Roles of Active Site Residues in *Pseudomonas aeruginosa* Phosphomannomutase/Phosphoglucomutase†

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Received April 28, 2003; Revised Manuscript Received June 24, 2003

ABSTRACT: In *Pseudomonas aeruginosa*, the dual-specificity enzyme phosphomannomutase/phosphoglucomutase catalyzes the transfer of a phosphoryl group from serine 108 to the hydroxyl group at the 1-position of the substrate, either mannose 6-P or glucose 6-P. The enzyme must then catalyze transfer of the phosphoryl group on the 6-position of the substrate back to the enzyme. Each phosphoryl transfer is expected to require general acid−base catalysis, provided by amino acid residues at the enzyme active site. An extensive survey of the active site residues by site-directed mutagenesis failed to identify a single key residue that mediates the proton transfers. Mutagenesis of active site residues Arg20, Lys118, Arg247, His308, and His329 to residues that do not contain ionizable groups produced proteins for which V_{max} was reduced to 4−12% of that of the wild type. The fact that no single residue decreased catalytic activity more significantly, and that several residues had similar effects on V_{max}, suggested that the ensemble of active site amino acids act by creating positive electrostatic potential, which serves to depress the p*K* of the substrate hydroxyl group so that it binds in ionized form at the active site. In this way, the necessity of positioning the reactive hydroxyl group near a specific amino acid residue is avoided, which may explain how the enzyme is able to promote catalysis of both phosphoryl transfers, even though the 1- and 6-positions do not occupy precisely the same position when the substrate binds in the two different orientations in the active site. When Ser108 is mutated, the enzyme retains a surprising amount of activity, which has led to the suggestion that an alternative residue becomes phosphorylated in the absence of Ser108. ^{31}P NMR spectra of the S108A protein confirm that it is phosphorylated. Although the S108A/H329N protein had no detectable catalytic activity, the ^{31}P NMR spectra were not consistent with a phosphohistidine residue.

In the bacterium *Pseudomonas aeruginosa*, the *algC* gene encodes the dual-specificity enzyme phosphomannomutase/phosphoglucomutase, which catalyzes the interconversion of mannose 6-P and mannose 1-P, as well as the interconversion of glucose 6-P and glucose 1-P. Genetic studies have established that the PMM[1] activity is required for both alginate and lipopolysaccharide biosynthesis, and that the PGM activity is required for LPS biosynthesis (*1*). Recently, it has been shown that *algC* is also required for rhamnolipid biosynthesis (*2*), and that rhamnolipids are required for mature biofilm formation (*3*). Since alginate, LPS, and rhamnolipid are all important virulence factors, PMM/PGM may be a useful target for pharmaceutical intervention in combating *P. aeruginosa* infections, and detailed characterization of PMM/PGM should facilitate the development of useful inhibitors.

Steady-state kinetic characterization of the PMM and PGM activities in the biosynthetically relevant direction established that mannose 6-P and glucose 6-P are accepted by PMM/PGM as substrates with equal efficiency (*4*). The active site has been identified through structural studies (*5*); Ser108 is phosphorylated in the resting enzyme, and the catalytic mechanism is believed to involve initial transfer of the phosphoryl group from Ser108 to the substrate to generate a bis-phosphorylated intermediate. To complete the catalytic cycle, the intermediate must reorient and then rephosphorylate Ser108 using the phosphoryl group that was originally on the substrate. The net effect of these group transfers is to move a phosphoryl group from the 6-position to the 1-position of the substrate and regenerate the active, phosphorylated enzyme. Each phosphoryl transfer is expected to require general base catalysis to increase the nucleophilicity of the hydroxyl group to be phosphorylated, and general acid catalysis to stabilize the group from which the phosphoryl group was transferred. With a structure of PMM/PGM containing ligands bound at the active site in hand, we have used site-directed mutagenesis and kinetic studies to identify the residues that serve as general acid−general base residues in the catalytic reaction. Surprisingly, we found that no single residue could be identified as playing a critical role in the catalytic reaction; rather, mutation of each of several residues led to significant decreases in V_{max}, but did not abolish activity. We also have investigated further the surprising observation we reported earlier (*4*), that mutation of Ser108 to alanine led to an only 20-fold reduction in V_{max}. We now

† Supported by NIH Grant GM59653.

