Inhibition of Fructose-1,6-bisphosphatase by a New Class of Allosteric Effectors

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Abstract
A highly constrained pseudo-tetrapeptide (OC252-324) further defines a new allosteric binding site located near the center of fructose-1,6-bisphosphatase. In a crystal structure, pairs of inhibitory molecules bind to opposite faces of the enzyme tetramer. Each ligand molecule is in contact with three of four subunits of the tetramer, hydrogen bonding with the side chain of Asp$^{187}$ and the backbone carbonyl of residue 71, and electrostatically interacting with the backbone carbonyl of residue 51. The ligated complex adopts a quaternary structure between the canonical R- and T-states of fructose-1,6-bisphosphatase, and yet a dynamic loop essential for catalysis (residues 52-72) is in a conformation identical to that of the T-state enzyme. Inhibition by the pseudo-tetrapeptide is cooperative (Hill coefficient of 2), synergistic with both AMP and fructose 2,6-bisphosphate, noncompetitive with respect to Mg$^{2+}$, and uncompetitive with respect to fructose 1,6-bisphosphate. The ligand dramatically lowers the concentration at which substrate inhibition dominates the kinetics of fructose-1,6-bisphosphatase. Elevated substrate concentrations employed in kinetic screens may have facilitated the discovery of this uncompetitive inhibitor. Moreover, the inhibitor could mimic an unknown natural effector of fructose-1,6-bisphosphatase, as it interacts strongly with a conserved residue of undetermined functional significance.

Keywords
Catalysis, Crystal structure, enzyme inhibition, gluconeogenesis, Fructose-Bisphosphatase, kinetics

Disciplines
Biochemistry | Chemistry | Molecular Biology

Comments
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A highly constrained pseudo-tetrapeptide (OC252–324) further defines a new allosteric binding site located near the center of fructose-1,6-bisphosphatase. In a crystal structure, pairs of inhibitory molecules bind to opposite faces of the enzyme tetramer. Each ligand molecule is in contact with three of four subunits of the tetramer, hydrogen bonding with the side chain of Asp187 and the backbone carbonyl of residue 71, and electrostatically interacting with the backbone carbonyl of residue 51. The ligated complex adopts a quaternary structure between the canonical R- and T-states of fructose-1,6-bisphosphatase, and yet a dynamic loop essential for catalysis (residues 52–72) is in a conformation identical to that of the T-state enzyme. Inhibition by the pseudo-tetrapeptide is cooperative (Hill coefficient of 2), synergistic with both AMP and fructose 2,6-bisphosphate, noncompetitive with respect to Mg²⁺, and uncompetitive with respect to fructose 1,6-bisphosphate. The ligand dramatically lowers the concentration at which substrate inhibition dominates the kinetics of fructose-1,6-bisphosphatase. Elevated substrate concentrations employed in kinetic screens may have facilitated the discovery of this competitive inhibitor. Moreover, the inhibitor could mimic an unknown natural effector of fructose-1,6-bisphosphatase, as it interacts strongly with a conserved residue of undetermined functional significance.

Fructose-1,6-bisphosphatase (α-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11; FBPase) catalyzes a tightly regulated step of gluconeogenesis, the hydrolysis of fructose 1,6-bisphosphate (F16P2) to fructose 6-phosphate (F6P) and P1 (1, 2). AMP and F26P2 (binding to allosteric and active sites, respectively) inhibit FBPase, while simultaneously activating its counterpart in glycolysis, fructose-6-phosphate 1-kinase (3, 4). Biosynthesis and degradation of F26P 2 is subject to hormonal control principally by glucagon and insulin (4, 5). F26P 2 enhances the binding of AMP to FBPase by up to an order of magnitude (6). Hence, although intracellular concentrations of AMP remain relatively constant, AMP becomes a more potent inhibitor of FBPase as concentrations of F26P 2 increase. AMP binds 28 Å away from the nearest active site and perhaps not surprisingly inhibits catalysis noncompetitively with respect to F16P 2. Yet AMP is a competitive inhibitor of catalysis with respect to essential divalent cations (Mg²⁺, Mn²⁺, or Zn²⁺), all of which are in proximity to (and probably coordinate with) the 1-phosphoryl group of F16P 2 (7–10).

