Human liver glycogen phosphorylase inhibitors bind at a new allosteric site

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Background: Glycogen phosphorylases catalyze the breakdown of glycogen to glucose-1-phosphate for glycolysis. Maintaining control of blood glucose levels is critical in minimizing the debilitating effects of diabetes, making liver glycogen phosphorylase a potential therapeutic target.

Results: The binding site in human liver glycogen phosphorylase (HLGP) for a class of promising antidiabetic agents was identified crystallographically. The site is novel and functions allosterically by stabilizing the inactive conformation of HLGP. The initial view of the complex revealed key structural information and inspired the design of a new class of inhibitors which bind with nanomolar affinity and whose crystal structure is also described.

Conclusions: We have identified the binding site of a new class of allosteric HLGP inhibitors. The crystal structure revealed the details of inhibitor binding, led to the design of a new class of compounds, and should accelerate efforts to develop therapeutically relevant molecules for the treatment of diabetes.

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Introduction

Non-insulin-dependent (type 2) diabetes mellitus is a disease characterized by high levels of glucose in the plasma and leads to complications such as nerve and kidney damage, blindness, premature atherosclerosis and heart disease. In the United States, 15.6 million people have been diagnosed with type 2 diabetes [1]. The molecular basis of the disease remains poorly understood but is characterized by peripheral insulin resistance and pancreatic defects in insulin secretion. Intensive control of blood glucose levels prevents and delays the onset of diabetic complications [2] but such control is rarely achieved with oral antidiabetic agents. Liver phosphorylase catalyzes glycogenolysis (the phosphorolysis of an α (1-4) glycosidic bond in glycogen to yield glucose-1-phosphate for metabolism) and plays an important role in hepatic glucose production. In diabetic subjects, glycogenolysis remains an important contributor to hepatic glucose output even when blood glucose levels are high, establishing a potential role for phosphorylase inhibitors in diabetes therapy.

Human liver glycogen phosphorylase (HLGP, a homodimer of 846 residues per subunit) is allosterically regulated by the binding of small molecule effectors and by phosphorylation of Ser14, both of which induce conformational switching ([3,4] and references therein). Allosteric effectors and phosphorylation alter enzyme activity by stabilizing either the active or the inactive conformation. The phosphorylated enzyme, HLGP*a*, may adopt both an active or inactive conformation, the unphosphorylated enzyme, HLGP*b*, exhibits low activity but may be weakly activated by AMP. The active conformation is stabilized by phosphorylation of Ser14 and binding of AMP. The inactive conformation is stabilized by dephosphorylation, glucose, and the binding of heterocyclic compounds, such as caffeine. Glucose binds at the catalytic site, caf-

feine binds near to the catalytic site, and AMP binds to a separate allosteric site. Glucose functions synergistically with caffeine to inhibit phosphorylase [5].

Inhibitors of phosphorylase include glucose analogs (active site) [6,7]; an AMP site inhibitor [8]; and hydroxylated piperidines and pyrrolidines [9]. We recently reported two series of indole-2-carboxamide inhibitors which have oral activity in an animal model of type 2 diabetes [10,11]. The indole-2-carboxamides show caffeine-like synergy with glucose, a desirable property which could minimize the risk of hypoglycemia, a potentially severe side effect of many antidiabetic agents. Here, we show that indole-2-carboxamides bind to a novel, allosteric site on HLGP*a* [12] and present the key features of the site which enabled the design of a second class of inhibitor with nanomolar affinity. During review of this manuscript, an independent report of the crystal structure of a complex of another member of this class of compounds (CP-320,626, [11]) with rabbit muscle glycogen phosphorylase *b* appeared [13] showing that the site also exists in the inactive, unphosphorylated form of the rabbit muscle enzyme, and that many of the interactions we now describe are conserved. We have also observed this binding site in the crystal structures of two other inhibitors of this class complexed to the phosphorylated form of rabbit muscle phosphorylase (VLR, personal communication).

