Fructose-1,6-bisphosphatase is a square planar tetramer of identical subunits, which exhibits cooperative allosteric inhibition of catalysis by AMP. Protocols for in vitro subunit exchange provide three of five possible hybrid tetramers of fructose-1,6-bisphosphatase in high purity. The two hybrid types with different subunits in the top and bottom halves of the tetramer co-purify. Hybrid tetramers, formed from subunits unable to bind AMP and subunits with wild-type properties, differ from the wild-type enzyme only in regard to their properties of AMP inhibition. Hybrid tetramers exhibit cooperative, potent, and complete (100%) AMP inhibition if at least one functional AMP binding site exists in the top and bottom halves of the tetramer. Furthermore, titrations of hybrid tetramers with AMP, monitored by a tryptophan reporter group, reveal cooperativity and fluorescence changes consistent with an R- to T-state transition, provided that again at least one functional AMP site exists in the top and bottom halves of the tetramer. In contrast, hybrid tetramers, which have functional AMP binding sites in only one half (top/bottom), exhibit an R- to T-state transition and complete AMP inhibition, but without cooperativity. Evidently, two pathways of allosteric inhibition of fructose-1,6-bisphosphatase are possible, only one of which is cooperative.
Fructose-1,6-bisphosphatase Cooperativity

**EXPERIMENTAL PROCEDURES**

**Materials**—F6P, F26P2, NADP+, and AMP were purchased from Sigma. Glucose-6-phosphate dehydrogenase and phosphoglucone isomerase came from Roche Molecular Biochemicals. Other chemicals were of Sigma. Glucose-6-phosphate dehydrogenase and phosphoglucose isomerase came from the Genetic Stock Center at Yale University. Plasmids 3F16P2, F26P2, NADP+ were purchased from Amersham. The letters p, q, and r refer to 2:2 hybrids with like subunits.

**Mutagenesis of Wild-Type FBPase**—Mutations were accomplished by the QuikChange site-directed mutagenesis kit (Stratagene). The following FBPases were expressed in a strain of *E. coli* deficient against 20 mM Tris-HCl, but FBPases with impaired AMP binding (constructs ii–vi) were dialyzed against first against 1 mM MglCl, followed by 20 mM Tris-HCl. The latter procedure removed F16P2 introduced during phosphocellulose chromatography. Protein purity and concentration was confirmed by SDS-polyacrylamide gel electrophoresis (30) and the Bradford assay (31), respectively.

**Formation and Purification of Hybrid Enzymes**—Hybridization protocols developed here employed only pure FBPases. Appropriate enzymes were combined overnight at 4 °C, after which the mixture was loaded onto a QSW-HR HPLC column, previously equilibrated with 20 mM Tris-HCl, pH 7.5. Individual hybrids were eluted with 20 mM Tris-HCl, pH 7.5, and a gradient from 0 to 0.3 M in NaCl. The nomenclature of 2:2 hybrids used here is that of Cornish-Bowden and Koshland (29). The letters p, q, and r refer to 2:2 hybrids with like subunits at positions C1/C2, C1/C4, and C1/C3 of the tetramer, respectively (Fig. 2). Differences in electrostatic charge of various tetramers due to different numbers of polyglutamyl tags permitted the partial assignment of peaks in the elution profile to specific hybrids that formed from protocol A of Fig. 2. Retention times should increase with the number of Glu-tagged subunits in a tetramer. Indeed, the retention times of pure wild-type homotetramer and pure Glu-tagged homotetramer correspond to those of the first and last peaks eluted from hybrid mixtures. The second and penultimate peaks were assigned to 3:1 hybrids (3 subunits wild-type to 1 subunit Glu-tagged) and to 1:3 hybrids (1 subunit wild-type to 3 subunits Glu-tagged), respectively. The three possible 2:2 hybrids resolved into two peaks. Assignments of hybrid type to these peaks entailed hybridization protocols B and C of Fig. 2. In the presence of F16P2, subunit exchange occurs only at the level of C1/C2 dimers (28). Hence, protocol B leads to hybrids 4:0, 0,4, and 2:2p. The corresponding elution profile has but three peaks, the assignment of two of which (hybrids 4:0 and 0:4) is unambiguous. The central peak of the elution profile of protocol B, which must be the 2:2p hybrid, corresponds in retention time to one of two 2:2 hybrid peaks from the elution profile of protocol A. Using a similar process, the corresponding peak eluted to a mixture of 2:2q and 2:2r hybrids. The corresponding elution profile again has three peaks, with the third peak being unambiguously due to the mixture of 2:2q/2:2r hybrids. The retention time of the 2:2q/2:2r hybrids from protocol C corresponds to the other 2:2 hybrid peak of protocol A. To arrest subunit exchange in purified hybrid enzymes, the hybrids were kept at room temperature (which slows subunit exchange) but monitored NADPH production by its fluorescence emission at 470 nm, using an excitation wavelength of 340 nm. All assays were performed at room temperature (22 °C). Initial kinetic rates were analyzed using programs written either in the MINITAB language, using an α value of 2.0 (33), or by the ENZFITTER program (34).