FBPase is a homotetramer (subunit Mr of 37,000 (11)) and exists in at least two distinct quaternary conformations called R and T (12–14). AMP induces the transition from the active R-state to the inactive (or less active) T-state. Substrates or products in combination with metal cations stabilize the R-state conformation. A proposed mechanism for allosteric regulation of catalysis involves three conformational states of loop 52–72 called engaged, disengaged, and disordered (15). AMP alone or with F26P 2 stabilizes a disengaged loop (16, 17), whereas metals with products stabilize an engaged loop (10, 17–19). In active forms of the enzyme, loop 52–72 probably cycles between its engaged and disordered conformations (15, 18). Fluorescence from a tryptophan reporter group at position 57 is consistent with the conformational states for loop 52–72, observed in crystal structures (20, 21). Presumably, the engaged, disengaged, and disordered conformations of loop 52–72 are possible in both the R- and T-states of FBPase, but only the engaged and disordered conformers of the R-state, and the disengaged conformer of the T-state, have been reported in crystalline complexes (17, 18, 22, 23).

A recent report (24) in the literature identifies a new inhibitor site on FBPase, distinct from the active and the AMP-binding sites. The new family of anilinoquinazolinone inhibitors was discovered by a search for potential drugs in the treatment of non-insulin-dependent diabetes. Although the site of binding on FBPase is clear, the kinetic mechanism of inhibition for this new class of inhibitor was not reported. Independent of the efforts above, a screen for new inhibitors of FBPase resulted in the discovery of a chemically distinct molecule (OC252–324,
Fig. 1. Covalent structure of OC252. Atoms C-8, C-10, C-12, and C-14 correspond to Co atoms of residues labeled 1–4. Atoms C-8, C-10, and C-12 adopt the L-configuration of a common amino acid. Residues 1 and 4 have tyrosyl side chains; residue 2 has a phenylalanine side chain bridged by a methylene group to its amide nitrogen atom (N-1), and residue 3 has an n-propyl side chain. In its crystalline complex, a close contact between atoms N-3 and O-3 infers a strong intramolecular bridge by a methylene group to its amide nitrogen atom (N-1), and atom N-2 through enhanced electrostatic interactions.

hereafter OC252, Fig. 1) that targets the same binding site on FBPase. Inhibition of FBPase by OC252 is cooperative (Hill coefficient of 2), synergistic with AMP and F26P2, noncompetitive with respect to Mg2+, but uncompetitive with respect to F16P2. OC252 greatly decreases the concentration at which substrate inhibition dominates the kinetics of FBPase. The crystal structure reveals a pair of OC252 molecules bound to each face of an FBPase tetramer. The quaternary conformation of the tetramer differs from the canonical R- and T-states, observed in the absence and presence of AMP, respectively, yet the loop (residues 52–72) is in its disengaged conformation. A strong hydrogen bond between OC252 and the side chain of a conserved aspartate residue of undetermined functional significance suggests the possibility of a binding site recognized by an unknown natural effector.

EXPERIMENTAL PROCEDURES

Materials—F16P2, F26P2, NADP+, and AMP were purchased from Sigma. Glucose-6-phosphate dehydrogenase and phosphoglucose isomerase came from Roche Applied Science. Other chemicals were of reagent grade or the equivalent. QSW-HR high pressure liquid chromatography resin came from Tosoh-Hass Bioseparations. FBPase-deficient Escherichia coli strain DF657 came from the Genetic Stock Center at Yale University. Plasmids used in the expression of wild-type FBPase came from a previous investigation (20). The inhibitor OC252 was provided by Ontogen Corp. (Carlsbad, CA).

