Mechanism of Irreversible Inactivation of Phosphomannose Isomerases by Silver Ions and Flamazine

Timothy N. C. Wells,* Paul Scully, Gerhard Paravicini, Amanda E. I. Proudfoot, and Mark A. Payton

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

Received January 18, 1995; Revised Manuscript Received March 29, 1995[⊗]

ABSTRACT: Silver ions and silver-containing compounds have been used as topical antimicrobial agents in a variety of clinical situations. We have previously shown that the enzyme phosphomannose isomerase (PMI) is essential for the biosynthesis of *Candida albicans* cell walls. In this study, we find that PMI can be inhibited by silver ions. This process is shown to be irreversible, and is a two-step process, involving an intermediate complex with a dissociation constant, K_i, of 59 ± 8 μM, and a maximum rate of inactivation of 0.25 ± 0.04 min^{-1} in 50 mM Hepes buffer, pH 8.0 at 37 °C. The enzyme can be protected against this inactivation by the substrate mannose 6-phosphate, with a dissociation constant of 0.31 ± 0.04 mM, close to its K_m value. Flamazine (silver sulfadiazine) is a silver-containing antibiotic which is used clinically as a topical antimicrobial and antifungal agent. We compared the ability of silver sulfadiazine and two other silver-containing compounds to irreversibly inactivate *C. albicans* PMI. The addition of the organic moiety increased the affinity of the compounds, with silver sulfadiazine showing a K_i of 190 ± 30 nM. In all cases, the maximum inhibition rate was similar, implying a similar rate-determining step. Silver sulfadiazine does not inhibit *Escherichia coli* PMI, and this suggests a role of the only free cysteine, Cys-150, in the inactivation process. To confirm this, we mutated this residue to alanine in *C. albicans* PMI. The resultant Cys150 → Ala mutant protein showed similar V_m and K_m values to the wild-type enzyme. However, it could not be inhibited by silver sulfadiazine, and was 1000-fold less sensitive to mercury inhibition. This confirms that Cys-150 is the site of action of the compounds in the *C. albicans* enzyme. All the silver-containing compounds inhibit human PMI with similar values for k_i/K_i, in the range 0.43−1.67 $\mu M^{-1} \cdot min^{-1}$. By modifying the organic moiety, we have changed the compounds from a 210-fold bias toward inhibiting the mammalian enzyme to only 1.3-fold. The region surrounding Cys-150 presumably contains residues which could be important in the design of selective PMI inhibitors.

Phosphomannose isomerase (PMI)[1] is a zinc metalloenzyme which catalyzes the reversible interconversion of fructose 6-phosphate and mannose 6-phosphate. The protein is a 48 kDa monomer, which contains one atom of zinc, which is absolutely required for activity. The reaction catalyzed 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 essential role in yeast cell wall biosynthesis (Orlean, 1990; Payton et al., 1991). This is confirmed by the fact that temperature-sensitive mutations in the *Saccharomyces cerevisiae* PMI gene cause cell lysis at the restrictive temperature (Smith et al., 1992). Potent inhibitors of the yeast PMI would therefore be possible candidates as antifungal agents. The gene coding for the *Candida albicans* PMI enzyme has recently been cloned (Smith et al., 1995) using probes based on the *S. cerevisiae* gene (Smith et al., 1992) and an ordered array genomic library from *C. albicans* DNA. The protein has been overexpressed in an *Escherichia coli* host, enabling the purification of milligram quantities of active enzyme.

Previous studies of the *S. cerevisiae* enzyme (Wells et al., 1993) showed that it could be inhibited by the metal ions zinc, cadmium, and mercury, and that this inhibition could be competed by the substrate mannose 6-phosphate. Kinetic evidence, based on Theorell−Yonetani double-inhibition plots, showed that the binding site for mercury ions was structurally distinct from the binding site for cadmium or zinc ions. Subsequent experiments (Proudfoot et al., 1994b; Wells et al., 1994) confirmed similar inhibition patterns for the *C. albicans* enzyme. Labeling studies with [^{14}C]-iodoacetate showed that there is only one free substrate-protectable surface thiol group in *C. albicans* PMI, namely, Cys-150 (Coulin et al., 1993). Studies with [^{203}Hg] showed only one atom of mercury binding per protein, under conditions where enzyme activity was lost. Preliminary X-ray crystallographic analysis of *C. albicans* PMI crystals soaked in $HgCl_2$ showed only one mercury bound per protein molecule, and that this is in close proximity with Cys-150 (Tolley et al., 1994; Ann Cleasby, unpublished results). The evidence for the role of this residue in the mercury binding site was further strengthened by the fact that the *E. coli* PMI, which contains no equivalent cysteine residue to Cys-150, requires almost 3 orders of magnitude higher concentrations of mercury ions to bring about inhibition.

Mercury or mercuric compounds would not make suitable *in vivo* inhibitors of *C. albicans* PMI due to their toxicity problems. However, several pharmaceutical agents have been produced which contain metal salts, which could conceivably bind to an active-site thiol group, such as Auranofin [containing gold(I)] and silver sulfadiazine [containing silver[(I)]. We therefore studied the inhibition of *C.*

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1995.

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

0006-2960/95/0434-7896$09.00/0

albicans PMI by silver ions, the silver-containing compounds silver sulfadiazine, and two other silver compounds originally made as DNA intercalators. All compounds are irreversible inactivators of *C. albicans* PMI, and they also inactivate the human enzyme. Using site-directed mutagenesis, we have changed Cys-150 to alanine in the *C. albicans* enzyme, and eliminated the susceptibility to irreversible inactivation by silver-containing compounds, confirming Cys-150 is the primary site of inhibition of the enzyme by such compounds.