Results and discussion

Discovery of the novel binding site

Initially, we thought that these glucose-sensitive inhibitors bound to the caffeine site. Further analysis revealed that they also exhibited synergism with caffeine [10], indicating that a different locus was involved. To identify this site, the crystal structure of HLGP*a* complexed with CP-403,700, one of these compelling inhibitors, was solved to 2.4 Å resolution (see Figure 1 and Table 1).

The electron density of the bound inhibitor was located in a difference map of a crystal of HLGP*a* grown in the presence of a glucose analog, *N*-acetyl-β-D-glucopyranosylamine (GlcNAc), and excess CP-403,700. GlcNAc was included because it is a potent inhibitor of rabbit muscle phosphorylase [8]. The compound binds to a new site on the enzyme within the solvent cavity which forms part of the dimer interface. Two molecules are identically bound, very close to the axis of the molecular 2-fold symmetry operator. Consequently, the two inhibitors are within 6 Å of each other and taken together, share one large, continuous, binding site.

Description of the site

The binding site for each inhibitor is made up of residues from both subunits and consists of two qualitatively different environments (Figure 2). One environment is hydrophobic, formed almost exclusively from one subunit, and houses the chloroindole moiety of the inhibitor; the other binds the carboxamide, phenylalanine and azetidine moieties, is both hydrophobic and polar, includes residues from both subunits, and extends into a solvent-filled cavity. The hydrophobic chloroindole pocket is formed from the aliphatic part of the side chains of Arg60 and Lys191, and the side chains of six other hydrophobic residues (Figure 2b). The chloroindole group is completely buried in the complex. This includes 266 Å^2 of hydrophobic surface area which makes a large contribution to the binding energy. Additionally, the indole nitrogen forms a hydrogen bond to a backbone carbonyl and the guanidinium group of Arg60 appears to have a favorable electrostatic interaction with the indole ring [14].

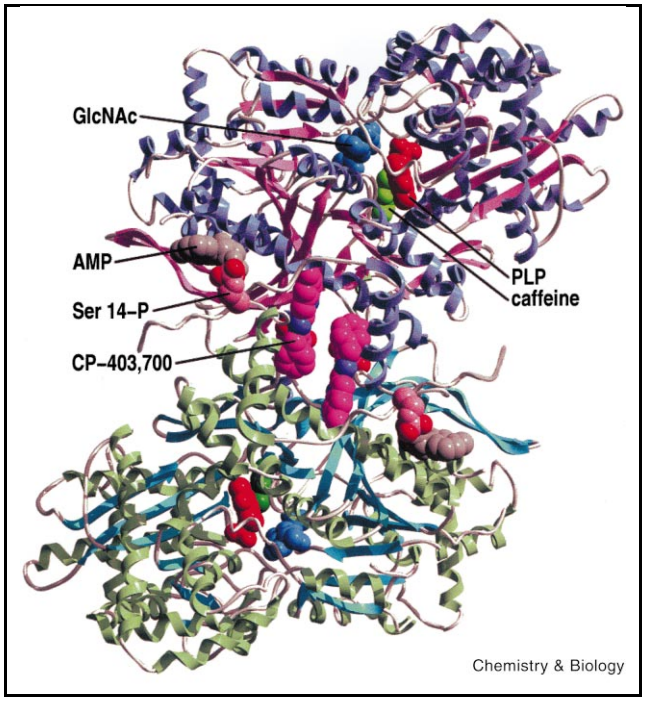


Figure 1. The new allosteric inhibitor site. HLGP*a* as a ribbon diagram, one subunit in purple (helices) and pink (sheets) and the other in green (helices) and blue (sheets). In CPK are AMP (gray); Ser14-P (pink, red phosphates); PLP (pyridoxal phosphate, the essential cofactor, in red); caffeine (green); GlcNAc (blue, marks the glucose binding site); and CP-403,700 (carbon, pink; nitrogen, blue; oxygen, red). Two-fold symmetry operator relating the subunits located between the two molecules of CP-403,700, orthogonal to the plane of the page. The binding sites for Ser14-P and AMP are located close to each other but do not overlap. To show all the binding sites in one image, a composite of three crystal structures was made; AMP and residues 5–22 (including Ser14-P) from the crystal structure of HLGP*a* complexed with AMP (Rath, V.L. et al., Molecular Cell, in press); caffeine, from crystals of HLGP*a* complexed with GlcNAc and CP-403,700 soaked in caffeine (manuscript in preparation); remainder from the complex of HLGP*a* complexed with GlcNAc and CP-403,700 described herein.

