Identification of Active Site Residues in Pyrophosphate-dependent Phosphofructo-1-kinase by Site-directed Mutagenesis*

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The primary structure of pyrophosphate-dependent phosphofructokinase (PFK) from Propionibacterium freudenreichii exhibits a low but significant level of sequence identity with Escherichia coli ATP-dependent PFK, permitting the tentative assignment of residues that may be involved in catalysis. Based on these assignments, the roles in catalysis of 2 aspartyl residues (Asp<sup>151</sup> and Asp<sup>152</sup>) and 2 lysyl residues (Lys<sup>80</sup> and Lys<sup>86</sup>) were examined. Mutagenesis of the Asp<sup>151</sup> to alanine and serine reduced k<sub>cat</sub> by a factors of 2 × 10<sup>4</sup> and 4 × 10<sup>3</sup>, respectively, while showing no change in K<sub>m</sub> for either substrate in the forward reaction or for metal ion in the back reaction. The k<sub>cat</sub> for Asp<sup>152</sup> was decreased by a factor of 700 with no change in K<sub>m</sub> for pyrophosphate and an increase of about 20-fold in K<sub>m</sub> for fructose 6-P and close to 4-fold for magnesium ion. That these changes in the mutants were not the result of global conformational changes was indicated by their identical behavior during substrate-specific elution chromatography, ion-exchange chromatography, and limited proteolysis by trypsin and subtilisin. Mutations of Lys<sup>80</sup> and Lys<sup>86</sup> showed no significant changes in kinetic parameters, suggesting no involvement in mechanism or substrate binding. These and other results permit preliminary modeling of the active site of pyrophosphate-dependent PFK.

The pyrophosphate-dependent phosphofructokinase (PP<sub>P</sub>-PFK)<sup>1</sup> from Propionibacterium freudenreichii catalyzes the inorganic pyrophosphate-dependent phosphorylation of the hydroxyl a C-1 of Fru 6-P to generate Fru 1,6-P<sub>2</sub> and inorganic phosphate. A number of recent studies have provided several mechanistic details of the reaction, indicating that the mechanism is rapid equilibrium random (1), that the phosphoryltransferase step is rate-limiting (2) and proceeds through a dissociative transition state (3), and that a proton-shuttle mechanism may be involved (4). The identification of actual residues involved in the reaction has been lacking, except for the identification by chemical modification of Lys<sup>816</sup> as a potential interaction site for the phosphoryl group of Fru 6-P (5). The recent availability of a recombinant clone of PP-PFK expressed in Escherichia coli (6) permits the use of site-directed mutagenesis to search for catalytically important amino acids.

The selection of appropriate candidates for site-directed mutagenesis can be based on our knowledge of the more extensively studied ATP-dependent PFK from E. coli (7) and on chemical modification studies of the PP-dependent enzyme. Although the PP-PFK from P. freudenreichii has been shown to differ substantially from the major family of ATP-dependent phosphofructokinases, a low but significant level of sequence identity with E. coli PFK has been established to indicate that the PP-dependent and the ATP-dependent enzymes derive from a common ancestor (6). Employing a computer-based alignment program coupled with a few adjustments made by eye, an optimal alignment of residues indicating an overall identity to the ATP-dependent enzyme of about 23% was achieved (6). Despite this low level of overall similarity, a number of residues that have been implicated by X-ray crystallographic studies (7) of the ATP-dependent E. coli PFK to be involved in either Fru 6-P binding or the phosphoryltransferase reaction have been tentatively aligned with residues in the PP-dependent enzyme (6). The longest common sequence between the PP<sub>P</sub> and ATP-dependent enzymes was 5 amino acids, Thr-Ile-Asp-Asn-Asp (T-I-D-N-D), which is significant because this sequence appears to be at the active site of ATP-PFK as determined from the structure derived from X-ray crystallography (7). Other than this evidence based upon an uncertain alignment of the ATP- and PP<sub>P</sub>-dependent enzymes, ambiguity remains regarding the identity of residues involved either in substrate binding or in the catalytic mechanism. Some suggestion of a binding region for pyrophosphate was provided by studies involving modification of PP-PFK by pyridoxal phosphate plus borohydride (5). These studies, although not establishing a definitive relationship between modification and activity loss, showed that partial protection against the reaction of pyridoxal phosphate with 2 lysyl residues, Lys<sup>80</sup> and Lys<sup>86</sup>, was afforded by MgPP<sub>P</sub>, suggesting that these residues may be located near the PP<sub>P</sub> binding site.