MATERIALS AND METHODS

Unless otherwise stated, chemicals were purchased from Sigma and enzymes from Boehringer Mannheim. Silver sulfadiazine (Flamazine, silver 4-amino-*N*-2-pyrimidinyl-benzenesulfonamide, Figure 2) was the kind gift of Dr. P. Salden, Solvay Duphar B. V., Weesp, NL. The compounds GR 131921 X and GR 123465 X were obtained from the Glaxo compound file (Figure 2). They were synthesized from the free bases by dissolving in 50% aqueous ethanol, containing dilute aqueous ammonia, and adding 1 molar equiv of $AgNO_3$. In both cases, the complexes precipitated and were removed by filtration. All three of the silver complexes were dissolved in dimethyl sulfoxide prior to use, and then diluted with buffer. In no case was the dimethyl sulfoxide concentration in the assay higher than 2%, and this had been previously shown to have no significant effect on the assay.

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 $NADP^+$ measured by the change in absorption at 340 nm (Slein, 1955; Wells et al., 1993); 0.25 unit of each of the coupling enzymes was normally used in each assay (total volume 250 μL), except at high concentrations of silver ions, where 10 times this quantity was used (2.5 units per assay). Unless otherwise stated, the buffer used was 50 mM Hepes, pH 8.0, with Na^+ as the counterion.

Enzyme Purification. Recombinant *C. albicans* PMI was overexpressed in *E. coli*, and was purified by a similar method to that described previously (Proudfoot et al., 1994a). The mutant Cys → Ala150 was purified using a smaller scale version of this protocol. Endogenous *E. coli* PMI was purified from 200 g of *E. coli* B cells using a protocol similar to those described for the yeast enzymes (Proudfoot et al., 1994b).

Analytical Methods. Protein concentrations were determined using the specific absorbance at 280 nm. (A 1 mg/mL solution gives an absorbance of 0.53.) The specific absorbance was calculated from the known amino acid composition of *C. albicans* PMI, as determined from the DNA sequence (Smith et al., 1995). SDS−PAGE was carried out on 10−15% Phast gradient gels (Pharmacia) or 12% polyacrylamide gels using the Biorad Protean-II minigel system, and the proteins were stained with Coomassie Brilliant Blue R250. Metal incorporation measurements were performed by incubating enzyme (1 mg/mL) in the presence of concentrations of $AgNO_3$ and 1 mM mannose 6-phosphate, and then gel-filtering using PD-10 columns (Pharmacia) to remove unincorporated metal ions. The metal content of the samples was measured by inductively-coupled plasma atomic absorption spectroscopy (ICP-AES) by Alan Cox at Sheffield Hallam University, U.K.

Time-Dependent Inhibition of the Enzyme by Silver Ions and Complexes. The time-dependent irreversible inactivation of PMI was studied using an integrated rate equation approach, similar to that used in the study of inhibition of PMI by mercury ions (Wells et al., 1994; Kitz & Wilson, 1962), and other enzyme inactivation studies (Tew, 1993). The enzyme was mixed with the substrate mannose 6-phosphate at a concentration close to its K_m value (normally 1 mM was used) in a 96-well microtiter plate. Coupling enzymes and buffers were added. At the beginning of the experiment, a concentration of silver ions or silver complexes was added to each well of the plate, and the plate was inserted into a thermostatically controlled microtiter plate reader (Thermomax, Molecular Devices, Palo Alto, CA). As the enzyme turns over, the substrate is converted into fructose 6-phosphate, which is finally converted to 6-phosphogluconate by the coupling enzymes, with a resultant increase in the absorbance at 340 nm. The progress of the reaction was monitored by reading the absorbance of each well every 9 s. These data were stored, and transferred via the spreadsheet program Excel (Microsoft) into the curve-fitting package Grafit 3.01 (Leatherbarrow, 1992).

The principle of the experiment is that any irreversible inhibitors will inactivate the enzyme over the course of the experiment. The observed velocity of the enzyme will therefore decrease exponentially. In practice, this means that the slope of a graph of absorbance plotted against time (Figure 2, Wells et al., 1994) will decrease as the experiment proceeds, due to inactivation of the enzyme. As discussed in this previous study, the slope of the plots of absorbance against time could in theory be measured, and thus the reaction velocity calculated directly at any time point in the experiment. However, this involves a loss of data points, and in practice, it is more accurate to integrate the equation describing the rate of change of enzyme activity, and fit *directly* to the data of absorbance (i.e., product formation) against time (Wells et al., 1994). Assuming that the time-dependent inhibition of the enzyme is a pseudo-first-order process for any given inhibitor concentration, the enzyme velocity at a time t, (v_t) can be described by

$$v_t = v_0 \exp(-k_i^{app} t) \quad (1)$$

where v_0 is a measure of the initial velocity of the enzyme-catalyzed reaction. However, we can only measure the absorbance at 340 nm, which is a measure of the amount of product formed during the course of the experiment. Since the absorbance at time t, $A_{340}(t)$ is related to the reaction velocity v_t by the equation $A_{340}(t) = A_{340}(0) + \int_0^t (v_t)\, dt$, inserting the expression describing the variation of the reaction velocity v_t with time gives

$$A_{340}(t) = A_{340}(0) + \int_0^t [v_0 \exp(-k_i^{app} t)]\, dt \quad (2)$$

$A_{340}(0)$ is the absorbance at the start of the experiment, and corresponds to the absorbance due to the plate itself and the various buffer components. Integrating eq 2 (Kitz & Wilson, 1962) gives

$$A_{340}(t) = A_{340}(0) + v_0/k_i^{app}[1 - \exp(-k_i^{app} t)] \quad (3)$$

The absorbance data from the microtiter platereader were fitted directly to this equation, and from these values for the

rate of inactivation of the enzyme under these conditions, k_i^{app}, and the initial velocity of the enzyme-catalyzed reaction, v_0, could be calculated.

