Glutamate 325 Is a General Acid−Base Catalyst in the Reaction Catalyzed by Fructose-2,6-bisphosphatase†

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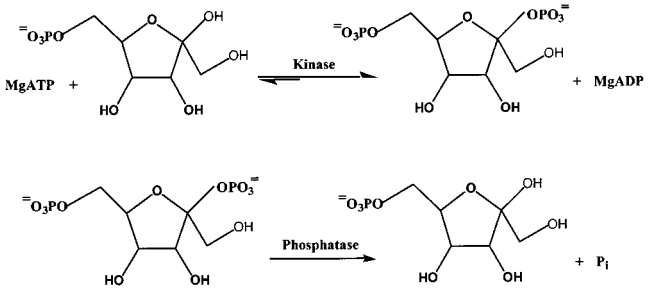
ABSTRACT: A bifunctional enzyme, fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase, catalyzes synthesis and hydrolysis of fructose 2,6-bisphosphate. The phosphatase reaction occurs in two steps: the formation of a phosphoenzyme intermediate and release of β-D-fructose 6-phosphate, followed by hydrolysis of the phosphoenzyme. The objective of this study was to determine whether E325 in the Fru 2,6-Pase active site is an acid−base catalyst. The pH−rate profile for k_{cat} for the wild-type enzyme exhibits p*K* values of 5.6 and 9.1. The pH dependence of k_{cat} for the E325A mutant enzyme gives an increase in the acidic p*K* from 5.6 to 6.1. Formate, acetate, propionate, and azide accelerate the rate of hydrolysis of the E325A mutant enzyme, but not of the wild-type enzyme. Azide and formate, the smallest of the weak acids tested, are the most potent activators. The k_{cat} vs pH profile of the E325A mutant enzyme in the presence of formate is similar to that of the wild-type enzyme. Taken together, these data are consistent with E325 serving an acid−base role in the phosphatase reaction. The exogenous low MW weak acids act as a replacement general base in the hydrolysis of the phosphoenzyme intermediate, rescuing some of the activity lost upon eliminating the glutamate side chain.

The bifunctional enzyme fructose-6-phosphate, 2-kinase: fructose-2,6-bisphosphatase catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, the most potent activator of phosphofructokinase, according to Scheme 1 (*1*).

The rat testis enzyme is a homodimer with a subunit M_r of 55 000. The enzyme monomer consists of two independent catalytic domains: a Fru 6P,2-kinase[1] domain in the N-terminal half and a Fru 2,6-Pase domain in the C-terminal half. The crystal structure of the enzyme has been determined (*2*).

The crystal structure (*3*), as well as the conservation of an active site histidine, indicates that the phosphatase domain is similar to the phosphoglycerate mutase family, which catalyzes its reaction via a phosphoenzyme intermediate (*4*−*7*). A histidine, His256 of the rat testis enzyme, has been shown to be phosphorylated (*9*). Results have led to the hypothesis that the phosphatase reaction proceeds via Scheme 2.

Scheme 1: Reactions Catalyzed by the Bifunctional Fructose-6-phosphate, 2-Kinase/Phosphatase



Scheme 2: Reaction Pathway for $F26P_2$ Phosphatase

Crystallographic data (*2*) have demonstrated that in addition to H256, other charged or polar amino acid side chains located in the active site of rat testis Fru 2,6-Pase are R255, E325, R305, Y336, Y365, and H390. Figure 1 shows a proposed reaction pathway based on the structure (*8*) and recent kinetic data (*9*). As shown in Figure 1, E325 is postulated as the general acid, which donates a proton to the leaving 2-hydroxyl of $F26P_2$ to form the F6P product. E325, once deprotonated, is then postulated to serve as a general base, activating water for the hydrolysis of the phosphoenzyme intermediate (*9*). One might expect E325 to be ionized under conditions where the enzyme activity is

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[1] Abbreviations: Fru 6P,2-kinase, fructose-6-phosphate, 2-kinase; Fru 2,6-Pase, fructose-2,6-bisphosphatase; F6P, β-D-fructose 6-phosphate; $F26P_2$, fructose 2,6-bisphosphate; DTT, dithiothreitol.

