Identification of Cys-150 in the Active Site of Phosphomannose Isomerase from *Candida albicans*

Florence Coulin, Edith Magnenat, Amanda E. I. Proudfoot, Mark A. Payton, Paul Scully, and Timothy N. C. Wells*

Glaxo Institute for Molecular Biology, 1228 Plan-les-Ouates, Geneva, Switzerland

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ABSTRACT: *Candida albicans* phosphomannose isomerase (PMI) (EC 5.3.1.8) has been recently cloned and overexpressed in *Escherichia coli*. The enzyme can be irreversibly inactivated by iodoacetate in 50 mM borate buffer, pH 9.0, in a time-dependent manner at a rate of 4.2 ± 0.03 min^{-1} M^{-1}. This inhibition can be prevented by the substrate mannose 6-phosphate with a K_s of 0.22 ± 0.05 mM, slightly lower than its K_m value. However, metals such as zinc and cadmium, which are reversible, competitive inhibitors for PMI, do not protect the enzyme against modification. The protein has been labeled by using [2-^{14}C]iodoacetate, in the presence or absence of substrate, and the protein is fully inactivated when 1.0 thiol group is modified per molecule of enzyme. Tryptic maps of the modified protein have been produced. The protected peptide has been identified and sequenced, and the phenylthiohydantoin amino acids have been collected. The modified amino acid is Cys-150. This cysteine residue is conserved in mammalian and yeast phosphomannose isomerases, but not in bacterial species where it is replaced with asparagine. We therefore purified PMI from *E. coli* and showed that this enzyme is not sensitive to inactivation by iodoacetate. The iodoacetate is presumably inhibiting PMI by sterically blocking the mannose 6-phosphate binding site. Multiple sequence alignment procedures were used to try to identify potential ligands of the zinc atom that is essential for enzyme activity and thus to delineate the active site region. There is a strong homology between residues 130–140 in phosphomannose isomerase and the zinc binding site of copper–zinc superoxide dismutase, which contains two of the zinc ligands. This implies that the sequence 130–150 forms a central part of the active site of the enzyme.

Phosphomannose isomerase (PMI)[1] catalyzes the reversible interconversion of fructose 6-phosphate and mannose 6-phosphate. This is the first step in the synthesis of cell wall mannoproteins and other N- and O-linked oligosaccharides from glycolytic intermediates. The enzyme therefore plays an important role in yeast cell wall biosynthesis (Orlean, 1990; Payton et al., 1991). The gene coding for this enzyme has recently been cloned (Smith et al., 1993) by using probes based on the *Saccharomyces cerevisiae* gene (Smith et al., 1992) and an ordered-array genomic library from a *Candida albicans* cDNA library.

Studies of the pH dependence of the enzyme have shown a bell-shaped dependence with two pK_a values of 6.0 and 9.7 for the enzyme–substrate complex (Wells et al., 1994). The higher pK_a is indicative of an arginine, a lysine, a cysteine, or a tyrosine. Initial studies with modifying reagents confirmed reactivity with phenylglyoxal and iodoacetate implying the presence of an arginine and a cysteine in the active site. Kinetic studies of the highly homologous *S. cerevisiae* enzyme showed that there is a ligand in the active site capable of binding an inhibitory zinc or cadmium ion with a pK_a of 9.2 (Wells et al., 1993). In view of the known affinity of metals of the d^{10} group (zinc, cadmium, and mercury) for thiol ligands, this adds further support to the idea of a cysteine residue being present in the enzyme active site. We have therefore identified the active site cysteine and further studied its role in the catalytic process of phosphomannose isomerase.

MATERIALS AND METHODS

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

Enzyme Assay. The activity of phosphomannose isomerase was measured at 37 °C using a coupled assay in which the product fructose 6-phosphate is converted into glucose 6-phosphate. This is then oxidized to 6-phosphogluconate, and the reduction of $NADP^+$ is measured by the change in absorption at 340 nm (Slein, 1955; Wells et al., 1993). Unless otherwise stated, the buffer was 50 mM Tris-HCl, pH 8.0.

Enzyme Purification. Recombinant *C. albicans* PMI overexpressed in *Escherichia coli* was purified as described previously (Smith et al., 1994). Endogenous *E. coli* PMI was purified from 200 g of *E. coli* B cells by using a protocol similar to those described for the yeast enzymes (Wells et al., 1994).

