FR258900, a potential anti-hyperglycemic drug, binds at the allosteric site of glycogen phosphorylase

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Abstract

FR258900 has been discovered as a novel inhibitor of human liver glycogen phosphorylase *a* and proved to suppress hepatic glycogen breakdown and reduce plasma glucose concentrations in diabetic mice models. To elucidate the mechanism of inhibition, we have determined the crystal structure of the cocrystallized rabbit muscle glycogen phosphorylase b–FR258900 complex and refined it to 2.2 Å resolution. The structure demonstrates that the inhibitor binds at the allosteric activator site, where the physiological activator AMP binds. The contacts from FR258900 to glycogen phosphorylase are dominated by nonpolar van der Waals interactions with Gln71, Gln72, Phe196, and Val45' (from the symmetry-related subunit), and also by ionic interactions from the carboxylate groups to the three arginine residues (Arg242, Arg309, and Arg310) that form the allosteric phosphate-recognition subsite. The binding of FR258900 to the protein promotes conformational changes that stabilize an inactive T-state quaternary conformation of the enzyme. The ligand-binding mode is different from those of the potent phenoxy-phthalate and acyl urea inhibitors, previously described, illustrating the broad specificity of the allosteric site.

Keywords: FR258900; glycogen phosphorylase; inhibition; X-ray crystallography; type 2 diabetes

Inhibition of glycogenolysis has been proposed as a therapeutic strategy for the treatment of type 2 diabetes. Glycogen phosphorylase (GP) catalyzes the first step in

glycogen degradation, and it is expected that inhibition of GP will inhibit glycogenolysis, reduce hepatic glucose production, and lower blood glucose, thereby providing a potential new treatment for type 2 diabetes (McCormack et al. 2001; Treadway et al. 2001; Sarabu and Tilley 2005; Baker et al. 2006). GP is physiologically regulated through small molecule allosteric effectors as well as through phosphorylation at Ser14, resulting in a structural switch between active (R-state) and inactive (T-state) conformation (for a review, see Johnson et al. 1989; Johnson 1992; Oikonomakos et al. 1992). Several binding sites on the enzyme such as the catalytic, allosteric, inhibitor, and the new allosteric site have been identified as specific targets for inhibitor binding (for a review, see Oikonomakos 2002).

The allosteric site, which binds the activator AMP (Barford et al. 1991; Sprang et al. 1991) and the natural inhibitor Glc-6-P (Johnson et al. 1993), has recently

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Abbreviations: GP, glycogen phosphorylase; 1,4- α -D-glucan, ortho-phosphate α -glucosyltransferase (EC 2.4.1.1); rmGPb, rabbit muscle glycogen phosphorylase *b*; rmGPa, rabbit muscle glycogen phosphorylase *a*; hGPa, human liver glycogen phosphorylase *a*; PLP, pyridoxal 5'-phosphate; glucose, α -D-glucose; Glc-1-P, α -D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate; FR258900, (2*R*,3*S*) 2,3-bis((*E*)-3-(4-hydroxyphenyl) acryloyloxy) pentanedioic acid; W1807, (–)(*S*)-3-isopropyl 4-(2-chlorophenyl)-1,4-dihydro-1-ethyl-2-methyl-pyridine-3,5,6-tricarboxylate; compound **4j**, 4-[2,4-Bis-(3-nitrobenzoylamino) phenoxy]phthalic acid; compound **21**, 1-(2-chloro-4-fluorobenzoyl)-3-(5-hydroxy-2-methoxyphenyl) urea; RMSD, root-mean-square deviation.

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attracted considerable interest. The site has been shown to bind the Bayer compound W1807 (Zographos et al. 1997; Oikonomakos et al. 1999; Tsitsanou et al. 2000) and several dihydropyridine diacid analogs (Ogawa et al. 2003), phenyl diacid analogs (Lu et al. 2003), and phenoxyphthalates (Kristiansen et al. 2004), which inhibited both the basal and the glucagon-induced glucose production in cultured primary hepatocytes, and also acyl ureas (Klabunde et al. 2005; Oikonomakos et al. 2005), which caused a significant reduction of the glucagon-induced hyperglycemic peak when administered to anesthetized Wistar rats. Ligands occupying this site are able to inhibit GP by either direct inhibition of AMP binding and/or indirect inhibition of substrate binding through stabilization of the T- or T'-state conformation of the enzyme.