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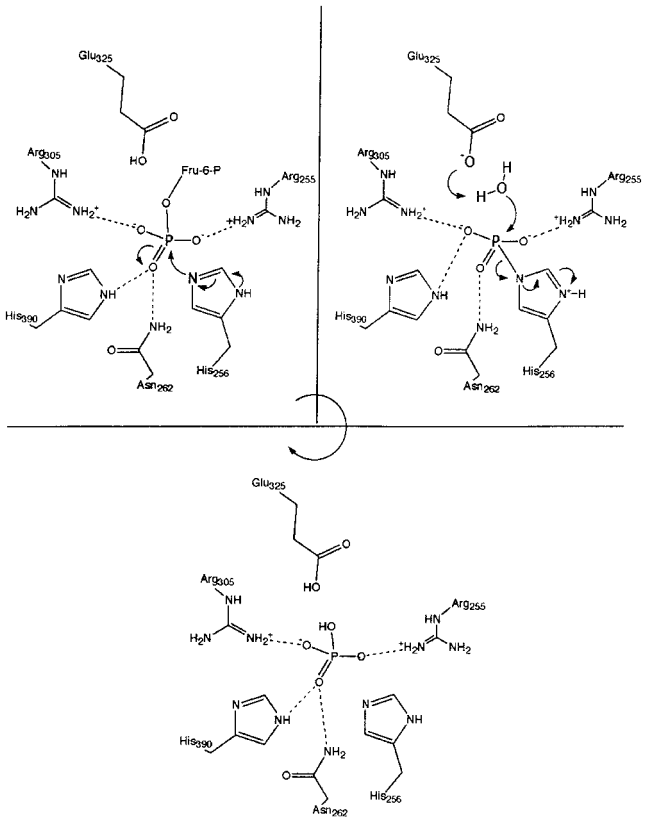


FIGURE 1: Proposed mechanism begins with formation of the E−F26P_2 complex shown in the first panel (top left). H256 attacks the 2-phosphate, resulting in a E∼P complex upon release of F6P. E325 then activates the water as a nucleophile to attack the E∼P intermediate as shown in the second panel (top right). Finally, the free phosphate remains bound in the 2-phosphate binding site (third panel, bottom) until it is displaced by another molecule of F26P_2, restarting the cycle.

optimal at pH 7. However, F26P_2 could selectively bind to enzyme with E325 protonated. Previously, Lin et al. (*10*) determined the pH dependence of k_{cat}/K_{F26P2} for the wild-type, H392A, and E327Q mutant enzymes of rat liver Fru 2,6-Pase. The authors reported the presence of two ionizable groups in the k_{cat}/K_{F26P2} pH profile with p*K* values of 6.1 on the acidic side and 8.4 on the basic side of the pH profile. They attributed the p*K* of 6.1 to the secondary p*K* of a phosphate of F26P_2 and the p*K* of 8.4 to an unidentified residue on the enzyme, respectively.

To gain insight into the role of E325, we have determined the pH dependence of k_{cat} for the phosphatase reaction. In addition, small organic acids have been used in rescue experiments. Data suggest that E325 is the general acid/base in the phosphatase reaction.

MATERIALS AND METHODS

Materials. The cDNA encoding the Trp-less mutant (RT2K-Wo) of the rat testis Fru 6P,2-kinase:Fru 2,6-Pase in which all four Trp residues of RT2K have been altered to Phe by site-directed mutagenesis was described previously (*11*). The pT7-7 RNA polymerase/promoter plasmid was a gift from Dr. Stan Tabor (Harvard Medical School) (*12*). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). The Quik Change Site-Directed Mutagenesis Kit was from Strategene. Synthetic oligonucleotides were purchased from Integrated DNA Technologies Inc. All other chemicals were of reagent grade and obtained from commercial sources.

Site-Directed Mutagenesis. Oligonucleotide-directed in vitro mutagenesis was performed using the Quik Change Site-Directed Mutagenesis Kit (Strategene).