In the current study, we examine by site-directed mutagenesis the significance of the 2 aspartyl residues in the T-I-D-N-D sequence of PP<sub>P</sub>-dependent PFK and find that both are crucial for the catalytic process. In addition, Lys<sup>80</sup> and Lys<sup>86</sup> were eliminated as candidates for roles either in the binding of PP<sub>P</sub> or in the stabilization of the transition state.

EXPERIMENTAL PROCEDURES

Oligonucleotide-directed Mutagenesis—An 1170-base pair HindIII/Smal fragment from pLG1 (6), encoding the entire reading frame for

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1 The abbreviations used are: PFK, 6-phosphofructo-1-kinase; Fru-6-P, fructose 6-phosphate; Fru-1,6-P<sub>2</sub>, fructose 1,6-bisphosphate; Tes, 2-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PP<sub>P</sub>, inorganic pyrophosphate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.
the 406 amino acids of the enzyme, was subcloned into HindIII/SmaI of pSELECT-1 (Promega Corp.). Cleavage at the SmaI site of pLG1 permitted the removal of approximately 400 base pairs from the sequence downstream of the PP-PFK coding sequence of the original insert. A single-stranded form of p-SELECT-1 containing the PP-PFK gene was generated by using the Altered Sites in vitro mutagenesis system (Promega Corp.) and used as a template. Cultures were grown in LB medium employing two mutagenic primers: one to produce the desired mutation in PP-PFK and a second that corrects a defect in the lactamase gene of pSELECT-1. The mutagenic oligonucleotides were synthesized by phosphoramidite chemistry in a Biosearch 8700 DNA synthesizer, deprotected and used without further purification. Various oligonucleotides for construction of the mutants are shown in Table 1. All mutants were obtained by this method and were identified by sequencing using the dideoxy chain termination method (8) using sequencing primers prepared on the Biosearch synthesizer.

**Protein Expression and Purification**—The 1170-base pair HindIII/EcoRI fragments of wild-type and mutant PP-PFKs were subcloned from pSELECT-1 into Bluescript II KS" HindIII/EcoRI site and transformed into E. coli strain DF1020 to overproduce the mutant protein. Strain DF1020 lacks both ATP-dependent PFKs of E. coli and makes a convenient host that prevents potential interference in enzyme assays by crude extracts. Cultures were grown in LB media containing 0.5 mM isopropylthiogalactoside, and the cells were harvested by centrifugation. Wild-type and mutant PP-PFK expressed in DF1020 were isolated and purified by a revision of the method described for the wild-type enzyme by Ladror et al. (6) in which a modification of the basic procedure described by Boehringer Mannheim. The technique involves substrate elution from phosphocellulose (Whatman P-11) by Fru 1,6-P2. The position of elution was reproducible, thus the enzyme could be readily purified whether or not significant activity was present. Fractions from the column were pooled based upon optical densities at 280 nm and where possible by activity assay. The enzyme in the pooled fractions was in most cases approximately 90% homogeneous but required an additional purification step. Mutant PP-PFK was precipitated from the pooled fractions by the addition of solid ammonium sulfate (43 g/100 ml). The precipitated enzyme was collected by centrifugation. The pellets were then dispersed in a small volume (2-3 ml) of 100 mM Tris/HCl buffer at pH 7.6 containing 0.1 mM EDTA. Insoluble particles were removed by centrifugation, and the clarified supernatant was dialyzed for 3 h against 500 ml of 20 mM Tris/HCl buffer, pH 7.6. The dialyzed enzyme solution was then loaded onto a Pharmacia FPLC Mono Q HR 5.5 column preequilibrated with 20 mM Tris/HCl buffer, pH 7.6. Elution was performed by a Pharmacia FPLC system at a flow rate of 0.5 ml/min. The column was eluted with 10 ml of 20 mM Tris/HCl, pH 7.6, followed by a 25-ml gradient from 50 to 100% 500 mM sodium chloride in 20 mM Tris/HCl, pH 7.6. Effluents were monitored at an absorbance of 280 nm, and fractions were collected at 1-min intervals (~0.5 ml). The enzyme eluted as a sharp single peak at a chloride concentration of ~350 mM. Fractions containing PP-PFK mutants K80A, K85A, D153A, and D151A were used directly in kinetic assays for 1 h. At the end of this time, 0.5-volume of a solution containing 0.1 M Tris/HCl, 0.1 M imidazole HCl, 30% glycerol, 6% sodium dodecyl sulfate, 1.5 M mercuric acetate, and 0.002% bromphenol blue 0.1 was added, and the solutions were heated for 2 min in a boiling water bath. These samples were used for SDS-PAGE. Differences in final protein concentrations in the proteolysis mixtures had no effect on the results.