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[1] Abbreviations: PMM/PGM, phosphomannomutase/phosphoglucomutase; LPS, lipopolysaccharide; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CD, circular dichroism; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

10.1021/bi034673g CCC: $25.00
Published on Web 07/26/2003

provide direct spectroscopic evidence that the S108A mutant protein is phosphorylated, although the site of phosphorylation remains obscure. The picture that emerges from these mutational studies is of an unusually plastic active site in which the reactivity of the substrate is modulated by an ensemble of amino acids, rather than one in which individual amino acids play unique, easily identifiable roles.

MATERIALS AND METHODS

Glucose 1-P, glucose 1,6-P_2, and glucose 6-P dehydrogenase from *Leuconostoc mesenteroides* were obtained from Sigma.

The *alg*C gene, encoding PMM/PGM, was subcloned into the pET-14b vector (Novagen) to allow for the production of PMM/PGM containing a His_6 tag at the N-terminus. *Escherichia coli* BL21(DE3) cells were transformed with the expression plasmid; cell growth, preparation, and treatment of the cell free extract with protamine sulfate and ammonium sulfate were performed as described previously (*4*). The ammonium sulfate pellet containing PMM/PGM was dissolved in 20 mM sodium phosphate buffer (pH 7.4) containing 10 mM imidazole and 0.5 M NaCl (buffer A). The sample was applied to a Chelating Sepharose (Pharmacia) column charged with Ni^{2+} and equilibrated in buffer A. The column was washed with buffer A until the absorbance of the eluate at 280 nm returned to the baseline. PMM/PGM was eluted from the column by washing with buffer A supplemented with 140 mM imidazole. Fractions containing PMM/PGM were identified by enzymatic activity or by SDS−PAGE. The desired fractions were pooled, concentrated by ultrafiltration, supplemented with glycerol to 10% (v/v), and stored at −80 °C.

Site-directed mutants of PMM/PGM were constructed using the QuikChange mutagenesis kit (Stratagene); the integrity of each mutated *alg*C gene was verified by automated DNA sequencing. Mutants were purified in the same manner as the wild-type protein. Purified proteins were characterized by CD spectroscopy to determine whether gross changes in secondary structure had been incurred by the mutations.

Wild-type PMM/PGM and the S108A mutant were characterized by MALDI-TOF, after in-gel trypsin digestion, at the University of Missouri Proteomics Center. The expected peptides and the masses were calculated with the Peptide-Mass algorithm at the ExPASy website (http://us.expasy.org/cgi-bin/peptide-mass.p1). The mass spectrometry analysis was conducted with approximately 10 μg of each protein sample.

^{31}P NMR spectra of PMM/PGM S108A were acquired using a Bruker DMX500 spectrometer operating at 203 MHz. The sample was dialyzed exhaustively to remove the phosphate buffer from the metal affinity chromatography step, and the protein was concentrated to 1 mM. The sample was prepared in H_2O in 50 mM MOPS (pH 7.4) containing 0.5 mM DTT; 50 μL of d_6-DMSO was added to the 0.6 mL sample to provide a lock signal. Spectra were obtained using an acquisition time of 0.5 s, a relaxation delay of 0.5 s, and approximately 50 000 scans. After the spectrum of the native protein had been acquired, NaOH was added to increase the pH to 12, and a spectrum of the denatured protein was obtained.

