expended to identify new therapies, giardiasis has been designated as a World Health Organization neglected disease [4]. In addition, the Center for Disease Control has classified *Giardia* as a

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bioterrorism category B organism. The drugs metronidazole and tinidazole, which are currently used to treat giardiasis, produce problematic side effects. Moreover, the rate of recurrence of infection is high, and the existence of resistant strains has been documented [5–8]. The cellular biology and biochemistry of *G. lamblia* are not well defined nor are its host adaptation and survival tactics. However, the *G. lamblia* genome sequence is now known [9] and, thus, understanding and controlling *G. lamblia* pathogenicity are tractable objectives.

Recent studies in our laboratories have focused on the identification, characterization and design of inhibitors of enzymes that constitute potential *G. lamblia* drug targets. From the outset, the class II fructose 1,6-bisphosphate aldolase (FBPA) was viewed as a particularly attractive target. FBPA catalyzes the reversible cleavage of D-fructose 1,6-bisphosphate (FBP) to dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (G3P) (Fig. 1), a key step in the Embden-Meyerhof-Parnas glycolytic pathway. Because *G. lamblia* lacks the components of oxidative energy metabolism, the generation of ATP via the glycolytic pathway is likely to be essential for trophozoite colonization of the human gut [10–12]. This assumption gains support from the finding that RNAi/antisense RNA FBPA gene silencing in

[☆] Inhibitor synthesis was carried out by Z. L., D. W. C., J. Z., M. G. and G. D. M under the direction of P. S. M.; protein preparations and kinetic studies were carried out by Z. L., L. Li and H. Z. under the direction of D.D.-M.; EXAFS studies were carried out by R. M. B. under the direction of D. L.T.; and the crystal structure determination was made by A. G. under the direction of O. H. The manuscript was written by P.S.M. and D.D.-M.

The atomic coordinates and structure factors have been deposited in the Protein Data Bank.

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Fig. 1. The reaction pathways catalyzed by class I and class II fructose 1,6-bisphosphate aldolases.



Fig. 2. A. Surface representation of the substrate-binding site observed in the structure of the *GI*FBPA–TBP complex (PDB: 3GAY). The enzyme nitrogen atoms are colored, carbons atoms white and oxygen atoms red. The Zn^{2+} cofactor is shown as a green sphere and the TBP ligand as yellow sticks. B. The stereoview of the *GI*FBPA–TBP complex (PDB: 3GAY) substrate-binding site. Hydrogen bonds are represented with black dashed lines. The carbon atoms of the TBP are colored yellow, oxygen atoms red and phosphorus atoms orange. C. Cartoon representation of the inhibitor design.

transfected *G. lamblia* trophozoites is lethal under standard culture conditions [13].

FBPA function is also essential to the human host. Nevertheless, through strategic design of mechanism-based inhibitors, it might be possible to eliminate the activity of the G. lamblia FBPA (GIFBPA) without interfering with the activity of the human FBPA. This proposal is based on the fact that the GIFBPA and human FBPA evolved within different protein fold families and thus possess different active site structures and, most importantly, they employ different catalytic mechanisms. *GI*FBPA is a class II aldolase [14], that uses a Zn^{2+} cofactor to activate FBP for retro-aldol cleavage [15–18] whereas the human FBPA is a class I aldolase that utilizes an active site lysine to activate cleavage of FBP via Schiff base formation [19-21] (Fig. 1). Enzymes that employ Zn²⁺ in substrate activation are susceptible to inhibition by small molecules that possess Zn²⁺-binding groups. Examples of Zn²⁺-dependent enzymes which have been successfully modulated in vivo with small molecule inhibitors equipped with a "Zn²⁺-warhead" include carbonic anhydrase [22], matrix metalloprotease [23] and histone deacetylase [24].

