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Essential Arginyl Residue at the Active Site of Pyrophosphate:Fructose 6-Phosphate 1-Phosphotransferase from Potato (*Solanum tuberosum*) Tuber

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The aim of this work was to test the proposal that the active site of pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP) contains an essential arginyl residue. Enzyme activity was inhibited equally in the glycolytic and gluconeogenic directions by arginine-modifying reagents. The second-order rate constants for 2,3-butanedione and phenylglyoxal were 13.1 ± 0.45 and 55.3 ± 1.3 M^{-1} min^{-1}, respectively. The corresponding values for the kinetic order of inactivation by these modifying reagents were 0.84 ± 0.049 for 2,3-butanedione and 0.89 ± 0.052 for phenylglyoxal. The substrates, fructose 6-phosphate and pyrophosphate, and a range of substrate analogs protected the enzyme from inactivation by 2,3-butanedione. These data suggest that modification of no more than one arginyl residue at, or close to, the active site is required to inhibit the enzyme. This result supports the proposal that the active site of PFP in plants is equivalent to that of the bacterial ATP-phosphofructokinase (S.M. Carlisle, S.D. Blakeley, S.M. Hemmingsen, S.J. Trevanion, T. Hiyoshi, N.J. Kruger, and D.T. Dennis [1990] J Biol Chem 265: 18366–18371).

PFP catalyzes the reversible interconversion of Fru-6-P and Fru-1,6-P_2, an important regulated step of both glycolysis and gluconeogenesis. In the glycolytic direction the reaction catalyzed by PFP is comparable to that of PFK, except that the former uses PPi as the phosphoryl donor, whereas the latter uses ATP. In many higher plant tissues the activity of PFP is equal to or greater than that of PFK. However, at present, the role of PFP in plant metabolism is uncertain (Stitt, 1990).

Despite the similarity between the reactions catalyzed by PFP and PFK, these enzymes are composed of separate, distinct polypeptides (Kruger and Hammond, 1988). PFP purified to homogeneity from potato (*Solanum tuberosum*) tubers has a native M_r of 265,000 and is a heterotetramer containing two α and two β polypeptides with M_r values of 65,000 and 60,000, respectively (Kruger and Dennis, 1987). The precise function of the two different subunits is unknown. However, the isolation from wheat leaves of a catalytically active form of the enzyme containing only PFP_β indicates that this polypeptide, at least, contains a functional active site (Yan and Tao, 1984).

We previously isolated nearly full-length cDNA clones for both PFP_α and PFP_β from potato (Carlisle et al., 1990). The deduced amino acid sequences of these clones are similar to those of bacterial and mammalian PFK. Moreover, many of the regions of greatest similarity include residues that are important for substrate binding or for catalytic competence within *Escherichia coli* PFK (Hellinga and Evans, 1987; Shirakihara and Evans, 1988; Berger and Evans, 1990). Bacterial PFK catalyzes an in-line nucleophilic attack of the 1-hydroxyl group of Fru-6-P on the γ-phosphoryl group of ATP (Shirakihara and Evans, 1988) during which a pentacoordinate γ-phosphoryl group transition state is formed (Fig. 1). All five residues involved in the proposed transition state of bacterial PFK appear in the same relative positions within PFP_β, whereas only two are identical in PFP_α. Neither Asp^{127} nor Arg^{72}, which are important for effective catalysis (Hellinga and Evans, 1987; Berger and Evans, 1990), are present in the corresponding positions in PFP_α. This has led us to suggest that PFP_β possesses an active site that is structurally equivalent to that of *E. coli* PFK (Carlisle et al., 1990).

An active site in PFP similar to that of bacterial PFK would contain an essential arginyl residue, and as a consequence the enzyme should be susceptible to inactivation by chemical modification using Arg-specific reagents. To test this we have studied the response of PFP to 2,3-butanedione and phenylglyoxal, compounds that are considered to react specifically with guanidino groups that occur in Arg (Takahashi, 1968; Yankeelov, 1972).

