Arginine 304 Is an Active Site Residue in Phosphomannose Isomerase from *Candida albicans*

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Received November 29, 1993; Revised Manuscript Received February 23, 1994®

ABSTRACT: The reaction catalyzed by *Candida albicans* phosphomannose isomerase (PMI) (EC 5.3.1.8) has a bell-shaped pH dependence, with pK_a's at 5.6 and 8.7. The enzyme can be inhibited in a time-dependent manner using the arginine-specific modification reagent phenylglyoxal. This modification takes place with a rate constant of 0.022 ± 0.002 min^{-1} mM^{-1} at 37 °C in 50 mM Hepes buffer, pH 8.5. The enzyme can be protected from this inactivation by the addition of the substrate mannose 6-phosphate at concentrations close to its K_m value. The pH dependence of the inactivation reaction shows a single pK_a at 9.1 ± 0.1, which is close to one of the values for the pH dependence of the enzyme-catalyzed reaction. Using [7-^{14}C]phenylglyoxal, it is shown that a single molecule is incorporated into the enzyme in the absence of substrate and that this inactivates the enzyme. This incorporation of radioactivity is prevented by the coincubation with substrate. The modified protein has then been reduced with sodium borohydride to fix the modification and then cleaved with Asp-N protease. The resultant peptides were separated by HPLC, and the radioactivity was counted. Sequencing of the peptide with the highest incorporation level identified it as DNVVRAGFTPKFK, which corresponds to amino acids 300–312 of phosphomannose isomerase. Radioactive counting of the phenylthiohydantoin amino acid derivatives confirmed that the modified amino acid was arginine 304. The role of this residue in the catalytic reaction of phosphomannose isomerase is discussed.

Phosphomannose isomerase (PMI)[1] catalyzes the interconversion of fructose 6-phosphate and mannose 6-phosphate. It is a metalloenzyme and contains one atom of zinc per protein molecule, and this metal is essential for activity (Gracy & Noltman, 1968). The enzyme is important in the biosynthesis of yeast cell walls, since it is the first step in the biosynthesis of cell wall mannoproteins from glycolytic intermediates. Its importance has been confirmed by the isolation of temperature-sensitive mutants of the PMI gene in *Saccharomyces cerevisiae*. These strains undergo cell lysis at the restrictive temperature (Smith et al., 1992). The gene coding for this enzyme has recently been cloned from an ordered array genomic library of *Candida albicans* cDNA (David J. Smith, unpublished results). Overexpression of the protein in *Escherichia coli* has enabled us to carry out detailed studies of the enzyme mechanism.

The enzyme shows a bell-shaped pH dependence with pK_a values of 5.6 and 8.7 for the free enzyme and 6.0 and 9.7 for the enzyme-substrate complex. One candidate for the amino acid whose side chain is responsible for the lower pK_a is histidine. Experiments with diethyl pyrocarbonate have shown the presence of a substrate-protectable histidine (Timothy N. C. Wells, unpublished results). Candidates for the high-pH group include lysine, arginine, cysteine, and the essential zinc atom. The analogous enzyme phosphoglucose isomerase has been shown to have two active site lysines, Lys-263 and Lys-577, by modification with pyridoxal phosphate (Marchand et al., 1989). However, no inhibition of phosphomannose isomerase could be obtained with pyridoxal phosphate (Timothy N. C. Wells, unpublished results). Phosphomannose isomerase can be irreversibly inhibited with the cysteine modification reagent iodoacetate (Coulin et al., 1993). The substrate-protectable group was shown to be Cys-150. However, this group is unlikely to be one of the catalytic bases. The residue is not conserved in phosphomannose isomerase from *E. coli* or *Salmonella typhimurium* (Proudfoot et al., 1994), and the *E. coli* enzyme is insensitive to iodoacetate (Coulin et al., 1993). Therefore the two most likely candidates for the residue responsible for the higher pK_a are an arginine or the essential zinc.

A further role for a group with pK_a = 9.2 has been shown in metal ion inhibition experiments (Wells et al., 1993). Zinc, cadmium, and mercury are micromolar inhibitors of the enzyme. pH-dependence studies of this inhibition showed that in the case of zinc and cadmium ions there is a group within the enzyme active site that has a pK_a of approximately 9.0 that inhibits the binding of both hydrated zinc and cadmium ions at pH values above the pK_a. We have therefore investigated the possibility that an active site arginine may be play a role in the active site of phosphomannose isomerase, by studying the effects of the arginine-specific chemical modification reagent phenylglyoxal (Takahashi, 1968).

MATERIALS AND METHODS

Unless otherwise stated, chemicals were purchased from Sigma and enzymes were purchased from Boehringer Mannheim.

