Inhibition of Phosphomannose Isomerase by Mercury Ions

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ABSTRACT: Mercury ions can inhibit *Candida albicans* phosphomannose isomerase (PMI) by two different processes at sub-micromolar concentrations. Kinetic studies show that mercury ions are in rapid equilibrium with the enzyme and cause a clear partial noncompetitive inhibition when mannose 6-phosphate is used as the substrate. The inhibition constants at 37 °C in 50 mM Hepes buffer, pH 8.0, are 35 and 57 nM for K_{ii} and K_{is}, respectively. In addition to this inhibition at rapid equilibrium, mercury ions also inactivate *C. albicans* PMI by a much slower process, involving an irreversible mechanism. This is shown to be a two-step process, proceeding via an intermediate complex with a dissociation constant of 5.6 μM, with a maximum rate of inactivation of 0.15 min^{-1}. The rate of irreversible inactivation can be slowed by the addition of the substrate, mannose 6-phosphate. Incubation of the enzyme with $[^{203}Hg]Cl_2$ causes the formation of a stable adduct with one atom of mercury incorporated into each enzyme molecule during the inactivation. Since cysteine-150 is the only iodoacetate-modifiable cysteine in the protein, we propose that this is where the mercury ion reacts during the irreversible inactivation process. In the *Escherichia coli* enzyme this cysteine is replaced by an asparagine, and the enzyme cannot be irreversibly inactivated by mercury ions. In view of the variation of the protein sequence found around this residue in phosphomannose isomerases from different species, we studied the effect of mercury ions on the enzyme purified from a further three species: *Saccharomyces cerevisiae*, human and porcine. We have previously shown that these enzymes have very similar physicochemical and kinetic properties, implying an almost total conservation of the active site residues. Despite these similarities, the bimolecular inactivation constants for these four enzymes by mercury ions span 2 orders of magnitude, with the human enzyme being most easily inactivated. Clearly there are significant differences in the amino acids side chains in the local environment of cysteine-150 between the different species studied. These differences open up possibilities for the design of compounds which will show species-selective inhibition of phosphomannose isomerases.

Phosphomannose isomerase (PMI)[1] catalyzes the reversible isomerisation of fructose 6-phosphate and mannose 6-phosphate. In yeasts this is the first step in the biosynthesis of the mannoprotein in the cell wall, from glycolytic intermediates as well as the O- and N-mannosylation (Orlean, 1990). Temperature-sensitive mutants of the *pmi* gene in *Saccharoymces cerevisiae* produce mannose-deficient cell walls and undergo lysis at the restrictive temperature (Payton et al., 1991). The activity of this enzyme is therefore essential for the survival of yeast. The enzyme has been shown to be a monomeric metalloenzyme, with a molecular mass of 45 000 Da and containing one atom of zinc per protein molecule (Gracy & Noltmann, 1968a,b)

A monofunctional gene for PMI has been identified in six species: *S. cerevisiae*, *Candida albicans*, *Aspergillus nidulans*, *Escherichia coli*, *Salmonella typhimurium*, and human (Proudfoot et al., 1994a). There is a high degree of variation between the various phosphomannose isomerases. For example, the human and fungal enzymes are 41% identical at the level of amino acid sequence. The proteins have been purified to homgeneity from five species (Gracy & Noltmann, 1968a,b; Proudfoot et al., 1994b). Despite the large numbers of different amino acid residues between the various species, kinetic and physicochemical studies of these enzymes have shown that the porcine, human, *C. albicans*, and *S. cerevisiae* enzymes are remarkably similar (Proudfoot et al., 1994b). Our long term aim is to identify inhibitors of the fungal enzyme which are nontoxic to mammalian cells. We are therefore using protein chemistry techniques to identify regions of the enzyme where there are significant differences in amino acid composition, but yet they still play a role in controlling the enzyme activity.

Chemical labeling studies showed that only one cysteine residue, Cys-150, in the *C. albicans* protein can be labeled by iodoacetate. This carboxymethylation inactivates the enzyme and could be prevented by coincubation with substrate (Coulin et al., 1993). Inhibition by mercury ions had also been seen for the *S. cerevisiae* enzyme (Wells et al., 1993) and at a site distinct from the site where zinc ions inhibit the enzyme. Since mercury atoms show a clear preference for thiolate ligands, we have investigated the inhibition of PMI by mercury ions in more detail. In this study, we show evidence that Cys-150 forms part of the binding site for mercury ions. In addition there are significant differences in the mechanism of inhibition for the mammalian, fungal, and *E. coli* enzymes by mercury ions. This suggests that Cys-150 is located in a region of the protein where there is significant variation in side-chain composition between species.