MATERIALS AND METHODS

Materials

PFP was purified from mature tubers of potato (*Solanum tuberosum* L. cv Maris Piper) by heat treatment, PEG precipitation, and DEAE-Sepharose chromatography, essentially as described previously (Trevanion and Kruger, 1991). The enzyme was purified about 700-fold to a specific activity of 13.56 ± 0.78 μmol min^{-1} mg^{-1} of protein (mean ± SE of three determinations) measured in the glycolytic direction and 12.66 ± 0.37 μmol min^{-1} mg^{-1} of protein measured in the gluconeogenic direction. The preparation did not contain detectable activity of PFK, Fru 1,6-bisphosphatase, inorganic pyrophosphatase, or aldolase. Activity of phosphogluco-

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Abbreviations: Fru-1,6-P_2, fructose-1,6-bisP; k, second-order rate constant for inactivation; k', pseudo-first-order rate constant for inactivation; K_d, dissociation constant; PFK, phosphofructokinase (EC 2.7.1.11); PFP, pyrophosphate: fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90).

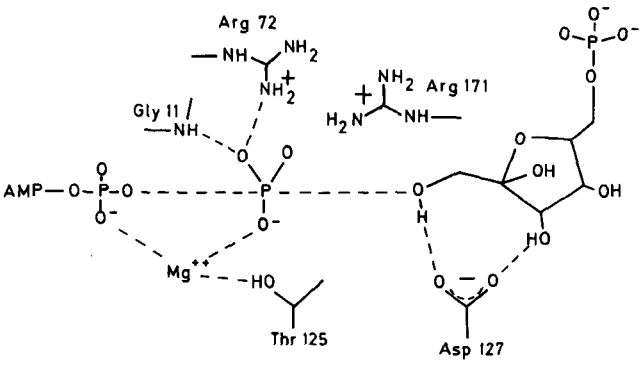

Figure 1. Schematic view of the proposed transition state of *E. coli* PFK based on studies described in Berger and Evans (1990).

isomerase was <1% of that of PFP and did not significantly interfere with the kinetic analysis.

All biochemicals and auxiliary enzymes were purchased from Sigma. DEAE-Sepharose was from Pharmacia-LKB.

Enzyme Assays

PFP activity was measured spectrophotometrically at 25°C as described previously (Kombrink et al., 1984). Ammonium sulfate was removed from the auxiliary enzyme before use. In the glycolytic direction the assay contained, in 1 mL: 100 mM Tris-Cl (pH 8.0), 2 mM $MgCl_2$, 5 mM Fru-6-P, 0.2 mM Na_4PPi, 2 μM Fru-2,6-bisP, 0.1 mM NADH, 1 IU of aldolase, 10 IU of triosephosphate isomerase, and 1.3 IU of glycerol 3-P dehydrogenase. In the gluconeogenic direction the composition of the assay mixture was, in 1 mL: 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM Fru-1,6-P_2, 8 mM Pi, 2 μM Fru-2,6-bisP, 0.1 mM $NADP^+$, 1 IU of phosphoglucoisomerase, and 1 IU of Glc-6-P dehydrogenase. In both directions the reaction was started by adding up to 2×10^{-3} IU of PFP. Variations in the composition of these assays are indicated in the text. All other enzyme activities were assayed as described by Kombrink et al. (1984). Protein was determined as described by Bradford (1976) using bovine γ-globulin as a standard.

Enzyme Inactivation

Purified potato PFP at a concentration of 5 μg mL^{-1} was incubated at 25°C with either 2,3-butanedione in 50 mM Na-borate buffer (pH 8.0) or phenylglyoxal in 50 mM K-Hepes buffer (pH 8.0). At appropriate times the incubation mixture was diluted 50-fold in PFP assay medium, and enzyme activity was measured immediately. Alternatively, excess modifying reagent was inactivated by adding 0.1 volume of 100 mM Arg in 100 mM Tris-Cl (pH 8.0), after which the enzyme mixture was stored on ice for up to 2.5 h before assaying PFP activity.

In protection experiments, PFP was incubated with the protecting compound at 25°C for 5 min before adding 2,3-butanedione. The degree of protection was calculated from the following formula.

$$\% \text{ protection} = 100 \times (V_{protected} - V_{residual})/(V_{control} - V_{residual})$$

in which $V_{control}$ is enzyme activity measured in the absence of inactivator and $V_{residual}$ and $V_{protected}$ represent that remaining following inactivation in the absence and presence of protector, respectively.