The second part of the inhibitor binding site is formed from both subunits, includes more hydrogen bonds, and is characterized by a less constrained fit with the compound. Direct hydrogen bonds are formed to a backbone carbonyl and to the side chain of Lys191. The phenylalanine side chain of the inhibitor forms van der Waals contacts with a Pro, Phe and His but is less buried (84%) than the chloroindole. The azetidine ring and its carboxylate are solvent-exposed; only 57% of the total solvent accessible surface area is buried in the complex. The carboxylate makes only water-mediated hydrogen bonds to the enzyme.

The binding site of CP-403,700 in the crystal structure of the active complex of HLGP*a* with AMP is masked by the side chains of Arg60, Val64 and Lys191. This was determined by superimposing the Cα carbons of one subunit (residues 30–830) of the CP-403,700-inhibited complex on the coordinates of the active complex of HLGP*a* with AMP (Rath, V.L. et al., Molecular Cell, in press) and examining the interactions between the side chains of active HLGP*a*/AMP and the compound. The results show that the side chains of Arg60, Val64 and Lys191 of active HLGP*a* lie within 0–1.2 Å of the inhibitor and must move to accommodate its binding. The required side chain movements could not be predicted in the absence of CP-403,700. Five other residues (38′, 40′, 53, 57 and 192) make smaller adjustments to eliminate close contacts with the inhibitor. Only one hydrogen bond (which is between the two subunits, Arg60:Thr38′) is disrupted on inhibitor binding, and both residues have new hydrogen bonding partners in the complex. Thr38′ forms a hydrogen bond to the indole nitrogen of the compound and the guanidinium group of Arg60 forms a hydrogen bond to a water molecule (in addition to stacking over the chloroindole moiety).

Inhibitors are allosteric effectors

CP-403,700 functions as a classic allosteric inhibitor in that it stabilizes the inactive conformation of the enzyme and exhibits synergy with other inhibitors [15]. Over the range of physiologic glucose concentrations (2.5–7.5 mM), the inhibitory potency of the compound is increased 3-fold (IC_{50} for CP-403,700 at 0 mM glucose, 0.145 μM; at 2.5 mM glucose, 0.054 μM; at 5.0 mM glucose, 0.028 μM; at 7.5 mM glucose, 0.0175 μM, see Materials and methods). CP-403,700 increases the rate of dephosphorylation 2-fold, as measured by the release of ^{32}P from Ser14 [^{32}P]HLGP*a*, consistent with results reported for other allosteric inhibitors of rabbit muscle glycogen phosphorylase *a* [5].

Design of a new class of inhibitors from the structure

We speculated that the most important structural component of CP-403,700 for binding was the chloroindole carboxamide group. On seeing the closely bound inhibitors, and aware of the potential in correctly joining two ligands [16], we reasoned that joining two chloroindole groups together with an appropriate linker could give a new type of inhibitor which would interact simultaneously with both chloroindole binding pockets. After estimating the length of a connector from the CP-403,700 X-ray structure, various bis-indolecarboxamides were synthesized by coupling two

Table 1
Crystallographic data and refinement statistics.