MATERIALS AND METHODS

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

Enzyme Purification and Assay. *S. cerevisiae* and porcine enzyme were purified as described in Proudfoot et al. (1994b). Recombinant human and *C. albicans* PMI were purified as described in Proudfoot et al. (1994a) and Smith et al. (1994). *E. coli* PMI was purified using similar protocols (Coulin et

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[1] Abbreviations: PMI, phosphomannose isomerase; Tris-HCl, tris-(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

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al., 1993). 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 using a Thermomax platereader (Molecular Devices, Palo Alto), with a total reaction volume of 300 μL (Slein, 1955; Wells et al., 1993). Unless otherwise stated, the buffer was 50 mM Hepes pH 8.0 and the temperature was maintained at 37 °C.

Analytical Methods. Protein concentrations during purification were determined using the Bio-Rad Protein Assay reagent, with BSA as the standard. For the pure protein, the absorbance at 280 nm was used, calculating the specific absorbance based on the amino acid composition (Proudfoot et al., 1994). 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.

Reversible Inhibition by Mercury Ions. The enzyme was incubated with a variety of substrate and inhibitor concentrations and the reaction was routinely monitored for 3–5 min. The reaction velocity was calculated using the platereader software using linear regression. To verify that no significant time-dependent inhibition had taken place during this period, the individual time points for each progress curve were also transferred using the spreadsheet Excel (Microsoft) to the curve fitting package Grafit 3.01 (Leatherbarrow, 1992). These data were fitted to the equation for the progress curve (see below) by multiple parameter nonlinear regression and values for the initial reaction velocity v_0 were calculated. No significant difference was seen between the reaction velocity calculated by the linear regression and by the progress curve analysis for the *C. albicans* enzyme at $HgCl_2$ concentrations of less than 10 μM.

The data for the variation of the reaction velocity, v_0, with substrate and inhibitor concentration were measured at metal ion concentrations between 0 and 1 μM. At each metal ion concentration, the initial rate of enzyme activity v_0 was measured by direct fit to the integrated progress curve, in the substrate range $K_m^{app}/10$ to $5 \times K_m^{app}$. After examination of the data, the entire data set was combined and fitted to the standard equations for competitive, uncompetitive, and mixed inhibition (Cleland, 1979).

$$v = \frac{V_m[S]}{K_m(1 + [I]/K_{is}) + [S]} \quad (1)$$

$$v = \frac{V_m[S]}{K_m + [S](1 + [I]/K_{ii})} \quad (2)$$

$$v = \frac{V_m[S]}{K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})} \quad (3)$$

Since the secondary plots showed significant deviation from linearity, the data were also fitted to a partial noncompetitive (hyperbolic) inhibition model (Segel, 1975, equation IV-23):

$$v = \frac{V_m[S](1 + \beta[I]/K_{ii})}{K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})} \quad (4)$$

The statistical validity of including the extra parameters in eqs 3 and 4 was calculated using an *F*-test on the reduced χ^2 values.

Time-Dependent Inhibition of the Enzyme. This was studied by incubating the enzyme with substrate ([S] = K_m) in the presence of varying concentrations of mercury ions. Under these conditions, the concentration of substrate was not significantly reduced during the course of the experiment. The principle of the experiment is that as the enzyme is irreversibly inactivated, so the observed rate of change of the absorbance will decrease. The amount of product formed during the reaction was measured by reading the absorbance every 9 s and transferring the entire data set (200 × 96 readings for a 30-min incubation) via the spreadsheet programme Excel (Microsoft) into Grafit. Assuming that the time-dependent inhibition of the enzyme is a pseudo-first-order process for any given inhibitor concentration, the enzyme velocity at time t, (v_t) is described by

$$v_t = v_0 \exp^{-k_i^{app} t} \quad (5)$$

The absorbance at 340 nm is a measure of the amount of product formed, and the absorbance at time t, $A_{340}(t)$, is given by

$$A_{340}(t) = A_{340}(0) + \int_0^t (v_0 \exp^{-k_i^{app} t})\, dt \quad (6)$$

where $A_{340}(0)$ is the absorbance at the start of the experiment. Integrating eq 6 (Kitz & Wilson, 1962) gives:

$$A_{340}(t) = A_{340}(0) + v_0/k_i^{app}(1 - \exp^{-k_i^{app} t}) \quad (7)$$

The absorbance data were fitted directly to this equation to give values for k_i^{app}.

Incorporation of [^{203}Hg] into Phosphomannose Isomerase. C. albicans PMI was concentrated to 160 μM (7.69 mg/mL) in a Centricon 10 centrifugal concentrator; 65 μL of this protein was incubated with 1 mM [^{203}Hg]Cl_2 (Amersham, 0.3–2 mCi/mgHg) in 50 mM Hepes pH 8.0 buffer at 37 °C. Then 1-μL aliquots of this incubation were removed at timed intervals and diluted into 1 mL of ice-cold buffer to prevent further reaction. In order to assay the amount of incorporation of radioactivity, three 200-μL aliquots of this dilution were filtered through prewetted nitrocellulose filters (25 mm, 0.22 μm pore size, Schleicher and Schuell). 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 diluted incubation were used under the standard assay conditions.