FR258900, (2*R*,3*S*) 2,3-bis[(*E*)-3-(4-hydroxyphenyl)acryloyloxy] pentanedioic acid, (Fig. 1), a novel glycogen phosphorylase inhibitor isolated from Fungus No. 138,354, was proved to stimulate glycogen synthesis and glycogen synthase activity in primary rat hepatocytes (Furukawa et al. 2005a). The compound exhibited a potent inhibitory action

on human liver GP (hlGP), and significantly reduced the plasma glucose concentrations in diabetic mice models. These effects were accompanied by increased liver glycogen contents (Furukawa et al. 2005b), suggesting that it may activate glycogen synthesis via glycogen phosphorylase inhibition and therefore provide a new potential anti-hyperglycemic agent.

Here we report the detailed X-ray crystallographic analysis of FR258900 in complex with rabbit muscle glycogen phosphorylase (rmGPb). The structural data show that the compound binds at the allosteric site of the enzyme and occupies a position similar to that of the allosteric activator AMP. Binding of FR258900 induces conformational changes in the vicinity of the site and stabilizes an inactive T-state conformation.

Results and Discussion

FR258900 was found to inhibit rmGPb with an IC_{50} value of $0.30 \pm 0.02 \mu\text{M}$ and a K_i value of $0.46 \pm 0.06 \mu\text{M}$ with respect to AMP (Fig. 2). Furukawa et al. (2005a) reported an IC_{50} value $2.5 \mu\text{M}$ for FR258900 inhibition of hlGP in the absence of AMP. In order to elucidate the structural basis of inhibition, we have determined the crystal structure of rmGPb in complex with FR258900. A summary of crystallographic data collection and refinement statistics for the rmGPb–FR258900 complex structure is given in Table 1. The $2F_o - F_c$ Fourier electron density map indicated that FR258900 bound at the allosteric site (Fig. 3), while a portion of the $2F_o - F_c$ electron density map for molecule FR258900 is shown in Figure 4. The molecule could be fitted unambiguously at the allosteric site, since clear density was present for almost all atoms of the inhibitor.

Ligand–enzyme interactions of FR258900

The allosteric site is situated where the C termini of the helices $\alpha 2$ (residues 47–78) and $\alpha 8$ (residues 289–314) come together (Barford et al. 1991). It is lined by strands of the central core of $\beta 4$ (residues 153–160) and $\beta 11$ (residues 237–247), and surrounded on the third side by the short $\beta 7$ strand (residues 191–193) and the following loop to residue 197. The site is closed by the cap' region (residues 36'–47') (the superscript prime refers to residues from the symmetry-related subunit). FR258900, 2-deoxy-3,4-bis-*O*-[(*2E*)-3-(4-hydroxyphenyl)prop-2-enoyl]-*L*-threo-pentamic acid or (2*R*,3*S*)-2,3-bis[(*E*)-3-(4-hydroxyphenyl)acryloyloxy]pentanedioic acid, can be considered as a diester derivative of 2-deoxy-*L*-threo-pentamic acid (an aldaric acid derivative of a sugar) or a substituted pentanedioic acid. The two ester groups can be named as [(*2E*)-3-(4-hydroxyphenyl)prop-2-enoyl]- or [(*E*)-3-(4-hydroxyphenyl)acryloyloxy]- groups. Thus, the molecule contains two phenolic (4-hydroxyphenyl-)

Figure 1. Chemical structures of compound FR258900 (showing the numbering system used), compound **4j**, and compound **21**.