Expression and Purification of Wild-type FBPase—Separate preparations of enzyme were used for the structural and kinetics investigations. Recombinant FBPase was expressed in a strain of E. coli deficient in endogenous FBPase and then purified to homogeneity. Cell-free extracts of the wild-type FBPases were subjected to heat treatment (65 °C for 5 min), followed by centrifugation. For enzyme used in kinetics investigations, the supernatant solution was loaded onto a Cibacron Blue-Sepharose column, previously equilibrated with 20 mM Tris-HCl, pH 7.5. For crystallographic experiments, after volume reduction by pressure concentration through an Amicon PM-30 membrane, the supernatant solution from centrifugation was passed through a CM-Sepharose column using a NaCl gradient (0–3 M). The purified enzyme was dialyzed extensively against 20 mM Tris-HCl, pH 7.5. For crystallization experiments, after volume reduction by pressure concentration through an Amicon PM-30 membrane, the supernatant solution from centrifugation was passed through a DEAE-Sepharose column and then eluted with a NaCl gradient (0–0.3 M). The purified enzyme was dialyzed extensively against 20 mM Tris-HCl, pH 7.5. For crystallization experiments, after volume reduction by pressure concentration through an Amicon PM-30 membrane, the supernatant solution from centrifugation was passed through a CM-Sepharose column using a NaCl gradient (20–400 mM) in 10 mM Tris malonate, pH 6.0, and then dialyzed against KP (20 mM, pH 7.0). Purity and protein concentrations of FBPase preparations were confirmed by SDS-PAGE (25) and the Bradford assay (26), respectively.

Kinetic Experiments—Assays for the determination of specific activity, kcat, and activity ratios at pH 7.5 and 9.5 employed the coupling enzymes, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase (1). The reduction of NADP+ to NADPH was monitored by absorbance spectroscopy at 340 nm. All other assays used the same coupling enzymes, but monitored NADPH production by its fluorescence emission at 470 nm, using an excitation wavelength of 340 nm. Kinetic assays were performed at room temperature (22 °C). Data fitting and analysis used the program DYNAPFF (27).

Crystallization of the Product Complex—Crystals of FBPase grew by the method of hanging drops. Equal parts of a protein solution (FBPase (10 mg/ml), KP, pH 7.4 (10 mM), MgCl2 (5 mM), FSP (5 mM), and OC252 (2 mM)) and a precipitant solution (Tris malonate, pH 7.4 (2.5 mM), polyethylene glycol 3350 (6% w/v)) were combined in a droplet of 4 μl volume. Wells contained 500 μl of the precipitant solution. Crystals of dimensions 0.4 × 0.4 × 0.3 mm grew in approximately 3 days at 20 °C. OC252 is relatively insoluble in aqueous solutions. Approximately 10 μg of pure inhibitor was dissolved initially in acetone, and appropriately prepared aliquots were distributed to empty vials. The acetone was removed by evaporation, and the protein solution above was added. The inhibitor dissolved after a brief time interval to provide the clear protein solution used in the crystallization experiments.

Data Collection—Data were collected at Iowa State University on a rotating anode/Siemens area detector at 120 K, using CuKα radiation passed through a graphite monochromator. Data were reduced by XENGEN (28).

Structure Determination, Model Building, and Refinement—Crystals grown for the present study are isomorphous to the AMP-Zn2+ product complex (17). Phase angles, used in the generation of initial electron density maps, were based on model 1EYJ of the Protein Data Bank, from which water molecules, metal cations, small molecule ligands, and residues 52–72 had been omitted. Residues 52–72 were built into the electron density of omit maps, with reference to the Ca coordinates of loop 52–72 from the AMP complex (17), using the program XTALVIEW.
In the absence of inhibitor and substrate, all enzyme is in an $E(Mg^{2+})_2$ complex (conditions of saturating $Mg^{2+}$). Each model assumes a Hill coefficient of 2 for OC252 inhibition and rapid equilibrium kinetics, in which the conversion of $E(Mg^{2+})_2$ to $E(Mg^{2+})_2F6P$ is rate-limiting. The Michaelis equilibrium, $E(Mg^{2+})_2 + F16P_2 \rightleftharpoons E(Mg^{2+})_2F16P_2$, is common to all mechanisms, and is not included. M, S, and I below represent $Mg^{2+}$, $F16P_2$, and OC252, respectively. RMSSD is the root mean squared deviation between observed velocities and those calculated from the model.