Table 1: Kinetic Parameters for PMM/PGM Site-Directed Mutants in the Conversion of Glucose 1-P to Glucose 6-P

protein	V_{max} (s^{-1})	V/K (s^{-1} μM^{-1})	K_m (μM)	K_i (μM)
wild type	22 ± 1	1.8 ± 0.2	12 ± 1	110 ± 10
H109Q	1.3 ± 0.1	0.15 ± 0.06	8 ± 3	−[a]
H308N	22 ± 2	2.1 ± 0.4	10 ± 2	160 ± 30
H329N	1.4 ± 0.3	0.09 ± 0.04	16 ± 6	170 ± 80
K118L	0.9 ± 0.2	0.12 ± 0.05	8 ± 3	170 ± 80
R20A[a]	2.6 ± 0.1	0.9 ± 0.2	2.8 ± 0.7	−[a]
R247A[a]	1.9 ± 0.1	0.22 ± 0.06	9 ± 2	−[a]
H308N/H329N[a]	1.02 ± 0.01	0.8 ± 0.4	1.3 ± 0.7	−[a]
K118L/H109Q[a]	1.1 ± 0.1	0.4 ± 0.1	2.7 ± 0.8	−[a]

[a] No substrate inhibition observed.

The activity of each mutant protein was quantitated by measuring the PGM activity in the direction of glucose 6-P formation, using a coupled assay with glucose 6-P dehydrogenase. The concentration of glucose 1-P was varied from 1 to 200 μM. NAD^+ was present at 0.9 mM. Glucose 1,6-P_2 was present at 0.5 μM. $MgSO_4$ was present at 1.5 mM. Reactions were carried out in 50 mM MOPS (pH 7.4) containing 1 mM DTT. The reaction was monitored by measuring the rate of production of NADH, based on its fluorescence at 430 nm when excited at 340 nm. Changes in the fluorescence signal per unit time were converted to concentration units by constructing a standard curve with authentic NADH. The kinetic data were fitted to either the Michaelis−Menten equation or eq 1 when substrate inhibition was observed.

$$v = \frac{VA}{K_a + A + A^2/K_I} \quad (1)$$

RESULTS

For the studies reported here, PMM/PGM was produced with a His tag, to ensure that the activity that was measured arose from PMM/PGM and not from contaminating *E. coli* PGM. All of the mutants were soluble, and their CD spectra were identical with those of the wild-type protein, suggesting that the mutations that were introduced did not cause dramatic changes in structure. A comparison of wild-type PMM/PGM with and without the His tag demonstrated that it did not affect the kinetic properties of the enzyme (data not shown). All of the proteins produced for this study were >95% pure after metal affinity chromatography, based on Coomassie Blue-stained SDS−PAGE gels.

The kinetic properties of the mutant proteins that were produced to examine the roles of potential acid−base catalytic residues are listed in Table 1. It can be seen that the H109Q, H329N, K118L, R20A, and R247A proteins have similar kinetic properties. In each instance, V_{max} and V/K are reduced to 5−10% of the values of the wild-type enzyme. The H308N protein has kinetic properties quite similar to those of the wild-type enzyme.

A second group of mutant enzymes was prepared in an attempt to identify the putative alternative phosphorylation site, that is, the residue that is phosphorylated in the absence of Ser108 (Table 2). The S108A protein exhibited a V_{max} that was greater than 10% of the value of the wild-type enzyme; V/K was unchanged from that of the wild type. When Ser108 was replaced with a charged residue (aspartate), V_{max} decreased 10-fold as in the S108A protein, and

Table 2: Kinetic Parameters for PMM/PGM Ser108 Mutants in the Conversion of Glucose 1-P to Glucose 6-P

protein	V_{max} (s^{-1})	V/K (s^{-1} μM^{-1})	K_m (μM)	K_i (μM)
wild type	22 ± 1	1.8 ± 0.2	12 ± 1	110 ± 10
S108A	2.6 ± 0.1	2.0 ± 0.5	1.3 ± 0.3	500 ± 190
S108V	0.22 ± 0.03	0.022 ± 0.006	10 ± 2	280 ± 100
S108D	1.52 ± 0.08	0.44 ± 0.09	3.4 ± 0.6	—[a]
S108A/H109Q	1.38 ± 0.06	0.6 ± 0.1	2.4 ± 0.4	—[a]
S108A/H308N	0.62 ± 0.07	0.12 ± 0.05	5 ± 2	160 ± 10
S108A/H329N	nd[b]	nd[b]	nd[b]	—[a]

[a] Substrate inhibition was not observed in these reactions. [b] Not detectable.

