The role of the 190's loop of fructose-1,6-bisphosphatase (Frutase) in the allosteric regulation of fructose-1,6-bisphosphatase has been investigated through kinetic studies on three mutant enzymes, Glu-192→Ala, Glu-192→Gln, and Asp-187→Ala. AMP is an allosteric inhibitor, which binds to the regulatory sites and induces the R- to T-state transition. For wild-type Frutase, AMP inhibition is cooperative with a Hill coefficient of 2.0. The replacement of Asp-187, which forms an interaction across the C1:C2 monomer-monomer interface, with alanine did not change the catalytic efficiency, and it had no effect on the cooperativity of AMP inhibition; however, the apparent dissociation constant for AMP increased more than 4-fold as compared to the value for the wild-type enzyme. The replacement of Glu-192, which forms interactions across the C1:C4 dimer-dimer interface, with Ala and Gln lowered $K_m$ from 21 s$^{-1}$ for wild-type enzyme to 15 s$^{-1}$ and 13 s$^{-1}$, respectively, for the mutant enzymes, while their respective $K_m$ values were not changed. However, these replacements did have dramatic effects on AMP inhibition; first, cooperative AMP inhibition was lost; second, the AMP inhibition was biphasic, which can be interpreted as due to AMP binding to two classes of binding sites. The high affinity class of sites corresponds to the regulatory sites, whereas the low affinity class of sites may be the active sites. The results reported here, combined with the structural and kinetic results from the Lys-42→Ala enzyme, strongly suggest that the C1:C4 dimer-dimer interface, rather than the C1:C2 monomer-monomer interface, is critical for the propagation of the allosteric signal between the AMP sites on different subunits; in addition, cooperative AMP inhibition is essential for the enzyme to be fully inhibited by the binding of AMP to the allosteric site.

Fructose-1,6-bisphosphatase (Frutase), a key regulatory enzyme in gluconeogenesis, catalyzes the hydrolysis of the α-anomer of fructose-1,6-bisphosphate (Frutase) to α-D-fructose 6-phosphate and inorganic phosphate in the presence of divalent metal ions. Pig kidney Frutase is an allosteric enzyme composed of four identical subunits, each with a molecular weight of 34,000 (2). Although the enzyme is normally in the active R state, upon the binding of AMP it is transformed into the completely inactive T state. X-ray structural data for both the R- and T-states are available for pig kidney Frutase. The AMP complex (3) both in the absence and the presence of fructose 6-phosphate are identified as T-state structures, while those complexes with bound fructose 6-phosphate (4) or fructose 2,6-bisphosphate (5) are identified as R-state structures. The tertiary structure of each monomer is composed of two folding domains (Fig. 1), the AMP domain with the AMP binding site and the FBP domain containing the active site.

The activity of pig kidney Frutase is negatively modulated by the allosteric inhibitor AMP and also by Frutase, which is a competitive inhibitor (6, 7). AMP binds to the allosteric site which is about 28 Å (3) from the active site and its inhibition is cooperative with a Hill coefficient of approximately 2.0. The enzyme requires divalent metal ions, such as Mg$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$, to achieve catalytic activity (6). Magnesium activation is also cooperative with a Hill coefficient of 2.0. There are two metal binding sites on each monomer. One metal binding site is defined as a structural site which is thought to be essential for subsequent substrate binding, and the other is defined as a catalytic site, thought to be essential for catalysis. The metal binding sites are located at the interface between the two domains of the enzyme (8) (Fig. 1).

During the transition from the R- to the T-state, the C1:C2 dimer rotates about 15° relative to the C3:C4 dimer, and within each monomer the AMP domain rotates about 1.9° and translates about 1.6 Å relative to the FBP domain. By comparing the T- and R-state structures, Lipscomb and co-workers (9) have proposed that, after the binding of AMP, the secondary structure of the AMP domain undergoes a series of rearrangements leading to a movement involving a relative rotation and translation of the AMP domain relative to the FBP domains. This movement disturbs the active site, which consists of a substrate binding region from the FBP domain and a metal binding region at the domain interface, in such a way that enzyme activity is inhibited. Inhibition is achieved by preventing coordinated binding of substrate and of one essential metal ion in the proper arrangement necessary for substrate hydrolysis. Despite progress made in explaining the allosteric mechanism, the mechanism of signal propagation between subunits during the allosteric transition remains unclear.

Structural data for the wild-type enzyme have suggested

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* This work was supported by National Science Foundation Grant MCB-9631143. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Frutase, fructose-1,6-bisphosphatase; Frutase, fructose 1,6-bisphosphatase; Frutase, fructose 2,6-bisphosphatase.