The X-ray structures of *Gl*FBPA bound with substrate and substrate analogs provide the needed insight into the steric and electrostatic features of the active site that govern ligand binding. Most helpful to new inhibitor design is the 1.8 Å resolution structure of *Gl*FBPA bound with the inert substrate analog tagatose 1,6-bisphosphate (TBP) (a C(4) epimer of FBP) [25]. This structure defines a solvent excluded pocket that accommodates both the Zn^{2+} cofactor and the TBP C(1)–C(3) unit (Fig. 2A) and shows that the TBP C(4)–C(6) unit extends along the protein surface. The polar interactions observed between the TBP ligand and the binding site residues are extensive (Fig. 2B). The Zn^{2+} cofactor is bound to the enzyme through the coordination bonds formed with the imidazole rings of His84, His178 and His210 (Fig. 2B).

The design of first generation of GIFBPA inhibitors described below centers on a synthetically accessible scaffold that positions two highly negatively charged, tetrahedral groups for interaction with the numerous substrate C(1) and C(6) phosphate binding residues, a hydrogen bond donor to the catalytic Asp83, and a Zn^{2+} binding group (Fig. 2C). In the effort described, alkyl phosphonate/phosphate substituted 3-hydroxy-2-pyridinones and a dihydroxypyridine were synthesized and evaluated. The competitive inhibition constants for these first generation inhibitors are reported and interpreted in the context of X-ray crystallographic and solution X-ray absorption (EXAFS) analyses of the inhibited enzyme. The major conclusions are that the FBPA Zn²⁺ cofactor-substrate binding is weak at the ground state but strong at the transition state and that effective inhibitor design based on the use of a Zn²⁺ warhead must take into account the steric and electrostatic properties of the Zn²⁺ ligand at the transition state.

2. Materials and methods

2.1. Determination of GIFBPA inhibition constants of 1-10

Initial velocities were measured for reactions initiated by the addition of FBP (2–20 μ M) to solutions containing *GI*FBPA (0.02 μ M), inhibitor (0–3K_i), 200 μ M NADH, 5 units of triosephosphate isomerase, and 2 units of glycerol-3-phosphate dehydrogenase in 50 mM K⁺HEPES (pH 7.5; 25 °C). The progress of the reaction was continuously monitored at 340 nM for conversion of NADH to NAD ($\Delta \epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$). The initial velocity data were fitted to Eq. (1).

$$V_0 = V_{\max}[S] / [K_m(1 + [I] / K_i) + [S]].$$
(1)

In Eq. (1), [S] = substrate concentration, [I] = inhibitor concentration V_0 = initial velocity, V_{max} = maximum velocity and K_m = Michaelis-Menten constant, and K_i = the competitive inhibition constant.

2.2. Crystallization and data collection

The crystals of the *GIFBPA*–inhibitor **8** complex were prepared by soaking TBP-liganded crystals [25] with inhibitor **8**. The TBP-liganded crystals were grown at room temperature in hanging drops using the vapor diffusion method. The protein solution containing 10 mM TBP was mixed with an equal volume of mother liquor containing 22% polyethylene glycol 3350 and 0.2 M NH₄NO₃. The crystals required a period of 2–3 weeks to appear. Ligand exchange was accomplished by 4 rounds of transferring the crystals with a loop to a fresh solution of mother liquor containing 20 mM inhibitor **8**. The crystals diffracted X-rays to a resolution of 2.3 Å. For data collection, the crystals were transferred to solutions containing mother liquor, 20 mM inhibitor **8** and 20% glycerol, and flash-cooled in liquid nitrogen.

Diffraction data were acquired at the Southeast Regional – Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source (APS, Argonne National Laboratory, Argonne, Illinois). For data acquisition, the SER-CAT beamline was equipped with a Quantex210 CCD detector. Data processing was carried out using CrystalClear version 1.3.6 (Rigaku MSC Inc.). The statistics of data collection are provided in Table 1.