In Vitro Mutagenesis. A 1.5 kb *Cla*I fragment containing the entire *C. albicans* PMI open reading frame was introduced into the single *Cla*I site of the pSelect vector of the Altered Sites *in vitro* mutagenesis kit (Promega, Madison, WI). The mutation Cys → Ala150 was introduced using the mutant oligonucleotide 5′-TTTTGAAGGGTTTGCT-GGGTTTAAACCTT-3′, according to the manufacturer's instructions. After the mutagenesis, the 1.5 kb *Cla*I fragment was reinserted into the vector CBA′, which depends on the temperature-sensitive *p*L induction system (Allet et al., 1988). The insert regions and the cloning sites were sequenced to verify that only the mutation coding for Cys150 → Ala had been introduced. The CBA′ vector construct was used to express the mutant protein in an *E. coli* B host under the control of the p_L promoter. Cells were grown in 500 mL cultures in 2 L shaker flasks, and induced in early logarithmic phase by a temperature shift from 37 °C to 42 °C. The mutant protein was purified to homogeneity using a similar protocol as that used for the wild-type enzyme (Proudfoot et al., 1994a).

RESULTS

Irreversible Inactivation of C. albicans PMI with Silver Ions. When *C. albicans* PMI is incubated in 50 mM Hepes buffer, pH 8.0, 37 °C, in the presence of silver nitrate, a time-dependent inhibition of the enzyme is observed. In order to make it easier to study the rate of this inactivation, we have measured the loss of enzyme activity using an integrated progress curve analysis method. The progress curves for the coupled enzyme reaction in the presence of silver ions or complexes are not linear and appear to reach a limiting value or plateau which is the result of inactivation of the enzyme rather than depletion of substrate or $NADP^+$. This plateauing effect is similar to that seen with mercury ions (Wells et al., 1994). From this, a rate constant for the inactivation of PMI can be calculated, provided that the decrease in reaction velocity can be shown to be due solely to the inhibition of PMI. Several control experiments indicate that this is the case. First, the decrease is not due to inactivation of the coupling system enzymes (G6PDH or PGI), since addition of excess coupling enzyme does not significantly alter the rate of inactivation of PMI at silver ion concentrations of less that 10 μM. The activity of the coupling system was further verified by the addition of a second aliquot of fresh PMI once the reaction had reached a plateau. This second aliquot was capable of producing a similar rate of change of absorbance as the first aliquot, indicating that the coupling enzymes had not been inactivated. Second, the inactivation is not due to thermolability of the PMI, which has been shown to be stable at 37 °C for the duration of the assay in Hepes buffer at pH 8.0. In the absence of inhibitors, the reaction has been run for 120 min with no major deviation from linearity. Finally, the inhibition is not due to product inhibition since the reaction product (6-phosphogluconate) is not inhibitory at these concentrations. It can therefore be concluded that the decrease in activity measured in these assays is due to the metal ion causing a time-dependent irreversible inhibition of PMI.

C. albicans PMI was inactivated in a time-dependent manner by a range of concentrations of silver ions, and the rate of irreversible inactivation (k_i^{app}) and the initial velocity (v_0) were calculated by directly fitting the variation of the absorbance at 340 nm data to eq 3 as described earlier. The variation of the rate of inactivation of *C. albicans* PMI (k_i^{app}) with silver ion concentration is shown in Figure 1a. The data show a hyperbolic dependence of the rate of inactivation on the concentration of inhibitor, which is consistent with a two-step model of inactivation (Wells et al., 1994):

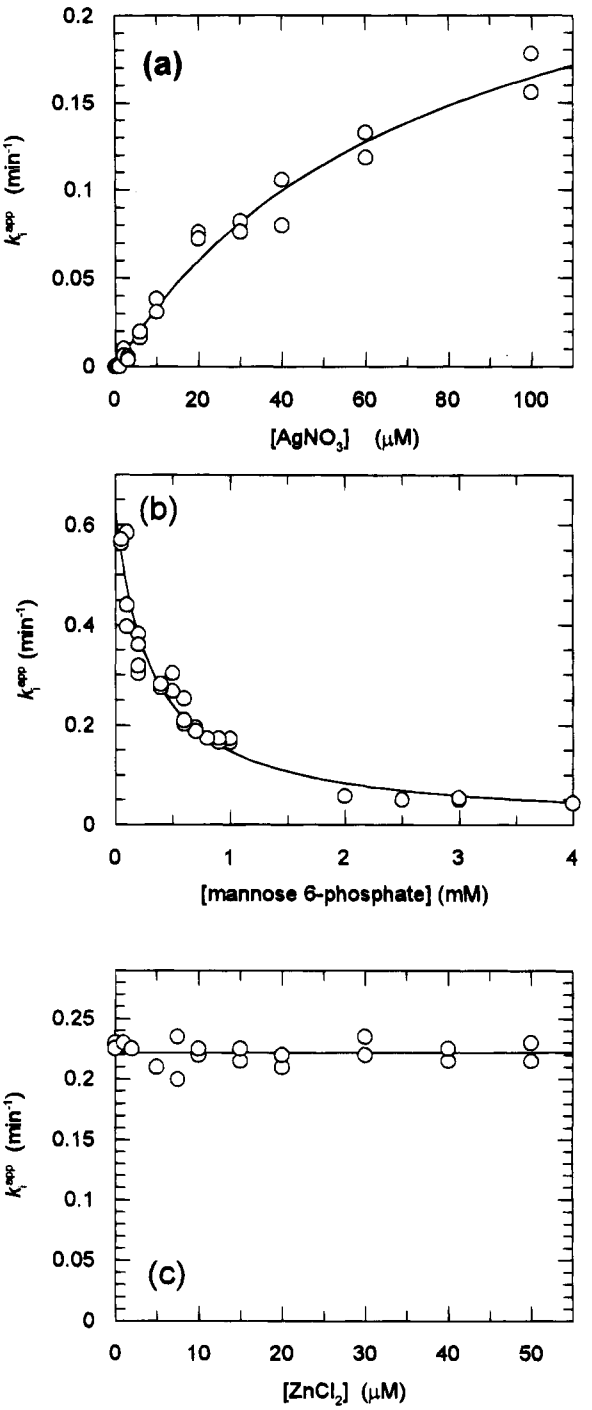


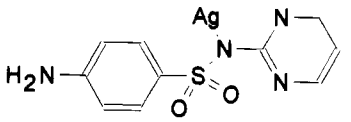
FIGURE 1: (a) Irreversible inhibition of *C. albicans* PMI by silver ions in 50 mM Hepes, pH 8.0, buffer at 37 °C. Progress curves were measured with mannose 6-phosphate at 1 mM. (b) Protection against the irreversible inactivation of *C. albicans* PMI by silver ions by the addition of mannose 6-phosphate, in the presence of 50 μM $AgNO_3$ in 50 μM Hepes, pH 8.0, buffer at 37 °C. (c) Lack of protection by zinc ions against inactivation by 50 μM $AgNO_3$ and 0.2 mM mannose 6-phosphate under similar conditions.