	HLGP*a*/1-GlcNAc/CP-403,700	HLGP*a*/1-GlcNAc/CP-526,423
Space group	$P3_1$ (dimer in asymmetric unit)	$P3_1$ (dimer in asymmetric unit)
Cell constants	$a=b=124.63$ Å; $c=124.06$ Å	$a=b=123.31$ Å; $c=122.32$ Å
	$\alpha=\beta=90.0°$; $\gamma=120.0°$	$\alpha=\beta=90.0°$; $\gamma=120.0°$
Data collection statistics		
Resolution	99.0–2.4 Å	99.0–2.2 Å
Unique reflections	81,403	97,977
Redundancy	5–6	2–3
Resolution (last shell)	99.0–2.4 (2.49–2.4)	99.0–2.2 (2.28–2.2)
Chi^2	1.04 (1.08)	1.07 (0.83)
R_{merge}	0.071 (0.378)	0.074 (0.359)
I/error	19.1 (2.8)	13.6 (2.0)
Completeness (%)	99.8 (99.0)	97.8 (87.1)
Refinement statistics		
Protein	23–249, 260–313, 326–830	22–249, 260–314, 324–830
Disordered	1–22, 250–259, 314–325, 831–846	1–22, 250–259, 315–323, 831–846
Ligands	two molecules 1-GlcNAc	two molecules 1-GlcNAc
(per dimer)	two molecules CP-403,700	two molecules CP-526,423
	two molecules MPD	one molecule MPD
Water molecules	333	737
Resolution	99.0–2.4 Å (2.55–2.4)	99.0–2.2 (2.34–2.2)
R	0.236 (0.283)	0.233 (0.307)
R_{free}	0.280 (0.334)	0.264 (0.340)
r.m.s.d. bond lengths	0.007 Å	0.006 Å
r.m.s.d. bond angles	1.22°	1.26°

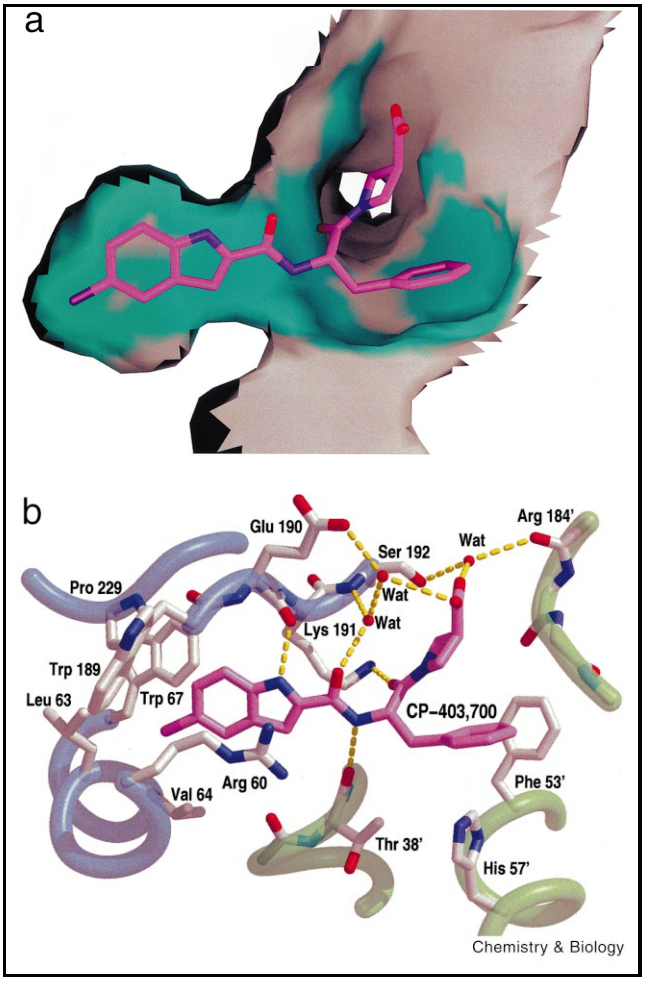


Figure 2. The binding site consists of two different protein environments. **(a)** Solvent accessible surface. On the left, the site is hydrophobic; on the right, the site is both hydrophobic and polar, and open to solvent at the top, bottom and into the page beyond the azetidine ring. The 2-fold symmetry operator relating the subunits runs vertically in the plane of the page. CP-403,700 (carbon, pink; nitrogen, blue; oxygen, red; chlorine, purple) shown with the solvent accessible surface area of the cavity (GRASP [23]) in gray, regions of van der Waals contact shown in turquoise. **(b)** Subunits colored blue and green as in Figure 1. Hydrogen bonds shown are 2.6–3.2 Å; water molecules, red spheres. Val40′, described in the text, is omitted for clarity.

equivalents of 5-chloroindole-2-carboxylic acid with different diamines, illustrated by CP-526,423 (Table 2). CP-526,423 inhibits HLGP*a* with an IC_{50} of 6 nM, consistent with productive, cooperative binding of both chloroindole groups in their respective pockets.