After 40 min of incubation, the remaining mixture was gel filtered by using a PD10 gel filtration column (Pharmacia) into 50 mM Hepes buffer, pH 8.0. The stability of the complex was measured by incubating it in either 50 mM Hepes buffer at pH 8.0 or buffer adjusted to pH 2.6 by the addition of KCl/HCl buffer. Samples were removed and the amount of bound [^{203}Hg] determined by filtration on nitrocellulose filters as described above. Two micrograms of Asp-N protease were mixed with 0.5 mg of modified protein and incubated overnight at 37 °C. The peptides obtained were separated, analyzed, and sequenced as described previously (Coulin et al., 1993).

RESULTS

Reversible Inhibition of C. albicans Phosphomannose Isomerase. Initial studies of the inhibition of *C. albicans* PMI with mercury ions showed that although the metal ion was a potent inhibitor, complete inhibition could not be achieved even at saturating concentrations of the metal ion. A complete analysis of the variation of rate with the

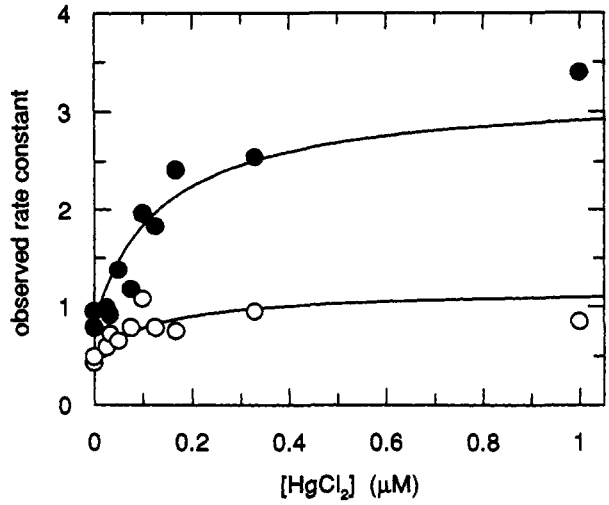

FIGURE 1: Inhibition of *C. albicans* PMI by mercury in 50 mM Hepes pH 8.0 buffer at 37 °C. Values for reciprocal rate constants have been expressed in units of $\mu mol^{-1} \cdot mg \cdot min$. At each metal ion concentration, the initial rate of enzyme activity v_0 was measured by direct fit to the integrated progress curve, in the substrate range $K_m^{app}/10$ to $5 \times K_m^{app}$. The secondary plots of the data show the variation of $(1/V_m)^{app}$ (●) and $(K_m/V_m)^{app}$ (○) with inhibitor. There is a clear hyperbolic inhibition pattern. The curves are calculated from the equation for partial noncompetitive inhibition, which is the model giving the lowest value for χ^2 (see Table 1).

concentration of the substrate at different inhibitor concentrations was therefore carried out. At each metal ion concentration, the initial rate of enzyme activity, v_0, was measured by direct fit to the integrated progress curve, in the substrate range $K_m^{app}/10$ to $5 \times K_m^{app}$. The apparent values for V_m and K_m were calculated at each concentration of metal ion, using the equation $v = V_m[S]/(K_m + [S])$. Plots of the variation of the observed values of $(1/V_m)^{app}$ and $(K_m/V_m)^{app}$ with inhibitor concentration (Figure 1) showed that both had substantial curvature and reached a limiting finite value at high concentrations of mercury ions. This behavior is typical of that for a partial noncompetitive inhibition (Segel, 1975). The entire data set was fitted to the equations for competitive, uncompetitive, noncompetitive and partial noncompetitive inhibitions using eqs 1–4. The reduced χ^2 values were lowest for the partial non-competitive model, and an F-test showed that the fit to this model was better than to any of the others, with $p < 0.0001$ (Table 1). The physical interpretation of this data is that the *C. albicans* enzyme can bind a mercury atom, but that this complex can still bind substrate and turn over, although with a reduced rate (βV_m).