Analytical Methods. Protein concentrations were determined by using the Bio-Rad protein assay reagent, with BSA as the standard. SDS–PAGE was carried out on 10–15% Phast gradient gels (Pharmacia) or 12% polyacrylamide gels using the Bio-Rad Protean-II minigel system, and the proteins were stained with Coomassie Brilliant Blue, R250.

Inactivation of C. albicans Phosphomannose Isomerase. Enzyme was incubated with 0–40 mM iodoacetate in 50 mM sodium borate buffer, pH 9.0, at 25 °C. At time intervals over 1 h, 10-μL samples were removed and assayed at 37 °C for remaining activity, using an assay cocktail containing 50 mM Tris-HCl, pH 8.0, buffer. Data were transferred to Grafit 3.0 (Leatherbarrow, 1992) and fitted to the equation for a pseudo-first-order inactivation, $v_t = v_0(\exp^{-k_i^{app}t})$, using proportional weighting. The lack of significant deviation from this equation was confirmed by examining residual plots. The ability of mannose 6-phosphate and zinc to prevent this

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[1] Abbreviations: PMI, phosphomannose isomerase; Tris-HCl, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; M6P, mannose 6-phosphate; Cu–ZnSOD, copper–zinc superoxide dismutase.

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inactivation was tested by incubating the enzyme with a fixed concentration of 10 mM iodoacetate and protective concentrations of either 0–2 mM $ZnCl_2$ or 0–25 mM mannose 6-phosphate at 25 °C in 50 mM sodium borate buffer, pH 9.0. The inactivation rate of the enzyme k_i^{app} was measured as described above.

Radiolabeling of Phosphomannose Isomerase and Identification of the Active Site Residue. Protein was concentrated to 20 mg/mL by using a Centricon 30 centrifugal concentrator (Amicon). One milligram of phosphomannose isomerase was then incubated with 2.5 mM [2-^{14}C]iodoacetate (Amersham, 56 Ci/mmol) at pH 9.0 in 50 mM sodium borate buffer, in the presence or absence of 20 mM mannose 6-phosphate. The total reaction volume was 100 μL. At six time intervals over a 1-h period, a 5-μL aliquot was removed and diluted to 1 mL with ice-cold distilled water containing 1 mM 2-mercaptoethanol to stop the reaction. In order to assay the extent of modification of the enzyme, three 200-μL aliquots of this dilution were filtered through prewetted nitrocellulose filters (25-mm diameter, Schleicher and Schuell) and washed four times with 5 mL of ice-cold buffer. The filters were dried under a heat lamp before being scintillation counted using Optiphase HiSafe II scintillant (LKB). To assay the residual activity of the enzyme, three 5-μL aliquots of the stopped reaction dilution were used under the standard assay conditions.

The time-course of the modification having been established, 1 mg of protein was modified in the presence or absence of mannose 6-phosphate for 30 min as described above. The proteins were desalted by using PD10 gel filtration columns (Pharmacia) in 100 mM Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$, and the excluded volume was collected as a single fraction. Fifty micrograms of trypsin (sequencing grade) was added to each tube, and the tubes were incubated overnight at 37 °C. The peptides were separated by using a gradient of 0–40% acetonitrile in 0.1% aqueous trifluoroacetic acid at 0.2 mL/min over 70 min on a 220 × 2.1 mm C18 column. The peaks were collected, dried, and redissolved in 100 μL 20% acetonitrile/0.1% trifluoroacetic acid. Two and one-half microliters of each peak was added to scintillation cocktail and counted to quantify the amount of radioactivity associated with each peak. The peptides were sequenced by Edman degradation using an Applied Biosystems 477A peptide sequencer, with on-line quantification of phenylthiohydantoin derivatives by reversed-phase HPLC. Thirty percent of the amino acid phenylthiohydantoins were collected in the internal fraction collector. These were added to 5 mL of scintillation cocktail and counted for 2 × 10 min.

