Phosphomannose Isomerase from *Saccharomyces cerevisiae* Contains Two Inhibitory Metal Ion Binding Sites

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ABSTRACT: Phosphomannose isomerase (PMI) from *Saccharomyces cerevisiae* is a zinc-dependent metalloenzyme. Besides its role in catalysis, zinc is also a potent inhibitor of the enzyme. The inhibition is competitive with the substrate mannose 6-phosphate, with K_{is} = 6.4 μM in 50 mM Tris-HCl buffer, pH 8.0, at 37 °C. This inhibition constant is 4 orders of magnitude smaller than for group II divalent cations, indicating that the binding is not primarily electrostatic. Micromolar inhibition is also observed with ions of the other metals of the electronic configuration d^{10}. Under identical conditions, cadmium is a predominantly competitive inhibitor with K_{is} = 19.5 μM. Inhibition by mercury is predominantly competitive with K_{is} = 6.0 μM but shows a hyperbolic Dixon plot. Theorell–Yonetani double-inhibition analysis shows that zinc and cadmium ions are mutually exclusive inhibitors against mannose 6-phosphate. However, analysis of zinc and mercury double inhibition shows that they can simultanaously bind in the mannose 6-phosphate binding pocket, with only a small mutual repulsion. Inhibition of the enzyme by cadmium and zinc ions is strongly pH dependent with pK_a = 9.2 for cadmium and one pK_a at 6.6 and two at 8.9 for zinc. The inhibitory species are the monohydroxide forms, $Zn(OH)^+$ and $Cd(OH)^+$. However, inhibition by mercury is relatively pH-independent, consistent with the neutral $Hg(OH)_2$ being the inhibitory species. In all three cases, the metal ion binding causes a conformational change in the enzyme as judged by tryptophan fluorescence. The K_s at pH 8.0 determined by fluorimetry is similar to the kinetic K_i for zinc and cadmium but is 5-fold higher for mercury. These studies demonstrate that the zinc triad of metal ions specifically inhibits phosphomannose isomerase. However, although all three metals are inhibitors with respect to the mannose 6-phosphate substrate, there are two distinct modes of inhibition, one for zinc and cadmium and the other for mercury.

Phosphomannose isomerase (PMI)[1] catalyzes the reversible isomerization of mannose 6-phosphate and fructose 6-phosphate. This is the first step in the synthesis of the carbohydrate moiety of glycoproteins from glycolytic intermediates in yeast. The enzyme, therefore, plays a key role in the biosynthesis of the yeast cell wall. The mannose 6-phosphate product is the substrate for the dolichol–phosphate–mannose (Dol-P-Man) biosynthetic pathway. The dolichol–phosphate–mannose is used as the donor in a wide variety of mannosylation reactions, including N- and O-linked glycosylation (Orlean, 1990). Temperature-sensitive mutants of the *pmi* gene in *Saccharomyces cerevisiae* produce cell walls deficient in mannose and undergo cell lysis at the restrictive temperature (Payton et al., 1991).

PMI has been purified from yeast (Gracy & Noltmann, 1968a,b) and is a monomeric protein (M_r = 45 000) which contains 1 mol of zinc/mol of enzyme that is required for activity. In addition, zinc can also be an inhibitor of yeast (Gracy & Noltmann, 1968b) and *Amorphophallus konjac* (Murata, 1975) phosphomannose isomerase when added in excess.

Several zinc-dependent proteases (for example, carboxypeptidase A, thermolysin, and collagenase) are inhibited by excess zinc in the concentration range 10^{-6}–10^{-5} M (Larsen & Auld, 1991). Apart from carboxypeptidase A, however, little is known about the properties of these zinc inhibitory sites. To further investigate the mechanism of zinc inhibition in phosphomannose isomerase from *S. cerevisiae*, we have studied the actions of a wide range of metal cations. Micromolar competitive inhibition is limited to zinc, cadmium, and mercury ions. The modes of inhibition of each metal ion, the hydrolysis state involved, and interdependence of inhibition by these three metal ions have been studied.

MATERIALS AND METHODS

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

Enzyme Purification. Phosphomannose isomerase (yeast) was purchased from Boehringer Mannheim and required further purification to obtain homogeneous material. All steps were carried out at 4 °C. It was dissolved in 50 mM potassium phosphate–1.7 M $(NH_4)_2SO_4$, pH 7.0, buffer, loaded onto a Hi-Load phenyl Superose 26/10 column (Pharmacia), and eluted with a gradient of 1.7–0 M $(NH_4)_2SO_4$ in 50 mM potassium phosphate buffer, pH 7.0. Active fractions were pooled and gel filtered on an XK50/100 column (Pharmacia) containing Sephadex S200 HR, using 100 mM Tris-HCl, pH 8.0, buffer. Active fractions were >98% pure as judged by SDS–PAGE using a Phast system (Pharmacia) and staining with coomassie brilliant blue. Amino acid composition analysis, sequencing of cyanogen bromide cleaved peptides, and kinetic analyses showed that this enzyme is identical to that purified from *S. cerevisiae* (Amanda E. I. Proudfoot, Mark A. Payton, and Timothy N. C. Wells, unpublished observations). The zinc content of the enzyme was 0.75–0.8 mol/mol of enzyme as judged by inductively-coupled plasma atomic absorption spectroscopy.