Figure 2. Kinetics of FR258900 inhibition of rmGPb with respect to AMP (0.01, 0.02, 0.03, 0.05, 0.1, and 1.0 mM) in the direction of the glycogen synthesis (30°C, pH 6.8). Double reciprocal plots of initial reaction velocity versus [AMP] at constant concentrations of Glc-1-P (10 mM) and glycogen (0.2% w/v) and various concentrations of FR258900. Inhibitor concentrations (mM) were as follows: 0 (○), 0.2 (●), 0.5 (■), and 1.0 μM (□). (Inset) Hill plots for AMP, which yielded the apparent K_m values for AMP and the Hill coefficient. The apparent K_m values and the Hill coefficients (top left) were 54 ± 1 μM (1.5), 70 ± 7 μM (1.6), 105 ± 12 μM (1.6), and 186 ± 11 μM (1.4). From the secondary plot (top right) of the apparent K_m (app) versus inhibitor concentration, a K_i value of 0.46 ± 0.06 μM was calculated (Leatherbarrow 1992).

rings, followed by a *trans*-acrylate (or *trans*-prop-2-enoyl-) moiety, while the core of the molecule is the pentanedioic acid group containing two protected hydroxyl substituents in a *threo* conformation and with 2*R*, 3*S* configuration.

FR258900 binds at the interface of the dimer forming the AMP site. One [(*E*)-3-(4-hydroxyphenyl)acryloyloxy] ester group (ester-1 group) is buried in the AMP allosteric site, while the other (ester-2 group) protrudes into the bulk solvent. FR258900 makes polar contacts to the protein, involving all potential hydrogen-bonding groups except the phenolic group O1. In the complex structure, FR258900 makes a total of 17 hydrogen bonds (Table 2) and 71 van der Waals interactions (three polar/polar, 45 polar/nonpolar, and 23 nonpolar/nonpolar interactions) (Table 3). There are 20 contacts to the symmetry-related subunit of which 10 are interactions between nonpolar atoms of the inhibitor and Val45' CG1. The hydrogen-bonding interactions formed between the ligand and the protein are illustrated in Figure 5. Specifically, the ester-2 group makes a water-mediated hydrogen-bonding interaction with Arg310 O and Ser313 O through its ester O3 and eight van der Waals contacts to Tyr75, Phe196, and Asn44' and Val45' from the symmetry-related subunit. The ester-1 group exploits numerous van der Waals contacts (30) that are dominated by the substantial contacts

made to Gln71 (9), Gln72 (8), and Val45' (6). The side chain of Gln72 stacks against the phenolic ring making some six van der Waals contacts. There are hydrogen bonds from the ester O8 to Gln71 NE2, Asp42', and Asn44' and from the phenolic OH (O10) to Asp42' OD1 and Asn44' ND2.

The carboxylate oxygens O4 and O5 of the central pentanedioic acid group exploit the allosteric effector phosphate-recognition subsite. The subsite is formed by the two arginine residues of the α8-helix, Arg309 and Arg310, and a more distant arginine located on the β11-strand, Arg242. The subsite recognizes the phosphate of a variety of phosphorylated compounds, such as AMP, ATP, Glc-6-P, and the carboxylate of the nonphysiological inhibitor W1807 (Zographos et al. 1997). The groups O6 and O7 of the other carboxylate group make hydrogen-bonding interactions with Thr240 OG1, Tyr155 OH, and also Arg193 and Asp227 (through water molecule Wat269).

The inhibitor becomes buried on forming the complex with rmGPb. The solvent accessibilities of the free and bound FR258900 molecule are $660 \text{ \AA}^2$ and $138 \text{ \AA}^2$, respectively, indicating that 79% of the ligand surface becomes buried or that a surface area of $522 \text{ \AA}^2$ becomes inaccessible to the solvent. Binding of FR258900 is associated with both

Table 1. Summary of diffraction data and refinement statistics for the rmGPb–FR258900 complex