Multiple-Sequence Alignment. Phosphomannose isomerase is a metalloenzyme (Gracy & Noltman, 1968), and so multiple-sequence analysis was carried out to try to identify the potential ligands for the zinc in the active site. The sequences of six phosphomannose isomerase species (human, *C. albicans*, *Aspergillus nidulans*, *S. cerevisiae*, *E. coli*, and *Salmonella typhimurium*) were aligned by using the GCG program Pileup (Feng & Doolittle, 1987; Proudfoot et al., 1994). Key amino acids which were potential ligands were tested initially by making position-specific scoring tables, known as profiles (Gribskov et al., 1987), using the GCG program Profilemake. These were then searched against the Swissprot database to detect any significant match with either a protein known to be a zinc-containing protein or one for which a three-dimensional structure was already known. In view of the size of the database, results were only considered significant where the *Z*-score of the fit (the number of standard deviations above background) was greater than 4.

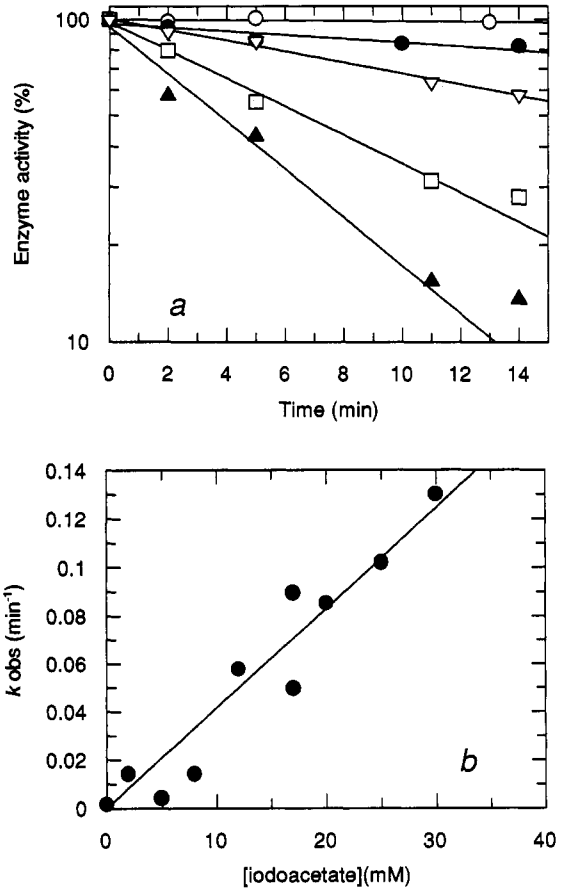
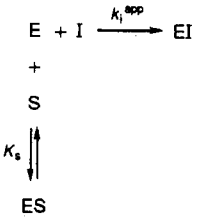


FIGURE 1: (a) Inactivation of *C. albicans* phosphomannose isomerase by iodoacetate at 25 °C in 50 mM borate buffer, pH 9.0. Iodoacetate concentrations were 0 (○), 8 (●), 17 (▽), 25 (□), and 30 mM (▲). (b) The inactivation rate constant k_i^{app} was calculated from the data in Figure 1a and is replotted as a function of iodoacetate concentration.

RESULTS

Absence of Saturation Kinetics for Inactivation by Iodoacetate. Recombinant *C. albicans* phosphomannose isomerase is inactivated in a time-dependent manner when incubated with an excess of iodoacetate at pH 9.0. There is a linear relationship between the observed rate of inactivation, k_i^{app}, and the iodoacetate concentration, as can be seen in Figure 1. The slope of this plot is a measure of the bimolecular rate constant for modification, 4.2 ± 0.03 min^{-1} M^{-1} at 25 °C and pH 9.0. There is no evidence of hyperbolic curvature at high concentrations of iodoacetate, implying that a kinetically significant enzyme–inhibitor complex does not form prior to inactivation.

Substrate Protection against Modification by Iodoacetate. If the residue that is being modified is in the active site of the enzyme, then the addition of substrate should protect against the modification and inactivation. The effect of mannose 6-phosphate on the kinetics of inactivation by 10 mM iodoacetate is shown in Figure 2. Assuming the simple scheme