Enzyme Purification and Assay. The enzyme was expressed in *E. coli* and purified using methods similar to those described previously (Proudfoot et al., 1994). The final protein was judged greater than 95% pure by SDS–PAGE. The activity of phosphomannose isomerase was measured routinely at 37 °C in a coupled assay where the product fructose 6-phosphate is converted into glucose 6-phosphate and then oxidized to 6-phosphogluconate (Slein, 1955; Wells et al., 1993). The concomitant reduction of $NADP^+$ is measured by the change

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

[1] Abbreviations: PMI, phosphomannose isomerase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; M6P, mannose 6-phosphate.

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in absorption at 340 nm using a thermostated 96-well microtiter plate reader (Thermomax, Molecular Devices, Palo Alto, CA). Protein concentrations were determined using the Bio-Rad protein assay reagent with BSA as the standard. SDS–PAGE was carried out using 10–15% Phast gradient gels (Pharmacia) or 12% polyacrylamide gels with the Bio-Rad Protean-II minigel system. The proteins were stained with Coomassie Brilliant Blue R250.

Inactivation of the C. albicans Phosphomannose Isomerase. Enzyme was incubated with 0–25 mM phenylglyoxal in 50 mM Hepes buffer, pH 8.0. At time intervals over 25 min, 10-μL samples were removed and assayed at 37 °C for remaining activity in 300 μL of assay cocktail at a final buffer concentration of 50 mM Tris-HCl, pH 8.0. The residual enzyme velocities over a 5-min period were calculated using the Thermomax plate reader software. Data were transferred from the plate reader via the spreadsheet Excel (Microsoft) to Grafit 3.01 (Leatherbarrow, 1993) and fitted to the equation for pseudo-first-order inactivation, $v_t = v_0(\exp^{-k_{obs}t})$, using simple weighting. The lack of significant deviation from this equation was confirmed by examining plots of the difference between the observed and calulated reaction velocities. No significant pattern was seen. The ability of the substrate mannose 6-phosphate and an inhibitor of catalysis, zinc ions, to prevent modification was tested by incubating the enzyme in 50 mM Hepes buffer, at pH 8.5 and 37 °C, at a fixed concentration of 10 mM phenylglyoxal and adding protective concentrations of 0–4 mM sugar phosphates and 0–100 μM $ZnCl_2$. The inactivation rate constant, k_{obs}, was measured as described above.

pH Dependence of the Inactivation Reaction. For the pH studies the buffers used were sodium phosphate (pH 6.0–8.0) and sodium borate (pH 8.0–10.0) at 37 °C. These buffers were used since primary amine containing buffers may react with the phenylglyoxal and interfere with the reaction. Stocks of buffers were made at 0.2 M and diluted 4-fold for the final assay. The ionic strength of each buffer was calculated, and NaCl was added to produce a constant ionic strength of I = 0.2 M. The pH of each buffer was then rechecked at its final dilution of 0.05 M using a Radiometer PHM83 pH meter. The enzyme was incubated in these buffers in the presence of 1 mM phenylglyoxal, and aliquots were removed over a 1-h time course. These were then diluted into the assay cocktail containing Tris-HCl, pH 8.0, and assayed immediately. The rate of enzyme inactivation, k_{obs}, was calculated as described above, assuming a first-order process. The effect of thermal denaturation on the loss of enzyme activity was not significant at these pH values over the time course of the assay. However, enzyme instability did preclude the accurate measurement of the inactivation rate constant at pH values greater than 10.0.

Radiolabeling of Phosphomannose Isomerase and Determination of the Stoichiometry of Modification. Protein was concentrated to 20 mg/mL using a Centricon 30 centrifugal concentrator (Amicon). One milligram of phosphomannose isomerase was then incubated with 2.5 mM [7-^{14}C]phenylglyoxal (Amersham, 23.1 mCi/mmol) at 37 °C in 50 mM Hepes buffer, pH 8.5, in the presence and absence of 16 mM mannose 6-phosphate. At five intervals over a 1-h period, a 1-μL aliquot was removed and diluted to 1 mL final volume with ice-cold distilled water. The extent of modification of the enzyme was assayed by filtering 200-μL aliquots of this dilution (in triplicate) through prewetted nitrocellulose filters (25-mm diameter, 0.45-μm pore size, Schleicher and Schuell). The filters were dried under a heat lamp before being scintillation counted using 5 mL of Optiphase HiSafe II scintillant (LKB). To assay the residual activity of the enzyme, three 5-μL aliquots of the diluted enzyme were assayed under the standard assay conditions. The modification reaction was terminated after 1 h by desalting using a PD10 gel-filtration column (Pharmacia) into a 50 mM NH_4HCO_3 buffer, pH 8.0. The modified arginine was then reduced by adding $NaBH_4$ to a final concentration of 5 mM and allowing the reaction mixture to stand for 3 h at 37 °C. The final product was separated by HPLC on a 220 × 2.1 mm C8 column using a gradient of 0–90% acetonitrile in 0.1% aqueous trifluroacetic acid over 15 min, with 90% acetonitrile for a further 10 min. The modified protein eluted at 15.4 min. The protein was lyophilized and stored at −20 °C.