Space group	$P4_32_12$
No. of images (°)	96 (0.8) ^a
Unit cell dimensions (Å)	$a = b = 128.78$, $c = 116.05$
Resolution range (Å)	90.91–2.2
No. of observations	674,217
No. of unique reflections	48,704
$\langle I/\sigma(I) \rangle$ (outermost shell) ^b	11.1 (4.7)
Completeness (outermost shell) (%)	97.2 (95.5)
R_m (outermost shell) ^c	0.107 (0.431)
Outermost shell (Å)	2.24–2.20
Redundancy (outermost shell)	6.3 (6.5)
Refinement (resolution) (Å)	90.91–2.20
No. of reflections used (free)	46,201 (2474)
Residues included	(12–254), (261–314), (324–835)
No. of protein atoms	6600
No. of water molecules	290
No. of ligand atoms	15 (PLP), 33 (FR258900)
Final R (R_{free}) (%) ^d	18.4 (21.3)
R (R_{free}) (outermost shell) (%)	20.3 (27.4)
Outermost shell in refinement (Å)	2.25–2.20
RMSD in bond lengths (Å)	0.007
RMSD in bond angles (°)	1.1
Average B (Å ²) for residues	(12–254), (261–314), (324–835)
Overall	31.1
CA, C, N, O	30.4
Side chain	31.8
Average B (Å ²) for ligands	19.1 (PLP), 50.2 (FR258900)
Average B (Å ²) for water molecules	36.2

^a0.8 is the rotation range per image.^b $\sigma(I)$ is the standard deviation of I .^c $R_{\text{merge}} = \sum_i \sum_h |I_{ih} - \langle I_h \rangle| / \sum_i \sum_h I_{ih}$, where $\langle I_h \rangle$ and I_{ih} are the mean and i th measurement of intensity for reflection h , respectively.^dCrystallographic $R = \sum ||F_o| - |F_c|| / \sum |F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. R_{free} is the corresponding R -value for a randomly chosen 5% of the reflections that were not included in the refinement.

subunits (surface areas of 410 Å² and 112 Å², respectively). While both polar and nonpolar groups of the inhibitor are buried, the greatest contribution comes from the nonpolar residues, which contribute 366 Å² (68.3%) of the surface that becomes inaccessible. On the protein surface, a total of 356 Å² (295 Å² in one subunit and 61 Å² in the symmetry-related subunit) of solvent-accessible surface area becomes inaccessible upon binding of the ligand. The total buried surface area (protein plus ligand) for the rmGPb–ligand complex is 878 Å².

Comparison with the native T-state structure

Superposition of the activation locus, residues 24–78, 94–111, and 118–125 from both subunits, as defined previously (Sprang et al. 1991), of the structure of the native T-state rmGPb with the activation locus of the structure of the rmGPb–FR258900 complex gave a root-mean-square deviation (RMSD) of 0.30 Å for C $_{\alpha}$ atoms, indicating that

Figure 3. A schematic diagram of the T-state rmGPb dimeric molecule, for residues 10–837, viewed down the molecular dyad, showing the positions for the catalytic and the allosteric binding sites. The catalytic site (marked by glucose, shown in gray), which includes the essential cofactor PLP (not shown), is buried at the center of the subunit accessible to the bulk solvent through a 15 Å-long channel. The allosteric site, which binds the activator AMP, and the allosteric inhibitors Glc-6-P, the Bayer compound W1807, the Novo compound **4j**, the Sanofi-Aventis compound **21**, and compound FR258900 (shown in gray), is situated at the subunit–subunit interface some 30 Å from the catalytic site.

the two structures have very similar overall conformations within the limits of the 2.2 Å resolution data. The major conformational changes on binding of FR258900 to rmGPb occur in the vicinity of the allosteric site. Shifts for main-chain atoms are observed for residues 45′–48′ (between 0.5 and 0.8 Å), and residues 193–196 (between 0.5 and 0.7 Å) that affect the subunit–subunit interface in

Figure 4. Stereo diagram of the $2F_o - F_c$ electron density map, contoured at 1 σ , for the bound compound FR258900 at the allosteric site. The electron density map was calculated using the standard protocol as implements in REFMAC (Murshudov et al. 1997) before incorporating ligand coordinates.

