Identification of Interactions That Stabilize the Transition State in Escherichia coli Phosphofructo-1-kinase*

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The ATP-dependent phosphofructo-1-kinase of Escherichia coli catalyzes the phosphorylation of Fru-6-P to produce Fru-1,6-P$_2$. The enzyme displays allosteric properties with cooperative binding of Fru-6-P that is influenced by the activators ADP or GDP or the inhibitor phosphoenolpyruvate. The steady state kinetics indicate a general adherence to the concerted allosteric mechanism (1) and a random mechanism that is nonequilibriating under some conditions (2, 3).

Deville-Bonne et al. (4) studied the pH dependence of the kinetic properties of the enzyme in the pH range of 6 to 9 and noted the catalytic rate constant to be controlled by the ionization of a critical group with a pK of approximately 6.6 in the presence of allosteric effectors. The critical group must be unprotonated for the enzyme to be active. The identity of this group has not been established but a number of functional group candidates can be inferred from crystallographic studies of the enzyme with products bound (5) and from site-directed mutagenesis studies (6, 7). Deville-Bonne et al. (4) suggested that the pH dependence of kcat could be due to a carboxyl or histidine residue and that Asp-127 was a prime candidate for the critical residue on the basis of results of Hellinga and Evans (6) who showed that mutation of the Asp-127 residue to serine reduces activity by more than 5 orders of magnitude. Laine et al. (8) examined the pH dependence of both Asp-127 and Asp-129 and concluded that the Asp-127 is not directly involved in the pH dependence of the enzyme and that a mutation of Asp-129 results in a shift of the pH response.

In the current study, we examined the pH dependence of a mutant involving both residues 127 and 129. We suggest that the pH dependence reflects the pK of a phosphoryl group of ATP that is interacting with a positively charged residue on the enzyme. Furthermore, single and double mutants involving basic residues reputed to be present in the active site of phosphofructo-1-kinase, Arg-72 and Arg-171, have been studied to identify the residues that are critical to charge stabilization in the transition state.

EXPERIMENTAL PROCEDURES

Strains—E. coli strain DF1020 pro-82, DppK301, recA56, D(rha-pkA)200, endA1, hsdR17, supE44). E. coli strain TG-1 is Dlac-pro AB), thi, sup E, D(ssr-1-recA506:Trn10tet) (F' tra D56, pro AB, lac I21D15).

Site-directed Mutagenesis—The preparation of the pRZ3 plasmid, which basically consists of the E. coli gene in pUC19, has been described previously (3). The pfr gene from pRZ3 was removed by digestion with BanHI and HindIII and ligated into M13 for site-direct mutagenesis by the method of Kunkel (9) using the Mutagen M13 mutagenesis kit from Bio-Rad. Uracil-containing single-stranded DNA was generated in the dUTP-ung mutant E. coli strain CJ236 and used as template to synthesize the mutated strand with the synthetic primer. The double mutant R72H/R171H was produced by using two mutagenic primers and wild type template uracil-containing DNA. The triple mutant D127S/D129N/R252Q was produced with two mutagenic primers, which basically consists of the E. coli gene in pUC19, has been described previously (3).

Expression and Purification—The pfi gene from pRZ3 was removed by digestion with BanHI and HindIII and ligated into M13 for site-directed mutagenesis. The obtained double-stranded DNA was transformed into wild type E. coli strain TG-1 to select against the nonmutagenized strand. DNA sequencing by Sequenase protocol was used to screen the mutants and to verify the entire sequence of one strand of the mutant DNA.

Expression and Purification of the Enzymes—Wild type E. coli phosphofructo-1-kinase and the mutant phosphofructo-1-kinases were expressed in DF1020 which is a mutant E. coli strain with pfr genes deleted. The bacteria were grown in a medium consisting of 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 5 mM ampicillin. Except where indicated, the enzymes were purified to homogeneity by the method of Kotlarz and Buc (10) as modified by Banas et al. (11) utilizing Blue Sepharose CL-6B affinity chromatography eluted with 0.5 M ATP. The purified enzymes were stored in the presence of 2 mM ATP and 50% glycerol at -20 °C. Before use, the enzyme stocks were dialyzed against 50 mM Tris-acetate, 0.1 mM EDTA, pH 7.4.