Data Analysis

Kinetic constants were determined by nonlinear regression analysis using the Marquardt algorithm, and the corresponding SEs were calculated by the matrix inversion method (Leatherbarrow, 1987). The apparent K_d for protection was calculated using the degree of protection, as defined above, rather than absolute enzyme activity.

Pseudo-first-order rate constants for enzyme inactivation (k') were calculated by nonlinear regression analysis as described above. Second-order rate constants of inactivation were obtained from the gradient of a plot of k' against [modifier]. The order of each reaction with respect to inactivator was derived from the gradient of a plot of log k' against log [modifier] (Levy et al., 1963). Both plots yielded straight lines. The best-fit estimates and corresponding SEs for these gradients were calculated by nonlinear regression analysis (Leatherbarrow, 1987). Hyperbolic curves fitted to these data were analyzed statistically using EZ-FIT (Perrella, 1988).

RESULTS

Both 2,3-butanedione and phenylglyoxal rapidly inactivated PFP. The extent of inactivation was dependent on the concentration of modifying reagent and was equivalent when measured in the glycolytic and gluconeogenic directions (Fig. 2). This inactivation was time dependent and followed pseudo-first-order reaction kinetics (Figs. 3 and 4). For both inactivators, plots of k' against [modifier] yielded straight lines that intersected the ordinate close to the origin and possessed high correlation coefficients (Figs. 5 and 6). From the gradients of these graphs we calculated that the second-order rate constants for inactivation by 2,3-butanedione and phenylglyoxal were 13.1 ± 0.45 and 55.3 ± 1.3 M^{-1} min^{-1}, respectively. The same data were used to derive the order of the reaction with respect to these modifiers. Plotting log k' against log [modifier] yielded straight lines with high correlation coefficients. The gradients of these lines were 0.84 ± 0.049 for 2,3-butanedione and 0.89 ± 0.052 for phenylglyoxal (Figs. 5 and 6). These gradients indicate that no more than one molecule of either modifier is required to inhibit each active site within the enzyme.

The analysis described above assumes that inactivation of the enzyme follows second-order reaction kinetics. Appreciable reversible binding of the modifier to the enzyme before inactivation would invalidate this assumption and distort interpretation of the results (Carlson, 1984). Such binding has been reported during the inactivation of malic enzyme by phenylglyoxal (Rao et al., 1991). However, detailed analysis of our data provides no evidence that inactivation exhibits saturation kinetics, which is indicative of reversible binding. Using nonlinear regression analysis, we are unable to fit the data presented in Figures 5 and 6 to statistically significant hyperbolic curves. For both 2,3-butanedione and phenylglyoxal, the curve of best fit failed a Runs statistics test (Swed