**Circular Dichroism Spectroscopy**—Circular dichroism spectra were recorded for wild-type and mutant FBPases at room temperature on a Jasco J710 CD spectrometer in a 1-cm cell, using a protein concentration of 0.35 mg/ml. Spectra were collected from 200 to 260 nm in increments of 1.5 nm. The spectra analyzed were an average of three independent scans. Each spectrum was blank-corrected using the software package provided with the instrument.

**Kinetic Experiments**—Assays for the determination of specific activity, kcat, and activity ratios at pH 7.5 and 9.5 employed the coupling enzymes, phosphoglucone 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). Initial kinetic rates were analyzed using programs written either in the MINITAB language, using an α value of 2.0 (33), or by the ENZFITTER program (34).

**Steady-state Fluorescence Measurements**—Fluorescence data were collected using an SLM 8100C Spectrofluorometer from Spectronic Instruments. The single tryptophan at position 57 was excited selectively using a wavelength of 285 nm. Fluorescence emission spectra were recorded in steps of 1 nm from 310 to 400 nm with a slit width of 2 nm.

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for both excitation and emission. The spectra presented are the average of three scans. Enzyme concentrations ranged from 0.2 to 0.3 mg/ml. As a control, the highest concentration of AMP used here caused no change in fluorescence emission from a solution of tryptophan (100 μM) or from the AMP/Trp57 homotetramer.

AMP titration data were analyzed by nonlinear least squares fits, using the following equation,

\[ \frac{\Delta F}{F_0} = \frac{(\Delta F_{\text{max}}/F_0) \times L^n}{K_d + L} \]  
(Eq. 1)

where \( \Delta F_{\text{max}} \) is the change in fluorescence caused upon addition of ligand \( L \), \( F_0 \) is the fluorescence in the absence of ligand, \( K_d \) is the dissociation constant, and \( n \) is the Hill coefficient.

**RESULTS**

Expression and Purification of Wild-type and Mutant Homotetramers—Expression and isolation procedures described above provided homotetramers of FBPase at least 95% pure by SDS-polyacrylamide gel electrophoresis (data not shown). AMP-impaired FBPases (constructs ii–vi described above) did not elute from the Cibacron blue column, using AMP concentrations up to 20 mM. A gradient in NaCl successfully eluted these enzymes, but an additional purification step (phosphocellulose chromatography) was necessary to achieve at least 95% purity.

Formation and Purification of Hybrid Tetramers—Subunit exchange between Glu-tagged (9-residue polyglutamyl extension to the C terminus) and AMP\(^-\) homotetramers, and between Glu-tagged and AMP\(^-\)/Trp57 homotetramers, occurs at rates comparable to that of Glu-tagged and wild-type homotetramers. By the strategy presented under “Experimental Procedures,” peaks with retention times of ~43 and 52 min (Fig. 2) correspond to a mixture of 2:2q and 2:2r hybrids and to a pure 2:2p hybrid, respectively (Fig. 3). The kinetic properties of the 2:2p hybrid created by protocol A or B (Fig. 2) are quantitatively identical. Over several preparations, the 2:2q/2:2r hybrid mixtures from protocols A and C differ reproducibly but not to a statistically significant extent. The 2:2q/2:2r hybrid mixture from protocol A has a lower Hill coefficient for AMP (1.4 ± 0.1) and a higher IC\(_{50}\) (11.3 ± 0.8) than that exhibited by the 2:2q/2:2r hybrid mixture from protocol C (Table I). Because the relative amounts of Glu-tagged and AMP\(^-\) subunits are different in protocols A and C, and because protocol C employs F16P2, whereas protocol A does not, the relative amounts of 2:2q and 2:2r need not be the same for each protocol.