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

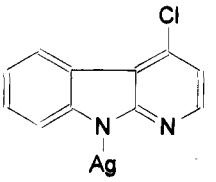
where E·I is an initial noncovalent complex between the enzyme and the inhibitor, and presumably represents the silver ion bound near to its final site of covalent attachment, but held by electrostatic forces. The term $E-I_{inactive}$ represents the final covalent complex with silver. The data were fitted to the equation $k_i^{app} = k_i[I]/(K_i + [I])$. The value calculated for K_i (the dissociation constant for the initial encounter complex between the enzyme and the metal ion) is 59 ± 8 μM. The value for k_i (the maximum rate of reaction of the metal ion with the enzyme) is 0.25 ± 0.04 min^{-1} in the presence of 1 mM mannose 6-phosphate substrate. Because of the difficulties of working with silver ion concentrations higher than 100 μM, it was impossible to go to extremely high concentrations of silver ions to prove that the data do have a hyperbolic dependence. However, if a linear fit is used, there is a clear pattern in the residuals, and an *F*-test shows that the fit to a hyperbola is significantly better than the fit to a straight line ($p < 0.0001$, $n = 18$). Similar data were seen when the reaction was carried out in sodium borate buffer, pH 8.0. In this case, K_i was found to be 17 ± 3 μM with $k_i = 0.29 \pm 0.05$ min^{-1}. No effect was seen on the initial rate constant, v_0, at the concentrations used. At silver ion concentrations higher than 5 μM, 10-fold higher quantities of coupling enzymes (2.5 units of each) had to be added to eliminate the possibility that inhibition of G6PDH or PGI was causing the observed loss of activity (Wells et al., 1994). In the range of concentrations used, the incorporation of silver into the proteins was found to be 0.8 silver atom per protein by ICP-AES analysis, and this incorporation did not cause the loss of the essential zinc atom.

The ability of mannose 6-phosphate to protect against this irreversible inhibition was studied by assaying the enzyme activity in the presence of 50 μM $AgNO_3$ and varying amounts of substrate (Figure 1b). Under these conditions an apparent binding constant can be calculated using the equation $k_i^{app} = k_i^{obs}/(1 + [S]/K_s^{app})$. This assumes that the enzyme which is complexed with substrate cannot bind the irreversible inhibitor. k_i^{obs} is the maximum rate of time-dependent inhibition for this concentration of silver ions, and can practically be measured at concentrations down to 50 μM mannose 6-phosphate. Under these conditions, k_i^{app} was calculated to be 0.62 ± 0.031 min^{-1} and K_s^{app} was 0.31 ± 0.036 mM, which is close to the value of K_m for mannose 6-phosphate in Hepes buffer at pH 8.0 (0.52 ± 0.06 mM). Since zinc ions were known to be competitive inhibitors of the *S. cerevisiae* enzyme (Wells et al., 1993; Proudfoot et al., 1994b), we investigated whether the addition of zinc ions would prevent the inactivation of the enzyme by silver ions. Consistent with previous results (Wells et al., 1993, 1994), the addition of zinc ions had no effect on the value of k_i^{app} (Figure 1c).

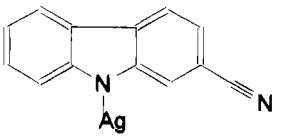
A search of the in-house compound databases revealed that several compounds were available in which a silver atom is bound to the nitrogen of an organic molecule. Two of these (GR 131921 X and GR 132465 X; see Figure 2) were made as part of a chemotherapy program as potential DNA intercalating agents. The third, Flamazine (silver sulfadiazine), is sold commercially as a topical antimicrobial agent, especially in the management of wound infection following burns (Nangia et al., 1987). When tested in the phosphomannose isomerase assay, all three of these compounds showed time-dependent irreversible inhibition. In addition, the rate of irreversible inhibition of the enzyme in each case showed a hyperbolic dependence on the concentration of the inhibitor used. The inactivation rate constants were therefore fitted to the equation $k_i^{app} = k_i[I]/(K_i + [I])$. The data are shown in Figure 3, and the rate constants are shown in Table 1. The effect of the organic moiety is clearly seen to alter the dissociation constant for the molecule (K_i) which varies over a 300-fold range, without having a significant effect on the maximum rate of inhibition (k_i) which stays constant in the range 0.23−0.30 min^{-1}. In all cases, the free organic base (without the silver present) was tested up to concentrations of 10 μM, and no signs of inhibition of the enzyme activity were seen. In all cases, the fit to a hyperbola was significantly better than to a simple bimolecular dependency



Silver sulfadiazine



GR 131921 X



GR 132465 X

FIGURE 2: Structures of silver-containing inhibitors used in this study.