2.3. Structure determination and refinement

The structure of the FBPA-inhibitor 8 complex was determined by using Molecular Replacement techniques with the computer program Phaser [26], employing the GIFBPA-phosphoglycolohydroxamate structure (PDB code 2ISW) as the search model rather than the GIFBPA-TBP structure in order to avoid phase bias. The Difference Fourier maps indicated some alternative tracing. Structure refinement was carried out using the CNS [27]. The final stages of refinement were performed with REFMAC [28]. The two molecules in the asymmetric unit were refined independently. The resulting models were inspected and modified on a graphics workstation by the using program 'O' [29]. Water molecules were added to the model based on the $F_0 - F_c$ Difference Fourier electron density map (where F_0 and F_c are the observed and calculated structure factors, respectively), using peaks with density $\geq 3\sigma$ as the acceptance criteria. Refinement statistics are provided in Table 1. PROCHECK [30] was used for analysis of geometry, QUANTA for molecular modeling and structural

 Table 1

 X-ray data collection and refinement statistics for the *GIFBPA*-inhibitor 8 complex.

Data collection	
Space group Cell dimension a, b, c (Å) Resolution range (Å) No. observations No. unique reflections Completeness (%) ^a	P2 ₁ 2 ₁ 2 ₁ 58.58, 64.95, 171.45 20.0–2.3 108,611 23,705 86.5(89.1)
Rmerre	0.079(0.248)
Refinement statistics	
No. reflections	22,497
No. residues	635
No. water molecules	339
R _{work} ^c	0.199
R _{free} ^d	0.274
RMS deviation	Bonds (Å) 0.014; angles (°) 1.5

^a The values in parentheses are for the highest resolution shell.

^b $R_{merge} = \sum_{hkl} [(\sum_{i} |I_i - \langle I \rangle |) / \sum_{i} |I_i|], \text{ for equivalent reflections.}$

^c $R_{work} = \sum_{hkl} |F_o| - |F_c| | / \sum_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factors included in the refinement, respectively.

 d R_{free} is computed for 5% of reflections that were randomly selected and omitted from the refinement.



Fig. 3. Chemdraw representation of the 5-coordinate, trigonal bipyramidal complex tris (pyrazoyl)borate–Zn²⁺–hydroxypyridinone complex adopted from reference [43].

alignment (Molecular Simulations Inc.), and PYMOL [31] for depiction of the structures.

2.4. X-ray absorption spectra measurement

Buffered (50 mM K⁺HEPES, pH 7.5) *GI*FBPA (3 mM) samples were prepared with 20% (v/v) glycerol, and loaded in Lucite cuvettes with 6 μ m polypropylene windows, before rapid freezing in liquid nitrogen. *GI*FBPA activity towards catalysis of FBP cleavage was assayed (*vide supra*) in the presence of the 20% (v/v) glycerol and found to be unchanged from that observed in buffer alone. The X-ray absorption spectra were measured at the National Synchrotron Light Source (Brookhaven National Lab, Upton, NY), beamline X3B, with a Si(111) double crystal monochromator; harmonic rejection was accomplished using a Ni focusing mirror. Data collection and reduction were accomplished according to published procedures [32]. The reported spectra represent the average of the 6–8 scans per sample.

Both raw and Fourier filtered EXAFS data were fitted utilizing theoretical amplitude and phase functions calculated with FEFF v. 8.00 [33]. The Zn–N scale factor and the threshold energy, ΔE_0 , were calibrated to the experimental spectrum for tetrakis-1-methylimidazole Zn(II) perchlorate, Zn(MeIm)₄ and held fixed at 0.78 and -16 eV, respectively, with E_0 set to 9675 eV. First shell fits were then obtained for all reasonable coordination numbers while allowing the absorber-scatterer distance, R_{as} , and the Debye–Waller factor, σ_{as}^2 , to vary. Fits to unfiltered EXAFS, presented in the Appendix A (Fig. SI1), gave identical results. In no case did inclusion of a mixed first shell, with distinct Zn–N and Zn–O scattering contributions, result in either a significant improvement in fit residual or resolvable Zn–N/Zn–O distances. Multiple scattering contributions from coordinated histidine residues were fitted using a set of combined multiple-scattering paths, according to published procedures [34].