‡ The four subunits of fructose-1,6-bisphosphatase are designated C1, C2, C3, and C4 and are labeled counter clockwise (see Fig. 1). The C1 and C2 subunits correspond to the upper dimer, and the C3 and C4 subunits correspond to the lower dimer.
that the 190’s loop\(^3\) (Fig. 1) may play a crucial role for allosteric regulation between subunits during the R- to T-state transition. Previously, Lys-42, which interacts at the dimer-dimer axis.

EXPERIMENTAL PROCEDURES

Materials

Agar, agarose, ampicillin, chloramphenicol, sodium dihydrogen phosphate, and magnesium chloride were purchased from Sigma. Tris and enzyme-grade ammonium sulfate were supplied by ICN Biomedicals. Tryptone and yeast extract were from Difco. The oligomers used to construct the Asp-187 → Ala mutation and some of the primers required for DNA sequencing were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by high pressure liquid chromatography employing a DuPont Zorbax Oligo ion-exchange column.

Methods

Oligonucleotide Synthesis—The oligonucleotide used to construct the Asp-187 → Ala mutation and some of the primers required for DNA sequencing were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by high pressure liquid chromatography employing a DuPont Zorbax Oligo ion-exchange column.

Construction of the Glu-192 → Ala, Gln-192 → Glu, and Asp-187 → Ala Mutations—Site-specific mutagenesis was performed on the Fru-1,6-P\(_2\)ase cDNA harbored on plasmid pEK284, employing the method of Kunkel (13, 14). Uracil-containing single-stranded DNA was obtained by infection of E. coli strain CJ236 containing pEK284 with the helper phage M13KO7 (15). Potential mutant candidates were initially identified by DNA sequence analysis (16). The entire cDNA of Fru-1,6-P\(_2\)ase of one candidate was sequenced to ensure that the site-specific mutagenesis had not introduced additional mutations other than the desired changes. The resultant plasmids, pEK281, pEK372, and pEK373 contained only the mutation at the codons corresponding to the Asp-187 → Ala, the Glu-192 → Ala, and the Gln-192 → Glu mutations, respectively.

Expression of Pig Kidney Fru-1,6-P\(_2\)ase in E. coli—In order to express the wild-type and mutant pig kidney Fru-1,6-P\(_2\)ases, the corresponding phagemids were transformed into E. coli strain EK1801. E. coli strain EK1601 has a deletion in the chromosomal fbp gene and can be induced to produce T7 RNA polymerase (12). In this manner the pig kidney Fru-1,6-P\(_2\)ase expressed from the plasmid could not be contaminated with the E. coli Fru-1,6-P\(_2\)ase expressed from the chromosome.

Enzyme Purification—Bacteria were cultured with vigorous agitation at 37 °C in M9 medium supplemented with 0.5% casamino acids and 0.5% ammonium chloride at 100 µg/ml. Induction of T7 RNA polymerase was initiated by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (Sigma). After further cultivation for 16–22 h, cells were harvested by centrifugation and broken open by a freeze-thaw procedure (17). Purification of mutant enzymes was accomplished by the method previously described (12).

Determination of Fru-1,6-P\(_2\)ase Activity and Data Analysis—A spectrophotometric, coupled-enzyme assay was employed to measure Fru-1,6-P\(_2\)ase activity (18). Standard conditions (2 mM magnesium chloride) were used to determine specific activity. Digital absorbance values were collected and fit to a straight line by computer, using data beyond the coupling lag period. In all assays, the enzyme was added last, after incubation and thermal equilibration of the coupling enzymes, magnesium (and inhibitor) concentration, and all the mutant enzymes were determined using the Lowry-Peterson method (20, 21) with bovine serum albumin as the standard.

Other Methods—SDS-polyacrylamide gel electrophoresis was used to judge enzyme homogeneity (22). Concentrations of Fru-1,6-P\(_2\)ase and NADP were checked by performance in the coupled assay, of AMP using 15.4 as the millimolar extinction coefficient at pH 7.0 and 259 nm, and of Fru-2,6-P\(_2\) by partial acid hydrolysis and analysis of fructose 6-phosphate (23).