Secondary Structure Analysis—The CD spectra of all homotetramers and hybrids are identical from 200 to 260 nm (data not shown), indicating little or no effect on the secondary structure of FBPase due to mutations and hybridization protocols.

Kinetics Experiments—Kinetic parameters of wild-type, Glu-tagged, AMP\(^-\), and Tyr\(^{57} \rightarrow \text{Trp} \) homotetramers, as well as...
specific hybrids from subunit exchange between Glu-tagged and AMP\(^-\) homotetramers are given in Table I. The Glu-tag does not perturb functional properties as evidenced by the nearly identical kinetic parameters for the wild-type and Glu-tagged homotetramers. The AMP\(^-\) homotetramer exhibits nearly a 12,000-fold decrease in AMP inhibition and some loss of AMP cooperativity with no change in any other kinetic property. The mechanism of AMP inhibition for the AMP\(^-\) homotetramer is uncertain. AMP may bind to the active site, and/or Na\(^+\) introduced along with the AMP may displace Mg\(^{2+}\) from the active site. Hence, the observed cooperativity (Hill coefficient of 1.4; Table I) for this low affinity interaction probably arises from a mechanism entirely different from that of the high affinity AMP inhibition. Other homotetramers, not fully characterized kinetically, revealed IC\(_{50}\) values for AMP of 4, 6, and 3 mM for Tyr\(^{113}\) → Phe, Tyr\(^{113}\) → Phe/Lys\(^{112}\) → Met, and Gly\(^{36}\) → Ile/Thr\(^{27}\) → Ile FBPases, respectively. The later double mutation confirms speculation regarding the loss of AMP recognition by chloroplast FBPGases (35). The Gly\(^{36}\) → Ile/Thr\(^{27}\) → Ile mutations create a chloroplast “AMP” pocket within the context of the porcine liver enzyme and a concomitant rise (3000-fold) of IC\(_{50}\) relative to that of the wild-type enzyme. The AMP\(^-\) homotetramer was used in subsequent subunit exchange experiments, because it has the highest IC\(_{50}\) for AMP while preserving all other kinetic properties of the wild-type enzyme.

Experiments in kinetics were performed immediately following the purification of hybrid enzymes. Hybrid tetramers were stable for several hours at 25°C (but not at 4°C). All the kinetic parameters of the hybrids were essentially identical to those of the wild-type enzyme, except those for AMP inhibition (Table I).

**Inhibition of Hybrid Tetramers by AMP—IC\(_{50}\) for the 2:2p hybrid is ~40 \(\mu\)M with a Hill coefficient of 1.0. Inhibition is complete upon saturation of the two high affinity AMP binding sites (Figs. 4 and 5). In contrast, AMP inhibition of the 2:2q/2:2r hybrid mixture is cooperative and 100% at saturation. The 2:2q/2:2r hybrid mixture has an IC\(_{50}\) of 6.1 \(\mu\)M with a Hill coefficient for AMP of 1.5. The hybrid enzyme with three functional AMP binding sites and a single AMP-impaired binding site (1:3 hybrid, Fig. 2) responds to AMP much like the 2:2q/2:2r hybrid mixture with a Hill coefficient of 1.6 and an IC\(_{50}\) for AMP of 6.8 \(\mu\)M.

**Steady-state Fluorescence—AMP-induced changes in the fluorescence of hybrid FBPGases are due to conformational changes in loop 52–72 of subunits, which do not possess functional AMP binding sites. Hence, the reporter groups sense conformational change due to AMP ligation of a neighboring subunit. This conformational change is most likely the transition from R- to T-state FBPGase.