V/K decreased 4-fold relative to that of the wild type. Replacement of Ser108 with valine had a more dramatic effect on V_{max}, which decreased to 1% of the wild-type value; *V/K* also decreased approximately 100-fold. The three active site histidine residues were each mutated in the S108A mutant protein, to create three double mutants. The S108A/H109Q and S108A/H308N proteins exhibited kinetic properties similar to those of the S108A and H109Q single mutants. However, the S108A/H329N double mutant exhibited no detectable kinetic activity.

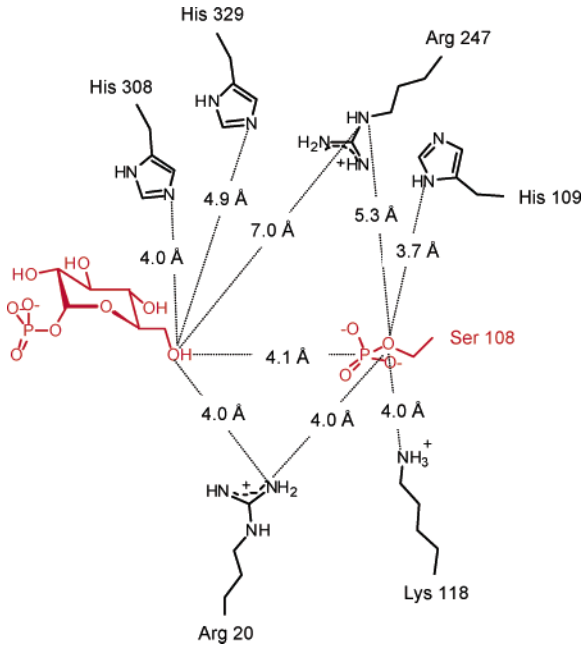
We confirmed that the S108A protein was phosphorylated by ^{31}P NMR. The spectrum of the native protein exhibited a broadened peak at −0.80 ppm, which we attribute to the phosphorylated protein, a peak at 2.2 ppm that we attribute to inorganic phosphate, and a peak at 5.4 ppm, the origin of which is unknown. Upon denaturation of the enzyme by addition of NaOH to increase the pH to 12, the peak at −0.80 ppm disappeared, and was replaced with a 47 Hz wide peak at 3.9 ppm.

We explored the possibility of determining the site of phosphorylation in the S108A mutant directly by mass spectrometry. Preliminary measurements of the mass of PMM/PGM and various mutants strongly suggested that the proteins were phosphorylated (data not shown). However, ambiguities arising from methionine oxidation and matrix effects led us to seek improved precision by characterizing tryptic peptides rather than the entire protein. The wild-type protein yielded a peptide with a mass of 2000.17 Da, which corresponds to the Ser108-containing peptide that extends from residue 101 to 118. The calculated mass of the phosphorylated peptide is 2000.10 Da, consistent with our expectation that Ser108 is phosphorylated. In the S108A protein, the peptide of residues 101−118, which was not expected to be phosphorylated, had an observed mass of 1903.22 Da, which compares well with the calculated mass of 1902.88 Da. On the basis of the kinetic analysis of the double mutants, we sought to determine whether His329 was phosphorylated in the S108A protein. His329 is contained in the peptide of residues 317−333 (peptide 317−333) that is generated by trypsin digestion. In the wild-type and S108A mutant proteins, the observed masses for peptide 317−333 were 1791.91 and 1792.12 Da, respectively. The calculated mass for the nonphosphorylated peptide is 1791.87 Da. Thus, the mass spectrometry analysis provided no evidence that His329 is phosphorylated in the S108A mutant.