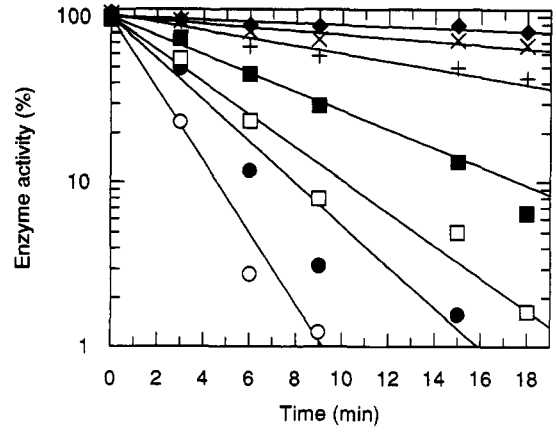

FIGURE 1: Inactivation of *C. albicans* phosphomannose isomerase by phenylglyoxal at 37 °C in 50 mM Hepes buffer, pH 8.5. Phenylglyoxal concentrations were (○) 25, (●) 15, (□) 10, (■) 5, (+) 2, (×) 1, and (◆) 0 mM. The inactivation rate constant k_{obs} was calculated from the data at each concentration as described in the text.

Peptide Mapping and Identification of the Substrate-Protectable Arginine Residue. The samples of PMI modified in the presence or absence of substrate were dissolved in 50 mM sodium phosphate buffer, pH 8.0. Two micrograms of Asp-N protease (Boehringer Mannheim, sequencing grade) was added, and the protein was left for 24 h at 37 °C. The peptides were separated using a gradient of 0–40% acetonitrile in 0.1% aqueous trifluroacetic acid over 90 min using a 220 × 2.1 mm C18 column. The peaks were collected, lyophilized, and redissolved in 100 μL of 20% acetonitrile. Two and one-half microliters of this material was added to 5 mL of scintillation cocktail and counted to quantify the amount of radioactivity associated with each peak. The fraction containing the majority of the radioactivity in the nonprotected sample was rechromatographed, using a similar gradient over 40 min, and a single radioactive peak was identified. This peptide was sequenced by Edman degradation using an Applied Biosystems 477A peptide sequencer, with on-line quantification of the phenylthiohydantoin derivatives by HPLC. Thirty percent of the amino acid phenylthiohydantoin derivatives were collected in the internal fraction collector. These were added to 5 mL of scintillation cocktail and were counted for 2 × 10 min.

RESULTS

Kinetics of Inactivation of C. albicans Phosphomannose Isomerase by Phenylglyoxal. The enzyme is inactivated in a time-dependent manner by the arginine-specific modifying reagent phenylglyoxal. The observed rate of inactivation shows a linear dependence on the concentration of phenylglyoxal added (Figure 1). At 37 °C the bimolecular rate constant for

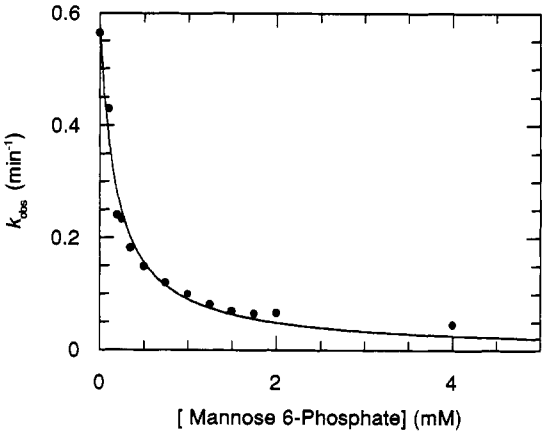

FIGURE 2: Protection of *C. albicans* phosphomannose isomerase by its substrate, mannose 6-phosphate, against inactivation by 20 mM phenylglyoxal at 37 °C in 50 mM Hepes buffer, pH 8.5.

this modification is 0.022 ± 0.002 min^{-1} mM^{-1} in 50 mM Hepes buffer, pH 8.5. The modification of arginine residues by phenylglyoxal is a reversible process (Takahashi, 1968). Incubation of the phenylglyoxal-inactivated enzyme with either 100 mM Tris-HCl, pH 8.0, buffer or 100 mM NH_4HCO_3 and incubation for 4 h at 37 °C caused reactivation of the enzyme giving up to 50% of the initial activity (data not shown). We were unable to use hydroxylamine in these experiments since it caused precipitation of the protein when added at concentrations greater than 1 mM, presumably by chelating the essential zinc atom.