Irreversible Inactivation of Phosphomannose Isomerase. Figure 2 shows a typical progress curve of the reduction of $NADP^+$ for the time-dependent inhibition of PMI with mercury ion at a fixed concentration of mannose 6-phosphate. It is typical of the progress curves used in this study. Clearly there is a time dependence to this inhibition, and so each time course was fitted to eq 7 (Kitz & Wilson, 1962). In order to visually inspect the quality of each fit, the data set was reduced by eliminating four out of every five points, and the velocity at any given time was calculated using the first derivative option within Grafit. The data can then be fitted to eq 5 and plotted on a logarithmic axis. An example of this is shown in Figure 2, where the raw data are shown as the primary plot and the calculated velocity is shown in the inset. The use of the integrated rate equation assumes that the only cause of loss of enzyme activity is due to the inactivation of phosphomannose isomerase. This has been verified by several control experiments. Firstly, addition of a 5-fold excess of coupling enzyme did not significantly alter the inactivation rate at mercury ion concentrations below 10 μM. This shows that the metal ion does not inactivate the coupling enzymes at these concentrations. Secondly, the product of the reaction sequence, 6-phosphogluconate is not inhibitory, as can be seen from the linearity of the progress curves in the absence of inhibitor. Thirdly, phosphomannose isomerase itself is stable at 37 °C in Hepes buffer at pH 8.0, although decay rates of 0.01–0.02 min^{-1} were seen in longer time course experiments in the absence of mercury ions. Control experiments where no inhibitor is added show a linear absorbance change for 1 absorbance unit, further confirming that the inhibition seen in the presence of mercury ions is not due to substrate or $NADP^+$ depletion.

The variation of the rate of inactivation of *C. albicans* PMI (k_i^{app}) with mercury ion concentration is shown in Figure 3a. The data show a hyperbolic dependence, consistent with a two-step model of inactivation:

$$E + I \overset{K_i'}{=} E{\cdot}I' \overset{k_i}{\rightarrow} E{-}I_{inactive}$$

where E·I′ is an initial noncovalent complex between the enzyme and the inhibitor and $E{-}I_{inactive}$ is the final inactive covalent complex with mercury. The data were fitted to the equation $k_i^{app} = k_i[I]/(K_i' + [I])$. The value calculated for K_i' is 5.6 ± 0.6 μM and k_i is 0.25 ± 0.04 min^{-1} (Table 2). The dissociation constant for mercury from this complex which leads to irreversible inactivation (K_i') is 2 orders of magnitude higher than the dissociation constants for the rapidly formed reversible enzyme mercury complex (K_{is} and K_{ii}).

The ability of mannose 6-phosphate to protect PMI against inactivation by $HgCl_2$ was studied by incubating the enzyme in 9 μM $HgCl_2$ (Figure 3b). Under these circumstances an apparent binding constant K_s^{app} for the substrate can be calculated using the equation $k_i^{app} = k_i^{obs}/(1+[S]/K_s^{app})$, where k_i^{obs} is the maximum rate of time-dependent inhibition for this concentration of mercury ions. It can be seen in Figure 3b that mannose 6-phosphate is able to protect the enzyme against inactivation by mercury ions. The value calculated for K_s^{app} is 1.0 ± 0.15 mM, at 37 °C in 50 mM Hepes buffer pH 8.0, which is similar to the K_m value for mannose 6-phosphate for this enzyme (0.7 mM).

For the *S. cerevisiae* enzyme, it has been shown that zinc ions can partially block reversible binding of mercury in the enzyme active site (Wells et al., 1993). The effect is for zinc to weaken the affinity of the enzyme for mercury by a factor of 11. We have therefore studied the ability of zinc ions to inhibit the irreversible inactivation of *C. albicans* PMI. The enzyme was incubated with 0.7 mM mannose 6-phosphate, 9 μM $HgCl_2$, and 0–200 μM $ZnCl_2$. The zinc ions did not protect the enzyme against modification.

Stoichiometry of Mercury Incorporation and Characterization of the Complex. The time course of incorporation of [^{203}Hg] into PMI is shown in Figure 4. The data show that 0.86 ± 0.08 atoms of mercury are incorporated into the protein with a rate constant of 0.33 ± 0.03 min^{-1}. This incorporation is paralleled by a loss of enzyme activity (data not shown). The rate of inactivation of the enzyme is higher in the absence of substrate, consistent with mannose 6-phosphate having a protective effect. The modified enzyme was stable at pH 8.0, where no significant decay could be detected at 25 °C. At pH 2.6, however, the half-life was estimated to be 450 min. In view of this stability, we attempted to isolate the mercury-containing peptide by protease digestion and RP-HPLC. In

Table 1: Inhibition of *C. albicans* PMI by Mercury Ions[a]

	V_m (μmol/min/mg)	K_m (mM)	K_{ii} (nM)	K_{is} (nM)	β	χ^2 (×10⁻⁴)
competitive	780 ± 30	0.33 ± 0.06		960 ± 404		6.06
uncompetitive	880 ± 50	0.43 ± 0.08	1.04 ± 0.31			6.02
noncompetitive	910 ± 50	0.44 ± 0.09	620 ± 318	nd[b]		6.04
partial noncompetitive	1200 ± 60	0.55 ± 0.10	35 ± 9	57 ± 21	0.26 ± 0.05	4.18

[a] The significance of the observed differences between the calculated χ^2 values was assessed using an *F*-test. The inclusion of the additional term in the partial noncompetitive model is significant at a value of $p \ll 0.0001$. [b] A value for this parameter was not resolved by the curve fitting.