Assay Method of Fru 2,6-Pase. This assay measures the time course for Fru 2,6-Pase continuously by coupling the production of F6P to the phosphoglucose isomerase (PGI) and glucose-6-phosphate dehydrogenase (G6PDH) reactions and measuring the appearance of NADPH fluorometically as described previously (*13*). The reaction mixture, in a final volume of 1 mL, contained 50 mM Bis-Tris-propane-HCl (pH 7.0), 0.2 mM EDTA, 50 μM NADP, 0.4 unit of G6PDH, 1 unit of PGI, and varying amounts of F26P_2. The reaction was initiated by the addition of phosphatase and followed at 25 °C. The appearance of NADPH was monitored at an emission wavelength of 452 nm upon excitation at 350 nm using a Ratio-2 fluorometer (Optical Technology Devices Co.). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADPH per minute under the above conditions. The enzyme activity at various pHs was determined using the above reaction with the exception that a saturating concentration of F26P_2 was used at all pH values. Saturation was accomplished by adding sufficient F26P_2 to generate a substrate-independent rate of reaction.

Measurement of pH-Dependent Activity. The Fru 2,6-Pase activity was determined between pH 5 and 9 under conditions in which F26P_2 was saturating at all pHs. Saturation was assured by measuring the rate of the reaction at what was believed to be a saturating concentration and again at a 2-fold higher concentration of F26P_2. In all cases, the rate was independent of the concentration of F26P_2. The pH of the reaction mixture was measured after the reaction. The enzyme activity, measured under acidic conditions, was corrected for the chemical degradation rate of F26P_2.

Data Processing. Initial velocity data obtained as a function of pH for Wo and mutant enzymes were fitted with the appropriate rate equation and a BASIC version of the Fortran programs developed by Cleland (*22*). pH profiles exhibiting slopes of 1 and −1 on acidic and basic limbs, respectively, were fitted using eq 1:

$$\log V = \log[C/(1 + H/K_1 + K_2/H)] \qquad (1)$$

where *V* represents the maximum velocity, *H* represents the hydrogen ion concentration, and K_1 and K_2 are acid dissociation constants for functional groups on enzyme required in a given protonation state for optimum reaction.

Other Methods The protein concentration was determined by the method of Bradford (*14*) using bovine serum albumin as a standard.

RESULTS

Steady-State Kinetics of Fructose-2,6-bisphosphatase. The kinetic constants of the rat testis Fru 2,6-Pase reaction of Wo and mutant enzymes are summarized in Table 1. RT2K Wo is the rat testis Fru 6P,2-kinase:Fru 2,6-Pase in which all four Trp residues have been substituted with Phe, but these mutations do not significantly alter the kinetic properties as compared with wild-type enzyme (*11*). Moreover, the E325A mutant enzyme was eluted from an affinity column

Table 1: Kinetic Parameters of the Fru 2,6-Pase Activity of Wild-Type and Mutant Enzymes

enzyme	K_{F26P2} (μM)	k_{cat} ($\times 10^{-3}$ s^{-1})	k_{cat}/K_{F26P2} ($\times 10^{3}$ M^{-1} s^{-1})
Wo	0.12	42	353
E325A[a]	9.1	1.9 (22)	0.21 (1765)

[a] E325A (14 μg) was used for assay. Fru 2,6-Pase activity was assayed as described under Materials and Methods using 0.5 μg of enzyme. The range of substrate concentrations used was 0.02−500 μM. The SEs on the parameters are less than 20%. Values in parentheses indicate the fold decrease in the respective parameter.

Table 2: Estimated pK Values for Wo and E325A

enzyme	p$K_1 \pm$ SE	p$K_2 \pm$ SE	k_{cat} (s^{-1})
Wo	5.5 ± 0.2	9.2 ± 0.2	0.052 ± 0.0004
E325A	6.5 ± 0.2	9.0 ± 0.2	0.0033 ± 0.0004
E325A + 50 mM formate	5.8 ± 0.2	8.8 ± 0.2	0.0087 ± 0.0004

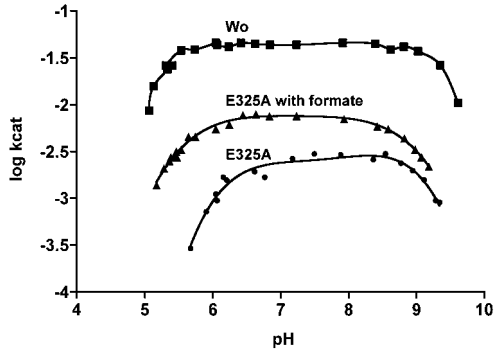


FIGURE 2: pH dependence of k_{cat} values of Wo and E325A mutant (in the absence and presence of 50 mM formate) Fru 2,6-Pases. Fru 2,6-Pase activity (0.6−42 μg of enzyme) was assayed as described under Materials and Methods with the exception that the pH was varied and that 10 μM $F26P_2$ was used for Wo and 75 μM $F26P_2$ was used for E325A. Wo was assayed using 5 μg of enzyme, while 42 μg of E325A was used. The $F26P_2$ concentration was maintained at least 4 times K_{F26P2}.