then the loss of enzyme activity can be described by the

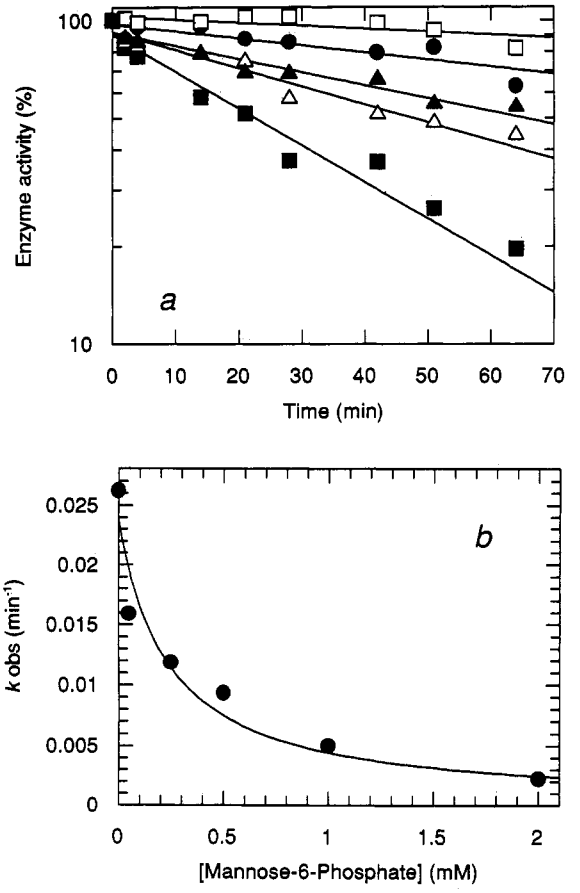


FIGURE 2: (a) Protection of *C. albicans* phosphomannose isomerase by its substrate, mannose 6-phosphate, against inactivation by 10 mM iodoacetate in 50 mM borate buffer, pH 9.0, at 25 °C. Mannose 6-phosphate concentrations were 0 (■), 0.25 (△), 0.5 (▲), 1 (●), and 2 mM (□). (b) Data replotted to show the dependence of the inactivation rate constant, k_i^{app}, on substrate competition.

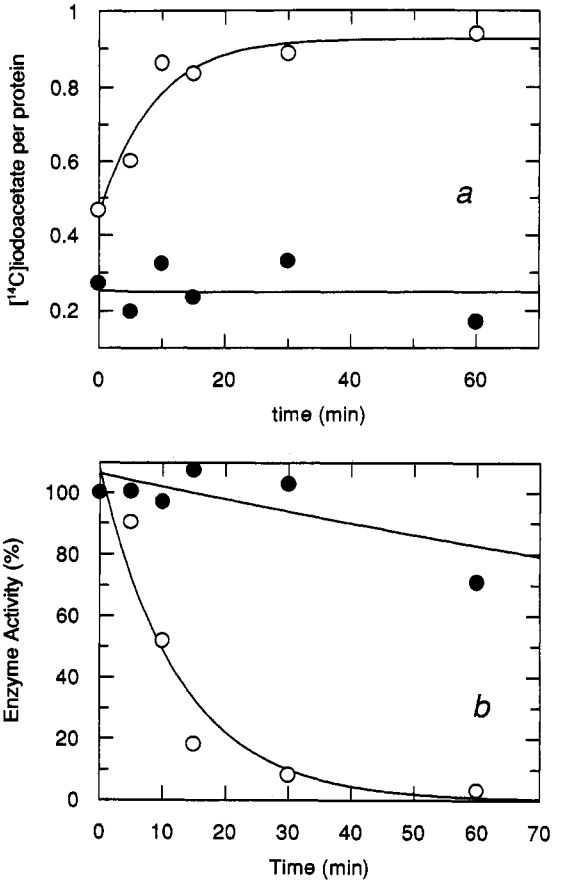


FIGURE 3: (a) Incorporation of radiolabel from 2.5 mM [^{14}C]-iodoacetate into phosphomannose isomerase, in 50 mM sodium borate buffer, pH 9.0 (○), and in buffer containing 20 mM mannose 6-phosphate (●). (b) Loss of enzyme activity after incubation with buffer containing iodoacetate in the absence (○) and in the presence of substrate (●).

equation

$$v_t = v_0 \exp^{-\{k_i app/(1 + [S]/K_s)\}}$$

The data fit well to this equation. The substrate shows a dissociation constant of $K_s = 0.22 \pm 0.05$ mM, which is slightly lower than the K_m value under these conditions. We had previously shown (Wells et al., 1993) that the metals zinc, cadmium, and mercury are all micromolar competitive inhibitors of phosphomannose isomerase from *S. cerevisiae*. Experiments using recombinant *C. albicans* phosphomannose isomerase showed that zinc and cadmium can also compete with mannose 6-phosphate (data not shown). However, we were unable to prevent iodoacetate modification of the enzyme by the addition of excess zinc, even up to millimolar concentrations.