The modification can be prevented by the addition of the substrate mannose 6-phosphate to the incubation mixture (Figure 2). The inactivation rate shows a linear dependence on the phenylglyoxal concentration. The substrate and inhibitor binding can therefore be described by the scheme

$$\begin{array}{ccc} E + I & \rightarrow & EI \\ + & & \\ S & & \\ K_s \downarrow\uparrow & & \\ ES & & \end{array}$$

The loss of enzyme activity can then be described by the equation $v_t = v_0(\exp^{-k_{obs}t})$, and the resultant values of k_{obs} can be fitted to the curve $k_{obs} = k_i'/(1 + [S]/K_s)$, where k_i' is the maximal rate of enzyme inactivation under these conditions (Coulin et al., 1993). The data fit well to this equation and show a dissociation constant K_s of 0.56 ± 0.04 mM. This is close to the value of K_m for the enzyme under these conditions. It has been previously shown that zinc ions can compete with mannose 6-phosphate for the *S. cerevisiae* and *C. albicans* phosphomannose isomerase, with K_i = 6.9 μM at pH 8.0 in 50 mM Tris-HCl buffer (Wells et al., 1993; Timothy N. C. Wells, unpublished results). However, no inhibition of the modification by phenylglyoxal was found using concentrations of zinc ions up to 100 μM (data not shown).

The pH-dependence data for the modification of PMI were fitted to the equation for a single ionization, $k_{obs} = k_1 + (k_2 - k_1)/[1 + 10(pK_a - pH)]$, where k_1 and k_2 are the limiting rates of modification at low and high pH values, respectively. The curve fitting (Figure 3) shows a single pK_a at 9.1 ± 0.1. This value is somewhat higher than the pK_a for the free enzyme (8.7 ± 0.1), although in previous studies of the pH dependance of PMI we have observed changes in kinetic constants by a factor of 2 simply by changing buffer type at constant pH. Since the phenylglyoxal modification was done in phosphate and borate buffers and the pH dependence study in secondary amine buffers, this difference in pK_a values is not considered significant.

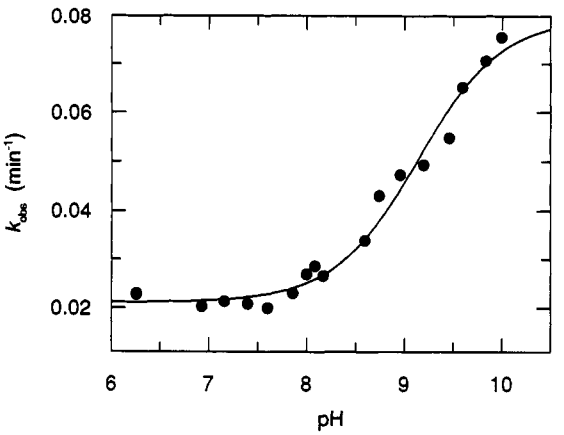

FIGURE 3: pH dependence of the rate of inactivation (k_{obs}) of phosphomannose isomerase at 37 °C in 50 mM (I = 0.05 M) buffer. The buffers used were sodium phosphate (pH 6.0–8.0) and sodium borate (pH 8.0–10.0). The data are fitted to an equation for the effects of a single pK_a, $k_{obs} = k_1 + (k_2 - k_1)/[1 + 10(pK_a - pH)]$.

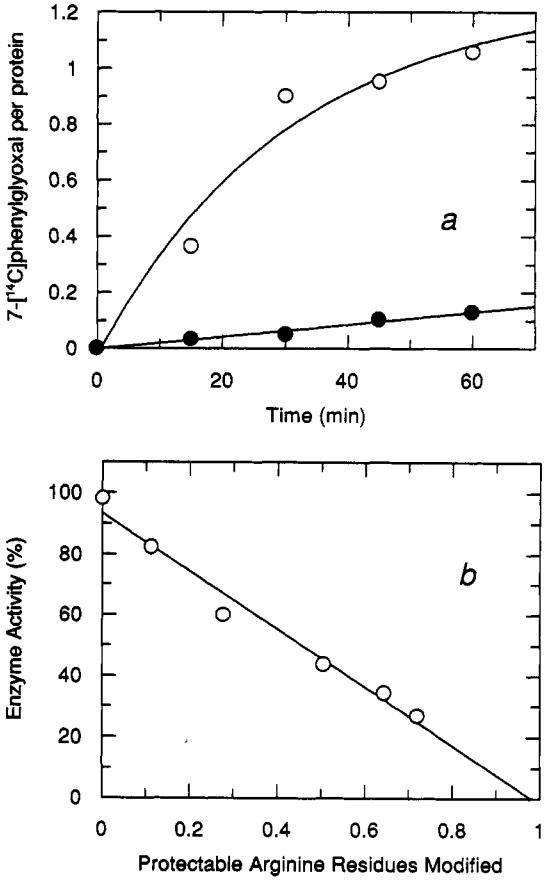

FIGURE 4: (a) Incorporation of radiolabel from 2.5 mM [7-^{14}C]-phenylglyoxal into phosphomannose isomerase in 50 mM Hepes buffer, pH 8.5, at 37 °C (○) and in buffer containing 16 mM mannose 6-phosphate (●). This data is typical of that seen in three equivalent experiments. (b) Relationship between the number of arginines modified and the loss of enzyme activity. The data have been fitted to the displayed straight line by linear regression.