[1] Abbreviations: PMI, phosphomannose isomerase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; Ampso, 3-[[dimethyl(hydroxymethyl)-methyl]amino]-2-hydroxypropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid.

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Kinetic Assays. The activity of phosphomannose isomerase was measured at 37 °C using a coupled assay in which the product fructose 6-phosphate is converted to glucose 6-phosphate. This is then oxidized to 6-phosphogluconate, and the concomitant reduction of the $NADP^+$ was monitored by the change in absorbance at 340 nm. Assays were carried out in a microtitre plate, in a total volume of 300 μL, containing buffer, 90 units of phosphoglucose isomerase (PGI), and 90 units of glucose-6-phosphate dehydrogenase (G6PDH) per assay. The rate of change of absorbance was measured using a Thermomax microtitre plate under (Molecular Devices, Palo Alto, CA), with a wavelength cut-off filter for 340 nm. Corrections for path length and filter effects were made by direct comparison of the results with those obtained using 1-mL cuvettes and a DU50 spectrophotometer (Beckman). The possibility that added metal ions would inhibit the coupling reaction was routinely tested for by adding five times the normal concentration of coupling enzymes. In no cases did this cause a significant increase in the rate observed. This indicates that even at high metal ion concentrations, the formation of product is not limited by the activity of the coupling enzymes.

Buffers. The reaction mixtures were buffered by zwitterionic compounds. For the pH studies, the buffers used were all alkyl sulfonic acids where the buffering group is either a secondary or tertiary amine. These buffers are not expected to form complexes with the metal ions at the concentrations used (Good et al., 1966). Stocks of buffers were made at 0.2 M which were diluted 4-fold in the assay. The ionic strength of each buffer was calculated, and NaCl was added to produce a constant ionic strength of $I = 0.2$ M. The pH of each buffer was then rechecked at its final dilution of 0.05 M with a glass-calomel electrode prior to use (Russell, Auchtermuchty, Scotland) on a Radiometer PHM83 pH meter.

Fluorescence Spectra. Fluorescence spectra were measured using a Perkin-Elmer LS-5 luminescence spectrometer. A 2.5-mL sample of 20 μg/mL of protein was incubated in 50 mM Hepes buffer, pH 8.0, and 37 °C with continual stirring. The tryptophan fluorescence was excited at 280 nm, and the emitted light was detected between 310 and 350 nm. In all cases maximal fluorescence was observed at 330 nm, and this was the signal used in subsequent analysis.

Data Analysis. Data were transferred to Grafit (Leatherbarrow, 1990) and fitted by multiple parameter nonlinear regression. Data were initially fitted to the standard equations (Cleland, 1979) for competitive, uncompetitive, and mixed inhibition, respectively.

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

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

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

The statistical validity of including the extra parameter in eq 3 was calculated by an F-test on the reduced χ^2 values for competitive and mixed inhibition. The data for mercury ion inhibition were also fitted to a partial noncompetitive (hyperbolic) inhibition model (Segel, 1975, IV-6):

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

The K_i values for the pH dependence studies were calculated from experiments where the metal ion concentration [I] was varied at fixed substrate concentration [S] for at least 15 values of [I]. Under these circumstances, eq 3 can be rearranged to the form

$$v = V_m^{obs}/(1 + [I]/K_i) \quad (5)$$

where $V_m^{obs} = V_{m'}[S]/(K_m = [S])$ and

$$1/K_i = 1/K_{is}\{K_m/([S] + K_m)\} + 1/K_{ii}\{[S]/([S] + K_m)\} \quad (6)$$

The K_m for mannose 6-phosphate does not vary significantly over the pH range studied. The inhibition by zinc, cadmium, and mercury ions remains predominantly competitive, with $K_{ii} > 10K_{is}$ (data not shown). Under these conditions, eq 6 reduced to $1/K_i = 0.375/K_{is}$ at [S] = 1 mM and $K_m = 0.6$ mM.