Ion Exchange Chromatography—For several mutants, the enzyme preparations were chromatographed on a Mono Q anion exchange column on a FPLC system. Prior to loading on the column, the preparations were dialyzed extensively against 20 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol, and 5 mM MgCl$_2$, all at the indicated pH. The Mono Q column was subsequently eluted with a gradient of 0 to 0.5 M NaCl in the dialysis buffer. The protein elution was monitored at 280 nm.

Enzyme Assay—The initial velocity of phosphofructo-1-kinase activity was determined spectrophotometrically by measuring the decrease in absorbance at 340 nm.
glycerophosphate dehydrogenase, and 1.12 mM NADH, 1 mM dithiothreitol, 1 mM EDTA, 27 pg of aldolase, 5 of Fru-1,G-P. The reaction was carried out in the presence of 0.2 mM of optical density at 340 nm, which represents indirectly the production isomerase in a 0.5-ml cuvette at 30 °C. The allosteric activator GDP was contained 0.1 &I MES, 0.051 added in all experiments. The buffer system used for all experiments NaOH. For substrate-dependent assays, one of the two substrates, ATP oranolamine. The pH was adjusted to the desired value with HCl or7.1 and the second at pH 8.1. Fig. 3 shows the elution profile from FPLC at pH 7.1; B, second run, pH 8.1. The dashed line indicates the salt gradient. The enzyme activity, which eluted at approximately 250 mM NaCl, is indicated by brackets.

RESULTS AND DISCUSSION

Purification of Mutants—With two exceptions, the wild type and mutant phosphofructo-1-kinases were purified to homogeneity by chromatography on Blue Sepharose. Mutant R72H bound weakly and could be eluted by salt, and R72H/R171H did not bind to the Blue Sepharose column. FPLC using a Mono Q anion exchange column was used to purify these two mutants as described under “Experimental Procedures.” For R72H, the elution buffer was adjusted to pH 8.1; for double mutant R72H/R171H, either the initial ammonium sulfate precipitate or enzyme from the initial effluent of the Blue Sepharose column was chromatographed twice, using the same buffer, once at pH 7.1 and the second at pH 8.1. Fig. 4A shows the elution profile from Mono Q of the double mutant R72H/R171H at pH 7.1, and the single major peak that was obtained after rechromatography at pH 8.1 (Fig. 4B). The SDS-PAGE gel shown in Fig. 2 describes the results from each step in the purification of mutant R72H/R171H. The purity and molecular mass for all mutants were verified by SDS-PAGE.

Circular Dichroism—To determine whether or not global changes in conformation were caused by the mutations at the active site of the enzyme, the circular dichroism spectra of the mutants employed in this study were determined. Differences in spectra of all mutants were very small, generally less than 5% at any given point. Because many of the mutants were studied at varying pH, the circular dichroism spectra were determined at pH 6.5, 7.5, and 8.5. No significant differences were seen among the wild type and mutant enzymes at any given pH, nor were significant differences promoted by the change in pH. The results for several of the mutants and wild type enzyme are shown in Fig. 3. The data suggest that there is no global conformational change in the mutant structures relative to wild type enzyme and that pH has no effect on the overall structure of either wild type or mutant enzymes.
Active Site Residues of Phosphofructo-1-kinase

**TABLE II**

| Enzyme                 | $K_w^{app}$ | $k_{cat}$ | $K_w^{app}$ | $k_{cat}$ |
|------------------------|-------------|-----------|-------------|-----------|
| Wild type              | $0.16 \pm 0.14$ | $167 \pm 11$ | $2.5 \pm 0.7$ | $4.0 \pm 0.5$ |
| D127S                  | $0.20 \pm 0.01$ | $7.6 \times 10^{-4}$ | $0.07 \pm 0.02$ | ND        |
| D127A                  | $0.35 \pm 0.01$ | $3.8 \times 10^{-3}$ | $4.1 \pm 0.9$ | $3.5 \times 10^{-3}$ |
| D127S/D129N/R252Q     | $0.6 \pm 0.1$ | $1.1 \times 10^{-3}$ | $7.2 \pm 1.9$ | $1.5 \times 10^{-3}$ |

**FIG. 3.** Circular dichroism spectra of wild type and mutant enzymes at pH 7.5. Mean residual ellipticity from six scans of each enzyme, wild type, R171H, R171H, R72H/R171H (HH), versus wavelength was plotted. Wave scan from 260 to 190 nm was carried out 0.2 nm/point. Potassium phosphate, 0.04 M, was used.