DISCUSSION

In each catalytic cycle, PMM/PGM must catalyze two chemical events, the first a phosphoryl transfer from the enzyme to the substrate and the second a phosphoryl transfer from the intermediate back to the enzyme. The chemistry of phosphoryl transfers has been studied extensively (*6*). Regardless of whether the group transfer proceeds via an associative or dissociative mechanism, the hydroxyl group that is being phosphorylated must be deprotonated, so one would expect the reaction to be facilitated by an amino acid residue acting as a general base. For example, the crystal structure of brain hexokinase I establishes that Asp657 is within 3 Å of the 6-OH group of glucose bound at the active site (*7*). The hydroxyl group must be deprotonated to allow phosphorylation of glucose by MgATP, and the activity of the D657A mutant protein is too low to characterize (*8*), suggesting that Asp657 serves as the general base in the hexokinase reaction. Phosphoryl group transfer should also be facilitated by general acid catalysis, to stabilize via protonation the alkoxide formed at the position from which the phosphoryl group departs. In the first half of the reaction catalyzed by PMM/PGM, the leaving group is the side chain of Ser108. An analogous reaction is that catalyzed by calcineurin, which catalyzes the dephosphorylation of phosphoserine and phosphothreonine residues; it has been proposed that His151 functions as a general acid to protonate the leaving serine or threonine residue (*9*).

Chart 1



Examination of the active site of PMM/PGM with a substrate bound reveals a rich array of amino acid residues that could potentially serve as general acid−base catalysts (Chart 1). Arg20, His109, and Lys118 are all appropriately located to act as a general acid to stabilize Ser108 during phosphorylation of the substrate, and to deprotonate the Ser108 side chain when it is being rephosphorylated by the reaction intermediate. His308 is 4 Å from the hydroxyl at the 6-position of the substrate, and appeared to be likely to act as a general base. Arg247 and His329 are 5−7 Å from the phosphorylation site, and were considered potential candidates as well. The disordered electron density for Arg20 and Arg247 indicates that those residues may be mobile, and therefore, we considered the possibility that dynamic motions

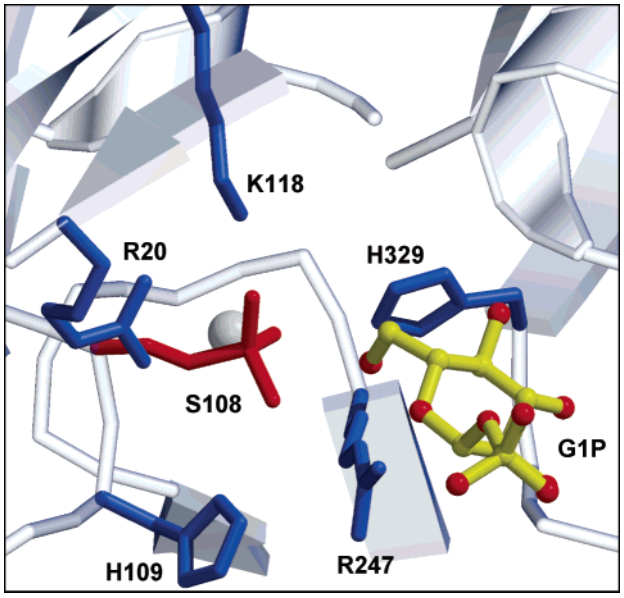


FIGURE 1: Close-up view of the PMM/PGM active site with glucose 1-P bound. Residues that are believed to play roles in the catalytic reaction are indicated.

during the catalytic cycle could bring one of them, or another residue, closer to the substrate.