3. Results

3.1. Inhibitor design

The steric and electrostatic features of the *GI*FBPA substratebinding site, defined by the structure of the *GI*FBPA–TBP complex [25] (Fig. 2A and B), guided the design of inhibitors (Fig. 2C). The multiple hydrogen bonds formed with the TBP C(1) and C(6) phosphate groups (Fig. 2B) suggested that the phosphate subsites supply a large fraction of the binding energy in the ground state complex ($K_i = 1 \mu M$ [25]; $\Delta G_{\text{binding}} = -8 \text{ kcal/mol}$). Therefore, the inhibitor must include comparable tetrahedral dianionic substituents in order to effectively displace the substrate FBP, which also employs the phosphate groups for tight binding [13]. Because the hydrogen bonds to TBP do not involve the bridging oxygen atoms of the phosphate groups, a phosphonate group, which provides greater chemical and metabolic stability than the phosphate ester [35], might be a useful replacement of the phosphate group.

The selection of the Zn^{2+} binding group (" Zn^{2+} warhead") was influenced by the demonstrated success of the hydroxypyridinonebased series of 6-membered ring Zn^{2+} chelating compounds developed by Cohen and his co-workers for targeting the Zn^{2+} center of matrix metalloproteases [36,37]. By first using the tris(pyrazoyl)borate- Zn^{2+} complex as a chemical model of the matrix metalloprotease Zn^{2+} center, these investigators showed that hydroxypyridinones displace the water ligand and engage in strong bidentate coordination with the caged Zn^{2+} to generate a 5-coordinate, trigonal bipyramidal complex (Fig. 3) [36]. Subsequently, the potencies of the hydroxypyridinone Zn^{2+} binding groups as inhibitors of the matrix metalloproteases were demonstrated, as were their metabolic stabilities and lack of toxicity [38].

In silico modeling of the 3-hydroxy-2-pyridinone to the *GI*FBPA active site [25] using the program AutoDock [39] indicated a good fit. Importantly, the close proximities of the ring carbonyl and hydroxyl functions with the Zn^{2+} and the Asp83, respectively, suggested the potential for strong binding interactions. The model also indicated that the attachment of the alkylphosphate/phosphonate substituents to the 3-hydroxy-2-pyridinone ring nitrogen and the *para*-carbon would place the anionic groups in the respective C(1) and C(6) phosphate-binding subsites. The structures of the first generation compounds **1–9**, prepared to test this design strategy, are given in Chart I. A close structural homolog, dihydroxypyridine **10**, was also prepared for evaluation.

3.2. Synthesis of inhibitors 1-10

The synthetic sequences used for the preparation of the hydroxypyridinones **1–9** and the hydroxypyridine **10** are shown in Schemes 1 and 2, respectively, and the complete experimental details can be found in Appendix A. The preparation of N-phosphonomethyl-3-hydroxy-2-pyridone **1** relied on initial N-alkylation of the benzyl-



Chart I. The chemical structures of inhibitors 1-10.



Scheme 1. The reaction sequences used in the syntheses of inhibitors 1-9.

protected pyridone **11** with diisopropyl bromomethylphosphonate followed by trimethylsilyl bromide promoted ester cleavage and hydrogenolysis. For preparation of 3-hydroxy-4-pyridones **2** and **6**, methoxymethyl-protected 2-chloro-3-hydroxypyridine **13** was converted to the benzyloxy analog **14**. Sequential C-4 formylation and olefination with tetraisopropyl methylene-bisphosphonate gave the 4-pyridyl vinylphosphonate diester **16**. Reduction of the alkene moiety in **16** was accompanied by hydrogenolytic cleavage of the benzyl ether to give **17**. Treatment of **17** with trimethylsilyl bromide then furnished inhibitor **2**. N-Alkylation of **17** with diisopropyl



Scheme 2. The reaction sequence used in the synthesis of inhibitor 10.

bromomethylphosphonate and ester deprotection led to inhibitor **6**. The pyridonyl ethylphosphonate **2** was prepared from intermediate **17** by treatment with trimethylsilyl bromide.

Reaction of the 4-pyridylcarboxaldehyde **15** with NaBH₄ served as the first step in the routes for preparation of 3-hydroxy-4-pyridones **3** and **7**. The alcohol **14**, formed in this manner, underwent O-alkylation when treated with diisopropyl bromomethylphosphonate to produce the corresponding ether **19**, which following hydrogenolytic cleavage formed **20**. Treatment of **20** with trimethylsilyl bromide afforded inhibitor **3**.