**FIG. 4.** pH dependence and $pK_d$ determination of wild type (A), D127A (B), and D127S/D129N/R252Q (C). The experiments were performed at 1 mM ATP, Fru-1,6-P$_2$, and GDP. Protein, 0.05 $\mu$g and 1 $\mu$g, were used in each measurement in the wild type and two mutants, respectively. The solid curves describe the theoretical titration of a single group with the $pK_d$ indicated on the figure.

spot in the native enzyme. A similar result was found in site-directed mutagenesis studies of the PP$_i$-dependent phosphofructo-1-kinase from Propionibacterium freudenreichii (15). In that enzyme, mutagenesis of Asp-151, a residue that is apparently homologous with Asp-127, resulted in a profound reduction in $k_{cat}$ (15). In that case also, the Ala mutant had much greater activity than the Ser mutant.

A comparison of the back reaction carried out by the two Asp-127 mutants is instructive. Hellinga and Evans (7) noted that the $K_w$ for the sugar bisphosphate is lower in the serine mutant as opposed to wild type enzyme. These results are confirmed by the data in Table II which indicate an apparent affinity of D127S for Fru-1,6-P$_2$ that is about 45 times higher than that of wild type, a value similar to that reported by Hellinga and Evans (7). This increase in apparent affinity of D127S is explained by the presumption that an Asp residue at this position does not favorably interact with the phosphoryl group on carbon 1 of the sugar phosphate whereas the Ser residue can form a favorable hydrogen bond. The D127A mutant, on the other hand, lacks the ability to form this bond and hence the apparent affinity of the Ala mutant for Fru-1,6-P$_2$ is much lower than that of the Ser mutant.

With the triple mutant, in which both Asp-127 and Asp-129 are converted to neutral residues, a profound decrease in $k_{cat}$ was observed, although the activity was somewhat higher than that seen with a single mutation at Asp-127. Note that in the mutants described in Table II, the $K_w$ values for fructose-6-P are nearly identical with that of wild type phosphofructo-1-kinase.

If either Asp-127 or Asp-129 represents the anionic residue involved in the rate-limiting step of catalysis or in stabilization of the transition state of the wild type enzyme, then the pH dependence of the mutants should be altered. The pH dependence of the mutants involving these residues should no longer describe the dissociation constant of a group in the range of 6 to 7 but should be shifted to that characteristic of one or more different new functional residues, unless, of course, the new critical residue(s) of the rate-limiting step has the same titration characteristics as the wild type enzyme. Fig. 4 describes the pH dependence of wild type phosphofructo-1-kinase and those of mutations at Asp-127, Asp-129, and Arg-252. Note that while maximal activity has been altered, the mutants display pH dependences that are not strikingly different from that of wild type. A shift of about 0.4 pH unit in seen in the $pK_d$ of the mutants relative to that of wild-type enzyme. The data on D127S are in agreement with that of Laine et al. (8) who also described a $pK_d$ of 7 for the pH dependence of $k_{cat}$ for this mutant. An examination of the active site of the enzyme as derived from x-ray crystallography (5) does not immediately suggest any other residue with a $pK_d$ in the range of 6 to 7. The only other negatively charged residue near the area is Asp-103, whose mutation to Ala by Berger and Evans (14) led to a modest reduction in $k_{cat}$ compared to the effects of mutation on Asp-127 and Asp-129, suggesting that Asp-103 does not play a major role in transition state stabilization. On the other hand, the secondary $pK_d$ of the terminal phosphoryl residue of the substrate ATP would be expected to be about 6.5. This leads to the suggestion that the pH dependence of the enzyme could be a
consequence of a stabilizing interaction of the negative charge on the terminal phosphate of ATP with a positively charged residue of the enzyme that is not titrated in the neutral range.