Surprisingly, mutagenesis of either Arg20, His109, Lys118, Arg247, or His329 to a residue that could not participate in general acid−base catalysis resulted in a substantial loss of catalytic activity, as reflected in V_{max}; however, none of the mutants lost so much activity that the mutated residue could be considered to be absolutely critical for catalysis. Of all the mutant proteins that were generated, only H308N was unchanged in its kinetic parameters from the wild-type enzyme. The V_{max} values in the other mutants varied from 4 to 12% of that of the wild-type enzyme; such changes are clearly within the limits of the accuracy of the kinetic experiments, yet are far smaller than what would be expected for loss of a residue that played a unique, required role in the reaction. For example, mutation of Glu165, which acts as the general base in the triose phosphate isomerase reaction, to an aspartate residue resulted in a decrease in V_{max} of 1000-fold (*10*), and mutation of His95, which acts as the general acid, caused a 400-fold decrease in V_{max} (*11*). A trivial explanation for the residual activity that was measured with PMM/PGM is that it arises from contaminating *E. coli* PGM, but we consider that unlikely since each mutant protein was purified by metal affinity chromatography and appeared to be pure as determined by SDS−PAGE.

Since no single residue could be identified as the expected general base or general acid, but rather several residues appeared to be important for full activity, we considered what the effect of the ensemble of amino acids would be. Figure 1 shows how Arg20, His109, Lys118, Arg247, and His329 are arranged at the active site. Figure 2 shows the electrostatic potential generated by the amino acid residues at the active site, and it can be seen that the phosphorylated serine residue and the reactive hydroxyl group on the substrate lie between two regions of positive electrostatic potential. This arrangement suggests a possible explanation for the kinetic data and a mechanism for facilitating phosphoryl transfer. In the presence of a positive electrostatic field, the p*K* of the substrate hydroxyl group should be decreased. If the effect is sufficiently substantial the hydroxyl group may ionize "spontaneously", i.e., the substrate may be present as the alkoxide at the active site, so no general base is necessary for phosphorylation. Similarly, the Ser108 alkoxide arising from phosphorylation of the substrate should be stabilized by the positive electrostatic field, so general acid catalysis would not be necessary. In this manner, the enzyme can enhance the nucleophilicity of the phosphorylation acceptor group and stabilize the leaving group, but without utilizing classic general acid−base catalysis. The catalytic function of the ensemble of amino acids would account for the substantial, but incomplete, loss of activity in each of the single mutants. The active site of PMM lies in a deep cleft, and only 5% of the surface of the substrate is solvent-exposed; as a result of sequestration of the substrate from solvent, electrostatic effects arising from the protein should be enhanced.

An alternative explanation of the mutagensis data is that the active site is constructed in a highly redundant fashion: if one residue is removed, another assumes its function. To evaluate that possibility, the K118L/H109Q double mutant was constructed. Lys118 and His109 are each appropriately located to act as the general acid to stabilize the Ser108 alkoxide formed by phosphorylation of the substrate. *A priori*, it seemed quite reasonable to suppose that either Lys118 or His109 could be the expected general acid. However, removal of either residue by mutagenesis did not decrease the catalytic activity as much as we expected. To explain these data, we hypothesized that in the artificial situation created by mutagenesis where one residue is absent, the other would substitute. However, the kinetic properties of the double mutant were quite similar to those of the two single mutants, demonstrating that the enzyme could capably function without either residue. Obviously, many other pairwise mutant possibilities are possible, and we have not explored them all; therefore, it is formally possible that each mutation induces a different residue to function as a general acid−base catalyst. Such a scenario does not really explain why each individual mutation causes a significant diminution in V_{max}, however, and therefore, we do not favor this explanation.

A potential rationale for the strategy of utilizing electrostatic forces to ionize the substrate is that the enzyme must bind each of its substrates in two orientations, and it must accommodate two different substrates, mannose 6-P and glucose 6-P. Perhaps by utilizing electrostatic forces to favor the ionization of the substrate hydroxyls, the necessity of precise positioning of each reactive hydroxyl within reach of a general base is avoided. Comparison of the structures of PMM/PGM with ligands bound shows that mannose 6-P and glucose 6-P do not place their reactive hydroxyl (at the 1-position) at the same position as mannose 1-P and glucose 1-P do (in which the reactive hydroxyl is at the 6-position).[2] However, in either orientation, the reactive hydroxyl group is placed within the electrostatic field created by the electropositive active site residues. The N-terminal end of the α-helix extending between residues His308 and Thr318

[2] C. Regni and L. Beamer, manuscript in preparation. The coordinates of the 1.6 Å structures of PMM/PGM with bound glucose 1-P and with bound glucose 6-P have been deposited with the Protein Data Bank as entries 1P5D and 1P5G, respectively.