**Other Methods**—The concentration of protein in crude fractions was determined by Bradford's dye binding assay with bovine serum albumin as the standard (10). Protein concentrations in purified fractions of PP-PFK were determined spectrophotometrically by measuring optical densities at 280 nm and using an E280 of 50.4 mm" cm"1. Gel electrophoresis of proteins was carried out using 7.5% sodium dodecyl sulfate, 1.5 M mercaptoethanol, and 0.002% bromphenol blue as the standard (11). Protein concentrations were calculated from values for K and Vmax obtained using the GraFit graphical analysis program. Values for Kmax were calculated from Vmax and total protein concentration.

**Results**

**Purification of Wild-Type and Mutant PFKs—Wild-type**

PP-PFK and the five mutants, D151A, D151S, D153A, K90A, and K85A, were purified from bacterial extracts as described under "Experimental Procedures." This procedure involves a very specific substrate elution of phosphocellulose that produces an enzyme that is nearly homogeneous. However, those mutants with very low activity could not be quantitated accurately during the purification procedure by direct enzymic activity assay, because trace-contaminating activities that contributed to a blank assay (without PP) interfered with the assay when it was necessary to increase the amount of protein added to the cuvette by 3-4 orders of magnitude. Nonetheless, the mutant enzyme could be detected in fractions eluted from the P-11 column by determining the absorbance at 280 nm. The absorbency profile indicated that the mutant enzymes eluted at volumes identical to those encountered during the purification of wild-type PFK. The presence of the mutant enzyme in the eluted fractions was confirmed by SDS-PAGE which showed the presence of the 43-kDa protein as the major component eluted from Fru 1,6-P2. It should be noted that all enzymes were eluted from the P-cellulose column by the same low concentration of the PFK reaction product, 0.5 mM Fru 1,6-P2. These results suggest that the integrity of the sugar bisphosphate binding site has been retained in the mutants. The existence of trace contaminants that interfered with assays at very high protein levels necessitated the further purification by ion-exchange chromatography of the enzyme eluted from the phosphocellulose column. Chromatography of

| Mutation | Oligonucleotide |
|----------|-----------------|
| D151A    | 5' TGTGTTGGAGCAGTGGTCT 3' |
| D151S    | 5' TGTGTTGGACAGTGGTCA 3' |
| K80A     | 5' GCACATGCGGCTTGGTCA 3' |
| K85A     | 5' ATCCGAGGCGCCACCGGGCA 3' |
| D153A    | 5' AACCGAGGCGCCACAGTGT 3' |
the pooled and concentrated eluate from the phosphocellulose column on the ion-exchanger, Mono-Q, separated the PP\textsubscript{I}-PFK from all interfering activities. All PFKs eluted from the Mono-Q ion-exchange column at the same salt concentration, 350 mM NaCl, indicating similar overall available charges on the wild-type and mutant proteins.