and an ion exchange column in the same position as the wild-type enzyme during purification, suggesting that there are no detectable gross changes in the global conformation. Data indicate that the k_{cat} of the E325A mutant enzyme is decreased 20-fold, while k_{cat}/K_{F26P2} is decreased about 1765-fold, suggesting E325 is a catalytically important residue.

pH Dependence of k_{cat} in Wo and Mutant Fru 2,6-Pase. Under k_{cat} conditions, hydrolysis of the phospho-His_{256} intermediate limits the overall reaction (*9*), reflecting the second panel of Figure 1. Thus, E325 is predicted to be unprotonated, and H390 could either be protonated or be unprotonated. The k_{cat} pH−rate profiles are shown in Figure 2, and the estimated pK values are shown in Table 2. The k_{cat} of Wo remains constant between pH 5.8 and 9, but decreases below and above these pHs with slopes of 1 and −1, respectively. pK values of 5.5 ± 0.2 and 9.2 ± 0.2 are obtained for Wo with a pH-independent value for k_{cat} of 0.052 ± 0.004 s^{-1}. The pH−rate profile for the E325A mutant enzyme is qualitatively identical to that obtained for Wo. The pK on the basic side of the pH−rate profile of the E325A mutant enzyme is within error identical to that of Wo, 9.0 ± 0.2, but the acidic pK is increased to 6.5 ± 0.2. The pH-independent value of k_{cat} of the E325A mutant enzyme is 0.0033 ± 0.0004. Data are summarized in Table 2.

Table 3: Activation of E325A by Weak Acids[a]

	wild type			E325A		
addition	V (mU/mg)	V (mU/mg)	fold activation	K_{act} (mM)	mol vol (Å^3)	solution pK
none	45	2	−	−	−	
formate	48	14	7	3	44.5	3.8
acetate	44	6	3	390	61.7	4.8
propionate	46	3.6	1.5	440	78	4.9
azide	43	10	5	0.1	37.6	3.7
oxalate	40	0.4	−5	−	−	

[a] Fru 2,6-Pase activity was assayed as described under Materials and Methods and Figure 3. The range of oxalate concentration was 0−100 mM. The SE on parameters is less than 20%. Data for acetate and propionate were fitted for the Michelis−Menten equation (*22*). Data for formate and acetate were fitted by eye, and K_{act} values were the concentration of the weak acid that gave $^1/_2V_{max}$ (obtained at the highest concentration used). Space left by Glu to Ala mutation is estimated as 59.2 Å^3.

Activation of E325A by Carboxylic Acids and Azide. If E325A is a general base catalyst as suggested by the pH−rate profiles, small nucleophiles with appropriate pK values could rescue the loss of function observed for the E325 mutant enzyme (*20*). Thus, a number of small acids were tested for their ability to activate the E325 mutant enzyme. Results are presented in Table 3. The most pronounced activation is observed with formate and azide added. Slight activation is observed with the larger acetate, while marginal activation is observed with propionate. Addition of oxalate inhibits, likely as a result of the additional negative charge. Of interest, saturating formate decreases the K_{F26P2} from 9 μM to 0.4 μM. The k_{cat} for E325A in the presence of formate is increased by 7-fold (ca. 30% that of Wo), while k_{cat}/K_{F26P2} is increased 630-fold (ca. 10% that of Wo).

To further characterize the activation of the E325A mutant enzyme, saturation curves were obtained at 75 μM (8 × K_{F26P2}) and increasing concentrations of the low MW acids. Intermediary plateaus are observed in the case of the two smallest activators, formate and azide (Figure 3). The plateau occurs at about 90% activation, which is close to the fractional saturation by $F26P_2$; i.e., about 90% of the enzyme will be in the E∼P form, and 10% will be in other forms, likely free E. Repeat of the experiment with $F26P_2$ at 180 μM (20 × K_m) gives a hyperbolic saturation curve for formate with a K_{act} of approximately 0.2 mM (Figure 4). Acetate and propionate, on the other hand, give hyperbolic saturation curves with k_{cat} values of 390 ± 40 and 440 ± 20 mM, repectively.