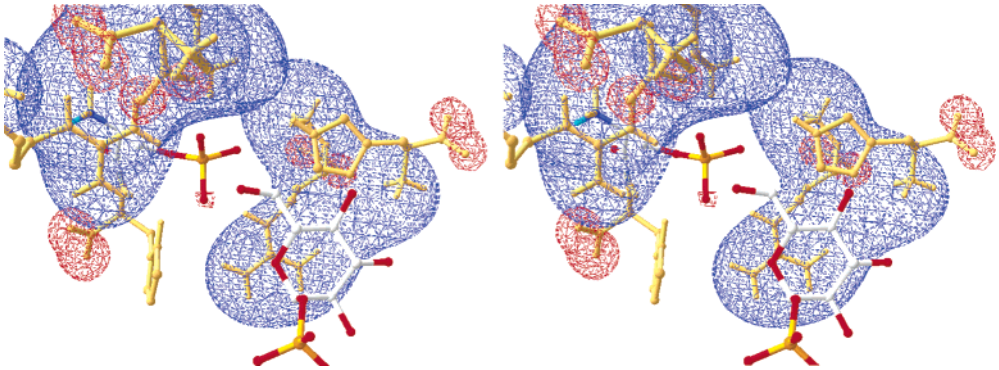


FIGURE 2: Electrostatic potential in the active site of PMM/PGM. The potentials from Coulombic interactions between the amino acid residues shown were calculated in DeepView and are contoured at ±2.55 kT/e; blue is positive potential, and red is negative potential.

also points into the active site, and the positive dipole moment arising from the helix could reinforce the effect caused by the active site residues.

Rabbit muscle phosphoglucomutase (PDB entry 1C47) has a similar constellation of amino acid residues arranged in a manner like that seen in PMM/PGM; the proximal phosphoryl group is encircled by Arg22, His117, Lys129, Arg292, and Lys388. A BLAST comparison of *P. aeruginosa* PMM/PGM with the 50 most closely related sequences reveals that Arg20, His109, Lys118, Arg247, and His329 (*P. aeruginosa* numbering) are conserved with only two or three exceptions. Since all of these enzymes, and rabbit muscle PGM, face the same task as PMM/PGM, the positive electrostatic ring around the phosphoryl group may be a general strategy adopted to enhance the nucleophilicity of the reactive hydroxyl groups.

It is interesting to note that the K_m for glucose 1-P did not change significantly in any of the mutant proteins. The values that were measured when H109, K118, H308, H329, and R247 were mutated were within experimental error of the value observed with the wild-type protein. The K_m in the R20A protein was lower than that observed in the wild-type protein. These results suggest that the determinants for binding are other amino acid residues at the active site.

Mutation of His109, Arg20, and Arg247 relieved the weak substrate inhibition that is observed with the wild-type enzyme. Since substrate inhibition arises from the competition between the substrate and the bis-phosphorylated intermediate for binding to the dephosphoenzyme, the loss of substrate inhibition in these mutants suggests that, in addition to their role in promoting phosphoryl transfer, these residues are important in the binding of the intermediate to the enzyme.