RESULTS

Kinetic Properties of the Asp-187 → Ala, Gln-192 → Ala, and Gln-192 → Gln Fru-1,6-P\(_2\)ases—The kinetic data for wild-type and the three mutant enzymes are summarized in Table I. For each enzyme, the substrate saturation curve is hyperbolic with inhibition exhibited at high substrate concentrations. Analysis

\(^3\) The 190’s loop corresponds to residues 187–192 in the AMP domain.
of the kinetic data was performed by using a nonlinear least squares method incorporating a term for substrate inhibition. The Asp-187 → Ala enzyme (Fig. 2) had the same kcat as the wild-type enzyme; however, the Km for this enzyme (0.7 μM) was slightly lower than the corresponding value for the wild-type enzyme (1.4 μM). Both the Glu-192 → Ala and the Glu-192 → Gln enzymes (Fig. 2) have lower kcat values, 15 s⁻¹ and 13 s⁻¹, respectively, compared to 21 s⁻¹ for the wild-type enzyme. However, the Km values of the mutant enzymes were close to that of the wild-type enzyme (Table I).

**Influence of Mg²⁺ on the Wild-type and the Mutant Fru-1,6-P₂ases**—Wild-type Fru-1,6-P₂ase requires divalent metal ions such as Mg²⁺ to achieve catalytic activity, and the activation effect is cooperative with a Hill coefficient of approximately 2 (6). For wild-type Fru-1,6-P₂ase, the concentration of Mg²⁺ needed to activate the enzyme to half its maximum activity is 0.34 mM; however, the corresponding values for mutant enzymes were 0.95 mM, 1.16 mM and 0.49 mM for the Glu-192 → Ala, Glu-192 → Gln, and Asp-187 → Ala enzymes, respectively. There were also slight differences in the values of the Hill coefficients between the wild-type and the mutant enzymes. The Hill coefficient of wild-type Fru-1,6-P₂ase was 1.8 ± 0.5; however, the corresponding values for the mutant enzymes were 1.4 ± 0.1, 1.4 ± 0.1, and 1.7 ± 0.1 for the Asp-187 → Ala, Glu-192 → Ala, and Glu-192 → Gln enzymes, respectively (Table I). The lower Hill coefficients indicate that the magnesium activation for the mutant enzymes were not as cooperative as it was for the wild-type enzyme.

**Influence of Fru-2,6-P₂ on the Wild-type and the Mutant Enzymes**—For the wild-type enzyme, Fru-2,6-P₂ is a competitive inhibitor (24), and all the mutant enzymes investigated here showed the same characteristic. The Kᵢ value for wild-type Fru-1,6-P₂ase was 0.065 μM, which is very close to the value determined for the Glu-192 → Ala enzyme (Kᵢ = 0.064 μM) and the Glu-192 → Gln enzyme (Kᵢ = 0.059 μM). However, the Kᵢ for the Asp-187 → Ala enzyme (0.11 μM), was slightly higher than that of the wild-type enzyme.

**Influence of AMP on the Wild-type and the Mutant Enzymes**—AMP is an allosteric inhibitor for pig kidney Fru-1,6-P₂ase, and it binds to an allosteric site, about 28 Å distant from the substrate binding site (3). For wild-type Fru-1,6-P₂ase, AMP inhibition is cooperative with a Hill coefficient of approximately 2 and a dissociation constant of 2.8 μM. For the Asp-187 → Ala enzyme (see Fig. 4), AMP inhibition was still cooperative with the same Hill coefficient as that of the wild-type enzyme. However, the dissociation constant was 13.4 μM, which is about 4-fold higher than the corresponding value for the wild-type enzyme.

In contrast to the Asp-187 → Ala enzyme, AMP inhibition was altered drastically for the Glu-192 → Gln and the Glu-192 → Ala enzymes (see Fig. 4). First, AMP inhibition was not cooperative for either of these mutant enzymes. Second, the inhibition was biphasic and the enzymes could only be fully inhibited at high concentrations of AMP. Thus, both the high and low affinity sites must be saturated in order for complete inhibition to be obtained. The high affinity binding sites could be saturated at approximately 200 μM of AMP, resulting in 90% inhibition of the Glu-192 → Ala enzyme. The AMP dissociation constant for the high affinity sites was 1.6 μM; however, the corresponding value for the low affinity sites could not be determined accurately due partially to the fact that the remaining activity of this phase was very low.

The Glu-192 → Gln enzyme also exhibited biphasic AMP inhibition; however, saturation of the high affinity binding sites resulted only in 40% inhibition, as compared with 90% for the Glu-192 → Ala enzyme. Moreover, the dissociation constant of the high affinity sites was 19 μM, which was about 10-fold higher than the corresponding value for the Glu-192 → Ala enzyme, while the dissociation constant for the low affinity binding sites was 14 μM.