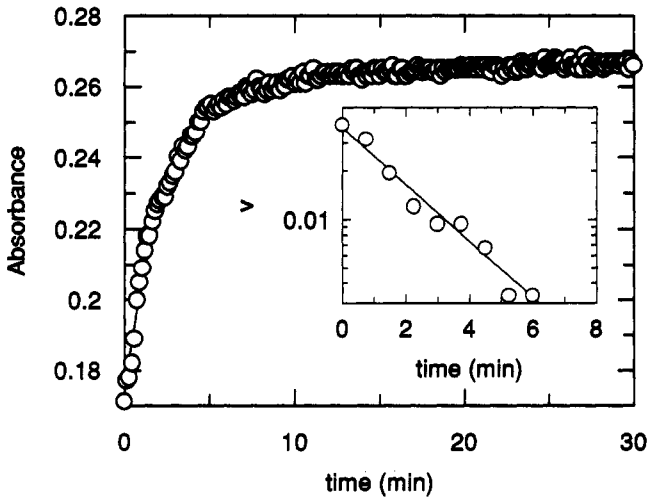

FIGURE 2: Typical inhibition experiment, showing the incubation of porcine phosphomannose isomerase with 250 μM $HgCl_2$ in 50 mM Hepes pH 8.0 buffer at 37 °C. The exponential rate of loss of enzyme activity was calculated by a direct fit of the absorbance data to the integrated rate equation as described in the text. For visual confirmation, the rate of change of absorbance was calculated for every fifth point using Grafit. This is plotted in the inset as a logarithmic transformation and shows good first-order kinetics over 4 half-lives.

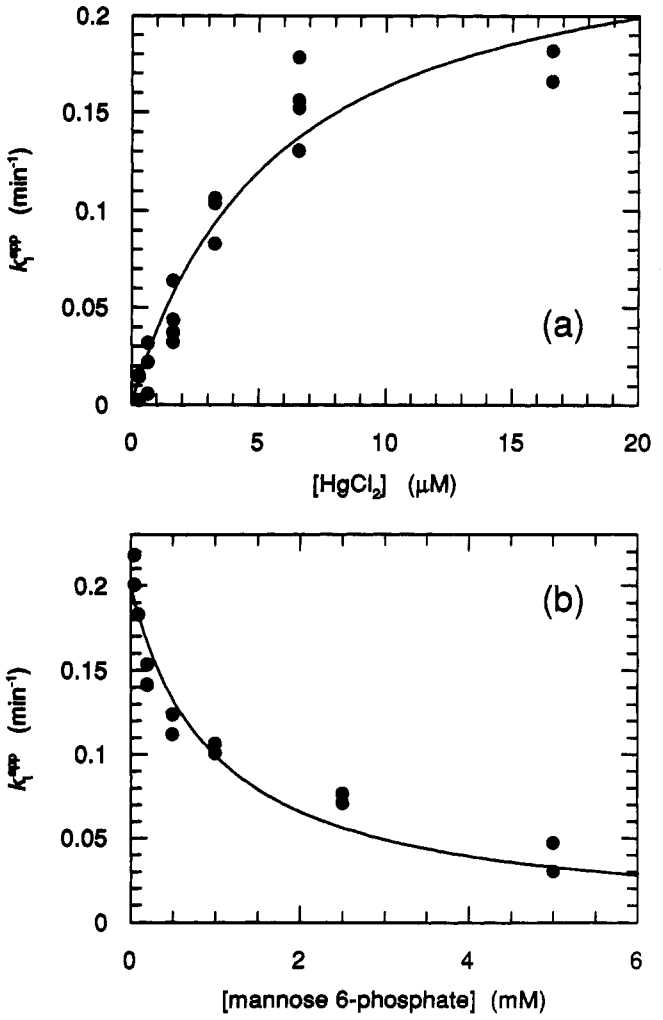

FIGURE 3: (a) Irreversible inhibition of *C. albicans* PMI by mercury ions in 50 mM Hepes pH 8.0 buffer at 37 °C. Progress curves were measured with mannose 6-phosphate at its K_m value, 0.7 mM. (b) Protection against the irreversible inactivation of *C. albicans* PMI by mercury ions by the addition of mannose 6-phosphate, in the presence of 9 μM $HgCl_2$ in 50 mM Hepes pH 8.0 buffer at 37 °C. (c) Protection by zinc ions against inactivation by 9 μM $HgCl_2$ for *C. albicans* in the presence of 0.7 mM mannose 6-phosphate.

three experiments, the radiolabel was found to be distributed between at least five peaks on the peptide map. The peak which was consistently labeled (with around 30% total radioactivity) was repurified and sequenced. It contained two sequences DFEGFXGFKPL (140 pmol) and DIIEXMAAS (35 pmol), where the residues X correspond to Cys-150 and Cys-295, respectively. We were unable to detect radioactivity in any of the phenylthiohydantoin derivatives. The radiolabel is presumably being scrambled between the cysteines within PMI as the protein is digested. The instability of the peptide–mercury complex means that we were unable to assign the radiolabeled amino acid with complete confidence.