Stoichiometry of Modification. The number of modifications carried out per enzyme molecule was determined by incubating enzyme with 20 mM [2-^{14}C]iodoacetate, in the presence or absence of 20 mM M6P. Figure 3 shows a clear incorporation of 1 mol of iodoacetate into the unprotected protein, although there is still some incorporation of radiolabel into the protected protein. The incorporation correlates well with the loss of enzyme activity (Figure 3b), confirming that only one thiol needs to be modified to inactivate the enzyme.

Separation and Analysis of the Tryptic Peptides. Following the treatment with trypsin, 43 peaks were obtained for each digest (Figure 4a). One peak, eluting at 52 min, showed a high level of incorporation of radioactivity. Three other peaks showed some radioactivity above background. Initially, 20% of the 52-min peptide was sequenced for 5 cycles and gave the sequence NYPDD... corresponding to amino acids 129–133 (Smith et al., 1994). On comparison with the gene sequence of phosphomannose isomerase, it seemed likely that this was a partial-digest peptide, with potential modification sites on histidine in cycle 7 and cysteine in cycle 22. Subsequently, the remaining material was sequenced through 26 cycles of Edman degradation and gave the sequence ^{1}NYPDD NHKPE ^{11}MAIAV TDFEG ^{21}FCGFK. At each stage the products of the Edman degradation, the amino acid phenylthiohydantoins (PTHs), were collected. The radioactive incorporation into the phenylthiohydantoins (Figure 4b) shows clear incorporation in cycle 22 (Cys-150). This is further confirmed by the presence of (carboxymethyl)cysteine on the on-line HPLC tracing. There is no incorporation in cycle 7 (His-135), the other possible site of carboxymethylation, and no *N*-(carboxymethyl)histidine in the PTH analysis. The radioactivity in cycle 23 is due to incomplete release of the PTH-cysteine in the previous cycle. The amount of radioactivity is proportional to the amount of (carboxymethyl)cysteine released in cycle 23. The other radioactive peaks were sequenced from the nonprotected incubation. In each case, several sequences were obtained, one of which corresponded to a tryptic peptide containing Cys-150.

The protection with mannose 6-phosphate was very clean. In the peptide map of the protein which had been protected by substrate, only one radioactive peak was found with more than 50 cpm of radioactivity in it, eluting at 8 min. On sequencing, this contained three short peptides, LFR, AG-