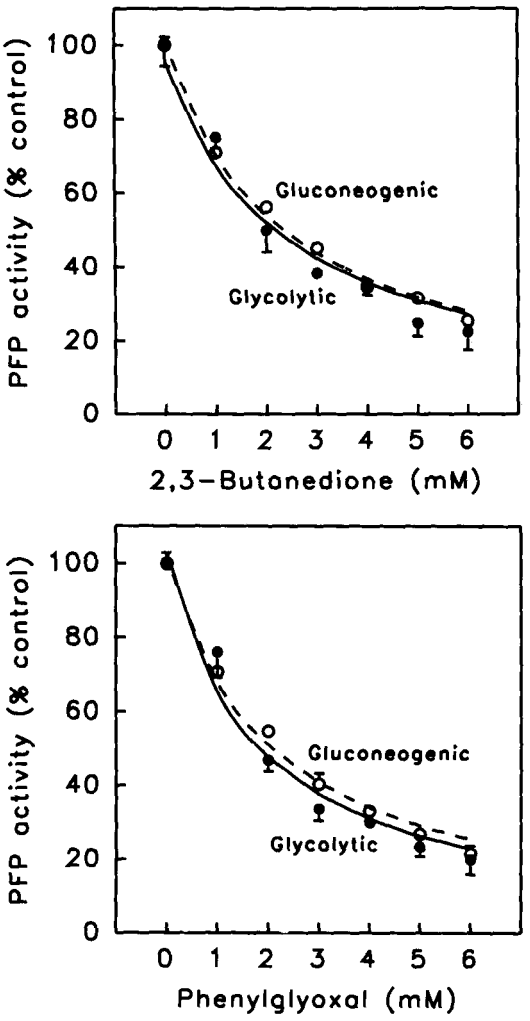

Figure 2. Effect of 2,3-butanedione and phenylglyoxal on PFP activity. PFP was incubated with either 2,3-butanedione (top) or phenylglyoxal (bottom). The concentration of modifier was varied as shown. After either 20 min (2,3-butanedione) or 5 min (phenylglyoxal) the mixtures were diluted 50-fold, and PFP activity was measured in either the glycolytic (●) or gluconeogenic (○) direction. Each value is the mean ± SE from three separate determinations. Omitted error bars are smaller than the corresponding symbols.

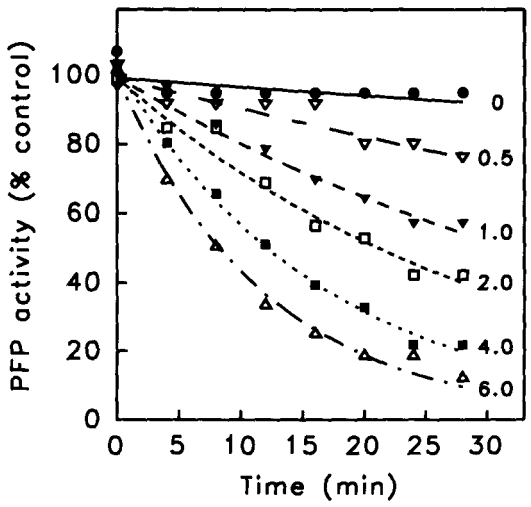

Figure 3. Time course of inactivation of PFP by 2,3-butanedione. PFP was incubated with 0 (●), 0.5 (▽), 1 (▼), 2 (□), 4 (■), or 6 (△) mM 2,3-butanedione in 50 mM Na-borate (pH 8.0) for up to 28 min. At times indicated the mixture was diluted 50-fold, and PFP activity was measured immediately in the glycolytic direction. Pseudo-first-order rate constants were obtained from these data by nonlinear regression analysis.

and Eisenhart, 1943), indicating systematic variation between the data and the fitted curve. Moreover, statistical analysis of the theoretical K_d (dissociation constant) and k_{max} (maximum apparent k) values derived from the curve revealed that neither was significantly different from zero ($P > 0.1$). Furthermore, the high correlation coefficients for linear regression in the analyses presented in Figures 5 and 6 (upper panels) are consistent with linear rather than hyperbolic kinetics. Based on these observations we assert that enzyme inactivation is not preceded by kinetically significant reversible binding of either modifier under the conditions used in this study.

In addition, we analyzed the kinetic properties of residual PFP activity following enzyme inactivation. In these experiments, inactivation was terminated by addition of a 2-fold excess of Arg to the enzyme mixture, which was then stored on ice for up to 2.5 h during the subsequent kinetic analysis. We checked that this treatment stopped the inactivation and prevented further modification of the enzyme. In an inactivation reaction terminated immediately after addition of the

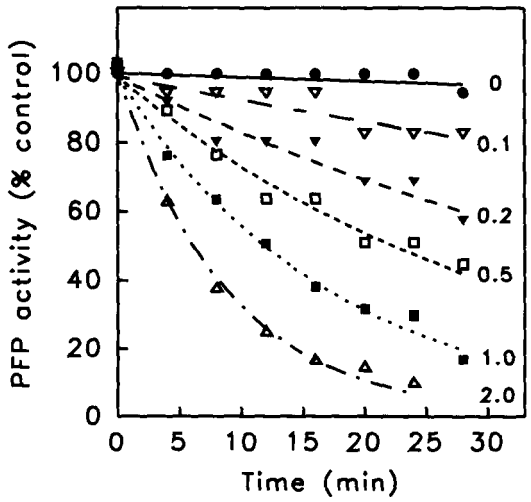

Figure 4. Time course of inactivation of PFP by phenylglyoxal. PFP was incubated with 0 (●), 0.1 (▽), 0.2 (▼), 0.5 (□), 1 (■), or 2 (△) mM phenylglyoxal in 50 mM Na-Hepes (pH 8.0) for up to 28 min. At times indicated the mixture was diluted 50-fold, and PFP activity was measured immediately in the glycolytic direction. Pseudo-first-order rate constants were obtained from these data by nonlinear regression analysis.