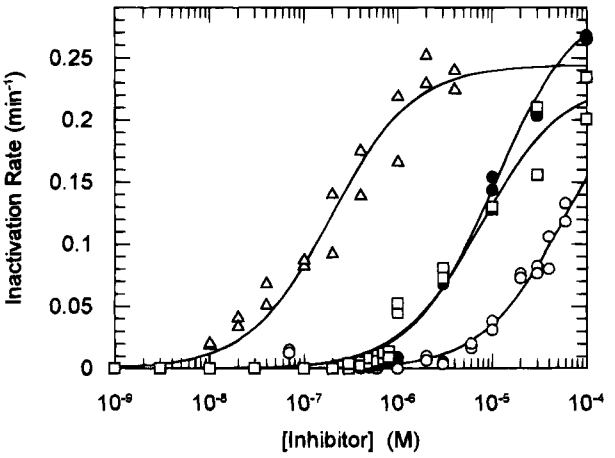


FIGURE 3: Irreversible inhibition of recombinant *C. albicans* PMI by silver ions and silver-containing compounds in 50 mM Hepes buffer at 37 °C. Time-dependent inactivation rate constants for (○) $AgNO_3$, (●) GR 131921, (□) GR 132465, and (△) silver sulfadiazine were measured in 50 mM Hepes, pH 8.0, buffer at 37 °C, with mannose 6-phosphate at 1 mM.

Table 1: Kinetic Constants for the Irreversible Inhibition of *C. albicans* and Human PMI by Silver Ions and adducts

inhibitor	species	k_i (min^{-1})	K_i (μM)	k_i/K_i (μM^{-1} min^{-1})	specificity index
$AgNO_3$	*C. albicans*	0.25 ± 0.04	59 ± 8	0.0042 ± 0.0004	210
	human	—[a]	—	0.88 ± 0.03	
GR 131921 X	*C. albicans*	0.30 ± 0.01	10.6 ± 0.8	0.028 ± 0.003	15.3
	human	—	—	0.43 ± 0.015	
GR 132465 X	*C. albicans*	0.23 ± 0.01	6.9 ± 0.9	0.033 ± 0.004	13.6
	human	—	—	0.45 ± 0.015	
silver sulfadiazine	*C. albicans*	0.24 ± 0.01	0.19 ± 0.03	1.25 ± 0.19	1.33
	human	—	—	1.67 ± 0.05	

[a] No evidence for saturation is seen in the experiments using human enzyme; therefore only the bimolecular rate constant k_i/K_i can be calculated.

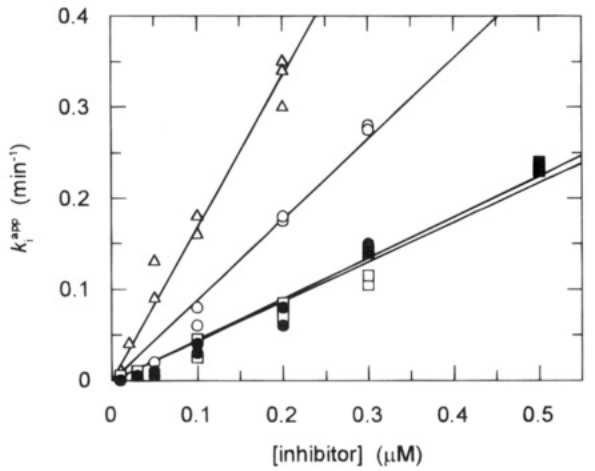


FIGURE 4: Irreversible inhibition of recombinant human PMI by silver ions and silver-containing compounds in 50 mM Hepes buffer at 37 °C. Time-dependent inactivation rate constants for (○) $AgNO_3$, (●) GR 131921, (□) GR 132465, and (△) silver sulfadiazine were measured in 50 mM Hepes, pH 8.0, buffer at 37 °C, with mannose 6-phosphate at 0.2 mM.

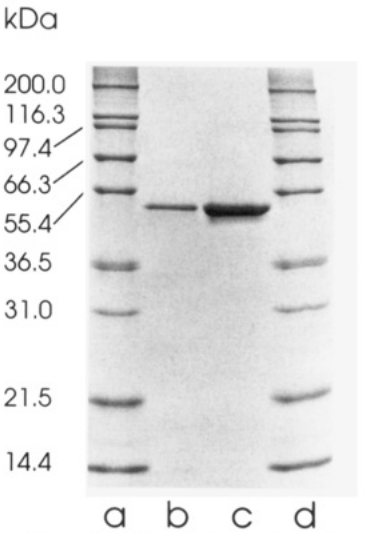


FIGURE 5: SDS−polyacrylamide gel electrophoresis analysis. The gel shows (a and d) molecular mass standards, (b) mutant PMI Cys150 → Ala, and (c) wild-type *C. albicans* PMI.

($p < 0.0005$). The use of higher concentrations of compounds was limited by their solubility.

Similar studies were carried out with recombinant human enzyme (Proudfoot et al., 1994a) in order to find out if there was any potential for species-selective inhibition with these compounds. $AgNO_3$ inhibits human phosphomannose isomerase, at much lower concentrations than the *C. albicans* enzyme (Figure 4). As can be seen from these graphs, the rate of irreversible inhibition is directly proportional to the concentration of silver ion added. In contrast to the effects seen with the *C. albicans* enzyme, the inhibition does not show signs of saturation within the range of concentrations used in this assay. In addition, the maximum rate of inhibition is much higher, with values as high as 0.6 min^{-1} observed (the practical limit for this type of assay). The inhibition data for the human enzyme were therefore fitted to the equation $k_i^{app} = (k_i/K_i)/[I]$ where k_i/K_i is the bimolecular rate constant for the association of the enzyme with the inhibitory metal, and this was treated as an single variable in the fitting process. No significant improvement in the χ^2 value was obtained when the data were fitted to a hyperbolic model. The results are summarized in Table 1, where it can be seen that for silver ions, the bimolecular rate constant for inhibition is 210-fold higher with the human enzyme than the *C. albicans* enzyme. This ratio of bimolecular reaction rates is the best comparison of the effects of compounds on the two enzymes, and we have called it a specificity index in Table 1. However, with the human enzyme, in contrast to the *C. albicans* enzyme, the addition of an organic moiety has very little influence on the bimolecular rate constant for inhibition, causing a 2-fold reduction in the rate in the case of GR 131921 X and GR 132465 X, and a 2-fold increase for silver sulfadiazine. The result of these changes is that the specificity index is reduced from 210 to 1.33 in the case of silver sulfadiazine. This means that whereas silver ions preferentially inhibit the human form, silver sulfadiazine is almost equally potent against both species of enzyme. This change in the bimolecular rate constant of inactivation is not due to the compounds binding at a different site, since the inhibition of the human and *C. albicans* enzymes by the silver-containing compounds can also be prevented by the addition of higher concentrations of the substrate mannose 6-phosphate to the incubation mixture.