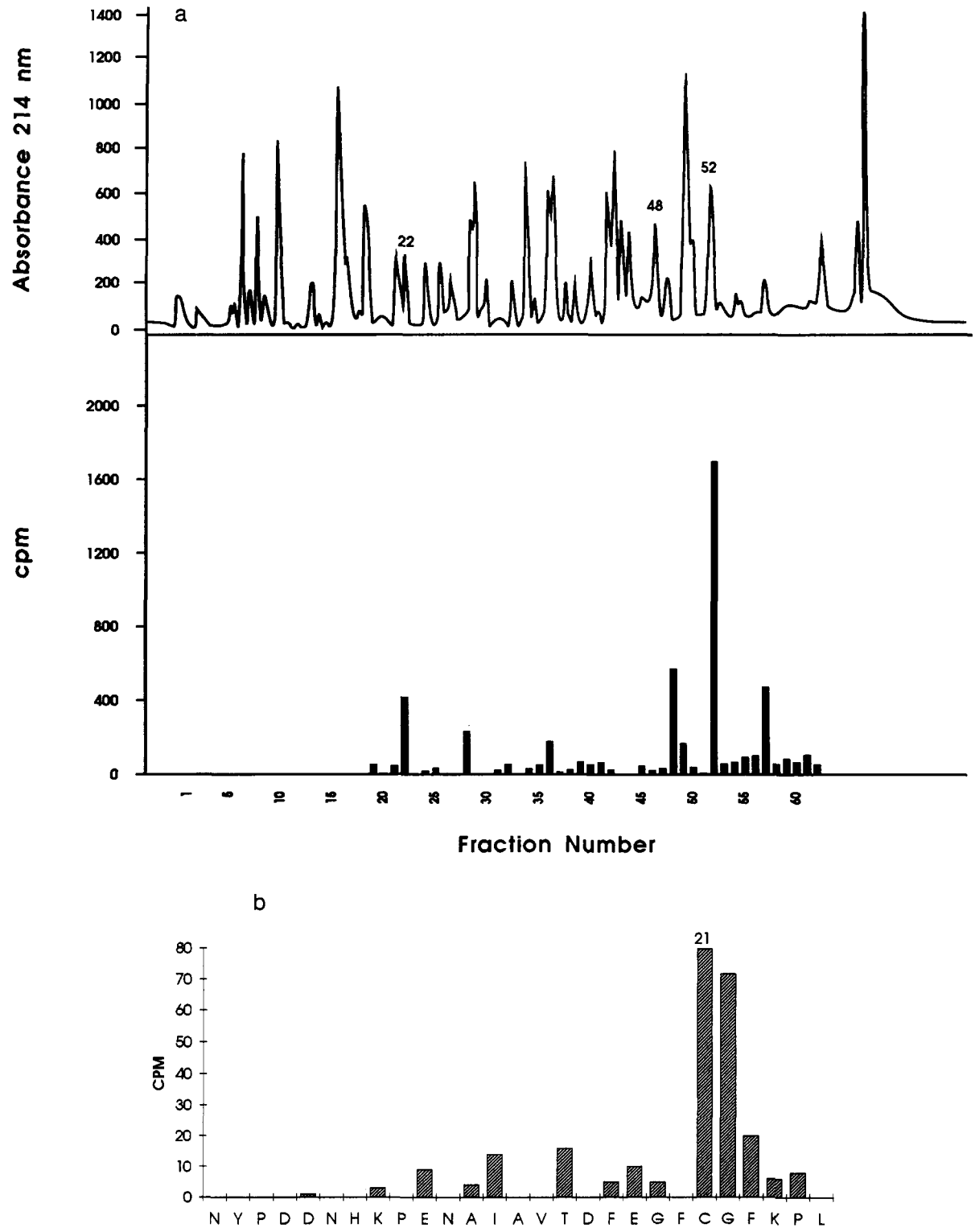


FIGURE 4: (a) Reversed-phase high-pressure liquid chromatograms of the tryptic digest of phosphomannose isomerase carboxymethylated by iodoacetate in the absence of substrate. (b) The radioactivity in each fraction is shown with the background subtracted. None of the peaks shown contained radioactivity when the incubation was carried out in the presence of mannose 6-phosphate.

FTPK, and SYESVEK, corresponding to amino acids 6–8, 305–310, and 324–330, respectively. However, since none of these peptides contain cysteine or histidine residues, it is assumed that the radioactivity is present as a non-peptide side product.

Phosphomannose Isomerase from E. coli Is Not Modified by Iodoacetate. Comparison of the sequence of the modified peptide with that of phosphomannose isomerase from other species shows that Cys-150 is conserved in mammalian and fungal phosphomannose isomerases. However, in the *E.coli* (Miles & Guest, 1984) and *S. typhimurium* (Collins & Hackett, 1991) enzymes this residue is replaced with an asparagine. It could therefore be predicted that the *E. coli* enzyme is not modified by iodoacetate. Incubation of *E. coli* phosphomannose isomerase with up to 20 mM iodoacetate does not cause any comparable loss of enzyme activity (Figure 5).

Prediction of Zinc Binding Site in Phosphomannose Isomerase. Phosphomannose isomerase from *S. cerevisiae* (Gracy & Noltman, 1968) and recombinant protein from *C.*

Table 1: Profile Sequences Used in Searching for Zinc-Binding Ligands

sequence used in profile[a]	residue nos.	Z-score[b]	rank order for Cu–Zn superoxide dismutase
PYAELWMGTHPSVPSKAID	45–63	c	n.d.
SIEKVLSIQHPDKKLGAQLH	103–123	c	n.d.
GEAMFLQAKDPHAYISGDIIECM	274–296	c	n.d.
PKNYPDDNHKPEMAIAVT	127–144	4.53	10
NYPDDNHKPEMAIAVT	129–144	4.97	10
YPDDNHKPEMAIAVT	130–144	5.29	7
PDDNHKPEMAIAVT	131–144	4.37	19
YPDDNHKPEMAIAV	130–143	5.69	3
YPDDNHKPEMAIA	130–142	5.71	3
YPDDNHKPEMAI	130–141	6.88	1
YPDDNHKPEMA	130–140	7.23	1
YPDDNHKPEM	130–139	7.24	1
YPDDNHKPE	130–138	6.28	1
GFPWTDDNHKGDLPALF[d]			