The synthetic sequence employed to prepare the N-phosphonomethyl phosphates **4** and **8** began with the protected dihydroxypyridine **14** (Scheme 1). Introduction of the C-4 hydroxyethyl group was performed by reaction of the C-4 anion of **18** with ethylene oxide. Hydrogenolytic removal of the benzyl group followed by N-phosphonomethylation gave the alcohol **29**. Transformation of **29** to the dibenzyl phosphate diester **30** was achieved by reaction with dibenzyl N,Ndiisopropylphosphite followed *meta*-chloroperoxybenzoic acid oxidation. Finally, a two-step deprotection sequence was used to convert **30** to inhibitor **8**. The phosphate analog **4** was produced from intermediate **21** by phosphorylation of the alcohol moiety and deprotection.

The route to **9R** began with Wittig olefination of aldehyde **15** to produce alkene **31**. Sharpless dihydroxylation [40] with AD-mix- β provided diol **32R** with a 95% ee. Differential protection of the hydroxyl groups in **32R** followed by hydrogenolysis generated pyridone **35R**, which was N-alkylated using dibenzyl phosphonomethyltriflate to form **36R**. Selective liberation of the terminal hydroxyl group provided alcohol **37R**, which was then converted to inhibitor **9R** by using a phosphorylation–deprotection sequence. Inhibitor **9S** was prepared by using the same route employed for **9R** except that the Sharpless AD-mix- α methodology was used to carry out dihydroxylation of **31**. The phosphate **5** was formed from intermediate **35R** by tert-butyldimethyl silyl deprotection, alcohol phosphorylation, and benzyl and methoxymethyl ether removal.

Table 2 *GI*FBPA inhibition constants (K_i) measured for compounds **1–10** vs FBP at pH 7.5 and 25 °C (see the experimental procedures in the Appendix for details).

Compound	K _i (μM)	Compound	K _i (μM)
1	1200 ± 200	7	110 ± 10
2	810 ± 50	8	14 ± 4
3	970 ± 50	9R	90 ± 6
4	900 ± 200	9 S	10 ± 2
5	2300 ± 500	10	15 ± 2
6	800 ± 100	TBP	1

Finally, the route used for preparation of the dihydroxypyridine derivative **10** was initiated by conversion of pyridoxal (**39**) to the protected pyridine **42**. Formylation of **42** followed by sequential phosphate and phosphonate introduction gave **46**, which undergoes hydrogenolysis to form inhibitor **10**.

3.3. Kinetic evaluation of compounds 1-10 as GIFBPA inhibitors

Steady-state kinetic methods were used to access the binding affinities of compounds 1-10 as inhibitors of the GIFBPA catalyzed conversion of FBP to DHAP and G3P at pH 7.5 and 25 °C. The initial velocity data, measured at varying FBP concentrations and changing fixed inhibitor concentrations, demonstrated that competitive inhibition was taking place in each case and vielded the inhibition constants listed in Table 2. Compounds 1-5, which possess only one of the phosphoryl groups, were observed to bind significantly less tightly than inhibitors 7–10, which possess two properly positioned phosphoryl groups. This finding shows that both alkylphosphate/phosphonate ring substituents (R_1 and R_2) contribute to inhibitor binding affinity. Comparisons of the inhibition constants of inhibitors 7-9 to that of inhibitor 6, indicate that the longer R₂ linker generates more binding energy. Elaboration of inhibitor **8** by incorporation of a (*S*)-hydroxyl substituent on the R₂ linker was found to have little effect on binding as shown by the inhibition constant of inhibitor 9(S). However, incorporation of a (R)-hydroxyl substituent caused a slight impairment in binding as noted for inhibitor 9 (Table 2). Replacement of the Zn^{2+} binding group O=C-C-OH in inhibitor **8** with the HO-C-C-OH moiety in inhibitor 10 did not alter the value of the inhibition constant.