**Limited Proteolysis of Wild-type and Mutant PPI-PFKs**—Wild-type proteins are relatively resistant to proteolysis and limited exposure to proteases under non-denaturing conditions generally produces a few discreet cleavages at available sites on the surface of the protein. The cleavage pattern is therefore under control of the overall folded design of the protein. Similar limited proteolysis patterns of wild-type and mutants of the same protein would suggest identical overall structures.

Wild-type PP\textsubscript{I}-PFK and the mutant enzymes D151A, D151S, and D153A, were digested with trypsin (20:1, PFK:trypsin) or subtilisin (100:1, PFK:subtilisin) for 1 h at 23 °C, as described under “Experimental Procedures.” The results for D151A and D151S, as analyzed by SDS-PAGE, are shown in Fig. 1. Wild-type PP\textsubscript{I}-PFK and both mutants were quite resistant to proteolysis by trypsin, with only a trace of a digested product with a mass of about 32-kDa appearing in all digestions. Subtilisin at a weight ratio of 100:1 digested the enzyme more extensively. It should be noted, however, that the same digestion patterns, three bands in the range of 20–27 kDa, were observed with wild-type and mutant enzymes. Not shown in the figure are the results of the digestion of D153A, which produced digestion patterns identical to those of the wild-type enzyme and the other two mutants.

**Kinetic Properties of Mutations at Asp\textsubscript{151} and Asp\textsubscript{153}**—Mutations at either Asp\textsubscript{151} or Asp\textsubscript{153} resulted in dramatic decreases in the catalytic activity of PP\textsubscript{I}-PFK. By assaying the enzyme at varying concentrations of substrate and cofactor, $k_{cat}$, $K_m$ for Fru 6-P, and the $K_m$ for PPi, were determined. These properties are presented in Table II. Mutation of Asp\textsubscript{151} to either serine or alanine had no effect on the apparent affinities for either substrate, PPi, or Fru 6-P. On the other hand, converting the acidic residue to alanine lowered $k_{cat}$ by a factor of $2 \times 10^4$ and converting it to serine reduced activity by $4 \times 10^3$.

When Asp\textsubscript{153} was mutated to alanine, the activity ($k_{cat}$) decreased by about 700-fold, indicating an important role in catalysis for this residue also. This mutation did not alter the affinity for PPi, but it decreased the binding affinity for Fru 6-P by about 20-fold.

**Effect of Mutation of Asp Residues on Metal Ion Affinity**—

![Figure 1. SDS-PAGE of limited proteolytic digests of wild-type and mutant PP\textsubscript{I}-PFK.](image)

**DISCUSSION**

Because changes in kinetic properties of the mutants at Asp\textsubscript{151} and Asp\textsubscript{153} could be the result of global changes in structure and not the result of specific effects of side chains of mutated amino acids, it was necessary to exclude possible overall structural changes before one can implicate specific residues in mechanism. Evidence for similar overall structures among the enzyme forms was provided by three lines of evidence. First, the integrity of all of the mutants with respect to sugar phosphate binding was demonstrated by their elution by a relative low concentration of Fru 1,6-P$_2$. Furthermore, that the overall charge distribution on the surfaces of the wild-type and mutant forms were similar was shown by the nearly identical elution position upon anion-exchange chromatography on Mono Q. All enzyme preparations eluted at NaCl concentrations in the range of 350 mM. The third and most convincing piece of evidence for similar secondary structures is that provided by limited proteolysis by trypsin and subtilisin. Wild-type and mutant PFKs were quite resistant to proteolysis by trypsin and the proteolysis that was achieved
generated a fragment of identical size for wild-type and mutant PFKs. Subtilisin digestion generated three fragments whose sizes were identical in the three mutations of aspartyl residues. These data indicate a common three-dimensional structure for the wild-type and mutant enzymes and suggest that the profound differences in kinetic properties were consequences of specific effects on catalysis produced by changing the side chains and not the result of global structural alterations.