Inhibition of PMI from Other Species by Mercury. In view of the differences on the sequence of phosphomannose isomerase in the region of Cys-150 (Table 3), we studied the effect of mercury ions on the inhibition of phosphomannose isomerase from four further species. In the case of the *E. coli* enzyme, studied at 1 mM mannose 6-phosphate substrate, there is an effect on the value of v_0, showing 50% inhibition at 52 μM (Figure 5a). This value is approximately a 100-fold weaker inhibition than seen for *C. albicans*. Since the *E. coli* measurements required such high concentrations of mercury, we had to use a 10-fold higher concentration of the coupling enzymes G6PDH and PGI, with 50-fold higher in the controls to eliminate mercury inhibition effects on the coupling system. It was therefore impossible to carry out a full analysis of the inhibition pattern with the *E. coli* enzyme. As can be seen in Figure 5b, there is no time-dependent irreversible inactivation of the *E. coli* enzyme by mercury ions. For *S. cerevisiae* PMI, mercury has already been shown to be a reversible inhibitor, with an inhibition constant of around 1 μM at pH 8.0 (Wells et al., 1993). However, it also causes a time-dependent irreversible inactivation of the enzyme at concentrations above 0.5 μM. As stated in the methods section, this irreversible inhibition will only significantly alter the measured value of the enzyme's initial velocity if the inactivation rate is higher than 0.2 min^{-1}. Finally, the two mammalian enzymes show completely different kinetics of inhibition by mercury ions. First, the irreversible inactivation of the human and porcine enzymes is much faster than the fungal enzyme, as can be seen from the bimolecular rate constant k_i/K_i'. The curves in Figure 5b show no convincing evidence of saturation when the observed inactivation rate reaches the limit of the assay (k_i^{app} = 0.6 min^{-1}) and therefore individual values of k_i and K_i, cannot be defined (Table 2). Second, we are unable

Table 2: Time-Dependent Inhibition of PMI by Mercury Ions[a]

	K_i' (μM)	k_i (min^{-1})	k_i/K_i' (min$^{-1}\cdot\mu$M^{-1})
C. albicans	5.65	0.25	0.044
S. cerevisiae	4.61	0.52	0.11
human	nd[b]	nd	4.16
porcine	nd	nd	1.80

[a] All values were determined with the substrate mannose 6-phosphate at its K_m concentration for each enzyme (Proudfoot et al., 1994b) in Tris-HCl pH 8.0 buffer at 37 °C. [b] The values of the individual rate and equilibrium constants could not be determined for the mammalian enzymes, as the data do not show any significant saturation. Only the bimolecular rate constant k_i/K_i can be calculated for these enzymes.

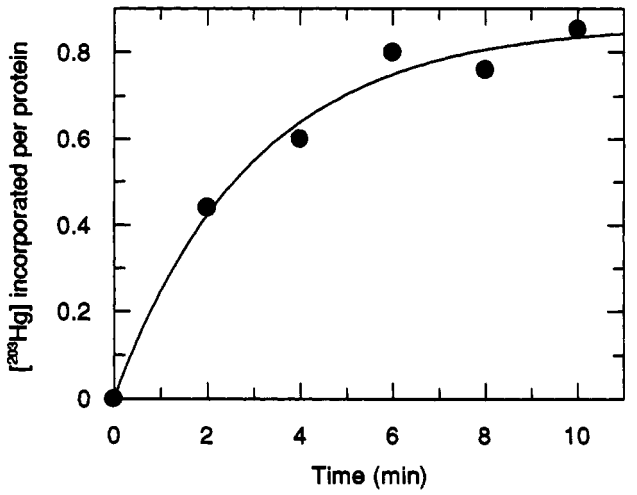

FIGURE 4: Incorporation of atoms of [^{203}Hg] into each *C. albicans* PMI molecule in 50 mM Hepes buffer, pH 8.0. The loss of enzyme activity correlates with the stable incorporation of the mercury ion. The experiment is the mean of three separate studies.