[a] The sequence quoted is that of the *C. albicans* enzyme. The equivalent portions of the other phosphomannose isomerases were taken from the alignment in Proudfoot et al. (1994). [b] The normalized Z-score is $Z = (s - \mu)/\sigma$, where μ and σ are the mean and the standard deviation of the scores obtained with randomly selected sequences of the same length. [c] For the first three profiles shown, there is no zinc-containing protein or protein with known tertiary structure with $Z > 4.0$. [d] The sequence of Cu–Zn superoxide dismutase from *Photobacterium leiognathi* is shown for comparison.

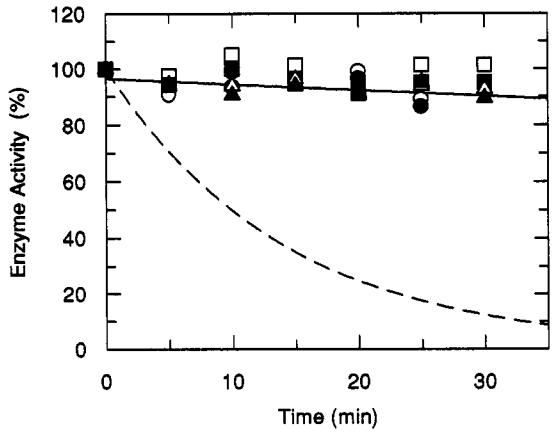


FIGURE 5: Incubation of *E. coli* phosphomannose isomerase with iodoacetate at 25 °C in 50 mM borate buffer, pH 9.0. The iodoacetate concentrations were 0 (O), 1 (●), 5 (□); 10 (■); 15 (△); and 20 mM (▲). The dotted line shows the effect of 2.5 mM iodoacetate on the *C. albicans* enzyme under identical conditions.

albicans (Amanda E. I. Proudfoot, unpublished) and human have been shown to be zinc-dependent enzymes. Knowing that Cys-150 plays a role in controlling the active site of *C. albicans* phosphomannose isomerase, we speculated that it may be close to the zinc binding pocket. However, phosphomannose isomerase shows no significant homology to any other known zinc-dependent protein. We therefore decided to search the database using profiles based on sections of the six available protein sequences for phosphomannose isomerase.

Comparison of the X-ray crystallographic structures of 12 zinc-containing enzymes (Valee & Auld, 1990) led to the conclusion that zinc binding sites in enzymes favor histidine as the principal ligand. In descending order of prevalence, other potential zinc ligands are glutamate > aspartate = cysteine. The zinc atom will have at least three ligands from the protein, of which two are normally separated by a short spacer of 1–3 amino acids, to allow formation of a primary bidentate complex during synthesis of the enzyme. We therefore aligned the six sequences (human, *C. albicans*, *A. nidulans*, *S. cerevisae*, *E. coli*, and *S. typhimurium*) as shown in Proudfoot et al. (1994). There are four completely conserved histidines (using the *C. albicans* numbering), His-54, His-113, His-135, and His-285. We created profiles (position-specific scoring tables; Gribskov et al., 1987) based on approximately 10 amino acids on either side of these histidines (see Table 1). These profiles represent an average of the amino acid types at each position and therefore allow us to search for sequences in the database which are similar to this consensus region in our target protein. Of the four profiles, three failed to show anything significant. However, the profile using residues 127–144 (centered on His-135) showed a significant homology to the copper–zinc superoxide dismutase of *Photobacterium leiognathi*, a luminescent marine symbiont (Steinman, 1987). Three other profiles were constructed against other regions of homology, which failed to find any interesting matches.

Although this homology is significant, there are nine other proteins having higher Z-scores. These were checked, and in no case is the histidine conserved. We therefore truncated the profile from each end, to remove sequences which are not important in the homology between the two proteins (Table 1). The final result of this is a 10 amino acid sequence, YPDDNHKPEM, which shows highest homology with WT-DDNHKGDL from the Cu–ZnSOD from *Photobacterium leiognathi*. This peptide corresponds to the peptide KDEER-HVGDL (amino acids 73–82) from the bovine Cu–ZnSOD (Steinman et al., 1974). The three-dimensional structure of this enzyme is known (Tainer et al., 1983), and the two "adjacent" ligands for the zinc are His-78 and Asp-81, which correspond to the ligands His-135 and Glu-138 in phosphomannose isomerase.