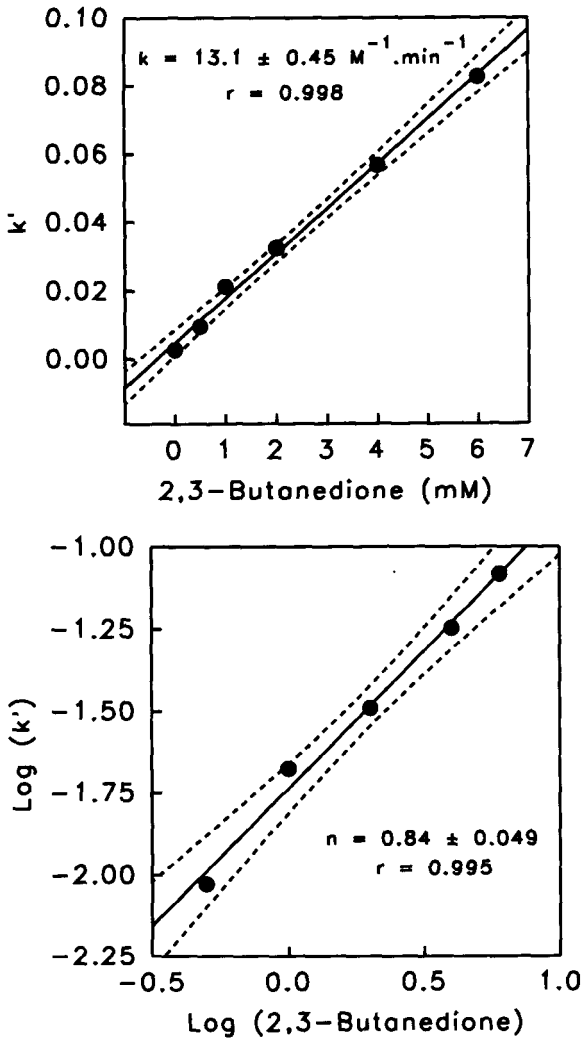

Figure 5. Kinetic analysis of inactivation of PFP by 2,3-butanedione. Pseudo-first-order rate constants derived from the data in Figure 3 were used to calculate the second-order rate constant for 2,3-butanedione (top) and to estimate the reaction order with respect to 2,3-butanedione (bottom). Dotted lines are 95% confidence limits for the lines of best fit.