Table 3: Alignment of Phosphomannose Isomerase Sequence Surrounding Cysteine-150

	141[a]	151
E. coli PMI[b]	FALTPFLAMN	AFREFSEVIS
C. albicans PMI	IAVTDFEGFC	GFKPLDQLAK
S. cerevisiae PMI	IAVTDFEGFC	GFKPLQEIAD
human PMI	IALTPFQGLC	GFRPVDEIVT

[a] Numbering is taken from the *C. albicans* sequence (Smith et al., 1994). [b] The sequences are taken from Miles & Guest, 1984 (*E. coli*); Smith et al., 1994 (*C. albicans*); Smith et al., 1992 (*S. cerevisiae*); and Proudfoot et al., 1994b (human).

to measure the formation of the rapidly formed, reversible enzyme–mercury complex for either of the two mammalian species. No significant effect on the v_0 value was seen at 5 x10^{-8} M $HgCl_2$ for the human enzyme and 10^{-7} M $HgCl_2$ for the porcine enzyme. Above these concentrations, the irreversible inactivation rate of the enzyme is greater than 0.2 min^{-1}, and therefore it is impossible to measure v_0 accurately.

DISCUSSION

The data presented here show that mercury ions are potent inhibitors of fungal phosphomannose isomerase and that they act by two different mechanisms. On the one hand, there is a reversible inhibition of enzyme activity, which in the case of *C. albicans* PMI has been shown to be a partial noncompetitive inhibition, with nanomolar inhibition constants. This shows there is a site on the enzyme where mercury ions can bind, causing a partial inhibition of the enzyme (26% of the maximum activity is achieved in the presence of high concentrations of substrate and inhibitor). This is a more clear case of partial inhibition than that seen for the *S. cerevisiae* enzyme (Wells et al., 1993).

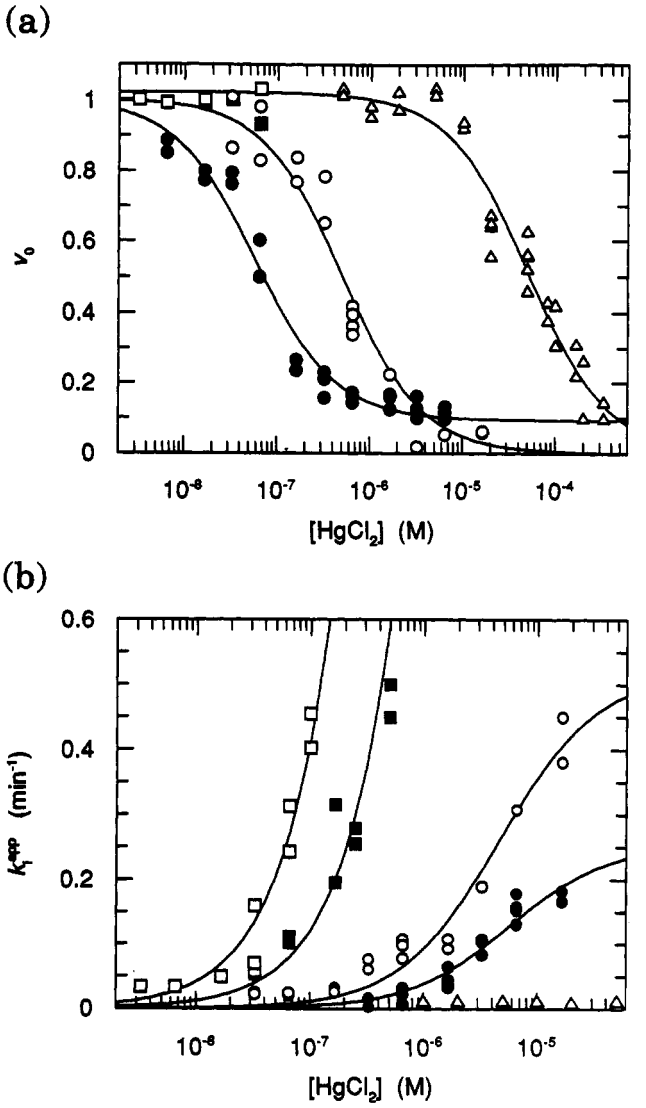

FIGURE 5: Reversible and irreversible inhibition of PMI by mercury ions. (a) Normalized initial velocities (v_0) for *C. albicans* (●), *S. cerevisiae* (○), porcine (■), human (□) and *E. coli* (△) PMI in the presence of varying concentrations of mercury ions, in 50 mM Tris-HCl pH 8.0 buffer at 37 °C. Mannose 6-phosphate is its K_m concentration in each case (Proudfoot et al., 1994b). v_0 values were calculated by fitting absorbance data to the integrated progress curves as described in the text. The error in v_0 values caused by time-dependent inactivation of the enzyme is less than ±10% for all data where k_i^{app} is less than 0.2 min^{-1}. (b) Time-dependent irreversible inactivation of *C. albicans* (●), *S. cerevisiae* (○), porcine (■), human (□), and E. coli (△) PMI by mercury ions under identical conditions.