**DISCUSSION**

Based on the x-ray structures of wild-type Fru-1,6-P₂ase (3, 4) and previous kinetic and x-ray crystal structure studies of the Lys-42 → Ala enzyme (10), we speculated that the 190’s loop (C1), which is located at the central part of the tetramer and forms multiple interactions with Lys-42 (C4), may be important for signal transduction across the interfaces during the R- to T-state transition (9). Both residues 187 and 192 from the
190’s loop form interface interactions with residues from the neighboring subunits (Fig. 3). Therefore, in order to elucidate the role of the 190’s loop in allosteric regulation, three mutant enzymes, the Glu-192 → Ala, the Glu-192 → Gln and the Asp-187 → Ala enzymes were generated and their kinetic characteristics were determined.

**Mutations at Residues 187 and 192 Have Only a Minor Effect on Catalytic Efficiency of the Mutant Enzymes**—In the structure of the wild-type enzyme, the side chain carboxyl group of Asp-187 forms hydrogen bonds across the C1:C2 monomer-monomer interface (9); the side chain of Glu-192 forms hydrogen bonds with Thr-39 (C4) and Lys-42 (C4) across the C1:C4 dimer-dimer interface in both the R- and T-states (see Fig. 3). Since the 190’s loop is about 30 Å distant from the C1:C4 dimer-dimer interface, rather than a pathway across the C1:C2 monomer-monomer interface, Glu-192 forms interactions only across the C1:C4 dimer-dimer interface with residues on the 190’s loop including 192, was mutated to Ala, and the resulting mutant enzyme also lost cooperativity associated with AMP inhibition. Grazi et al. (25) studied the kinetic properties of matrix-bound Fru-1,6-P_{2}ases. They found that the matrix-bound tetramer retained most of the catalytic activity but became half-desensitized to AMP. All these results strongly suggest that signal transduction between the AMP binding sites of Fru-1,6-P_{2}ase occurs mostly via a pathway involving Glu-192 and Lys-42 across the C1:C4 dimer-dimer interface, rather than a pathway across the C1:C2 interface.

**The C1:C4 Dimer-Dimer Interface Is Important for Signal Transduction between AMP Binding Sites**—Based on a comparison of the wild-type R- and T-state structural data, Zhang et al. (9) found that AMP domains have extensive interactions with each other across the interfaces. They also found that during the R- to T-state transition almost all the secondary structural changes occur within the AMP domain. These authors proposed that small initial changes at any one of the AMP domains could be quickly and efficiently transmitted to neighboring AMP domains (9). However, the pathway for intersubunit signal transduction, whether it be through the C1:C2 monomer-monomer interface and or the C1:C4 dimer-dimer interface cannot be determined by merely evaluating the structural data for the wild-type enzyme.

By combining the structural data from the wild-type enzyme with functional data obtained from mutant enzymes, it is possible to shed light on the mechanism of signal transduction in pig kidney Fru-1,6-P_{2}ase. For the Asp-187 → Ala enzyme, AMP inhibition is cooperative (Fig. 4), although the enzyme is about 4-fold less sensitive to AMP than the wild-type enzyme. For the Glu-192 → Ala and the Glu-192 → Gln enzymes (Fig. 4), AMP inhibition was altered more significantly. First, for these two enzymes AMP inhibition was no longer cooperative, and second, AMP inhibition was biphasic as has been seen previously for the Lys-42 → Ala enzyme (10).

The two residues mutated in this work participate in different intersubunit interactions. Asp-187 forms interface interactions exclusively at the C1:C2 monomer-monomer interface, whereas Glu-192 forms interactions only across the C1:C4 dimer-dimer interface. The Asp-187 → Ala mutation had no effect on the cooperativity associated with AMP inhibition, while the two mutations at Glu-192 abolished the cooperativity associated with AMP inhibition. Previously, Lys-42 (10), which forms hydrogen bonds across the C1:C4 dimer-dimer interface with residues on the 190’s loop including 192, was mutated to Ala, and the resulting mutant enzyme also lost cooperativity associated with AMP inhibition. The C1:C4 dimer-dimer interface is important for signal transduction between the AMP binding sites of Fru-1,6-P_{2}ase.
allosteric site, which suggests that comparable binding of AMP to the active site at high concentrations might be possible. Lu et al. (10) concluded that the high affinity binding sites were associated with the binding of AMP to the regulatory sites, while the low affinity sites were associated with the binding of AMP to the active sites. However, for wild-type enzyme the binding of AMP at the active site cannot be detected by standard activity measurements because the enzyme is completely inactivated at concentrations of AMP that would not be sufficient to bind significantly to the active sites. For the Glu-192 → Ala and Glu-192 → Gln enzymes, AMP binding at the allosteric sites cannot completely inactivate the enzyme. Only by binding of AMP at both the high and low affinity sites can these mutant enzymes be completely inhibited.