Stoichiometry of Modification. The number of substrate-protectable molecules of [7-^{14}C]phenylglyoxal incorporated per protein molecule was monitored during the reaction time course. As can be seen from Figure 4a, the curve shows an exponential approach to a limiting value of 1.2 arginines modified in the absence of substrate. This value compares

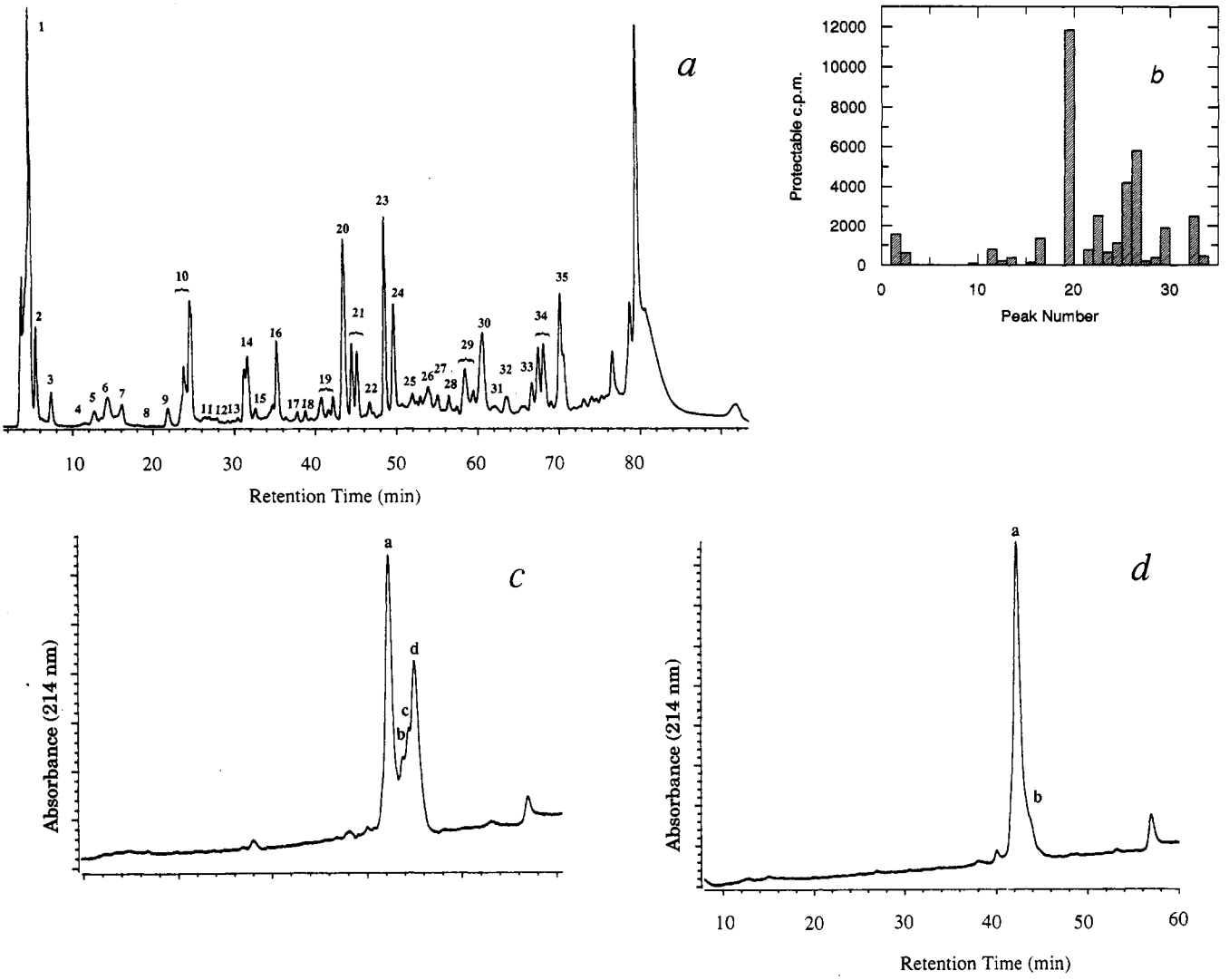

FIGURE 5: (a) Reversed-phase high-pressure liquid chromatograms of the Asp-N digest of phosphomannose isomerase modified with phenylglyoxal. The peaks were detected by absorption at 214 nm and were collected as numbered. (b) Radioactivity associated with each peak. The counts found in the substrate-protected modification have been subtracted for each peak. Peak numbers correspond to the peaks in the HPLC trace above. (c) Rechromatography of peak 19 from the nonprotected digest. The radioactivity is in peak 19d. (d) Rechromatography of peak 19 from the protected digest showing an absence of a peak with a retention time equivalent to that of peak 19d in (c).

with 0.2 arginine modified in a parallel experiment in the presence of the substrate M6P. A graph of remaining enzyme activity against the number of arginines modified (Figure 4b) shows clearly that the modification of only one arginine residue is needed to inactivate the protein.