Fluorescence spectra of Trp\(^{57}\) FBPGase (Fig. 6) are consistent with three conformational states of loop 52–72, observed in previous work (17, 18). All hybrids exhibit identical fluorescence spectra in the presence of products and divalent cations (R-state, loop engaged), and in the absence of all ligands (R-state, loop disordered). Hybrids 0:4, 1:3, 2:2p, and 2:2q/2:2r are sensitive to AMP, and at saturating levels of AMP attain nearly equivalent fluorescence spectra. In contrast, the fluorescence spectrum of AMP\(^-\)/Trp\(^{57}\) hybrids 3:1 and 4:0 are insensitive to AMP. Evidently, loops 52–72 of these FBPGase hybrids cannot achieve the T-state, disengaged loop conformation.

Changes in fluorescence in response to the titration of AMP confirm the absence of cooperativity in 2:2p hybrid tetramer and the existence of cooperativity in AMP-ligation of the 2:2q/2:2r and 1:3 hybrid tetramers (Table II). Dissociation constants determined by fluorescence spectroscopy mirror the variation in IC\(_{50}\) values. The \(K_c\) values for the 2:2q/2:2r and 1:3 hybrids are substantially lower than that of the 2:2p hybrid tetramer.

**DISCUSSION**

Kinetic parameters (\(k_{cat}\), ratio of activities at pH 7.5 and 9.5, \(K_m\) for F16P\(_2\), \(K_p\) for Mg\(^{2+}\), Hill coefficient for Mg\(^{2+}\), and \(K_p\) for F6P\(_2\)) and kinetic mechanisms of inhibition are virtually identical for hybrid and wild-type enzymes (Table I). Henceforth the discussion will focus on the two phenomena that differ: AMP cooperativity and AMP inhibition.

Cooperativity can be a property of the entire oligomer, in which case the Hill coefficient varies smoothly with the number of functional ligand binding sites in hybrid constructs. Such is the case for hybrids of lactate dehydrogenase from Bacillus licheniformis (26). On the other hand, if the mechanism of cooperativity relies on the integrity of a specific subunit interface, then mutations at that interface should eliminate cooperativity, whereas mutations elsewhere should have no effect. Cooperativity in the binding of NAD to tetrameric glyceraldehyde-3-phosphate dehydrogenase, for instance, is sensitive to the O–P interface (36).

Neither of these scenarios, however, applies to FBPGase. The lack of cooperativity in AMP inhibition of the 2:2p hybrid indicates no participation by the C1–C2 interface in this phenomenon. On the other hand, the 0:4 hybrid (Glu-tagged homotetramer) and the 1:3 hybrid (1 subunit AMP \(^-\) · 3 subunits Glu-tagged) have Hill coefficients for AMP of 2.0 and 1.6, respectively, suggesting some correlation between the number of functional binding sites and the Hill coefficient. If 2:2 hybrids can have cooperativity no greater than that of the 1:3 hybrid, then the Hill coefficient of 1.5 for the 2:2q/2:2r hybrid mixture requires one either of the hybrids (2:2q or 2:2r) is dominant and cooperative or that both hybrids are cooperative.

Of the two possibilities, mentioned above, the latter is most probable. To have the formation of say the 2:2q hybrid, but not the 2:2r hybrid, requires either a bias in the equilibrium distribution of hybrids or a bias in the kinetics of subunit exchange. Although the relative areas of peaks in the elution profile of Fig. 2A are not consistent with a binomial distribution, we see no evidence that would suggest a thermodynamic bias so large as to exclude the formation of any specific hybrid. Furthermore, we can envision a mechanism of subunit exchange that discriminates against the formation of both hybrids 2:2q and 2:2r, but no mechanism that leads to the formation of just one of these hybrids. Therefore, the data here infer comparable levels of each hybrid in the 2:2q/2:2r mixture and, hence, that both the 2:2q and 2:2r hybrids must have nearly equal AMP cooperativity.