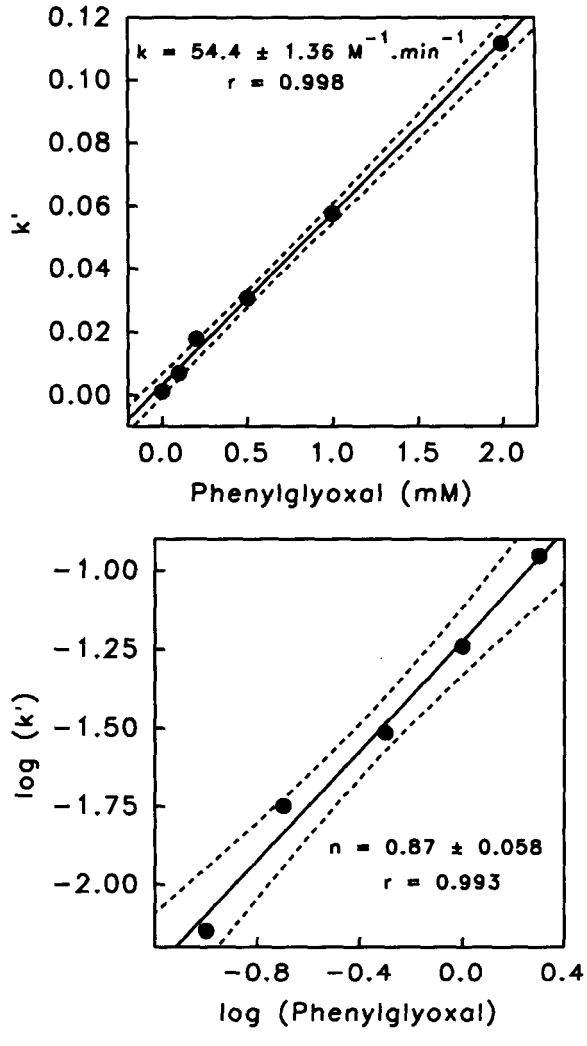

Figure 6. Kinetic analysis of inactivation of PFP by phenylglyoxal. Pseudo-first-order rate constants derived from the data in Figure 4 were used to calculate the second-order rate constant for phenylglyoxal (top) and to estimate the reaction order with respect to phenylglyoxal (bottom). Dotted lines are 95% confidence limits for the lines of best fit.

modifying reagent, we detected no significant decrease in enzyme activity for at least 3.5 h. The kinetic properties of enzyme that had been inactivated by >80% using either 2,3-butanedione or phenylglyoxal are presented in Table I. Neither modifier has any major effect on the affinity of the enzyme for its substrates. The residual PFP activity exhibits hyperbolic kinetics with respect to all reactants. In the glycolytic direction the apparent K_m values for both Fru-6-P and PPi are similar to those of the unmodified enzyme. In the gluconeogenic direction, the apparent K_m for Pi was not significantly affected, whereas that for Fru-1,6-P_2 approximately doubled. This suggests that inactivation is due largely to a decrease in the catalytic constant of the enzyme.

To examine whether the modified arginyl residue is close to the active site we examined the ability of reactants to protect the enzyme against inactivation by 2,3-butanedione.

Table I. *Kinetic constants of residual PFP activity after inactivation*

PFP was inactivated by incubation with either 5 mM 2,3-butanedione for 30 min or 5 mM phenylglyoxal for 10 min. Both of these treatments resulted in >80% inhibition. The apparent K_m of the residual activity for each reactant was determined. The control represents PFP incubated in 100 mM borate in the absence of 2,3-butanedione for 20 min. Similar constants were obtained after incubation of the enzyme in 100 mM Hepes for 5 min. Each value is the best-fit estimate ± SE from at least eight measurements.

Treatment	Apparent K_m			
	Fru-6-P	PPi	Fru-1,6-P_2	Pi
			μM	
Control	335 ± 15	19 ± 2.8	43 ± 5.1	1057 ± 152
2,3-Butanedione	288 ± 49	17 ± 2.2	72 ± 15.6	1393 ± 229
Phenylglyoxal	298 ± 49	16 ± 1.4	75 ± 12.4	937 ± 209

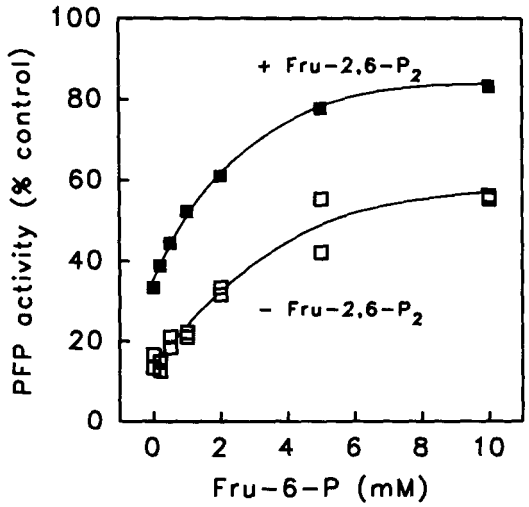

Figure 7. Effect of Fru-6-P on inactivation of PFP by 2,3-butanedione. PFP was incubated with 5 mM 2,3-butanedione for 20 min in the presence (■) or absence (□) of 1 μM Fru-2,6-bisP. The concentration of Fru-6-P was varied as shown. The mixture was diluted 50-fold, and PFP activity was measured in the glycolytic direction. Residual activity is expressed as a percentage of the initial activity measured in the absence of 2,3-butanedione.