<table>
<thead>
<tr>
<th>Model</th>
<th>Equilibria &amp; inhibitor constants</th>
<th>Inhibition type</th>
<th>RMSSD of fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Competitive</td>
<td>2.609</td>
</tr>
<tr>
<td>B</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Competitive with induced substrate inhibition</td>
<td>0.235</td>
</tr>
<tr>
<td>C</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Noncompetitive</td>
<td>0.686</td>
</tr>
<tr>
<td>D</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Noncompetitive with induced substrate inhibition</td>
<td>0.470</td>
</tr>
<tr>
<td>E</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Mixed</td>
<td>0.235</td>
</tr>
<tr>
<td>F</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Mixed with induced substrate inhibition</td>
<td>0.426</td>
</tr>
<tr>
<td>G</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Uncompetitive</td>
<td>0.234</td>
</tr>
<tr>
<td>H</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Uncompetitive with induced substrate inhibition</td>
<td>0.426</td>
</tr>
<tr>
<td>I</td>
<td>$E(Mg)_2 + 2I \rightleftharpoons E(Mg)_2I_2; K_I$</td>
<td>Uncompetitive with induced partial substrate inhibition</td>
<td>0.266</td>
</tr>
</tbody>
</table>

$$V/V_m = I_{0.5}/(I_{0.5} - I)$$ (Eq. 1)

where $n$ is the Hill coefficient, $V_m$ the initial velocity in the absence of inhibitor, $V_i$ the initial velocity at a specific inhibitor concentration, $I$ the concentration of OC252, and $I_{0.5}$ the concentration of OC252 that causes 50% inhibition.

Kinetics data were taken over broad concentration ranges of $Mg^{2+}$ (0.2 – 5 mM), $F16P_2$ (1 – 20 $\mu$M), and OC252 (0 – 20 $\mu$M) in order to determine the kinetic mechanism of inhibition. Plots of reciprocal velocity against $1/[Mg^{2+}]^2$ ($F16P_2$ saturating, but below concentrations that cause significant substrate inhibition) and $1/[F16P_2]$ ($Mg^{2+}$ saturating) indicate noncompetitive inhibition with respect to $Mg^{2+}$ and uncompetitive inhibition with respect to $F16P_2$ (Fig. 3). As is evident from Fig. 3A, substrate inhibition increases significantly with rising inhibitor concentration. Data from Fig. 3A were fit to nine different models.

**RESULTS**

Expression and Purification of Wild-type FBPase—Expression and isolation procedures described above provide FBPase in at least 95% purity, as judged by SDS-PAGE (data not shown). The $K_{eq}$ value (22 ± 1 s$^{-1}$) and the ratio of specific activities at pH 7.5 to 9.5 (3.3) indicate high purity and little or no proteolysis of the purified enzyme, consistent with the results from electrophoresis.

Kinetics Experiments—By using fixed concentrations of $MgCl_2$ (0.5 mM, approximately the $K_m$ for $Mg^{2+}$) and $F16P_2$ (20 $\mu$M, saturating), initial velocity varies as the inverse square of OC252 concentration (Hill coefficient of 1.97 ± 0.1) with an $I_{0.5}$ (concentration of OC252 that causes 50% inhibition) of 1.87 ± 0.07 $\mu$M (Fig. 2). $F26P_2$, AMP, and OC252 inhibit FBPase synergistically; $I_{0.5}$ values for OC252 are 0.63 ± 0.04 $\mu$M (Hill coefficient, 1.6 ± 0.1) in the presence of 1.5 $\mu$M AMP and 0.48 ± 0.01 $\mu$M (Hill coefficient, 1.7 ± 0.1) in the presence of 0.45 $\mu$M $F26P_2$. The numerical values above result from a fit of data to Equation 1, V/V_m = I_{0.5}/(I_{0.5} - I) (Eq. 1)
kinetic models using the program DYNAFIT (27), combining mechanisms of competitive, noncompetitive, and uncompetitive inhibition by OC252, with and without pathways for inhibitor-induced substrate inhibition or partial inhibitor-induced substrate inhibition (Table I). (Inhibitor-induced substrate inhibition requires the binding of OC252 prior to the binding of the inhibitory F16P2 molecule. Inhibitor-induced partial substrate inhibition allows turnover of the substrate-inhibited enzyme at a reduced maximal velocity.) All successful kinetic models include the association of two molecules of (inhibitor-induced partial substrate inhibition) are not.