Mutations of Asp$^{141}$ and Asp$^{153}$ greatly diminished $k_{cat}$ values for the phosphorylation of Fru 6-P. Because there was virtually no change in $K_m$ for substrates in the 151 mutant and very little in the 153 mutant, one can ascribe the change to the catalytic step, such as decreased stability of the transition state. Another possibility is that product release is the rate-limiting step and that the aspartyl residues facilitate this process. Their mutation to uncharged residues would destroy their role in facilitating release of the negatively charge product.

Hellinga and Evans (12) have suggested, on the basis of site-directed mutagenesis studies of the ATP-dependent E. coli PFK, that Asp$^{129}$ (the first Asp in the T-I-D-N-D sequence in that enzyme) acts as a general base interacting with the proton on the OH of C-1 of Fru 6-P. Asp$^{141}$ occupies the identical position within the sequence T-I-D-N-D of PP$_i$-PFK; the longest identical sequence found between these two distantly related structures. It is very likely that Asp$^{153}$ plays the same critical role of a general base in PP$_i$-PFK that Asp$^{129}$ performs in the ATP-dependent enzyme. It is curious that the alanine mutant of Asp$^{153}$ has 200 times more activity than the serine mutation. Perhaps the alanine mutation permits a role of nucleophile) of the carboxyl group of Asp that occupies the same critical role of a general base in PP$_i$-PFK that (or the Asp$^{129}$ mutation of E. coli PFK) acts as a general base interacting with the sugar phosphate binding site. Their mutation to uncharged residues would destroy the role of these groups in PP$_i$-PFK, the longest identical sequence found between these two enzymes. The data indicate a common three-dimensional structure for the wild-type and mutant enzymes and suggest that the homologous Asp at position 129 interacts with Mg$^{2+}$ which in turn interacts with the two terminal phosphates ATP (12). Thus, if a similar role is projected for Asp$^{153}$, the predicted effect of the mutation in the PP$_i$-dependent enzyme might be that of a change in PP$_i$ affinity or in Mg$^{2+}$ affinity. But no change in the $K_m$ for PPF, was seen and the increase in the $K_m$ for Mg$^{2+}$ as determined in the back reaction from Fru 1,6-P$_2$ to Fru 6-P was less than 4-fold. Similar effects were seen with an analogous mutation of Asp$^{159}$ in the E. coli ATP-dependent PFK. The D129S mutant of ATP-dependent PFK shows a 1000-fold reduction in $k_{cat}$, a 10-fold increase in $S_{0.5}$ for Fru 6-P, and no significant change in ATP binding. The lack of a strong influence of the Asp$^{153}$ mutation (or the Asp$^{159}$ mutation of E. coli PFK) on substrate $K_m$ values suggests that this aspartyl residue does not play a particularly significant role in substrate binding and is perhaps only one of many ligands involved in metal ion binding. This role must become more significant in stabilizing the transition state as indicated by its effect on $k_{cat}$.

Fig. 2 describes a model for the transition state based on the mutagenesis data herein, the data of Halkides (3) indicating a dissociative transition state, the chemical modification data of Green et al. (5), and the model of the active site for ATP-dependent PFK as described by Hellinga and Evans (12). Asp$^{153}$ is shown acting as a base to abstract the proton on the C-1 hydroxyl of Fru 6-P, whereas Asp$^{129}$ interacts with the phosphotransferase reacting through the metal ion. Also shown is Lys$^{315}$ which has been shown from chemical modification studies to interact at the sugar phosphate binding site (5).

Shown also in Fig. 2 are 2 of the several unidentified basic residues that one would presume to be involved in either PP$_i$ binding and or transition state stabilization. Mutagenesis of Lys$^{315}$ and Lys$^{318}$ have shown that these particular basic residues are not involved in any manner in the mechanism of the enzyme and that the identity of the mechanistically important basic residues must await future studies. On the basis of the earlier chemical modification studies that showed some protection of these groups by PP, (5), it is likely that Lys$^{315}$ and Lys$^{318}$ will be found near the PP$_i$ site and that electrostatic repulsion or shielding accounted for the partial protection in the chemical modification studies.

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