It is possible to determine the catalytically active ionization state of formate by comparing the activity at two different pHs. As shown in Figure 5, the slope of the activation curve for formate is approximately 2.5 times greater at pH 6 than it is at pH 5.5. Data suggest that ionized formate is the active form, consistent with formate acting as a general base in the hydrolysis of E∼P.

The pH profile for E325A in the presence of formate is also of importance, since it might be expected to become closer to that of Wo. Figure 2 shows the pH−rate profile in the presence of formate. The pK obtained from the acidic limb of the curve for the E325A mutant enzyme has decreased from 6.5 ± 0.2 in the absence of formate to 5.8 ± 0.2, closer to that of Wo. However, the pK on the basic side of the profile, 8.8 ± 0.2, has changed only slightly,

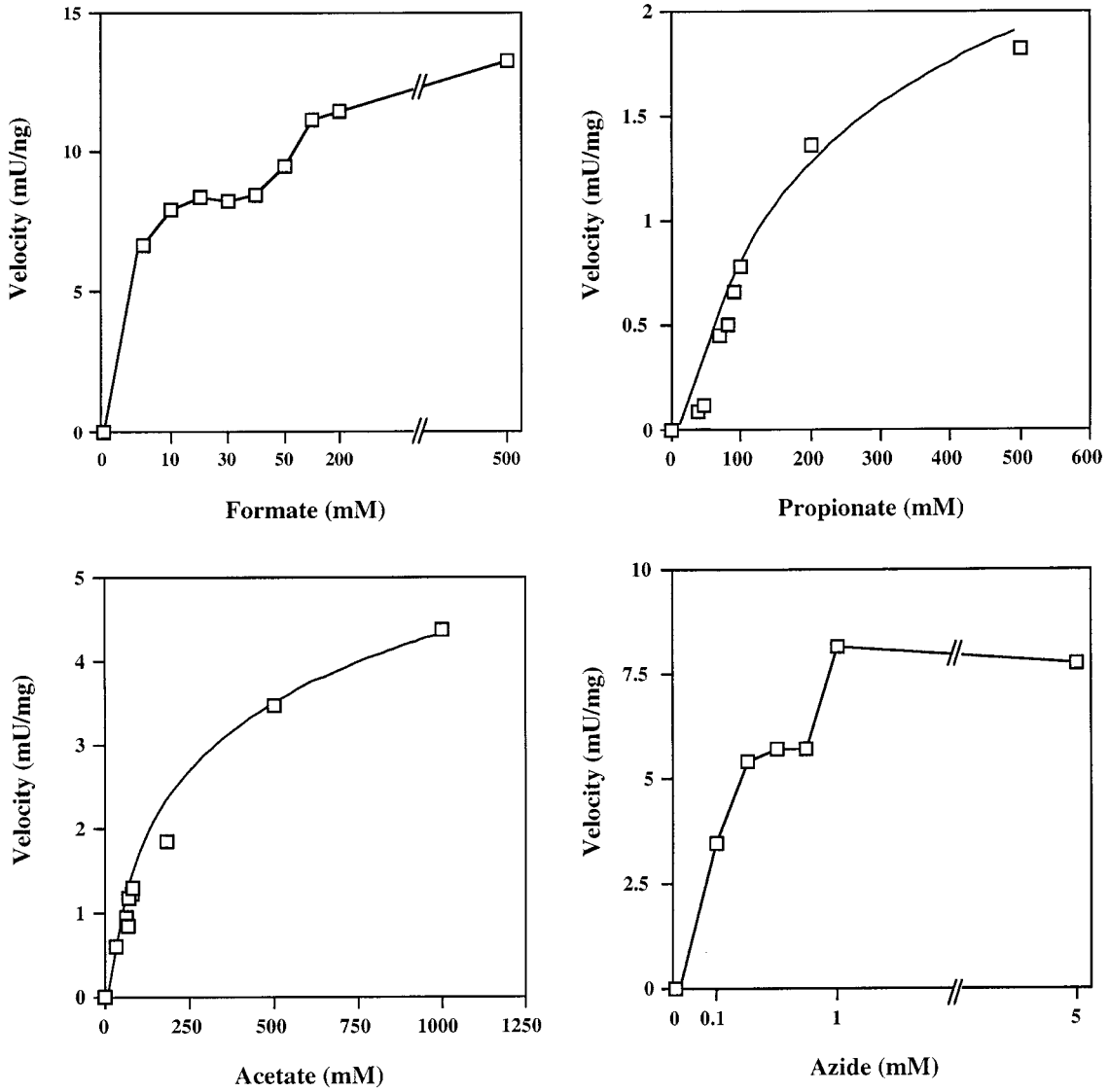


FIGURE 3: Activation of the E325A mutant enzyme as a frunction of the concentration of weak acid. Fru 2,6-Pase activity was assayed as described under Materials and Methods with the exception of 75 μM $F26P_2$ ($\sim 8 \times K_m$) and the indicated concentration of each of the weak acids. Curves are drawn by eye.

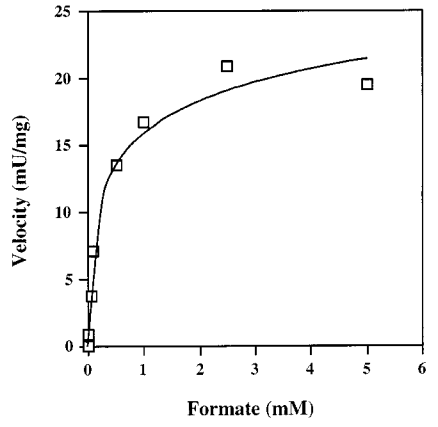


FIGURE 4: Activation of E325A mutant enzyme of formate at saturating concentration of $F26P_2$. Fru 2,6-Pase was assayed as in Figure 3, except 180 μM $F26P_2$ was added.