To verify this hypothesis, CP-526,423 was co-crystallized with HLGP*a* and the X-ray structure solved by molecular replacement to 2.2 Å resolution (Table 1). CP-526,423 is well ordered in the electron density map, demonstrating that a single molecule of CP-526,423 indeed spans the two inhibitor sites (Figure 3). After superposition of the Cα carbons of residues 30–830 of the respective phosphorylase monomers, the root mean square deviation (r.m.s.d.) of the chloroindole moieties of CP-403,700 and CP-526,423 is 0.34 Å.

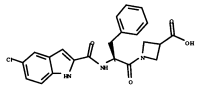
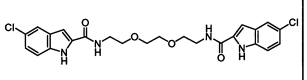
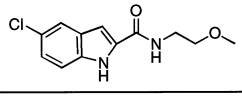
The increase in potency derived from joining two ligands together to give CP-526,423 is made apparent by comparing the potency of CP-305,494 (Table 2). CP-305,494 is expected to be able to make the same favorable interactions as CP-526,423, except through two molecules, rather than one, per HLGP homodimer. In effect, by linking two molecules of CP-305,494 to make CP-526,423, the IC_{50} is improved 2000-fold to 6 nM.

The solvent-filled gap between the two subunits of the phosphorylase dimer may be necessary to allow the two subunits to rotate (by 7°) with respect to each other when the enzyme is activated (Rath, V.L. et al., Molecular Cell, in press). We have shown that a new allosteric binding site exists within this gap for the chloroindole carboxamide class of glycogen phosphorylase inhibitor. A representative of this class reduces enzyme activity by stabilizing the inactive conformation of the enzyme, like other known allosteric inhibitors of phosphorylase. We have determined the atomic resolution details of the interactions between the compound and HLGP*a* which led to the discovery of a new class of simpler, more potent inhibitors. These crystal structures provide the foundation for further design of glycogen phosphorylase inhibitors for treating diabetes.

Significance

Non-insulin-dependent (type 2) diabetes mellitus affects

Table 2
Structures and IC_{50} values for inhibitors.

Inhibitor	Structure	IC_{50}, nM[a]
CP-403,700[b]		45
CP-526,423		6
CP-305,494		12,500

[a]Average of two experiments.
[b]Sodium salt.

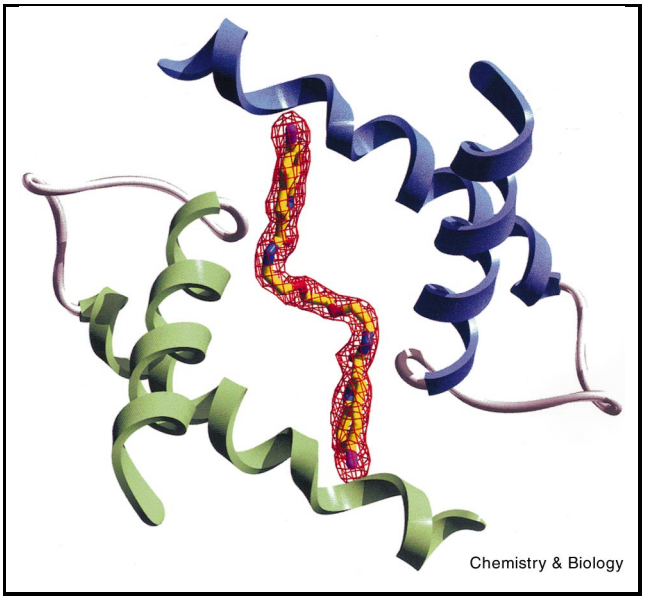


Figure 3. The structure of the HLGP*a* complexed with CP-526,423. CP-526,423 and its associated electron density (in orange, from a $2F_o-F_c$ map) are shown spanning the two chloroindole binding sites.