More than one-half of the initial PFP activity was protected in the presence of 10 mM Fru-6-P under conditions normally resulting in about 90% inactivation (Fig. 7). This degree of protection was enhanced to >80% by the presence of 1 μM Fru-2,6-bisP, which alone provided only modest protection (Fig. 7). In separate experiments the extent of protection provided by 10 μM Fru-2,6-bisP was 16.1 ± 1.1% (mean ± SE, $n = 4$). From the data presented in Figure 7, the apparent K_d for protection of the enzyme by Fru-6-P was 2.3 ± 0.19 mM (best-fit estimate ± SE) in the presence of Fru-2,6-bisP. The corresponding value in the absence of Fru-2,6-bisP was 4.6 ± 0.62 mM. Under similar conditions, the protection conferred by 0.1 mM PPi was 61.3 ± 1.6% (mean ± SE, $n = 4$). From data obtained by varying PPi between 10 and 1000 μM, the apparent K_d for protection of PFP by PPi was 21.8 ± 3.09 μM (best-fit estimate ± SE from 18 measurements). The extent of protection by PPi was not significantly affected by 1 μM Fru-2,6-bisP. To test the specificity of protection, PFP was incubated with 2,3-butanedione in the presence of a range of phosphorylated compounds, each at 1 mM (Fig. 8). At this concentration, tripolyphosphate and ATP provided a degree of protection equivalent to that displayed by PPi. The extent of protection conferred by trimetaphosphate and ADP was 84 and 63%, respectively, of that obtained using PPi. Methylene diphosphonate provided only modest protection, and Pi was the least effective of the compounds tested.

DISCUSSION

The susceptibility of plant PFP to inactivation by both 2,3-butanedione and phenylglyoxal is entirely consistent with the proposed reaction mechanism of this enzyme. At present we cannot identify specifically which arginyl residues are affected by this treatment. However, the kinetics of inactivation suggest that no more than one arginyl residue needs to be modified to inhibit the enzyme. Moreover, the ability of substrates and substrate analogs to protect against inactivation implies that the susceptible residue is at, or near, the active site of the enzyme.

This view is strengthened by our demonstration that Fru-2,6-bisP enhances protection of PFP by Fru-6-P. This enhancement is due, in part, to an increase in the affinity of the enzyme for Fru-6-P. Previous kinetic analysis has shown that not only is Fru-2,6-bisP a potent activator of PFP, but also that one of the principal effects of this metabolite on PFP, is a decrease in the apparent K_m for Fru-6-P (Kombrink et al., 1984). This correlation suggests that the protection displayed by Fru-6-P results from binding of the substrate at the active site of the enzyme. Similarly, the protection conferred by tripolyphosphate, trimetaphosphate, and methylene diphosphonate is consistent with these compounds being reversible inhibitors of PFP (Bertagnolli and Cook, 1984). However, as yet, we are unable to explain the strong protection conferred by ATP and ADP.

Kinetic analysis has not revealed any detectable effect of ATP on PFP activity at saturating levels of Fru-2,6-bisP (Kombrink and Kruger, 1984; P. Montavon and N.J. Kruger, unpublished results). Similar effects have been observed with the tonoplast pyrophosphatase from mung bean. Inactivation of this enzyme by phenylglyoxal is prevented by the presence

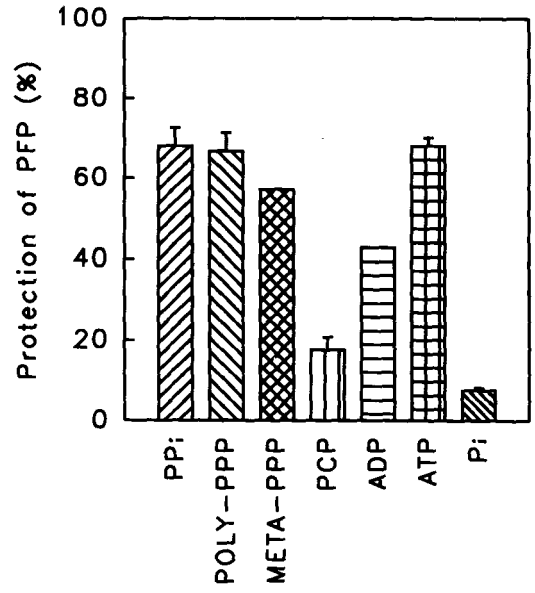

Figure 8. Specificity of protection of PFP against inactivation by 2,3-butanedione. PFP was incubated with 5 mM butanedione for 20 min in the presence of PPi, tripolyphosphate (poly-PPP), trimetaphosphate (meta-PPP), methylene diphosphonate (PCP), ADP, ATP, or Pi, each at 1 mM, after which the residual activity was measured in the glycolytic direction. The extent of protection, expressed as a percentage of initial activity, represents the mean ± SE from at least three determinations.