Directed mutations that alter AMP cooperativity in a homotetramer of FBPGase generally do not support a definitive conclusion regarding the mechanism of cooperativity (37–42). Mutations of Arg\(^{49}\) or Lys\(^{50}\) (residues located at the C1–C2 interface) eliminate AMP cooperativity (41, 42), but they also change the kinetic mechanism of AMP inhibition from noncompetitive to uncompetitive with respect to F16P\(_2\) and from competitive to noncompetitive with respect to Mg\(^{2+}\). One can infer little regarding cooperativity in a kinetic mechanism, if the
The names of the FBPases are defined under “Experimental Procedures” and Fig. 2.

<table>
<thead>
<tr>
<th>FBPase</th>
<th>Activity ratio</th>
<th>kcat (^b)</th>
<th>K-Mg(^{2+}) (^c)</th>
<th>K-Mg(^{2+})</th>
<th>IC(_{50})-AMP (^e)</th>
<th>Hill coeff. AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3.3</td>
<td>22 ± 1</td>
<td>1.8 ± 0.1</td>
<td>0.67 ± 0.04</td>
<td>1.9 ± 0.1</td>
<td>1.02 ± 0.01</td>
</tr>
<tr>
<td>Trp(^{57})</td>
<td>3.3</td>
<td>24 ± 1</td>
<td>3.4 ± 0.1</td>
<td>0.53 ± 0.06</td>
<td>1.9 ± 0.1</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>Glu-tagged</td>
<td>3.5</td>
<td>25 ± 1</td>
<td>2.2 ± 0.1</td>
<td>0.65 ± 0.05</td>
<td>2.1 ± 0.1</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>1:3</td>
<td>3.5</td>
<td>22 ± 2</td>
<td>2.2 ± 0.2</td>
<td>0.35 ± 0.03</td>
<td>2.2 ± 0.1</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>2:2q/2:2r</td>
<td>3.3</td>
<td>21 ± 1</td>
<td>1.8 ± 0.1</td>
<td>0.59 ± 0.04</td>
<td>2.0 ± 0.1</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>2:2p</td>
<td>3.4</td>
<td>23 ± 2</td>
<td>1.7 ± 0.1</td>
<td>0.41 ± 0.05</td>
<td>2.0 ± 0.1</td>
<td>0.183 ± 0.02</td>
</tr>
<tr>
<td>3:1</td>
<td>3.2</td>
<td>22 ± 1</td>
<td>1.8 ± 0.2</td>
<td>0.39 ± 0.02</td>
<td>2.2 ± 0.1</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>AMP(^{7})</td>
<td>3.2</td>
<td>21 ± 1</td>
<td>2.7 ± 0.1</td>
<td>0.46 ± 0.03</td>
<td>2.1 ± 0.1</td>
<td>0.15 ± 0.01</td>
</tr>
</tbody>
</table>

\(^a\) Ratio of specific activities at pH 7.5 and 9.5.
\(^b\) Determined at saturating substrate concentrations.
\(^c\) Determined at a Mg\(^{2+}\) concentration of 5 mM.
\(^d\) Determined at an F16P\(_2\) concentration of 20 \(\mu M\).
\(^e\) Determined at a Mg\(^{2+}\) concentration equal to the \(K_m\)-Mg\(^{2+}\) of each enzyme and a F16P\(_2\) concentration of 20 \(\mu M\).
\(^f\) Value calculated using normalized data shown in the inset of Fig. 5.

Mechanisms of AMP inhibition and, on the basis of fluorescence data, there are cases (39, 40) in which the introduction of a specific subunit interface in the mechanism of AMP cooperation. Met\(^{22}\) FBPase exhibits no cooperation in AMP inhibition yet retains all other wild-type kinetic mechanisms and parameters (37). Arg\(^{22}\) (of subunit C1) hydrogen bonds with backbone carbonyl 27 (of subunit C4) in the T-state of wild-type FBPase. This intersubunit hydrogen bond is absent, however, in the R-state. The absence of AMP cooperativity in Met\(^{22}\) FBPase implicates the top/bottom (C1–C4/C2–C3) interface in the phenomenon. As the top/bottom interface extends the entire width of the FBPase tetramer (Fig. 1), the coupling mechanism responsible for cooperativity can involve any pair of AMP molecules bound to opposite sides of the interface. The observation of cooperativity in the 2:2q/2:2r hybrid mixture and its absence in the 2:2p hybrid is fully consistent with a mutation that evidently severs communications between the top and bottom halves of the FBPase tetramer.