15 million people in the United States alone. The genetic basis of this chronic metabolic disorder is poorly understood but is characterized by defects in insulin secretion and insulin action, leading to serious complications such as nerve and kidney damage, blindness and heart disease. In type 2 diabetics, the liver produces excess glucose and is a major contributor to diabetic hyperglycemia. Recent data suggest that tight blood glucose control is critical to prevent or delay the onset of diabetic complications. The liver produces glucose by gluconeogenesis (de novo synthesis of glucose) and by glycogenolysis, the breakdown of glycogen by liver phosphorylase. Although diabetes is not caused by defects in liver phosphorylase, its inhibition may provide a means of controlling glucose levels in circulating blood, an effect which has been validated in an animal model of diabetes.

We present two unreported compounds which bind to a highly specific, novel site on glycogen phosphorylase, an enzyme which has been studied biochemically for over 60 years and crystallographically for 30 years. This is the first description of the diabetes target, human liver phosphorylase, complexed with an allosteric inhibitor. Compounds which bind to this site are the most potent inhibitors of liver phosphorylase identified to date and function, like other known inhibitors, by stabilizing the inactive conformation of the enzyme. Hence, these compounds are synergistic with active site inhibitors and promote dephosphorylation of the enzyme by the phosphatase, rendering it inactive. The site involves unique structural elements and reveals a new way the inactive conformation can be stabilized from a different locus on the protein. The details of the binding site have been exploited to design a second generation, higher affinity inhibitor which also demonstrates the concept of linking two ligands to create an exponentially more potent one.

Materials and methods

Figures were made using Midas Plus [17] except where noted.

Crystallization, data measurement, structure solution

Baculovirus-derived HLGP*a* was purified, crystallized with a 4-fold molar excess of CP-403,700 sodium salt, and frozen as described [12]. The synthesis of CP-403,700 has also been described [12]. Crystals of the complex with CP-526,423 (synthesis to be described elsewhere) were prepared in the same way. We were not able to obtain crystals with CP-403,700 alone under these conditions, however little effort was made to do so. Although we did obtain crystals of HLGP*a* complexed with the glucose analog alone, they were less reproducible and took much longer to grow. The best crystals (largest, fastest growing) were obtained when both CP-403,700 and the glucose analog, GlcNAc [18], were present. Data from crystals of both complexes were measured at beamline X12C of Brookhaven National Laboratories. The complex with CP-403,700 was solved by molecular replacement using HLGP*a*/AMP coordinates (Rath, V.L. et al., Molecular Cell, in press); CP-526,423 was solved using the CP-403,700 complex coordinates; solutions and refinement in X-*PLOR* [19] or CNS [20] using standard methods; models built in O [21]. Coordinates have been deposited (CP-403,700 complex, PDB entry 1EXV; CP-526,423 complex, PDB entry 1EMG).

Activity assays

HLGP*a* activity was measured as described [10], and the IC_{50} (concentration at which the enzyme activity is 50% inhibited) values are the average of duplicate determinations, performed in triplicate. Briefly, HLGP*a* (85 ng) activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate at 22°C in 100 μl of buffer containing 50 mM HEPES (pH 7.2), 100 mM KCl, 2.5 mM EGTA, 2.5 mM $MgCl_2$, 0.5 mM glucose-1-phosphate and 1 mg/ml glycogen. Phosphate was measured at 620 nm, 20 min after the addition of 150 μl of 1 M HCl containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green. Test compounds were added to the assay in 5 μl of 14% dimethyl sulfoxide (DMSO).

Rate of dephosphorylation

HLGP*b* was converted to HLGP*a* by reaction with [^{32}P]ATP and phosphorylase kinase [10]. [^{32}P]GP*a* was incubated with CP-403,700 or CP-526,423 (1 μM) or DMSO (control), in the presence or absence of 40 mM glucose. Protein phosphatase 1 (PP1) was added to initiate dephosphorylation, and at each time point, an aliquot removed for SDS–PAGE determination of the remaining [^{32}P]HLGP*a*. The HLGP bands were cut from dried gels and [^{32}P]HLGP*a* quantitated by scintillation counting (modification of [22]). Results expressed as % remaining [^{32}P]HLGP*a* relative to the 0 min (pre-PP1 addition) time point. The experiment was independently performed three times with results given as the mean of duplicate determinations from a representative experiment.

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