Another surprising feature of *P. aeruginosa* PMM/PGM is the extensive activity of the proteins in which Ser108 is mutated. The kinetic behavior of the enzyme strongly supports the proposed role of phosphoserine in the catalytic reaction (*4*), and the crystal structure of the enzyme demonstrates that Ser108 is phosphorylated (*5*). However, the S108A mutant retained a value of V_{max} that was 12% of that of the wild type, and the S108D mutant had a V_{max} that was 7% that of that of wild type. We reported these observations earlier and suggested that in the absence of Ser108 another residue at the active site became phosphorylated (*4*). Examination of the active site suggests that one of several histidine residues might be appropriately positioned to act as a surrogate for Ser108. To further investigate the origin of the activity in the absence of Ser108, we have constructed a series of double mutants, the kinetic properties of which are listed in Table 2. While the S108A/H109Q and S108A/H308N double mutants did not differ substantially in their kinetic properties from either the S108A or H109Q single mutant, the S108A/H329N double mutant had no detectable catalytic activity. Its CD spectrum was identical to that of the wild-type protein, so it appears that its lack of catalytic activity is not a consequence of misfolding. Therefore, these data suggested that His329 is the alternative site of phosphorylation in PMM/PGM.

However, the ^{31}P NMR data allow us to rule out phosphorylation of His329, because the signal arising from the phosporylated residue appears too far downfield. 3-*N*-Phosphohistidine and 1-*N*-phosphohistidine resonate at −3.9 and −4.7 ppm, respectively.(*12*). Pyruvate phosphate dikinase is a well-characterized enzyme that has a phosphohistidine residue. The native protein is characterized by a ^{31}P NMR peak at −4.0 ppm, which shifts to −3.9 ppm when the protein is denatured in 5 M urea (*13*). In comparison, the ^{31}P NMR signal for PMM/PGM S108A shifts from −0.8 ppm in the native protein downfield to 3.9 ppm in the denatured protein. Clearly, His329 plays a critical role in the reaction catalyzed by the S108A mutant, but it does not appear to be the site of phosphorylation.

In summary, the results of the mutagenesis experiments described here yield a picture of PMM/PGM as a surprisingly elastic and resilient enzyme. The alternative phosphorylation site is probably a quirk of the enzyme, with no mechanistic or physiological significance. The strategy that is utilized for the required proton transfers is, however, probably mechanistically significant. By constructing an active site in which substrate ionization can occur without precise positioning of the reactive hydroxyl near one particular residue, the enzyme has developed the means of accommodating the distinct geometrical orientations of the substrate that occur during the catalytic cycle.

REFERENCES

1. Ye, R. W., Zielinski, N. A., and Chakrabarty, A. M. (1994) *J. Bacteriol. 176* (16), 4851−4857.
2. Olvera, C., Goldberg, J. B., Sanchez, R., and Soberon-Chavez, G. (1999) *FEMS Microbiol. Lett. 179*, 85−90.
3. Davey, M. E., Caiazza, N. C., and O'Toole, G. A. (2003) *J. Bacteriol. 185* (3), 1027−1036.
4. Naught, N. E., and Tipton, P. A. (2001) *Arch. Biochem. Biophys. 396*, 111−118.

5. Regni, C., Tipton, P. A., and Beamer, L. J. (2002) *Structure 10*, 269−279.
6. Cleland, W. W., and Hengge, A. C. (1995) *FASEB J. 9*, 1585−1594.
7. Aleshin, A. E., Kirby, C., Liu, X., Bourenkov, G. P., Bartunik, H. D., Fromm, H. J., and Honzatko, R. B. (2000) *J. Mol. Biol. 296*, 1001−1015.
8. Arora, K. K., Filburn, C. R., and Petersen, P. L. (1991) *J. Biol. Chem. 266* (9), 5359−5362.
9. Rusnak, F., and Mertz, P. (2000) *Physiol. Rev. 80* (4), 1483−1521.
10. Straus, D., Raines, R., Kawashima, E., Knowles, J. R., and Gilbert, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2272−2276.
11. Nickbarg, E. B., Davenport, R. C., Petsko, G. A., and Knowles, J. R. (1988) *Biochemistry 27*, 5948−5960.
12. Gassner, M., Stehlik, D., Schrecker, O., Hengstenberg, W., Maurer, W., and Ruterjans, H. (1977) *Eur. J. Biochem. 75*, 287−296.
13. Thrall, S. H., Mehl, A. F., Carroll, L. J., and Dunaway-Mariano, D. (1993) *Biochemistry 32*, 1803−1809.

BI034673G