From Scheme I we derived the following relationship (Equation 2) assuming rapid-equilibrium kinetics under initial velocity conditions,

\[
1/V = (1/V_m)(1 + K_iA^2 + K_i/B + (K_{iis}K_i)/(A^2B) + (K_{iis}F_6P^2)/(A^2A^2))
\]

where A, B, and I are concentrations of free Mg\(^{2+}\), F16P\(_2\), and OC252, respectively; \(V_m\) is the maximal velocity, and \(K_{iis}\), \(K_i\), \(K_{iir}\), and \(K_{iia}\) are dissociation constants for two Mg\(^{2+}\) atoms from the E(\(\text{Mg}^{2+}\))\(_2\)F16P\(_2\) complex, for F16P\(_2\) from the E(\(\text{Mg}^{2+}\))\(_2\)F16P\(_2\) complex, for two OC252 molecules from the.

**TABLE II**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Numerical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_m)</td>
<td>18.4 ± 0.3 Å/s</td>
</tr>
<tr>
<td>(K_a)</td>
<td>2.16 ± 0.08 mM</td>
</tr>
<tr>
<td>(K_i)</td>
<td>25 ± 2 mM</td>
</tr>
<tr>
<td>(K_{iis})</td>
<td>7.6 ± 1 mM</td>
</tr>
<tr>
<td>(V_m)</td>
<td>17.1 ± 0.8 Å/s</td>
</tr>
<tr>
<td>(K_a)</td>
<td>0.38 ± 0.02 mM</td>
</tr>
<tr>
<td>(K_i)</td>
<td>3.65 ± 0.6 mM</td>
</tr>
</tbody>
</table>

**Equation 3**

\[
E(\text{Mg}^{2+})_2 \rightarrow E(\text{Mg}^{2+})_2\cdot F16P_2 \rightarrow E(\text{Mg}^{2+})_2\cdot F16P_2\cdot (OC252)_2 \rightarrow \text{Products}
\]

**Scheme I**

Kinetic parameters determined by fits of Equations 3 and 4 to the data of Fig. 3. Maximum velocity is expressed in terms of the change in fluorescence (units not defined) with time.
weak or absent electron density include residues 1–8 and 63–70. The model begins at residue 9 and continues to the last residue of the sequence, but segment 63–70 is unreliable, as evidenced by high thermal parameters. Thermal parameters vary from 5 to 57 Å². As determined by PROCHECK (32), the model has stereochemistry generally comparable with that of structures derived from data of nominal resolution 2.0 Å. Statistics for data collection and refinement are in Table III, and an overview of the complex appears in Fig. 4.

Although the two subunits of the OC252 complex are similar, the ligation of each active site by metals differs. In the subunit labeled chain B, strong electron density is at metal site 1. The thermal parameter of Mg²⁺ at full occupancy here refines to a value of 10 Å², which is significantly less than the average value (20 Å²) for atoms of the protein ligated to that metal. Hence Mg²⁺ and a heavier metal (Zn²⁺, on the basis of prior experience (19)) probably co-occupy site 1 in chain B. Site 2 of chain B has diffuse electron density that may represent disordered water molecules or the combination of water molecules and Mg²⁺ at low occupancy; Mg²⁺ at full occupancy refined at metal site 2 with a thermal parameter of 32 Å². In the subunit labeled chain A, Mg²⁺ refines with thermal parameters of 18 and 29 Å² at sites 1 and 2, respectively. Metal site 2 in chain A may be occupied partially by Mg²⁺. The conformations of side chains near metal site 2 also differ in each of the symmetry unique subunits of the crystal. In addition, molecules of P_i are at partial occupancy at each active site, but molecules of F6P are at full occupancy.

OC252 is arguably a highly constrained pseudo-tetrapeptide (Fig. 1); the first and fourth “residues” have tyrosyl side chains, a methylene group in residue 2 covalently bridges the phenylalanyl side chain to its amide nitrogen, and an n-propyl group is the side chain of residue 3. The N-2 atom of OC252 corresponds to the backbone nitrogen atom of the first and third residues, and the terminal carboxyl group is absent. Atoms C-8, C-10, and C-12 have the L-configuration of a common amino acid.
acid. In their FBPase complex, two molecules of OC252 are in mutual contact, related to each other by the molecular 2-fold axis that projects through the face of the FBPase tetramer. Most of the contacts between inhibitor and protein are apolar; however, the phenol oxygen atoms of residues 1 and 4 of OC252 hydrogen-bond with backbone carbonyl 71 and the side chain of Asp51, respectively. Furthermore, the C-O bond axis of backbone carbonyl 51 is normal to the plane defined by atoms C-8, N-2, C-11, and C-12 of OC252, with its oxygen atom 3.2 Å from atom N-2. The latter suggests an electronic resonance state of the site of the FBPase molecule that stabilizes this close contact (Fig. 1).