Identification of the Modified Amino Acid. Following Asp-N protease digestion of protein which had been modified in the presence or absence of substrate, 34 major peaks were separated by HPLC. Fractions with identical retention times were collected for the two incubation conditions in the presence and absence of M6P and the radioactivity compared. Fraction 19, which eluted between 41 and 42.5 min, contained the most radioactivity (Figure 5a). There was 7 times less radioactivity incorporated into the mannose 6-phosphate protected fraction 19 than into the nonprotected fraction 19. Both fractions were rechromatographed. For the nonprotected sample, two major peaks and two shoulders were collected (Figure 5c). The radioactive counts were present in one peak, fraction 19d. This fraction was not present in the rechromatography of fraction 19 from the mannose 6-phosphate protected sample, consistent with it containing the site of modification of PMI by phenylglyoxal.

When this peptide was sequenced, the phenylthiohydantoin yields were as follows (in pmol): Asp, 175; Asn, 136; Val, 172; Val, 165; Arg, 21.6; Ala, 118; Gly, 92; Phe, 85; Thr, 41; Pro, 45; Lys, 34; Phe, 33; Lys, 20. This sequence corresponds to amino acids 300–312 in the sequence of *C. albicans* phosphomannose isomerase (Proudfoot et al., 1994). A fraction of the amino acid derivatives were collected in the sequenator and scintillation counted (Figure 6a). The data shows that the radioactivity was cleaved from the peptide in cycle 5, which corresponds to amino acid 304, an arginine. There was some carry-over of radioactivity into subsequent fractions, which is probably due to incomplete cleavage of the modified arginine in cycle 5. In addition, there was some release of radioactivity in the first fraction. Since Asp-300 could not be modified with phenylglyoxal, we attribute this peak of radioactivity to phenylglyoxal which had not been reduced onto Arg-304 by the sodium borohydride treatment. In addition, there was a peak on the chromatogram of phenylthiohydantoin amino acids in cycle 5 that did not correlate with any of the amino acid standards and that eluted at 16.91 min, just before cysteine (Figure 6b) at 17.34 min. It is presumed that this corresponds to the phenylthiohydantoin of the reduced arginine/phenylglyoxal Schiff base. The peak

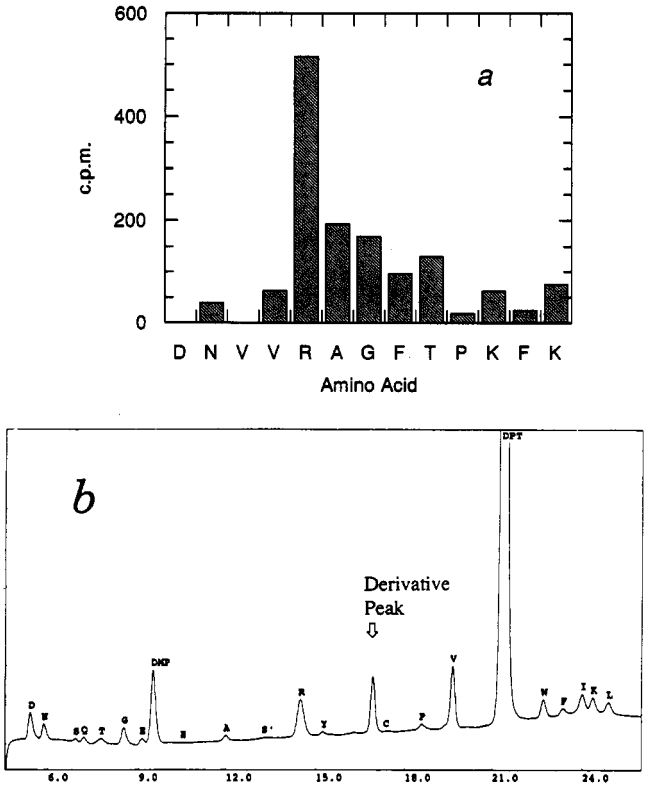

FIGURE 6: (a) Histogram showing the radioactivity released during each cycle of Edman degradation of the radiolabeled peptide 19d. The sequence corresponds to amino acids 300–312 in the sequence of *C. albicans* phosphomannose isomerase (Smith et al., 1994). (b) Reversed-phase high-pressure liquid chromatogram of the phenylthiohydantoin amino acids released in cycle 5 (corresponding to Arg-304). One peak, eluting at 16.91 min (0.43 min ahead of the cysteine elution time), does not correspond to any of the known amino acid standards. Its peak height correlates with the radioactivity released in cycles 5–9, and therefore it is most likely to be the modified arginine derivative.

height for this peak decreased in subsequent cycles, and this peak height correlated well with the amount of radioactivity released in each cycle.