Although AMP cooperativity may not be inseparably linked to allosteric inhibition, as noted above, AMP affinity and cooperativity are correlated phenomena. Cooperativity is highest and IC\(_{50}\) for AMP is lowest in the 0:4 hybrid (Glu-tagged homotetramer). As the number of AMP\(^{7}\) subunits increases in the hybrid, the Hill coefficient declines and IC\(_{50}\) for AMP rises. The 1:3 hybrid and the 2:2q/2:2r hybrid mixture are the exceptions to this trend. In fact, the kinetic properties of the 1:3 hybrid and the 2:2q/2:2r hybrid mixture are nearly identical, whereas the 2:2p hybrid has no cooperativity and an IC\(_{50}\) for AMP some 6-fold higher. Evidently, potent and cooperative AMP inhibition of FBPase requires at least one AMP molecule bound to each half (top/bottom) of the tetramer. This finding is in harmony with evidence for pairs of high and low affinity sites for AMP on wild-type FBPase (46).

The fluorescence data infer a common R-state conformation for hybrids and a global conformational change induced by saturating concentrations of AMP. The Trp\(^{57}\) reporter group behaves as if the 1:3 and 2:2 hybrids are undergoing the same R- to T-state transition as observed in the wild-type enzyme. The 12-fold rise in \(K_d\) for AMP, exhibited by the 2:2p hybrid mechanism itself is changed by the experiment. Indeed, the mutation of Lys\(^{50}\) to methionine has no effect on kinetic mechanisms and little or no effect on kinetic parameters, including AMP cooperativity (41). Mutations of Arg\(^{22}\) and Lys\(^{50}\) that result in significant functional perturbations are due most likely to their influence on the conformational dynamics of loop 52–72. Mutations of Asn\(^{64}\), Asp\(^{54}\), and Glu\(^{98}\), residues in or near loop 52–72, eliminate AMP cooperativity (43, 44).

The introduction of proline at position 50 dramatically alters the mechanism of AMP inhibition and, on the basis of fluorescence spectroscopy, greatly changes the conformational dynamics of loop 52–72 (45). In contrast, the 2:2p hybrid preserves all of the kinetic properties of the wild-type enzyme, save AMP cooperativity. There are, however, mutations that eliminate AMP cooperativity with only modest effects on other kinetic parameters. Mutations of Lys\(^{42}\), Ile\(^{190}\), Gly\(^{191}\), and Glu\(^{192}\) (residues at the C1–C4 interface) are such examples (39, 40). The above mutants of FBPase exhibit biphasic inhibition by AMP; maximal inhibition of substantially less than 100% at low concentrations of AMP (<10 \(\mu M\)), followed by complete inhibition at concentrations of AMP >10 mM. Liu et al. (38) concluded that such biphasic AMP inhibition is a consequence of lost AMP cooperativity. The 2:2p hybrid, however, has no AMP cooperativity and no biphasic AMP inhibition. An alternative model by Nelson et al. (13) accounts for biphasic AMP inhibition as a failure to stabilize the disengaged conformation of loop 52–72. Hence, catalysis in the presence of AMP arises from the T-state of FBPase, and the observed maximal inhibition at low concentrations of AMP is due to the transition from an active R-state to a less active T-state. The later model accounts for biphasic AMP inhibition but says nothing regarding the mechanism of AMP cooperativity.

Of the several mutations that eliminate AMP cooperativity in homotetramers of FBPase, the mutation of Arg\(^{22}\) to methionine perhaps makes the most compelling case for the participation of a specific subunit interface in the mechanism of AMP cooperation. Met\(^{22}\) FBPase exhibits no cooperation in AMP inhibition yet retains all other wild-type kinetic mechanisms and parameters (37).