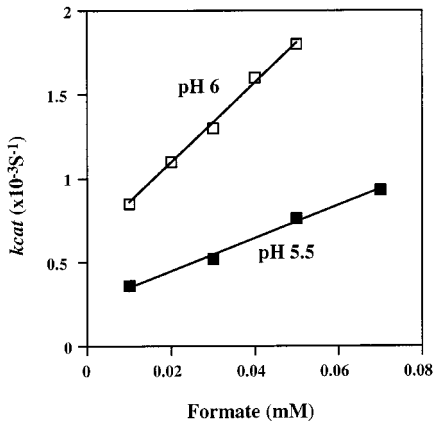


FIGURE 5: Dependence of the activity of Fru 2,6-Pase on the concentration of formate at pH 5.5 and 6.0. Fru 2,6-Pase activity was assayed as described under Materials and Methods with the exception that 75 μM $F26P_2$ and 21 μg of enzyme were used at pH 5.5 and 6.0. The ionic strength of the reaction mixture was adjusted to 0.05 with NaCl.

indicating a specific effect for the group with a p*K* of 5.6.

Activation of E325Q by Low MW Weak Acids. The same carboxylic acids and azide also activate the E325Q mutant enzymes (Table 4). This result was somewhat surprising since the glutamine side chain is expected to occupy about the same space as glutamate, leaving little room for a rescuing

Table 4: Activation of E325Q by Weak Acids[a]

additions	V (mU/mg)	fold activation	K_{act} (mM)
none	0.4	–	–
formate	6	15	36
acetate	0.7	1.8	33
propionate	0.7	1.8	130
azide	10.9	27	11

[a] Fru 2,6-Pase activity was assayed as described under Materials and Methods using 57 μg of enzyme. Values were calculated from plots of activity against weak acid concentration. The SE on parameters is less than 20%.

weak acid. The kinetics of activation differ for the two mutant enzymes, with hyperbolic plots of k_{cat} vs activator concentration obtained for all weak acids with the E325Q mutant enzyme.