DISCUSSION

Early studies of the yeast phosphomannose isomerase showed that it had a bell-shaped pH dependence (Gracy & Noltman, 1968a,b). This has been subsequently confirmed for the enzyme from *C. albicans*, where there are two sets of pK_a values, 5.6 and 8.7 for the free enzyme and substrate and 6.0 and 9.7 for the enzyme–substrate complex (Timothy N. C. Wells, unpublished results). The lower pK_a led to studies which identified substrate-protectable histidine residues in PMI (Timothy N. C. Wells, unpublished results). The higher pK_a was originally assigned at pH 7.8 (Gracy et al., 1968b), although we have subsequently shown that this value is experimentally underestimated (Wells et al., 1993). Additional quantities of coupling enzymes must be used in the assay at high pH values to obtain correct values for the kinetic parameters of phosphomannose isomerase.

There are no obvious sequence identities between phosphomannose isomerase and other isomerase enzymes, and so there is no indication of the active site residue by comparison with other known enzymes. Candidates for the high-pK_a group include lysine, arginine, cysteine, tyrosine, and the essential zinc in the protein. Previous experiments (Coulin et al., 1993) have studied the involvement of a cysteine or a lysine, and

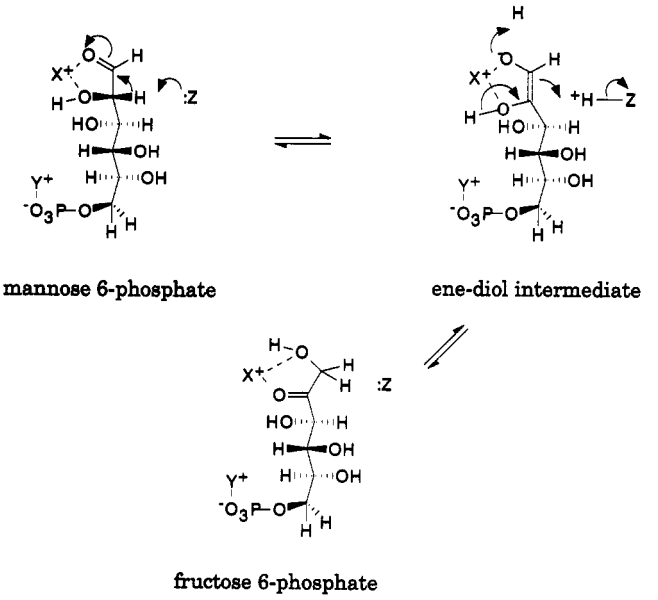

FIGURE 7: Proposed mechanism for the phosphomannose isomerase catalyzed reaction [after Gracy and Noltmann (1968b)]. The role of nucleophile Z could be fulfilled by an active site histidine (Timothy N. C. Wells, unpublished results). The role of Arg-304 could be to act as the base X assisting the isomerization. Alternatively, this role could be performed by the essential zinc atom, and the Arg-304 could provide part of the binding site for the sugar phosphate (group Y). Electron shifts are drawn for the reaction proceeding from mannose 6-phosphate to fructose 6-phosphate. The sugars are represented as the straight chain forms, implying enzyme catalyzed ring opening prior to isomerization.

therefore we turned our attention to the possibility of an active site arginine residue.

Using phenylglyoxal modification, we have determined the presence of an arginine residue, which when modified covalently results in the inactivation of the enzyme. Stoichiometry studies show that this modification involves a single residue, which can be protected by the addition of high concentrations of the substrate mannose 6-phosphate. The pK_a of this modification was shown to be 9.1 ± 0.1, which is approximately that of the second ionization of phosphomannose isomerase. The peptide mapping of the protein, which had been labeled with radioactive phenylglyoxal, enabled us to find a peptide which was preferentially labeled in the absence of substrate. This sequence of this peptide was determined and found to be DNVVRAGFTPKFK, corresponding to amino acids 300–312 of *C. albicans* PMI. The identification of the labeled amino acid is supported by three lines of evidence. First, the phenythiohydantoin residue corresponding to the cycle where the modified arginine should elute contains radioactivity. Second, the yield of unmodified arginine in this cycle was uncharacteristically low. Third, a peak was found on the chromatogram which does not correspond to one of the known amino acids, and this presumably represents the derivatized arginine. This confirms that the residue modified is Arg-304. This residue is conserved among all of the type I phosphomannose isomerases studied to date (Proudfoot et al., 1994) (Table 1). The amino acid sequence for *C. albicans* phosphomannose isomerase contains nine arginines, but this is the only one which is completely conserved.