Analysis of the Cys → Ala150 Mutant. The selective inhibition of human PMI compared with the fungal enzyme by mercury ions has been previously reported (Wells et al., 1994). In that study, the time-dependent inhibition was shown to involve one mercury atom. Several lines of evidence including protein chemistry and X-ray crystallography suggested that the mercury binding site was Cys-150. This residue had been previously shown to be the only accessible thiol in the protein (Coulin et al., 1993). In order to test whether Cys-150 was the site required for inhibition by silver and the silver-containing compounds, we made a mutant of the *C. albicans* enzyme where this cysteine had been replaced with alanine (Cys150 → Ala). The enzyme was expressed at similar levels to the wild-type protein, and was purified to homogeneity from small quantities of cells grown in 500 mL cultures in 2 L shaker flasks (Figure 5).

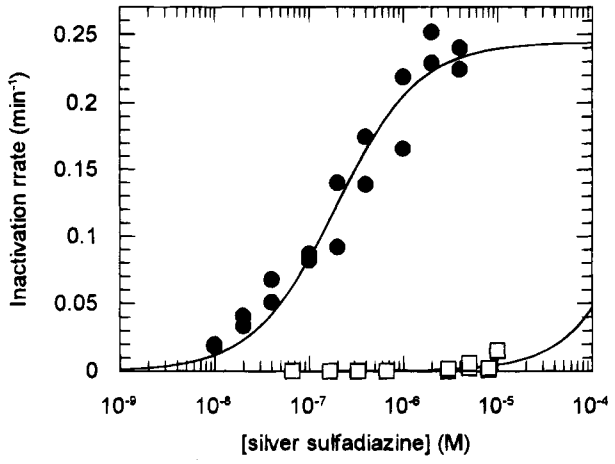


FIGURE 6: Lack of irreversible inactivation of the *C. albicans* PMI mutant C150A, when incubated with silver sulfadiazine (□), as compared with the wild type enzyme (●). The reactions were carried out at 37 °C in 50 mM Hepes buffer, pH 8.0, in the presence of 0.5 mM mannose 6-phosphate.

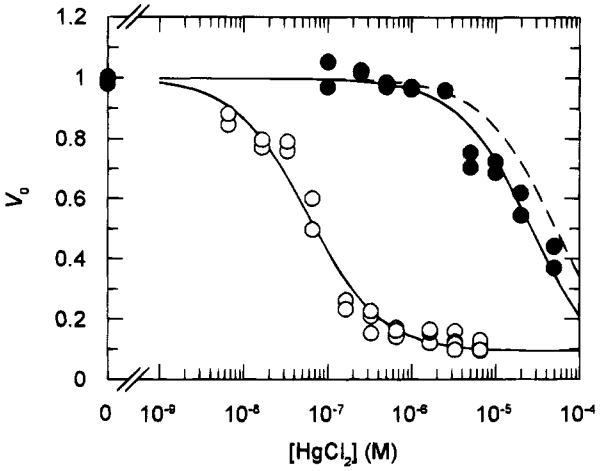


FIGURE 7: Reversible inhibition of the *C. albicans* PMI mutant C150A when assayed in the presence of mercury ions (●) compared with the wild-type enzyme (○). The equivalent curve for the *E. coli* enzyme, which also lacks the thiol at residue 150 (Wells et al., 1994), is shown as the dashed line. The reactions were carried out at 37 °C in 50 mM Hepes buffer, pH 8.0, in the presence of 0.5 mM mannose 6-phosphate.

The kinetic parameters of the purified Cys150 → Ala enzyme were measured by studying the reaction in 50 mM Hepes buffer, pH 8.0 at 37 °C, using the coupled assay procedure (Wells et al., 1993). Kinetic parameters were calculated by fitting to the equation $v = V_m[S]/(K_m + [S])$. The mutant and wild-type enzymes showed similar properties, with K_m(mannose 6-phosphate) = 0.46 ± 0.05 mM for Cys150 → Ala and K_m(mannose 6-phosphate) = 0.52 ± 0.06 for the wild type. The V_m value for Cys150 → Ala was 57% that of the wild-type enzyme under these conditions. The ability of zinc and cadmium ions to inhibit both wild-type and mutant enzymes was also compared. A fixed concentration of 0.5 mM mannose 6-phosphate (approximately corresponding to [S] = K_m; Wells et al., 1993) was used, and the data were fitted to an equation describing reversible inhibition: $v = V_m^{obs}/(1 + [I]/K_i^{obs})$. The metal ions inhibited both enzymes with a similar potency, and showed inhibition constants of 12.2 ± 1.3 μM for the wild-type enzyme and 21.4 ± 2.4 μM for the Cys150 → Ala mutant enzyme, when zinc ions were used. Again, the cadmium ions inhibited both enzymes to a similar degree, showing inhibition constants of 123 ± 12 μM for the wild-type enzyme and 120 ± 15 μM for the Cys150 → Ala150A mutant.

Effect of Silver Ions and Silver Sulfadiazine on the Cys → Ala150 Mutant. The ability of silver sulfadiazine to inhibit the mutant Cys150 → Ala phosphomannose isomerase was tested in the integrated time-dependent irreversible inactivation assay, using 1 mM mannose 6-phosphate as substrate, and 50 mM Hepes buffer, pH 8.0 at 37 °C. Silver sulfadiazine was not able to produce a significant time-dependent loss of enzyme activity below concentrations of 10^{-5} M (Figure 6). In comparison, the wild-type enzyme shows a maximal rate of irreversible inactivation at these concentrations. Silver ions were also incapable of irreversibly inactivating the mutant enzyme up to concentrations of 100 μM (data not shown).