3.4. X-ray crystallographic structure determination of GIFBPA bound with compound ${\bf 8}$

In order gain further insight into the structural determinants of inhibitor binding, the X-ray structure of *GI*FBPA, complexed with inhibitor **8**, was determined. The 2.3Å resolution structure was obtained by first soaking *GI*FBPA–TBP crystals [25] with inhibitor **8** to replace the TBP ligand. The crystallization and refinement statistics are listed in Table 1, and the stereo-picture depicting of the ligand electron density is shown in Fig. 4A. As predicted by analysis of the docking model, the CH₂PO₃ group of inhibitor **8** fits nicely in the desolvated pocket where the TBP (C1)phosphate binds [25]. Likewise, the CH₂CH₂OPO₃ substituent of inhibitor **8** is located in the depression at the protein surface with its phosphate group bound by the residues that form the TBP C(6)phosphate binding site (Fig. 4B). Comparison of the *GI*FBPA active site binding interactions observed with inhibitor **8** (Fig. 4C) to those occurring with TBP (Fig. 2B) leads to the



Fig. 4. A. Stereoviews of the *Gl*FBPA–inhibitor **8** complex electron density map showing the electron density of inhibitor **8** and the Zn²⁺ cofactor. B. Superposition of the structures of the *Gl*FBPA–TBP and *Gl*FBPA–inhibitor **8** complexes. The FBP is colored yellow and the inhibitor **8** is colored cyan (carbon), red (oxygen) and orange (phosphorus). The surface of the *Gl*FBPA–inhibitor **8** complex is shown in gray. C. Chemdraw representation of the nonbonding interactions observed for inhibitor **8** in the *Gl*FBPA–inhibitor **8** complex. The distances are provided in Ås.

conclusion that inhibitor scaffold design is successful. Specifically, each of the targeted hydrogen bond donors or acceptors observed in the *GI*FBPA–TBP complex along with the Asp83, which forms a hydrogen bond to the pyridone ring OH, are observed to favorably interact with the ligand in the *GI*FBPA–inhibitor **8** complex, although no additional binding energy is gained through incorporation of the hydroxyl substituent on the first carbon atom of the $CH_2CH_2OPO_3$ group (compounds **9S** and **9R**) (Table 2).

The binding of inhibitor **8** to *Gl*FBPA, in analogy to substrate binding, induced the crucial conformational change that locks down the active site and places the Zn^{2+} in the proximity of the reaction center.¹ This change in Zn^{2+} coordination is depicted in Fig. 5. In order

to gain binding energy through interaction of the Zn^{2+} cofactor with the 3-hydroxy-2-pyridinone unit, the Zn^{2+} must form short coordination bonds (2.1 Å) with the O=C-C-OH oxygen atoms. The high B factor observed for the Zn^{2+} , coupled with elongated bond distances between Zn^{2+} and the three histidine ligands (2.4–2.8 Å) and the



Fig. 5. Chemdraw representation of the anticipated change in the *GI*FBPA Zn^{+2} coordination state upon inhibitor **8** association.

¹ The change in the Zn²⁺ coordination depicted in Fig. 5 is based on the comparison of the structures of *H. pylori apo* FBPA (determined at 1.8 Å resolution; PDB accession code 3C4U) and the corresponding liganded structures (determined at 2.3 Å resolution; PDB accession codes 3C56 and 3C52) as well as on the comparison of the structure of *G. intestinalis apo* FBPA (determined at 2.9 Å resolution; PDB accession code 3GAK) and the TBP liganded structure (determined at 1.8 Å resolution; PDB accession code 2ISW).



Fig. 6. Fourier transformed Zn K-edge EXAFS (solid lines) and corresponding best fits (open symbols, see Table 3) measured for unliganded *GI*FBPA (labeled "wild-type") and *GI*FBPA complexes of inhibitor **8**, PGH and TBP. See the experimental procedures in the Appendix for details.

O=C-C-OH group of compound **8** (2.7–2.9 Å), indicated that the Zn²⁺ site is not well ordered in the structure. Therefore, we interrogated the Zn²⁺ coordination state in the *Gl*FBPA–inhibitor **8** complex in solution (see below).