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relative to the Trp<sup>57</sup> homotetramer, reflects most likely the diversion of more AMP binding energy to promote the R- to T-state transition. Hence, one molecule of AMP ligated to each half (top/bottom) of the tetramer is more effective in driving the R- to T-state transition, than a pair of AMP molecules bound to the same half of the tetramer. A 3:1 hybrid (3 subunits AMP/1 Glu-tagged) of FBPase is poorly inhibited by AMP (IC<sub>50</sub> of 340 M) and, on the basis of fluorescence spectroscopy, cannot drive the R- to T-transition. Data here from thermodynamics and kinetics then are in substantial agreement: Potent and cooperative inhibition of FBPase occurs by the binding of one AMP molecule to each of the bottom and top halves of the tetramer, driving an R- to T-state transition.

These findings indicate two possible pathways of allosteric inhibition: a cooperative pathway, triggered by the binding of a pair of AMP molecules to opposites sides of the top/bottom interface, and a noncooperative pathway, triggered by the binding of a pair of AMP molecules to the same side of the top/bottom interface. Formycin A 5'-mono-
phosphate (FMP) and 2-(3’)-O-(trinitrophenyl)adenosine 5’-monophosphate, however, cause complete allosteric inhibition with no cooperation (7, 45). For reasons unknown, the later effectors may prefer binding to the same side of the top/bottom interface of the wild-type tetramer, and thus the absence of cooperation in allosteric inhibition. Crystal structures of human FBPase, fully ligated with AMP and with FMP, however, reveal no discernable differences (47). Hence, differences in AMP and FMP complexes of FBPase must lie in partially ligated states of the enzyme, a conclusion at least consistent with the observed functional properties of the 2:2 hybrid tetramers.

Ackers et al. (48) have proposed a “symmetry rule” governing the transition of the hemoglobin from its T- to R-states. The hemoglobin tetramer is composed of two αβ dimers. The association of at least one ligand to each of the αβ dimers of the hemoglobin tetramer favors a T- to R-state transition. In contrast, the R- to T-state transition for FBPase occurs whether ligands bind to opposite halves or to the same half of the tetramer.

How then is it possible for AMP to promote an R- to T-state transition in FBPase with and without cooperation? A possible explanation appears in Fig. 7, where ΔΓp is the free energy change in the ligation of AMP to an “isolated subunit” of FBPase, ΔΓtext is the free energy penalty in overcoming tertiary conformational restraints due to the assembly of the “isolated subunit” into an R-state relative to a T-state tetramer, and ΔΓRT is the free energy penalty in overcoming quaternary conformational restraints in the R- to T-state transition.

Because unligated and singly ligated tetramers favor the R-state, ΔΓRT > ΔΓtext. Because doubly ligated tetramers are in the T-state, however, 2ΔΓtext > ΔΓRT. The last two inequalities can be combined: 2ΔΓtext > 2ΔΓtext > 2ΔΓtext > 2ΔΓRT - ΔΓtext, which clearly agrees with the relative free energies of unligated, singly ligated, and doubly ligated FBPases.

To account for noncooperative binding of AMP, we assume a thermodynamic penalty (ΔΓp, in Fig. 7) in the binding of two AMP molecules to the same half of the tetramer. Such a thermodynamic penalty is consistent with the weaker binding affinity of the third and fourth molecules of AMP to the wild-type tetramer (46). Noncooperative binding of AMP then requires ΔΓtext = ΔΓtext = ΔΓtext = ΔΓp, or equivalently, 2ΔΓtext = ΔΓtext + ΔΓtext. This latter equality and the former combined inequality (2ΔΓtext > 2ΔΓtext > 2ΔΓtext) require only that ΔΓtext > ΔΓp > 0, which is a plausible condition. Hence, the same allosteric effector of FBPase can promote an R- to T-state transition by way of either cooperative or noncooperative binding pathways.

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Hybrid Tetramers of Porcine Liver Fructose-1,6-bisphosphatase Reveal Multiple Pathways of Allosteric Inhibition
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doi: 10.1074/jbc.M112304200 originally published online February 19, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M112304200

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