Cooperative AMP Inhibition Is Essential for Full Allosteric Inhibition of Fru-1,6-P2ase—As shown in Fig. 4, even though the apparent AMP dissociation constant of the Glu-192 → Ala enzyme for the allosteric sites is about half the value for the wild-type enzyme, activity could not be fully inhibited by saturating the high affinity allosteric sites. The corresponding value for the Asp-187 → Ala enzyme is more than four-fold higher than the wild-type enzyme; however, it could be fully inhibited by binding of AMP to the allosteric site and it retained the cooperativity associated with AMP inhibition. Previously, kinetic studies on the Arg-22 → Ala enzyme (19) have shown that although this mutant enzyme’s apparent dissociation constant is about 10-fold higher than the wild-type enzyme, AMP inhibition is still cooperative with a Hill coefficient of 2. Furthermore, the mutant enzyme could be completely inhibited by saturating the allosteric sites with AMP. All these results strongly support the notion that cooperativity is required for complete inactivation of Fru-1,6-P2ase by the binding of AMP to the allosteric sites.

AMP inhibition of the Glu-192 → Gln and the Lys 42 → Ala enzymes is so similar that their inhibition curves are almost superimposable (Fig. 5), which suggests that these two mutations had almost the same effects on the allosteric regulation between the allosteric and the metal binding sites. However, the replacement of Glu-192 by Ala or Gln results in two mutant enzymes that are almost identical kinetically, except for their inhibition by AMP. Besides the approximate 10-fold difference between their apparent AMP dissociation constants for the allosteric site, the extend of inhibition is about 90% for Glu-192 → Ala enzyme whereas it is about 40% for the Glu-192 → Gln enzyme. These mutations would alter the hydrogen bond interaction between Glu-192 (C1) and Lys-42 (C4), which is important not only for allosteric regulation between the AMP binding sites across the dimer-dimer interface, but also for signal transduction between the AMP binding site and the metal binding sites within each subunit. In addition, because of the differences in their side chains, these mutations would have different effects on the charge neutralization of the two closely spaced Lys-42 residues. In this manner, the signal transduction pathway connecting the AMP binding site and the metal binding sites is interrupted differently in the Glu-192 → Ala and Glu-192 → Gln enzymes.

It is important to maintain the distinction between cooperative and allosteric phenomena, while recognizing that they are often found together (26). By definition, allostery allows one kind of small molecule to regulate the action of a protein on another kind of molecule. For the Glu-192 → Ala and the Glu-192 → Gln enzymes, even though AMP no longer inhibits cooperatively, within each subunit the AMP binding site can still communicate with the metal binding sites, and for this reason AMP can still inhibit the enzyme. However, the mutations at Glu-192 do affect the communication pathways in such a way that the binding of AMP at the allosteric site cannot completely inhibit these mutant enzymes. Thus, the enzyme requires cooperative AMP inhibition in order to achieve the full regulation effect between the allosteric site and the metal cation binding sites.

Mutations at the 190’s Loop Affect the Allosteric Regulation between Metal Binding Sites—x-ray crystallographic structural data for wild-type Fru-1,6-P2ase showed that there are two metal binding sites in each monomer, which are located between the FBP and the AMP domains (Fig. 1). Direct binding (27, 28) and steady-state kinetic studies (6) have established the sequence of binding of metal cations and the substrate in the order: structural metal cation, substrate, then catalytic metal cation. All three mutant enzymes presented here exhibit dramatic altered AMP inhibition, but are also less sensitive to magnesium activation, for which their Hill coefficients are decreased from 2 for wild-type enzyme to around 1.5 for these mutant enzymes. The alteration of residue 187 and 192 might cause a small local structural reorientation of the 190’s loop, which would result in the observed changes in the magnesium activation of the mutant enzymes.

The mechanism of allosteric regulation between the magnesium sites has been studied before. Direct binding studies (28) showed that after the binding of four structural metal ions per tetramer, 2 moles of ligand result in the binding of 4 catalytic metal ions, suggesting a positive cooperative interaction between sites on different subunits. Nevertheless, since within each subunit the structural metal binding site must be occu-
pied before the second metal cation can bind to the catalytic site (6), intrasubunit cooperativity cannot be ruled out.

Summary—The results presented here, combined with the previous kinetic and structural results from Lys-42 → Ala enzyme (10), strongly suggest that the signal transduction pathway between the AMP binding sites takes place across the C1:C4 dimer-dimer interface, mediated by residues of the 190’s loop. Furthermore, cooperative AMP inhibition is required in order to achieve full communication between the allosteric and the metal binding sites in fructose-1,6-bisphosphatase.

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