3.5. X-ray absorption spectral determination of GIFBPA complexes

Solution X-ray absorption spectroscopy, which offers an alternative method for examining Zn²⁺ coordination in protein complexes, was used as an independent method to determine if compound 8 coordinates to the GIFBPA Zn²⁺. X-ray absorption spectra were obtained for the unliganded enzyme and for GIFBPA bound with inhibitor 8, as well as for *GIFBPA* bound with the inhibitors phosphoglycolohydroxamate (PGH) [13] and TBP [25]. The Fourier transformed Zn K-edge EXAFS spectra are shown in Fig. 6 and curve fitting results are summarized in Table 3 and in Appendix A (Table SI1); XANES spectra and fits to raw EXAFS data are presented in Fig. SI1 and Table SI2 (Appendix A), respectively. Although high-resolution X-ray data are not available for the unliganded GIFBPA, it is reasonable to assume that the 4-coordinate geometry observed for the unliganded *H. pylori* FBPA (three His ligands plus the Glu ligand) [25] is representative of that of the unliganded GIFBPA (Fig. 5). The XANES and EXAFS data for unliganded *GI*FBPA (Figs. 6 and SI1 (Appendix A), Table 3) support this expectation. The best fit average bond length of 2.03 Å is wholly consistent with those determined crystallograhically for four-coordinate Zn(II) complexes containing all low-Z (N/O) donors. High-resolution (1.8 Å) X-ray structures are available for the GIFBPA-PGH [13] and GIFBPA-TBP [25] complexes, and these structures clearly define a 4-coordinate Zn^{2+} , consisting of three histidine ligands (H210, H178, H84) and an oxygen atom from the inhibitor ligand. The XANES spectrum, and the EXAFS-determined bond length of 2.03 Å for GIFBPA- TBP are nearly indistinguishable from the unliganded enzyme. The XANES and EXAFS for *GI*FBPA–PGH show some minor perturbations relative to the unliganded enzyme. However, the slight (0.04 Å) increase in first shell bond length is insufficient to suggest an increase in coordination number, although it may be indicative of slight geometric differences in the TBP and PGH complexes. The data for the *GI*FBPA–inhibitor **8** complex show minimal perturbation of the XANES and only 0.007 Å difference in average first shell bond length, which we take to indicate that Zn^{2+} in the *GI*FBPA–**8** complex coordinates to one oxygen atom of the pyridinone O=C–C–OH unit of inhibitor **8**.

4. Discussion

The EXAFS data measured for the *GI*FBPA-inhibitor 8 complex suggests that the Zn²⁺ cofactor does engage one of the pyridinone ring oxygen atoms in coordination. However, based on inspection of recent X-ray structures of liganded bacterial class II FBPAs we surmise that the coordination bond will be weak. Specifically, it was reported by Fonvielle et al. [41] that N-(3-hydroxypropyl)-glycolohydroxamic acid bisphosphate (PGH-PrP), a hydroxamic acid analog of FBP, binds to the *H. pylori* FPBA with high affinity ($K_i = 0.01 \mu M$) yet without the anticipated short-bond, in-plane, bidentate coordination geometry between the hydroxamic group and the Zn²⁺ cofactor. The long-range (C=O at 2.9 Å and N-OH at 2.5 Å), "out-of-plane" interaction between Zn^{2+} and the hydroxamic acid group indicates that the binding energy is primarily derived from hydrogen bonding interactions between active site residues and the phosphonate and hydroxamic acid groups. PGH-PrP presents both a flexible, substrate-like scaffold and a powerful Zn^{2+} binding group. The absence of tight, bidentate Zn^{2+} coordination in this complex is striking, but can be rationalized in light of the respective structures of the Mycobacterium tuberculosis FBPA bound with FBP or the charged enediolate form of DHAP reported by Mesecar et al. [42]. Whereas the Zn^{2+} is observed to be centered above the plane of FBP O = C(2) - C(3)(OH) - C(4)OH moiety and thus not engaged in strong coordination to any one of the three potential oxygen ligands, the DHAP enediolate participates in strong, in-plane bidentate coordination of Zn^{2+} (C(1)O at 2.1 Å and C(2)O at 2.2 Å). The DHAP enediolate is the reaction intermediate formed by the C(3)-C(4) cleavage step of FBPA catalysis (Fig. 1).