of an Mg-ATP complex (Kuo and Pan, 1990). However, purified pyrophosphatase activity is not inhibited by similar concentrations of Mg-ATP (Maeshima and Yoshida, 1989). The mechanism for this protection is unknown.

The kinetic properties of the inactivated PFP suggest that the principal effect of modification is a large reduction in catalytic capacity, which is accompanied by a small, although significant decrease in the affinity of the enzyme for Fru-1,6-P_2 (Table I). These properties are strikingly similar to those of a recombinant *E. coli* PFK in which residue 72 has been converted from Arg to Ser by site-directed mutagenesis (Berger and Evans, 1990). Modification of the bacterial enzyme results in a 97% decrease in the catalytic constant and a 20-fold increase in the $S_{0.5}$ value for Fru-1,6-P_2 with no significant change in the affinity of the enzyme for any of the other reactants. Thus, for both plant PFP and bacterial PFK the marked decrease in enzyme activity is due largely to a reduction in the catalytic ability of enzyme, supplemented by a decrease in the affinity of the enzyme for Fru-1,6-P_2. From this comparison we suggest that Arg^{115}, the residue in PFP_β corresponding to Arg^{72} in bacterial PFK, may be the principal site for inactivation by 2,3-butanedione or phenylglyoxal.

Three separate lines of evidence support the proposal that the catalytic site of plant PFP is equivalent to that of bacterial PFK. First, many residues thought to be important in the catalytic function of bacterial PFK are conserved in PFP_β. Based on other studies, it is this subunit that is likely to contain the active site of PFP. In contrast, far fewer identical residues are found in PFP_α, even though PFP_α and PFP_β have about the same degree of similarity to *E. coli* PFK. Second, both bacterial PFK and plant PFP are specific for β-D-fructofuranose-6-P (Bertagnolli and Cook, 1984; Younathan et al., 1981) and the equivalent divalent metal cation forms of their respective phosphate donors, namely, $MgATP^{2-}$ and $MgPPi^{2-}$ (Uyeda, 1979; Montavon and Kruger, 1992). Third, the data presented in this paper indicate that PFP, like PFK, contains an essential arginyl residue at, or near, the active site of the enzyme. Moreover, in both enzymes the arginyl group appears to be required for catalysis rather than binding of the substrates necessary for Fru-1,6-P_2 formation.

Recently, the gene encoding the homodimeric PFP from *Propionibacterium freudenreichii* has been isolated (Ladror et al., 1991). This gene appears to be related to those of higher plant PFP polypeptides and the bacterial and mammalian PFK. Based on the deduced amino acid sequence, about 30% of the residues of the amino-terminal half of *P. freudenreichii* PFP are identical with those of *E. coli* PFK, and 27% are identical with those of potato PFP_β (Ladror et al., 1991). The regions of greatest similarity are those close to the active site of the bacterial PFK. Seven of the 11 residues implicated in the binding of Fru-6-P and four residues that interact with the β- and γ-phosphate groups of ATP in the enzyme from *E. coli* are conserved in the same relative positions within *P. freudenreichii* PFP.

We appreciate that, despite the similarities noted above, the active sites of PFK and PFP must differ. Not only are these enzymes incapable of using their reciprocal phosphoryl donors, but also they differ quantitatively in their ability to bind a range of Fru-6-P analogs (Younathan et al., 1981; Bertagnolli and Cook, 1984). However, we believe that the transition state depicted in Figure 1 provides a useful model for explaining the catalytic mechanism of PFP. The presence of equivalent residues in the same relative positions within PFP from *P. freudenreichii* (Ladror et al., 1991) provides additional evidence that PFP and PFK share a common reaction mechanism. Further experiments are required to test this proposal.

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