Table 2. Hydrogen-bond interactions between compound FR258900 and residues of the allosteric site of rmGPb

Inhibitor atom	GPb–FR258900 complex	
	Protein atom	Distance (Å)
O3	Wat287	2.7
O4	Arg242 NH1	3.0
	Arg242 NH2	3.1
	Arg309 NH1	3.2
	Arg310 NH1	2.9
O5	Arg309 NH1	3.0
	Arg309 NH2	3.3
	Arg310 NE	2.8
	Arg310 NH1	3.3
O6	Thr240 OG1	2.7
	Wat269	2.7
O7	Tyr155 OH	3.3
	Wat 272	2.7
O8	Gln71 NE2	3.0
O10	Asp42' OD2	2.7
	Asp42' OD1	(3.4)
	Asn44' ND2	3.0

Wat269 is hydrogen bonded to Arg193 NH1 (2.8 Å) and NE (3.3 Å), Asp227 OD1 (2.6 Å), and Wat282 (2.5 Å). Wat282 is hydrogen bonded to Gln71 OE1 (3.2 Å) and Val40' from the symmetry related subunit through Wat270' (2.7 Å). Wat272 is hydrogen bonded to Ala154 O and Thr240 N through Wat137 (3.3 Å), and also to Val238 O and Thr240 OG1 through Wat267 (2.8 Å). Wat287 is hydrogen bonded to Arg310 O and Ser313 O through Wat273 (3.2 Å).

the region between the cap' and the loop between $\beta 7$ (residues 191–193) and $\beta 8$ (residues 198–209) strands. The greatest changes include shifts of the side-chain atoms of residues 193–196 by ~ 0.8 – 4.0 Å, of residue 45' by ~ 1.0 – 1.5 Å, and also shifts of the side-chain atoms of residues 47'–49' of ~ 0.6 – 1.1 Å. Similar shifts were observed previously on binding of Glc-6-P (Johnson et al. 1993), W1807 (Zographos et al. 1997), and acylureas (Klabunde et al. 2005; Oikonomakos et al. 2005) to the allosteric sites of both rmGPb and rmGPa. These shifts take place without a change in the quaternary structure, and the ligand-induced conformational changes are characteristic of a modified T state that is more tensed than the T state. A comparison of the two structures in the vicinity of the allosteric site is shown in Figure 6A.

Comparison with 4-[2,4-bis-(3-nitrobenzoylamino)phenoxy]phthalic acid binding

4-[2,4-Bis-(3-nitrobenzoylamino)phenoxy]phthalic acid (**4j**) (Fig. 1) was found recently to be a potent inhibitor of pig liver GPa (with an IC_{50} value of 74 nM) that binds at the AMP allosteric site (Kristiansen et al. 2004). Upon binding to rmGPb, **4j** induces conformational changes to the enzyme and stabilizes a conformation similar to that of the rmGPb–FR258900 complex. The superposition of the rmGPb–**4j** complex with the rmGPb–FR258900 com-

plex over the activation loci gave an RMSD of 0.377 Å for C_{α} atoms. The **4j** ring B overlaps with the 4-hydroxyphenyl ring of ester-1 group of FR258900, while the plane of the ring A is at an angle of $\sim 45^{\circ}$ to the plane of the pentanedioic acid group of FR258900 (Fig. 6B).

Comparison with 1-(2-chloro-4-fluorobenzoyl)-3-(5-hydroxy-2-methoxy-phenyl) urea (compound **21**)

Compound **21** (Fig. 1), with an enzymic activity of $IC_{50} = 23 \pm 1$ nM, and a cellular activity of $IC_{50} = 6.2$ μ M, when tested in rat hepatocytes, is one of the most potent inhibitors of human liver glycogen phosphorylase (hlGPa) that binds at the AMP allosteric site (Klabunde et al. 2005). In the complex with hlGPa, the 2-chloro-4-fluoro-substituted benzoyl ring is buried in a

Table 3. Van der Waals interactions between compound FR258900 and residues at the allosteric site of rmGP