DISCUSSION

The catalytic mechanism of Fru 2,6-Pase involves a two-step process: (1) formation of the phosphoryl-H256 intermediate and release of fructose 6-phosphate; and (2) hydrolysis of the phosphoenzyme intermediate (*8*, *9*). We suggest that in the course of the hydrolysis of $F26P_2$, E325 has two roles (*8*). It first acts as a general acid catalyst, responsible for protonation of the leaving 2-hydroxyl of F6P, and second as a general base, responsible for activating water in the hydrolysis of the phosphoenzyme intermediate to generate P_i (Figure 1). Previous studies (*15*) suggest that the rate-limiting step in the Fru 2,6-Pase reaction is the release of F6P, but F6P was not measured in those experiments. Results from this laboratory (*9*), however, demonstrate that when the rates of formation and release of all products and intermediates including F6P, P_i, and the phosphoenzyme intermediate were measured, hydrolysis of the phosphoenzyme intermediate is rate-limiting. Thus, measurement of k_{cat}, as was carried out in these studies, is limited to the hydrolysis of the phosphoenzyme intermediate.

Prior to these studies, Pilkis et al. (*15*) had suggested that H392 in the rat liver enzyme (H390 in the rat testis enzyme) acts as a proton donor to maintain E327 in the rat liver enzyme (E325 in the rat tesis enzyme) in a protonated state. However, according to the structure of the rate testis enzyme (*8*), H390 is not properly oriented to donate H^+ to E325. It has recently been shown that the H256A mutant enzyme, which has eliminated the active site nucleophile, exhibits a decrease in k_{cat} to a value 17% that of the wild-type enzyme (*9*). Elimination of the H256 nucleophilic catalyst by mutation results in a change in the reaction mechanism, whereby H390 likely acts as a general base to directly activate water to hydrolyze the 2-phosphate of $F26P_2$. Thus, in the absence of E325, H390 may be expected to serve as an auxiliary catalyst, perhaps via intermediacy of H_2O molecule. Consistent with this interpretation, the pK for the general base in the E325A mutant enzyme is 1 pH unit higher than that in Wo, likely reflecting the pK for H390.

There are several pieces of information obtained from the present studies that indicate the involvement of E325 as a base in the hydrolysis of the phosphoenzyme intermediate. First, the kinetic data in Table 1 show a 20-fold decrease in k_{cat} and a 1765-fold decrease in k_{cat}/K_{F26P2} when E325 is replaced with A. Second, the titrable group on the acid side of the pH−rate profile of Wo is increased by a pH unit in the E325A mutant enzyme. Finally, the use of low MW weak acids rescues some of the activity lost upon replacement of E325 with A.

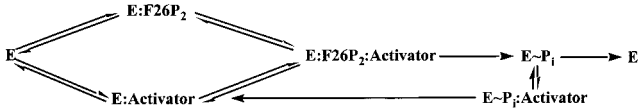
Exogenous Nucleophiles. Replacement of E325 with A is expected to generate a small cavity in the active site that could be capable of binding a small weak acid. Such binding is presumably further aided by release of F6P prior to hydrolysis of the phosphoenzyme intermediate. The weak acids could thus bind and serve as an exogenous catalyst in place of E325 in the E325A mutant enzyme, but not the wild-type enzyme. The following lines of evidence support the above conclusion: (a) there is no activity in the absence of E325A and the presence of the weak acid alone; (b) formate and azide restored k_{cat} and k_{cat}/K_{F26P2} to 30% (7-fold) and 10% (630-fold), respectively, those of the wild-type Fru 2,6-Pase activity; and (c) the pK value on the acidic limb of the pH−rate profile is decreased to approximate that of Wo.

The E325A mutation creates a cavity of about 60 Å^3 based on estimates from the crystal structure (*2*). Formate and azide have molecular volumes of 45 and 38 Å^3, respectively, and it is thus possible to model these molecules into the cavity. More bulky acids such as acetate and propionate showed higher activation constants and lower fold activation, a reflection of their poorer fit into the cavity. In the case of the weak acids that activate E325A, the pK does not appear to play a significant role, but this is likely a result of fit into the cavity being the overwhelming determinant of activation. For example, acetate (61.7 Å^3), propionate (78 Å^3), and azide (37.6 Å^3) have about the same pK value (Table 3), yet azide is 15 times more potent as an activator than the other two. Moreover, the pK of formate is lower than the acids, yet it is at least 8 times more potent. The conclusion is further supported by the fact that log k_{cat} is inversely proportional to the molecular volume but only poorly related to pK. It is thus reasonable to conclude that formate and azide are able to occupy the space vacated by the replacement of E325 with A and activate water to hydrolyze the phosphoenzyme intermediate.