Mutation of Candidate Positively Charged Residues on the Enzyme—On the basis of his crystallographic data, Evans’ laboratory (6, 16) has indicated that Arg-72 and Arg-171 are appropriately positioned to interact with the terminal phosphate of ATP. Mutation of these Arg residues to Ser (6, 7) produced reductions in $k_\text{cat}$ of 34- and 3-fold for the Arg-72 and Arg-171 mutants, respectively, without substantially influencing the $K_\text{m}$ for either substrate. We reasoned that if either of these 2 basic residues was involved in transition state stabilization by neutralizing the negative charge on the terminal phosphate of ATP, then a mutation should influence the pH-dependent behavior of $k_\text{cat}$. A mutation of the Arg residues to His was thought to provide the positive charge to neutralize the negative charge of the phosphate while providing a vastly different dependence upon pH. There are problems of geometry between His and Arg residues, although these difficulties are lessened by the fact that electrostatic interactions operate over greater distances than other types of interactions. The kinetic parameters of the R72H, R171H, and the double mutant, R72H/R171H, are given in Table III. Mutations in either of the basic residues produced very small changes in $K_\text{m}$ for both ATP and Fru-6-P. Furthermore, pH had a relatively small effect on apparent substrate affinity for either substrate. The largest differences were less than 2.5-fold, as seen in the affinity of the wild type enzyme for Fru-6-P. On the other hand, mutation of Arg-72 led to a decrease in $k_\text{m}$ of more than 100-fold, while mutation in Arg-171 led to a relatively modest decrease in activity. Note that the maximal activity of wild type enzyme is greater at pH 6.5, whereas the activity of the Arg-72 mutant is greater at pH 6.5.

A more complete description of the effect of pH on the activity of the Arg mutants is provided by Fig. 5, the data of which should be compared with the wild type pH dependence described in Fig. 4. Mutation of Arg-72 to histidine led to a change in the pH dependence of the reaction by shifting the maximum to a lower pH. The pH maximum was shifted to approximately pH 6.2, suggesting that the activity is for the most part controlled by ionization of the terminal phosphate leading to the ascending limb of the curve and deionization of histidine leading to the descending limb on the alkaline side of the histidine dissociation. The pH dependence of the activity of R72H plateaus at a value near one-half of the maximal activity in the region of pH 8, indicating the influence of a second positively charged group, possibly Arg-171. The double mutant in which both Arg-72 and Arg-171 have both been changed to histidine displays an even greater decrease in $k_\text{cat}$ on the alkaline side, eliminating the plateau seen in the single mutant, presumably as a result of the elimination of the secondary influence of Arg-171.

The kinetic data obtained with the above described mutants confirm the important role of Asp-127 in the mechanism of phosphofructo-1-kinase. However, the rate-controlling step appears to involve the chemical step transferring the phosphoryl group to the acceptor sugar phosphate. In nonenzymatic reactions involving phosphate transfer, a distinction is made between associative and dissociative mechanisms, but the distinctions are somewhat blurred in enzymatic reactions where donating and accepting molecules are both in place at the time of the chemical event (17). The transfer of the metaphosphate ion generated in the dissociative mechanism would be impeded by a nearby cationic group. An associative mechanism would receive assistance from a positive charge (18). The results with mutant R72H indicate the importance of charge neutralization in the transition state and hence favor the associative mechanism. The reaction rate is highest when the negative charge on transferring phosphate is neutralized by the positive charge at position 72. In wild type enzyme, position 72 is occupied by Arg which will be positively charged below pH 12, and the negative charge on the phosphoryl group will be titrated at about pH 6.5. In the R72H mutant, the histidine will lose a positive charge in the same pH range that the phosphoryl group will gain a negative charge, leading to a pH optimum between the pK values of the two groups. This generates the pH-dependent curve shown in Fig. 4 which confirms the importance of the interaction between the negative charge on the terminal phosphoryl group of ATP and the positive charge on the arginine residue at position 72 of phosphofructo-1-kinase.

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