Inhibitor atom	Protein atom	No. of contacts
C2	Tyr75 CZ,OH	1
C3	Val45' CG1	1
C4	Val45' CG1	1
C5	Val45' CG1	1
C6	Asn44' O	1
C7	Val45' CG1	1
C8	Wat287	1
C9	Phe196 CZ; Wat287	2
C10	Wat287	1
C11	Phe196 CZ; Arg309 NH1; Arg310 NE, CZ, NH1; Wat287	6
C13	Arg193 NH1; Wat269	2
C14	Ty155 CE1; Thr240 OG1; Wat269; Wat272	4
C15	Gln71 CB, NE2	2
C16	Gln71 O, CB, NE2	3
C17	Gln71 C, O; Tyr75 CB	3
C18	Gln72 CA; Val45' CG1	2
C19	Gln72 N; Val45' CG1	2
C20	Gln72 CB; Asp42' OD2; Val45' CG1	3
C21	Gln72 CG; Asp42' OD1, OD2; Val45' CG1	4
C22	Gln72 CG; Val45' CG1	2
C23	Gln72 CA; Val45' CG1	2
O2	Phe196 CZ, CE2	2
O4	Phe196 CZ, CE2; Arg242 CZ; Arg310 NE, CZ	5
O5	Arg309 CZ; Arg310 CG, CD,CZ	4
O6	Gln71 CD, OE1; Thr240 CB, CG2; Wat267; Wat272	6
O7	Ty155 CZ, CE1	2
O8	Gln71 CB, CD	2
O10	Gln72 CG, CD; Asp42' CG; Asn44' CB, CG	5
Total		71

Figure 5. Interactions between FR258900 and residues of rmGPb in the vicinity of the allosteric site.

narrow side pocket deep in the AMP site and tightly packs against Trp67, Arg193, Val40', and Lys41'. The central acyl urea moiety is hydrogen bonded with the carbonyl group of Val40', the backbone amide group of Asp42', and an ordered water molecule in the upper part of the AMP pocket. In addition to these interactions, the phenolic ring, which points toward the entrance of the allosteric site, reveals hydrophobic van der Waals interactions with Gln72. The additional van der Waals interactions are mediated by the methoxy substituent positioned near Tyr75, and the 5-hydroxy-2-methoxy-phenyl ring induces additional hydrogen bonds between the side chains of Asp42' and Asn44' and the phenolic hydroxyl group (Klabunde et al. 2005). These interactions provide a structural explanation for the potency of **21** as an allosteric inhibitor of hGPb. The superposition of the structure of the hGPb–**21** complex with the rmGPb–FR258900 complex structure over the activation loci gave an RMSD of 0.507 Å for C $_{\alpha}$ atoms. The 5-hydroxy-2-methoxy-phenyl ring of **21** overlaps with the phenolic ring of the ester-1 group of FR258900, while the central acyl urea moiety and 2-chloro-4-fluoro-substituted benzoyl ring of **21** do not overlap with FR258900 (Fig. 6C).

Comparison with R-state hGPb

Comparison of the rmGPb–FR258900 complex with the R-state hGPb–AMP complex (Rath et al. 2000) suggests that the inhibitor is likely to have lower affinity for the R-state conformation. Superposition of the activation loci of the structure of the R-state hGPb–AMP (subunit A) with the activation locus of structure of the rmGPb–FR258900 complex gave an RMSD of 1.50 Å for C $_{\alpha}$ atoms. The transformation that allows superposition of

the R-state rmGPb–AMP complex structure to the rmGPb–FR258900 complex structure involves a rotation of one subunit by $\sim 5.6^{\circ}$ so as to bring the two subunits closer together at the twofold axis of the dimer (the corresponding calculated value for the rmGPb–FR258900/native T-state rmGPb pair is 0.05°). If FR258900 were to be superimposed into the R-state hGPb–AMP complex structure, the phosphate would overlap partially with the central pentanedioic acid group, and the ribose and the adenine moieties would overlap partially with the [(*E*)-3-(4-hydroxyphenyl)acryloyloxy] ester-1. In addition, superposition of FR258900 into the allosteric site of the R-state hGPb–AMP complex would result in clashes with the side chains of residues Asp42' (CD2), Asn44' (ND2, CG), Arg309 (CZ, NH2), and Arg310 (NH1) (Fig. 6D). The positions of these residues would prevent binding of FR258900, but movements of these residues that would enable binding of the ligand, as seen in the rmGPb–FR258900 complex, appear to be suppressed by the subunit–subunit contacts that promote the R state. Hence, it would be anticipated that the affinity of FR258900 for the R state would be less than that for the T state, but there is no experimental structural evidence for this.