It is significant, however, that the larger acids activate the E325A enzyme at all, and additionally that the E325Q mutant enzyme is activated by the same weak acids, yet the putative cavity generated in the E325A mutant enzyme is not present. The most plausible explanation for activation of E325Q is that since the hydrolysis of the phosphoenzyme is being monitored, F6P has already dissociated from the active site, providing more space for the base to occupy. In agreement with this suggestion, the kinetics of activation differ, depending on the mutant enzyme activated and the activator. In addition, it is always difficult to know, in the absence of a structure, whether the glutamine side chain occupies the same space as that of the glutamate side chain in Wo.

In the case of acetate and propionate as activators of the E325A enzyme and for all activators of the E325Q enzyme, the saturation curves for the activators are hyperbolic, while for formate and azide as activators of the E325A mutant enzyme the saturation curves exhibit an intermediary plateau. The difference in the shape of these curves has mechanistic implications. The intermediary plateau suggests two kinetic pathways for activation, while the hyperbolic saturation curves suggest only one. Data are consistent with the conditions of the assay. In these assays, a fixed concentration of 75 μM $F26P_2$ was used. The concentration is only about

Scheme 3: Mechanism of Activation by Low MW Weak Acids



90% saturating, so that in the steady state, a mixture of enzyme forms will be present. This will depend on accuracy of data, and will likely be a range in the estimated proportion of enzymes in the two forms. Approximately 90% of the enzyme will be the E∼P form present after phosphorylation of H256 since hydrolysis of the phosphoenzyme intermediate limits the overall reaction under k_{cat} conditions. The remaining enzyme will likely be E or E−F26P_2 (see Scheme III). A hyperbolic saturation curve is expected for combination of the activator with the E∼P enzyme form given the amount of space available for binding. This is the case for all of the larger weak acids for E325A mutant enzyme and all weak acids activating the E325Q mutant enzyme. However, because they are small enough, formate and azide can also activate by combining with E and E−F26P_2. If the pathway for combination of the activator to E followed by addition of F26P_2 is faster than that for combination of F26P_2 first, the mechanism will approximate a steady-state random one, and the saturation curve for the activator will be sigmoidal. Since the F26P_2 is relatively high compared to its K_m, the latter is likely the case. The saturation curves for formate and azide then should be the sum of a hyperbolic saturation curve and a sigmoidal curve, with the two components weighted by the distribution of enzyme in the E∼P, and E plus E−F26P_2 forms. In agreement with this hypothesis, saturation with F26P_2 eliminates the plateau in the forma saturation curve, and one observes binding only to the E−P enzyme form.

Other Enzymes. There are several published examples of chemical rescue of enzymatic proton transfer reactions catalyzed by small exogenously added acids or bases. Schirch and Jenkins (*16*) demonstrated general base catalysis of the serine hydroxymethyltransferase reaction by added ammonia. Burger and Ray (*17*) demonstrated general acid catalysis of the decomposition of the pyruvate−NAD adduct of lactate dehydrogenase by ammonium ion. Silverman and Tu (*18*) showed that carbonic anhydrase is accelerated by buffer ions. Smith and Hartman (*19*) demonstrated that an inactive mutant enzyme of ribulosebisphosphate carboxylase/oxygenase is specifically activated by aminomethanesulfonate. Toney and Kirsch (*20*) performed an extensive Bronsted analysis of the aspartate aminotransferase reaction catalyzed by various exogenous amines. More recently, Macleod et al. (*21*), demonstrated rate acceleration of Glu to Ala mutant of the exoglucanase/xylanase by azide and other nucleophiles. These examples of exogenous catalysis of enzymatic reactions demonstrate their usefulness in the study of enzyme reaction mechanisms.

A number of questions still remain about the mechanism of the phosphatase reaction. One of these concerns the role of E325 as a general acid in the first half of the reaction. Based on the crystal structure (*8*), the protonated form of E325 should be required to protonate the leaving 2-hydroxyl of F6P. However, this must occur at neutral pH, and the solution p*K* of E325 is expected to be around 5. The solution p*K* is not necessarily a reflection of the p*K* on the enzyme. The p*K* can be considerably higher depending on environment. A good example is the p*K* of about 7.5 for the catalytic glutamate in triose isomerase (*23*). Other questions are concerned with the identity of the group observed on the other side of the pH−rate profiles. These questions will have to await further study.

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