In an attempt to further define the role of this residue, we carried out some sequence-identity searches. Sequence comparisons using the whole of the phosphomannose isomerase protein sequence against either Swissprot or Genbank databases using FastA (Pearson, 1990) revealed no proteins with significant identity. Therefore, searches were carried out using

Table 1: Alignment of Multiple Type I Phosphomannose Isomerases, Showing Active Site Arginine 304

Stpmi[a]	[281]AETPH AYLQG	VALE VMANSD	NVLRAGLTPK	Y I D I PE LVAN[320]
Ecpmi	[281]AETPH AYLQG	VALE VMANSD	NVLRAGLTPK	Y I D I PE LVAN[320]
Scpmi	[281]AKDPH AY ISG	D IMECMAASD	NVVRAGFTPK	FK D VKNLVSM[320]
Capmi	[281]AKDPH AY ISG	D I I ECMAASD	NVVRAGFTPK	FK D VKNLVEM[320]
Anpmi	[281]ADD I HAY ISG	D I I ECMAS SD	NVVRAGFTPK	F K D VDTLTEM[320]
Hupmi	[281]ANVPHAYLKG	DCVECMAC SD	NTVRAGLTPK	F I D VPT L CEM[320]

[a] Sequences: Stpmi, *S. typhimurium* (Collins & Hackett, 1991); Ecpmi, *E. coli* (Miles & Guest, 1984); Capmi, *C. albicans*; Anpmi, *Aspergillus nidulans* (D. Smith, unpublished results); Hupmi, human (Proudfoot et al., 1994). Residues which are conserved among all six species are shown in bold type.

conserved fragments surrounding Arg-304, IECMAASDNV VRAGFTPKFK D and NVVRAGFTPK FK, using the programs Profilesearch (Gribskow et al., 1987) and FastA. Not surprisingly, the results contain a large number of phosphate binding proteins. Two cases which show some significant regions of identity are RecA (Story et al., 1992) and phosphofructokinase (Evans & Hudson, 1979).

There are two possible roles for this arginine residue. First, it could be involved in the binding of the phosphate moiety of the substrate. The use of arginine to stabilize the binding of phosphates is quite a common occurrence in proteins for which the three-dimensional structure is known, such as phosphofructokinase (Evans & Hudson, 1979) and glycogen phosphorylase b (Hajdu et al., 1987). In addition, it has been recently shown by site-directed mutagenesis that two arginine residues are involved in the binding of mannose 6-phosphate to the bovine cation-independent mannose 6-phosphate/insulin-like growth factor II (M6P/IGF-II) receptor (Dahms et al., 1993).

Second, the Arg-304 could be used to polarize the carbonyl group at C-1 in mannose 6-phosphate and C-2 in fructose 6-phosphate, and thus facilitate the catalytic process (Figure 7). In the enzyme triose phosphate isomerase, it has been shown that this polarization is brought about by the side chain of Lys-13. Glucose 6-phosphate isomerase from pig muscle shows a similar bell-shaped pH dependence, with a higher pK_a value at 9.5–10.5. It was possible to label two lysines (Achari et al., 1981; Palmieri et al., 1982), Lys-263 and Lys-577 (Marchand et al., 1989), by using pyridoxal 5′-phosphate, indicating that the polarization of the carbonyl in this enzyme may also be carried out by lysine. Despite the similarity in the reactions catalyzed by glucose 6-phosphate isomerase and phosphomannose isomerase, there is no clear homology in the protein sequence which would allow an equivalent lysine to be identified. Phosphomannose isomerase does not react with pyridoxal phosphate (Timothy N. C. Wells, unpublished results). It could therefore be that the role of the catalytic lysine has been partly assumed by Arg-304 in phosphomannose isomerase. However, it was originally suggested (Gracy & Noltmann, 1968b), on the basis of similarities with the nonenzymic reaction, that the polarization was carried out by the essential zinc atom. It is perfectly possible, however, to have the zinc being entirely responsible for the structural integrity of the protein, rather than being part of the catalytic machinery *per se*. The final identification of the role of Arg-304 in phosphomannose isomerase, and with it the exact nature of the role of essential zinc in the active site, must await the solution of the three-dimensional structure. This is currently in progress (Tolley et al., 1994).

ACKNOWLEDGMENT

We would like to thank Dr. Alain Bernard for fermentation of the *E. coli* strains, Drs. Amanda Proudfoot and Mark Payton for discussions, and Dr. Susan Wells for proofreading the manuscript.

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