A comparison of the positions of AMP (R-state hGPb), **4j** (T-state rmGPb), **21** (T-state hGPb), and FR258900 (T-state rmGPb) bound at the allosteric site is shown in Figure 7.

Conclusions

The X-ray crystallographic study of the rmGPb in complex with FR258900 inhibitor showed that the compound binds at the allosteric site, at the subunit–subunit interface of the dimer with a high affinity ($K_i = 0.4 \mu\text{M}$). In contrast,

an IC_{50} value of 2.5 μ M was found for FR258900 inhibition of hGPa as expected for the binding of a T-state inhibitor to GPa that is predominantly R state (Oikonomakos 2002). FR258900, on binding to rmGPb, forms direct hydrogen bonds with the three arginines of the phosphate recognition subsite (Arg242, Arg309, and Arg310), Thr240, Tyr155, Gln71, Asp42', Asn44', water-mediated interactions with Arg193, and Asp227, and makes extensive nonpolar interactions with the enzyme that involve the side chains of Tyr75, Phe196, Tyr155, Gln71, Gln72, and Val45' from the symmetry-related subunit. These interactions may explain why FR258900 is a nanomolar inhibitor of the enzyme.

The major conformational changes upon binding of FR258900 to rmGPb occur in the vicinity of the allosteric site. Shifts for C_{α} atoms were observed for residues 45', and 47'–49' (between 0.5 and 0.8 Å), and residues 193–

196 (between 0.5 and 0.7 Å) that affect the plasticity of the site and appear important in stabilizing an inactive T state. The position of the inhibitor is partially overlapping with the position of AMP bound to the R state of the enzyme (Fig. 7A). The binding of FR258900 and AMP is therefore mutually exclusive. Overall, we suggest that FR258900 inhibits the enzyme directly by preventing binding of the allosteric activator AMP and allosterically by stabilizing the T state.

The structural results obtained with the FR258900 can be further exploited by means of chemical modifications to yield new potent inhibitors with improved anti-hyperglycemic properties. In particular, partial hydrolysis of the ester-2 group (the 4-*O*-substituent of the *L*-threopentamic acid), which is the group that protrudes into the bulk solvent in the crystal structure, may provide a hydroxyl handle for further derivatization, leading to

Figure 6. (Continued on next page)

structures with enhanced interactions within the allosteric site (targeting Phe196, Tyr75, Asn44', or Val45').

The crystallographic results with several dihydropyridine diacid analogs (Zographos et al. 1997; Ogawa et al. 2003), phenyl diacid analogs (Lu et al. 2003), phenoxy-phthalates (Kristiansen et al. 2004), acyl ureas (Klabunde et al. 2005; Oikonomakos et al. 2005), and FR258900 (this study) demonstrate the ability of the allosteric site to distinguish among various classes of inhibitors. The remarkable property of this binding site to recognize these classes by using nearly the same residues appears to originate from its conformational plasticity, which enables structural rearrangements such as the ones described above. Understanding this conformational plasticity will be important in structure-based design and optimization of inhibitors.

Materials and Methods

FR258900 was kindly provided by Astellas, Pharma Inc. RmGPb was isolated, purified, recrystallized, and assayed as previously described (Anagnostou et al. 2006). Kinetic studies performed in the direction of glycogen synthesis with 5 $\mu\text{g/mL}$ enzyme, constant concentrations of glycogen (0.2% w/v), Glc-1-P (2 mM), AMP (100 μM), and various concentrations of inhibitor FR258900 (0.1–1.0 μM) showed that the compound exhibited an IC_{50} value of $0.30 (\pm 0.02) \mu\text{M}$. More detailed kinetic experiments were performed with 5 $\mu\text{g/mL}$ enzyme, constant concentrations of glycogen (0.2% w/v), Glc-1-P (10 mM), and various concentrations of AMP (0.01 mM, 0.02 mM, 0.03 mM, 0.05 mM, 0.1 mM, and 1 mM) and inhibitor (0.2 μM , 0.5 μM , and 1.0 μM), in 30 mM imidazole/HCl buffer (pH 6.8), 60 mM KCl, 0.6 mM EDTA, and 0.6 mM dithiothreitol. Kinetic data presented in the form of Hill plots were treated by linear-regression analysis as previously described (Oikonomakos