DISCUSSION

The catalytic activity of phosphomannose isomerase is clearly inhibited by incubation with iodoacetate at pH 9.0. This reaction is time dependent and irreversible, implying covalent modification. The ability of the substrate, mannose 6-phosphate, to protect the enzyme against modification shows that the residue modified either is in the active site of the enzyme or becomes buried upon the conformational change which accompanies substrate binding. Incubation with radiolabeled iodoacetate confirms that only one residue is modified. The analysis of the tryptic peptides produced after modification and subsequent sequencing and the collected PTH-amino acids confirmed that the residue modified is Cys-150.

There are two possible roles for this cysteine in the function of the enzyme. First, it could be involved in the catalytic process of the enzyme itself. The enzyme-catalyzed reaction has a pK_a of 9.7, which is well within the pK_a range of thiol groups found in protein active sites. However, this is unlikely, since the equivalent residue in *E. coli* and *S. typhimurium* phosphomannose isomerases is an asparagine (Proudfoot et

al., 1994). In addition, the higher pK_a group in isomerases is usually lysine or arginine. We confirmed that the role of the active site cysteine in the bacterial phosphomannose isomerase is not played by another cysteine, since the *E. coli* enzyme is not inhibited by incubation with iodoacetate.

Second, it could be that Cys-150 is not *directly* involved in the mechanism, even though it is in the active site. The modification of Cys-150 causes steric blocking of the active site such that the substrate can no longer bind. Such steric blocking is quite common [see, for example, Mann et al. (1989)]. Although carboxymethylation only adds four atoms to the cysteine, this is the most likely explanation for the inactivation. The mutant protein where this side chain is replaced with an alanine is currently being made to investigate these hypotheses. There are interesting parallels with the enzyme L-histidinol dehydrogenase (EC 1.1.1.23), which is also a zinc enzyme inhibitable by a thiol-modifying reagent (Grubmeyer & Gray, 1986). Here again, the active site cysteine identified is not absolutely conserved between species and has been shown not to be essential by site-directed mutagenesis (Teng et al., 1993).

Inhibition studies on the *S. cerevisiae* (Wells et al., 1993) and *C. albicans* (Wells et al., 1994) enzymes have shown that zinc and mercury are competitive inhibitors of the enzyme. Initially we attempted to study the effect of zinc on protection against iodoacetate inactivation of the enzyme. The lack of any protective effect implies that Cys-150 is not one of the ligands for this inhibitory zinc species. Initial studies on *S. cerevisiae* enzyme using Theorell–Yonetani analysis showed that although zinc and mercury are both competitive inhibitors with respect to substrate, they are not mutually exclusive. This opens up the possibility that Cys-150 is involved in the binding of mercury ions. Unfortunately, it is impossible to carry out the same competition studies with the *C. albicans* enzyme. In this case, the inhibition by mercury ions is time dependent and irreversible (Wells et al., 1994). We have demonstrated that a single mercury atom is responsible for this inactivation (Timothy N. C. Wells and P. Scully, unpublished observation). We are therefore currently trying to isolate the mercury-binding peptide from the *C. albicans* enzyme using radioactive mercury.

One further possible role of Cys-150 *in vivo* could be to allow the control of enzyme activity as the redox potential of the cell changes (Gilbert, 1990; Wells & Saxty, 1992), since the free cysteine would be capable of forming disulfide bonds with other intracellular thiols. In view of the poor reactivity of Cys-150, this is unlikely.

Since the implication is that Cys-150 is close to the active site, we used profile searching with homologous sequences to attempt to identify the essential zinc binding site in phosphomannose isomerase. After some refinement, a highly significant homology was found between phosphomannose isomerase and the copper–zinc superoxide dismutase. Not only was there a high degree of homology, but the homology region identified was a part of the zinc binding site of the superoxide dismutase. This homology region finishes nine amino acids upstream of the reactive Cys-150. It is therefore tempting to speculate that this region is all part of a loop involved in the active site of phosphomannose isomerase. We are currently attempting to test this and other hypotheses on the mechanism of the enzyme by solving its three-dimensional structure using X-ray crystallography.

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