Effects of Mercury Ions on the Cys → Ala150 Mutant. Mercury ions cause time-dependent irreversible inactivation of *C. albicans* PMI. Previous studies had suggested that the site of interaction of the mercury ion with the enzyme is Cys-150 (Coulin et al., 1993; Wells et al., 1994). The Cys150 → Ala PMI mutant shows no significant irreversible inhibition by concentrations of mercury ions up to 50 μM in 50 mM Hepes, pH 8.0, buffer, containing 0.5 mM mannose 6-phosphate. However, some reversible inhibition is seen (Figure 7), showing 50% inhibition at a concentration of 2.7×10^{-5} M mercury ions. This value is of a similar order of magnitude to the 5.2×10^{-5} M required for 50% inhibition of the *E. coli* enzyme (which has the equivalent residue to Cys-150 replaced by an asparagine). It contrasts with the value of 5.8×10^{-8} M measured for the wild-type *C. albicans* enzyme (Wells et al., 1994).

DISCUSSION

In our search for inhibitors of the activity of *C. albicans* phosphomannose isomerase, we have found that silver ions are effective inhibitors at submicromolar concentrations. Detailed kinetic analysis has shown that this is a time-dependent irreversible inhibition, and that this irreversible inhibition shows saturatable (hyperbolic) kinetics. The simplest explanation of the hyperbolic dependence of the rate constant for inactivation with silver ion concentration is a stepwise reaction, with an initial complex formed, followed by covalent modification of the enzyme. The attacking species could either be Ag^+ or perhaps a complex between the silver ion and the Hepes base. However, the latter suggestion is much less likely since similar hyperbolic kinetics, and similar parameters, can be obtained if the reaction is carried out in sodium borate buffer at pH 8.0.

The enzyme can be protected from inhibition by silver ions by the addition of the substrate mannose 6-phosphate, and the K_d value is similar to the K_m value obtained under these conditions. This implies either that (i) the binding site for the silver ion is blocked by the addition of the substrate directly or that (ii) the binding of mannose 6-phosphate causes a conformational change in the enzyme which renders the binding site for the silver ion inaccessible. Support for the second of these hypotheses comes from the intrinsic tryptophan fluorescence changes seen in PMI when metal ions or substrate bind (Timothy N. C. Wells, unpublished results), indicating some conformational change. However,

the enzyme cannot be protected from inactivation by the addition of zinc ions. These are also competitive inhibitors of PMI when mannose 6-phosphate is used as the substrate. This implies that even though both metal ions are competitive inhibitors, the binding site for silver ions is different from that for zinc ions.

The data showing inhibition of PMI by silver ions are similar to those seen using mercury ions. The values obtained for the maximum rate of inhibition and for the dissociation constant for the initial complex between the silver ion and the enzyme are similar. However, silver offers the advantage that a number of covalent complexes exist between the metal and an organic base, which offers a chance to probe further the chemistry of the environment surrounding the silver ion binding site. One of these molecules, silver sulfadiazine, is used commercially as a topical antiinfective agent. The other two molecules selected, GR 131921 X and GR 132465 X, were originally made as potential antiproliferative agents in chemotherapy. The addition of the organic moiety to the silver ion reduced the dissociation constant of the silver complex for the enzyme by a factor of 200-fold, without significantly altering the maximum rate constant for inhibition, at around 0.25 min^{-1}. This implies that the organic moieties are able to interact with the enzyme. The rate constant of 0.25 min^{-1} may correspond to formation of a covalent bond to the enzyme, or else a conformational change in the enzyme. However, the current kinetic data cannot distinguish between the possibilities that the organic moiety either remains attached to the silver atom or dissociates after inactivation of the enzyme.

On the basis of the chemistry of silver, there are two main types of covalent interaction which could be envisaged, either to the imidazole nitrogen of histidine (Chambers et al., 1974) or else to a free sulfur on cysteine. In view of the similarity between the silver ion and the mercury ion inhibition data, we hypothesized that the silver ion binding site would also be Cys-150. However, addition of silver salts to crystals of *C. albicans* PMI caused them to crack, and so direct observation of the silver ion has not been possible. The high energy of the radioactive isotope [^{110m}Ag] precluded radioactive studies. We therefore made the mutation Cys150 → Ala in *C. albicans* PMI, thus removing the only accessible cysteine. This mutation had no significant effect on the catalytic properties (K_m and V_m) or on the inhibition constants for the competitive inhibitors cadmium and zinc ions. However, the removal of the Cys-150 side chain eliminates the inhibition caused by silver sulfadiazine and mercury, indicating that this thiol group is the site of modification. At high concentrations of silver ions (greater than 200 μM), histidine modifications probably do occur, since more than one silver atom is incorporated per protein, and this is accompanied by loss of the essential zinc atom.

The identification of Cys-150 as the primary binding site for silver ions and silver-containing compounds opened up the possibility of controlling species-selective inhibition. The linear sequence of the protein around Cys-150 is not as well conserved as the remainder of the protein between human and *C. albicans* PMI. Although this does not prove that the three-dimensional arrangement of residues around Cys-150 will also be similar for the two species, it does suggest that there may be differences here that can be used in finding species-selective inhibitors of PMI. The silver ions and silver-containing compounds were therefore tested against the human enzyme. The results show two key differences from the *C. albicans* enzyme. First, the maximal rate of inhibition is much faster (>0.6 min^{-1}), and the concentration dependence of the bimolecular inhibition rate constant (k_i/K_i) is linear. In addition, this rate constant of inactivation shows only a 4-fold variation across the four experiments. This implies that the organic part of the compounds is unable to make additional significant favorable binding interactions with the human enzyme, in contrast to what is seen with the *C. albicans* enzyme. It is therefore likely that the binding site for silver is in a region where there are significant amino acid differences between the human and the fungal enzymes.