In addition to this, we have also detected irreversible inhibition of the *C. albicans* enzyme by mercury ions. This takes place by a two-step process, the first step involving the formation of an enzyme mercury complex, with a dissociation constant of 5.6 μM, and the second step being the irreversible inactivation. This second step is presumably the covalent modification of the enzyme by the metal ion. Studies with radioactive mercury show that a single atom of the metal ion is incorporated and that this correlates with the inactivation of the enzyme. The addition of the substrate, mannose 6-phosphate, reduces the rate of modification, further confirming the importance of the modified amino acid side chain.

Three lines of evidence indicate that the site of irreversible inactivation is Cys-150. Firstly, cysteine is the preferred ligand for mercury. The ability of mercury ions to irreversibly inhibit enzyme activities has been well characterized in serine proteases (Betzel et al., 1988; Baggier et al., 1991), where they form covalent links with active site thiol groups. *C. albicans* PMI has five cysteines, but only one of them, Cys-150, is accessible to iodoacetate (Coulin et al., 1993). Carboxymethylation of Cys-150 leads to inactivation of PMI, and the enzyme can be protected against modification by the addition of mannose 6-phosphate, but not by the addition of excess zinc ions. This situation is exactly paralleled for the time-dependent inactivation of PMI by mercury ions, only one molecule of inactivator is required, and this inactivation is slowed by the substrate at its K_m value, but not by reversible binding of zinc ions. The *E. coli* enzyme has an asparagine residue in place of the Cys-150. Incubation of *E. coli* PMI with $HgCl_2$ does not cause any time-dependent inhibition of the enzyme, even at concentrations as high as 200 μM.

Secondly, the isolation of peptides from the inactivated *C. albicans* PMI showed that although there was a large amount of scrambling of the radiolabel during the digestion of the enzyme, the principal radioactive peak contained peptides bearing mainly Cys-150, with some Cys-295. Thirdly, we have recently crystallized the protein (Tolley et al., 1994). Mercury salts were used in the search for heavy atom derivatives to aid the solution of the three-dimensional structure by multiple isomorphous replacement. Difference Fourier analysis shows that the Hg atom is very close to the sulfur of Cys-150 in the crystal. Since only one mercury atom is seen in the structural studies, it is possible that under the conditions used for irreversible inactivation there is no binding of mercury ions to the site responsible for reversible hyperbolic noncompetitive inhibition.

The level of amino acid sequence identity around Cys-150 is about the same as the average for the whole protein (Table 3). Only four out of the twenty residues surrounding Cys-150 are conserved between the four species shown, and only eight are identical between the *C. albicans* and human enzymes. This contrasts with the sequence surrounding Arg-304, the other active site residue identified by chemical modification studies (Wells et al., 1994). In the region surrounding Arg-304, 14 out of 20 amino acids are totally conserved between the two species. This led us to speculate that the environment of the mercury binding site may be different between mammalian and fungal species. The study of the inhibition of mammalian PMI by mercury ions showed that much lower concentrations of mercury ions were required for irreversible inhibition. The bimolecular inhibition constants k_i/K_i' are 2 orders of magnitude higher for human PMI compared to *C. albicans* PMI. It is unlikely that the increased reactivity of the human enzyme is due to binding of mercury to another thiol residue, since the rate of inactivation of the human enzyme can also be reduced by adding mannose 6-phosphate at its K_m concentration. The enhanced reactivity of mercury toward the mammalian enzymes is most likely to be caused by differences in the local amino acid composition around the mercury binding site, cysteine-150.

In the design of therapeutic agents, this is an important factor. Our goal is to find molecules which selectively inhibit the fungal PMI, and this requires that there are significant differences in the protein sequences of the human and fungal forms of the enzyme in regions which can control enzyme activity. Previous studies of the fungal and mammalian enzymes failed to find any significant differences in the physicochemical or kinetic properties of phosphomannose isomerases (Proudfoot et al., 1994b). This implies that most of the active site residues are conserved between the species. The kinetic differences seen in this study imply that there are significant differences between the fungal and mammalian enzymes in the region around Cys-150 and it may be a potential binding site for species-selective compounds. Such a molecule would need to be nontoxic and also contain a ligand which used the amino acid differences in this region to selectively inhibit the fungal enzyme. Other thiol binding metals have already been used in therapeutic molecules, such as gold in Auranofin and silver in the topic antifungal silver sulfadiazine. Once the three-dimensional structure of this region has been elucidated, it may be possible to obtain a selective inhibitor by fine tuning the interactions of the organic parts of such organometallic complexes, to selectively bind the fungal form of the enzyme.

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