Although the OC252 and AMP crystalline complexes are isomorphous, the two FBPase tetramers adopt different quaternary conformations. Superpositions of C1–C2 subunit pairs from the OC252, T-state, and R-state complexes give comparable root mean squared deviations (Table IV). (See Fig. 4 for the convention adopted in the labeling of subunits.) On the other hand, superpositions of tetramers give significantly larger deviations, suggesting different quaternary structures. Indeed, the C3–C4 subunit pair in the OC252 complex rotates ~14° relative to the C1–C2 subunit pair, falling some 3° short of the canonical T-state (Fig. 5). Hereafter, we refer to the quaternary state of FBPase induced by OC252 as the I-state.

The I-state must be due to the specific effects of the OC252 inhibitor. First, the conditions of crystallization for the T-, R-, and I-states of FBPase differ only by the presence or absence of allosteric effectors. The T-state crystallizes in the presence of AMP (17), the I-state in the presence of OC252, and the R-state in the absence of allosteric effectors (10, 17). Hence, ligation of FBPase by OC252 favors neither the T- nor R-state. OC252, however, stabilizes the disengaged conformation of loop 52–72, which prior to this work has been seen only in AMP-ligated complexes. Moreover, rotation of the side chain of His55, about which prior to this work has been seen only in AMP-ligated complexes, in a possible electrostatic contact with the oxygen atom of the substrate, is unusual but not unprecedented. The mutation of Arg49 to cysteine changes the kinetic mechanism of FBPase with respect to Mg2+, whereas the phenylthiazole moiety overlaps residue 3 of the other OC252 molecule. No part of the anilinoquinazoline molecule corresponds to residue 4 of OC252, the side chain of which hydrogen-bonds Asp187. In fact, the phenylthiazole moiety, through stacking interactions, positions the side chain of His55 over the pocket occupied by the tyrosyl side chain of OC252 residue 4. The anilinoquinazoline is roughly crescent-shaped; the ends of the crescent contact the surface of the FBPase tetramer with the curve of the crescent projecting outward. Residues 3 (n-propyl side chain) and 4 (tyrosyl side chain) of OC252, on the other hand, penetrate the interior of the tetramer. Hence, the number of contacts between OC252 and FBPase greatly exceeds that of the anilinoquinazoline. Each OC252 molecule interacts with residues from three of four subunits of FBPase, whereas each anilinoquinazoline molecule interacts with residues from a pair of subunits (Fig. 7).

Curiously, despite all the differences above, both inhibitor types foster stabilizing interactions with backbone carbonyl 51 of FBPase. For the anilinoquinazoline, the hydroxyl group of the phenylthiazole moiety hydrogen-bonds directly with backbone carbonyl 51, whereas atom N-2 of the OC252 molecule is in a possible electrostatic contact with the oxygen atom of the backbone carbonyl 51.

Inhibitors of wild-type FBPases are either competitive (such as F2P6P2) or noncompetitive (such as AMP) with respect to F16P2. Uncompetitive inhibition of FBPase by OC252 with respect to the substrate is unusual but not unprecedented. The mutation of Arg49 to cysteine changes the kinetic mechanism of AMP inhibition with respect to F16P2, from noncompetitive to uncompetitive, but retains the competitive mechanism of AMP inhibition with respect to Mg2+ (33). Evidently, AMP best inhibits Cys49 FBPase at low concentrations of Mg2+ and at high concentrations of F16P2. The side chain of Arg49 partici-
Cys49 FBPase in the R state. AMP may bind to the T state relative to the R state. (and the superimposed OC252 complex (heavy lines)).