Taken together, the structures of FBPA bound with substrate (FBP) or substrate mimics (TBA, PGH-PrP and inhibitor **8**) provide solid evidence that the Zn^{2+} cofactor does not engage the substrate in strong coordination bonding and, thus, it does not significantly contribute to the substrate binding energy. The structure of FBPA bound with the DHAP enediolate is definitive proof that the Zn^{2+} cofactor engages in strong coordination bonding as the substrate changes to product along the reaction coordinate. The ability of a Zn^{2+} cofactor to modify its coordination with the reactant as it is transformed along the enzyme catalyzed reaction coordinate is well documented [43].

Table 3	
Best fits to EXAFS data measured for apo GIFBPA and GIFBPA-ligand c	omplexes.

Model	Zn–N/O	Zn-His ^b				R_{f}^{c}	R _u ^c
apo GlFBPA	4 at 2.030 (6.7)	2.91 (3.4)	3.17 (3.8)	4.22 (14)	4.41 (16)	106	448
GIFBPA-8	4 at 2.023 (6.4)	2.92 (2.6)	3.19 (1.7)	4.20 (24)	4.42 (19)	77	357
GIFBPA-PGH	4 at 2.068 (4.6)	2.92 (5.4)	3.20 (2.1)	4.18 (8.3)	4.40 (18)	78	366
GIFBPA-TBP	4 at 2.031 (8.5)	2.95 (10)	3.17 (2.9)	4.20 (11)	4.40 (16)	102	467

^a Distances (Å) and disorder parameters (in parentheses, $\sigma^2 (10^{-3} \text{ Å}^2)$) shown derive from integer coordination number fits to filtered EXAFS data [$\Delta k = 1-11 \text{ Å}^{-1}$; $\Delta R = 0.3-4.0 \text{ Å}$].

^b Multiple scattering paths represent combined paths, as described previously (see Materials and methods).

^c Goodness of fit (R_f for fits to filtered data; R_u for fits to raw data) defined as $1000 \frac{\sum_{l=1}^{N} \left\{ [\text{Re}(\chi_{loak})]^2 + [\text{Im}(\chi_{loak})]^2 \right\}}{\sum_{l=1}^{N} \left\{ [\text{Re}(\chi_{loak})]^2 + [\text{Im}(\chi_{loak})]^2 \right\}}$, where N is the number of data points.

5. Conclusions

In conclusion, we propose that the $GlFBPA Zn^{2+}$ cofactor functions in transition state/intermediate stabilization and not in the stabilization of the enzyme-substrate complex. Substrate binding induces a conformational change that dissociates the Glu135 ligand as the Zn²⁺ moves to the reaction center (Fig. 5). This induced-fit mechanism presents a challenge to inhibitor design strategies, which rely on recruiting the binding energy associated with Zn²⁺ coordination because substrate binding is used to drive the required conformational change rather than engage the Zn^{2+} in strong coordination. Consequently, for optimal binding a GIFBPA inhibitor should include a phosphonate substituent to fill the C(1)phosphate binding site (Fig. 2) and induce the conformational change, while at the same time containing a negatively charged substituent, such as a thiolate, to form a strong coordination bond to the Zn^{2+} cofactor.

Abbreviations

- FBP Fructose 1,6 bisphosphate
- fructose 1,6 bisphosphate aldolase **FBPA**
- Giardia lamblia fructose 1,6 bisphosphate aldolase **GlFBPA**
- TBP tagatose 1,6 bisphosphate
- G3P glyceraldehyde 3-phosphate
- DHAP dihydroxyacetone phosphate
- HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **EXAFS** extended X-ray absorption fine structure
- HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- extended x-ray absorption spectroscopy **EXAFS**

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2010.12.012.

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