Figure 6. Comparison between the rmGPb–FR258900 complex (shown in dark gray) and (A) the T-state rmGPb (shown in light gray), (B) the T-state rmGPb–4j (shown in light gray), and (C) the T-state hIGPa–21 (shown in light gray) in the vicinity of the allosteric site. (D) HIGPa–AMP complex in the R state (shown in dark gray) viewed in a similar orientation to that of the T-state rmGPb–FR258900 complex; the position of FR258900 is superimposed.

Figure 7. Comparison of the position of the inhibitor FR258900 as compared to those of (A) the activator AMP, (B) the inhibitor 4j, and (C) the inhibitor 21 at the allosteric site, after superimposing the R-state hGPα-AMP, the T-state rmGPb-4j, and the T-state hGPα-21 complexes, respectively, onto the rmGPb-FR258900 complex structure.

et al. 1995) by providing an explicit value for the standard deviation of each rate ("explicit weighting"). The K_i value was then determined by plotting $K_m(\text{app})$ versus inhibitor concentration using the same type of analysis.

Binding studies performed by diffusion of FR258900 into preformed rmGPb crystals, grown as previously (Oikonomakos et al. 1985), resulted in crystal cracking. Crystal cracking was overcome by cocrystallization studies. Indeed, the rmGPb complex with FR258900 was cocrystallized in a medium consisting of 23–25 mg/mL enzyme, 1 mM FR258900, 3 mM dithiothreitol, 10 mM Bis, 0.1 mM EDTA, and 0.02% sodium azide (pH 6.7). Crystallographic data were collected from a single cocrystal on SRS PX14.1 ($\lambda = 1.488 \text{ \AA}$). Crystal orientation,

integration of reflections, interframe scaling, partial reflection summation, data reduction, and post-refinement were all performed using DENZO and SCALEPACK (Otwinowski and Minor 1997).

Crystallographic refinement of the complex was performed by maximum-likelihood methods using REFMAC (Murshudov et al. 1997). The starting model used for the refinement of the complex was the structure of the native T-state rmGPb complex determined at 1.9 Å resolution (data not shown). The $2F_o - F_c$ and $F_o - F_c$ electron density maps calculated were visualized using the program for molecular graphics O (Jones et al. 1991). A ligand model was fitted to the electron density maps after adjustment of their torsion angles. Alternate cycles of manual rebuilding with O and refinement with REFMAC improved the quality of the model.

The stereochemistry of the protein residues was validated by PROCHECK (Laskowski et al. 1993; Collaborative Computational Project, Number 4, 1994). Hydrogen bonds and van der Waals interactions were calculated with the program CONTACT as implemented in CCP4 (Collaborative Computational Project, Number 4, 1994) applying a distance cutoff of 3.3 Å and 4.0 Å, respectively. Protein structures were superimposed using LSQKAB (Collaborative Computational Project, Number 4, 1994). Solvent-accessible areas were calculated with the program NACCESS (Hubbard and Thornton 1993). All the figures were prepared with the program MolScript (Kraulis 1991) and rendered with Raster3D (Merritt and Bacon 1997). Coordinate sets for comparison were: T-state rmGPb-4j (code 1Z6Q), T-state hGPα-21 (code 2ATI), and R-state hGPα-AMP complex (code 1FA9). The coordinates of the new structure (rmGPb-FR258900 complex) have been deposited with the RCSB Protein Data Bank (<http://www.rcsb.org/pdb>) with the code 2OFF.

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