The quaternary state of FBPase stabilized by OC252 (the I-state) may be similar to this hypothetical F16P2-stabilized state of Cys49 FBPase. First, uncompetitive inhibition by OC252 with respect to F16P2 reflects binding synergism; ligation of the active site by F16P2 enhances the affinity of OC252 for its binding site (see Scheme I). Moreover, the binding of OC252 induces F16P2 inhibition (Fig. 3B). Binding synergism between OC252 and F16P2 would occur if each ligand were to bind at distinct sites and stabilize a common conformational state of FBPase. Hence, the I-state observed in the OC252 complex may be similar to the quaternary state of FBPase in the presence of inhibitory concentrations of substrate. Ligation of FBPase by OC252 excludes the engaged conformation of loop 52–72, which is presumably required for catalysis. The I-state itself may disfavor the engaged conformation of loop 52–72, thereby accounting for the phenomenon of substrate inhibition of FBPase at concentrations of F16P2 in excess of 30 μM.

The I-state may also be the basis for synergistic inhibition of FBPase by OC252 and F26P2. Elevated levels of F26P2 (200 μM) cause a transition from the loop-engaged R-state to a conformation similar but not identical to the T-state, as measured by fluorescence from a Trp57 reporter group (20). Perhaps high concentrations of F26P2 stabilize the I-state, and indeed the tetramer does adopt the I-state in a preliminary crystal structure of an F26P2-FBPase complex. As OC252 and F26P2 stabilize the same quaternary conformation by interactions at topologically distinct sites, they must exhibit functional synergism in rapid equilibrium kinetics.

An explanation of the synergism between AMP and OC252 is an only slightly more complex. OC252 clearly excludes the engaged conformation of loop 52–72 and evidently stabilizes the disengaged loop present in the AMP complex of FBPase. Moreover, the I-state probably lies on or near the free energy pathway connecting the R- and T-states. So by stabilizing the I-state, OC252 does much of the work that AMP normally accomplishes in driving the R- to T-state transition.

But is the I-state of FBPase an appropriate target for the development of new drugs? Estimates of the free concentrations of F16P2 in liver (1 μM) are well below those that cause substrate inhibition (30 μM and above) of FBPase. Similarly, concentrations of F26P2 (200 μM) and products (250 μM) that stabilize the I-state in fluorescence experiments are far in excess of those in vivo. On the other hand, AMP and F26P2 are the principal metabolic effectors of FBPase. Given that OC252 inhibits FBPase synergistically with AMP and F26P2 at physiological concentrations of substrate, the central allosteric

\[ J.-Y. \) Choe and R. B. Honzatko, unpublished results. \]
These conditions exclude inhibitors that compete with F16P2 and bias the screen in favor of ligands that inhibit FBPase only in the presence of bound F16P2. Although this strategy has led to the discovery of molecules that do not bind to the active site, it has the disadvantage of selecting ligands that require high concentrations of F16P2 for potent inhibition. For instance, I0.5 is low (∼6 μM) for OC252 in the presence of 100 μM F16P2 but rises to 70 μM at a concentration of 2 μM F16P2. Hence, an initial screen using high concentrations of F16P2 may identify new leads that target the central allosteric site of FBPase, but further kinetic analysis at low concentrations of F16P2 may be more appropriate in optimizing the lead compound as a potent inhibitor in vivo.

The above also assumes that no consequence beyond inhibition will result from the ligation of the central allosteric pocket of FBPase. Such an assumption, however, may be unwarranted. Available sequences of FBPase reveal Asp187 as an invariant residue. The mutation of Asp187 to alanine has only a modest effect on the functional properties of FBPase (36). Its mutation to phenylalanine has no greater effect on FBPase function than that due to the alanine mutation. What selective pressure then resists aspartate at position 187 and is it just coincidence that OC252 hydrogen-bonds to this side chain? The conservation of Asp187 suggests a role in the recognition of a natural effector. Several reports (37–40) are in the literature, for instance, regarding interactions between FBPase and aldolase. The C-terminal residues of aldolase are putatively essential to these interactions, and the consensus sequences for both type A and B isozymes of aldolase end in tyrosine, which matches residue 4 of OC252. Hence, a potential drug may have to compete with a natural effector for the central allosteric pocket, or possibly FBPase itself may act as an effector in some other physiological process. Further clarification of these issues may be critical to the success of efforts to develop drugs that effectively target FBPase.

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