Even with this increase in relative potency for the *C. albicans* enzyme, we still have not obtained a selective inhibitor of the fungal enzyme. We define the specificity index as the ratio of the bimolecular inhibition constant for the human enzyme compared to the fungal enzyme. For silver ions, this ratio is 210, in favor of inhibiting the mammalian enzyme. The addition of the sulfadiazine base to silver can lower this specificity ratio down to 1.3. Ideally, we would like a compound that shows a specificity index less than 1. It could be envisaged that after further structure−activity studies compounds could be designed which would be selective against the fungal enzyme. This design would be facilitated by the availability of the X-ray structure, which is currently in progress (Tolley et al., 1994; Bernard et al., 1995).

The safety of using silver-containing compounds in mammals has been extensively debated. Silver nitrate is used topically for a variety of indications, especially intraoral ulceration. One of the proposed mechanisms for the antibacterial action of silver ions is via the modification of DNA, which inhibits the unwinding of the helical DNA during replication. However, although silver ions have been shown to be mutagenic *in vitro*, there is no clear evidence for carcinogenicity *in vivo* (Maibach & Newell, 1980). However, bacteria such as *Pseudomonas stutzeri* have been shown to be able to develop resistance to silver accumulation (Gadd et al., 1989), by conversion of the metal ion to Ag^0.

We have also shown that the ability of silver-containing compounds to inhibit PMI depends on the covalent modification of a residue which is not essential for the optimal activity of the enzyme. This limits the utility of such molecules as therapeutic agents. In the presence of silver ions or silver sulfadiazine, the fungus would be under selective pressure to replace cysteine-150 with a nonmodifiable amino acid such as alanine or asparagine, thus creating a resistant PMI. A good example of such development of resistance in the clinic are the non-nucleoside reverse transcriptase inhibitors of the human immunodeficiency virus (Larder et al., 1993). In this case, the enzyme is able to mutate out the drug binding site without compromising the catalytic activity of the enzyme. A similar situation could be envisaged for PMI inhibitors if they used Cys-150 as their principal target.

We have demonstrated irreversible inactivation of fungal and mammalian phosphomannose isomerases using silver ions and silver-containing compounds. This inhibition by silver ions shows species selectivity in that the mammalian enzymes are preferentially inhibited. However, the selectivity can be controlled by the introduction of organic bases, showing that there are binding pockets in the enzyme, which differ between human and *C. albicans*. We hope to exploit

these binding sites in our search for more selective inhibitors of phosphomannose isomerase.

ACKNOWLEDGMENT

We thank Frederick Borlat for help with the protein purification, Mariastella DeTiani for help with the mutagenesis, and Mr. Alan Cox of Sheffield Hallam University, U.K. for the ICP-AES measurements of metal ion content.

REFERENCES

Allet, B. A., Payton, M., Mattaliano, R. J., Gronenborn, A. M., Clore, G. M., & Wingfield, P. T. (1988) *Gene 65*, 259−268.

Bernard, A., Wells, T. N. C., Cleasby, A., Borlat, F., Payton, M. A., & Proudfoot, A. E. I. (1995) *Eur. J. Biochem.* (in press).

Chambers, J. L., Christoph, G. G., Krieger, M., Kay, L., & Stroud, R. M. (1974) *Biochem. Biophys. Res. Commun. 59*, 70−75.

Coulin, F., Magnenat, E., Proudfoot, A. E. I., Payton, M., Scully, P., & Wells, T. N. C. (1993) *Biochemistry 32*, 14139 −14144.

Gadd, G. M., Laurence, O. S., Briscoe, P. A., & Trevors, J. T. (1989) *Biol. Met. 2*, 168−173.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245−3249.

Larder, B. A., Kellam, P., & Kemp, S. D. (1993) *Nature 365*, 451−453.

Leatherbarrow, R. J. (1992) *GraFit Version 3.01*, Erithacus Software Ltd., Staines, U.K.

Nangia, A. K., Hung, C. T., & Lim, J. K. C. (1987) *Med. Actual. 23*, 21−30.

Orlean, P. (1990) *Mol. Cell. Biol. 10*, 5796−5805.

Payton, M. A., Rheinecker, M., Klig, L. S., DeTiani, M., & Bowden, E. (1991) *J. Bacteriol. 173*, 2006−2010.

Proudfoot, A. E. I., Turcatti, G., Wells, T. N. C., Payton, M. A., & Smith, D. J. (1994a) *Eur. J. Biochem. 219*, 415−423.

Proudfoot, A. E. I., Payton, M. A., & Wells, T. N. C. (1994b) *J. Protein Chem. 13*, 219−227.

Slein, M. W. (1955) *Methods Enzymol. 1*, 299−306.

Smith, D. J., Proudfoot, A. E. I., Friedli, L., Klig, L. S., Paravicini, G., & Payton, M. A. (1992) *Mol. Cell. Biol. 12*, 2924−2930.

Smith, D. J., Proudfoot, A. E. I., DeTiani, M., Wells, T. N. C., & Payton, M. A. (1995) *Yeast 11*, 301−310.

Srere, P. A. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 57−101.

Tew, D. G. (1993) *Biochemistry 32*, 10209−10215.

Tolley, S., Davies G., Hubbard, R. E., Smith, D. J., Proudfoot, A. E. I., Payton, M. A., Cleasby, A., Wonacott, A., & Wells, T. N. C. (1994) *J. Mol. Biol. 237; 349*−350.

Wells, T. N. C., Coulin, F., Payton, M. A., & Proudfoot, A. E. I (1993) *Biochemistry 32*, 1294−1301.

Wells, T. N. C., Payton, M. A., & Proudfoot A. E. I. (1994) *Biochemistry 33*, 7641−7646.

BI950112G