Table I: Inhibition of PMI by Metal Ions[a]

	V_m[b]	K_m (mM)	K_{ii} (μM)	K_{is} (μM)	χ^2
		Zinc			
competitive	118 ± 4	0.68 ± 0.07		6.4 ± 0.7	49.4
uncompetitive	143 ± 8	1.34 ± 0.15	14.3 ± 1.5		76.1
mixed	118 ± 5	0.69 ± 0.08	nd[a]	6.7 ± 1.2	49.4
		Cadmium			
competitive	103 ± 3	0.46 ± 0.04		13.9 ± 1.7	32.5
uncompetitive	127 ± 6	1.03 ± 0.11	51.9 ± 6.2		56.9
mixed	107 ± 3	0.54 ± 0.06	256 ± 99	19.5 ± 4.0	30.9
		Mercury			
competitive[c]	86 ± 2	0.32 ± 0.05		4.2 ± 0.8	15.6
uncompetitive	109 ± 5	0.97 ± 0.11	22.1 ± 3.4		32.1
mixed	90 ± 3	0.40 ± 0.07	122 ± 61	6.0 ± 1.7	15.0
mixed[d]	112 ± 4	0.59 ± 0.08	25.9 ± 7.1	7.22 ± 2.1	56.3

[a] The significance of the observed differences between the calculated χ^2 values was assessed using an F-test. [b] For the inhibition by zinc ions, K_{ii} is insignificant in the curve fitting, since the best fit required values $>10^5$ μM (187 degrees of freedom). For cadmium ions, the probability that K_{ii} is insignificant in the curve fitting $P < 0.001$ (282 degrees of freedom). For mercury ions, the probability that K_{ii} is insignificant in the curve fitting $P = 0.004$ (174 degrees of freedom). [c] Reduced data set with mercury ion concentration >1.9 μM (linear region of $1/v$ secondary plot in Figure 3c). [d] The full data set was used in this data fitting.

RESULTS

Inhibition by Divalent Cations. The effect of divalent cations on the PMI reaction was assayed in 50 mM Tris-HCl, pH 8.0, buffer at 37 °C ($I = 0.05$) containing 1.0 mM mannose 6-phosphate. Similar results were obtained using 50 mM Hepes, pH 8.0, as a buffer.

We tested a range of metal ions for their ability to inhibit phosphomannose isomerase. This initial search gave micromolar inhibition constants for zinc, cadmium, and mercury (Table I). All the group II metals were tested at concentrations up to 10 mM and showed inhibition constants some 1000-fold higher than values for the zinc triad. Inhibition was not detected with 10 mM Ba^{2+} and Sr^{2+}, and higher concentrations caused precipitation in the assay. Other transition metal ions also have millimolar inhibition constants. Inhibition experiments with Co(II) and Mn(II) (which are both readily oxidized) were carried out with enzyme-free controls, because of their tendency to reduce to $NADP^+$ in the coupling assay,

and this produces an enzyme-independent rate. No inhibition was seen using $CuCl_2$ or $PbCl_2$. NaCl and KCl can inhibit PMI but only at very high concentrations (K_i > 200 mM), indicating that the inhibition observed with the transition metal ions is not caused by the anion. This result is confirmed with K_2SO_4 (K_i = 28 mM) and K_2HPO_4 (K_i = 36 mM), showing that the anion effect is negligible in these studies.

Characterization of Zinc and Cadmium Inhibition. The effect of added metal chloride on the isomerization of mannose 6-phosphate in 50 mM Tris-HCl buffer, pH 8.0, at 37 °C is shown in Figure 1a. Inhibition studies were carried out varying the substrate between 0.05 and 5 mM (K_m = 0.6 mM), and the metal ion concentration varied between 0 and 200 μM. The use of higher substrate concentrations was precluded by the observation of product inhibition effects.

Fitting of the data to competitive inhibition (eq 1) gave values of 0.68 mM for K_m(M6P) and K_{is} = (6.4 ± 0.7) μM for inhibition by zinc (Table I). The value of χ^2 (the sum of the squares of the differences between the observed and calculated rates) did not change when a K_{ii} term was included in the equation (the model for mixed inhibition). The data is shown in the form of a Lineweaver–Burk plot in Figure 1a, which clearly shows the competitive inhibition. (The high inhibitor and low substrate values have been eliminated from the plot for reasons of clarity, but were included in the data fitting.) The replots of $(1/V_m)_{app}$ against [I] and $(K_m/V_m)_{app}$ against [I] are shown as Figure 1, parts b and c, respectively.

The data for the inhibition of phosphomannose isomerase by cadmium ions are shown as a Lineweaver–Burk plot in Figure 2a, where the plots intersect slightly to the left of the $1/v$ axis. The best fit to the data (Table I) was obtained with the mixed inhibition model giving K_m = (0.54 ± 0.06) mM, K_{is} = (19.5 ± 4) μM, and K_{ii} = (256 ± 99) μM. The replots of $(1/V_m)_{app}$ against [I] and $(K_m/V_m)_{app}$ against [I] are shown as Figure 2, parts b and c, respectively. Although the value for K_{ii} contains a large error term, the existence of an enzyme–substrate–inhibitor complex is shown by a decrease in χ^2 from 32.5 to 30.9 when the K_{ii} term is allowed in the curve fitting. An F-test shows that the probability of K_{ii} being insignificant in the curve fitting is $P < 0.001$ (282 degrees of freedom).

Characterization of Mercury Inhibition. The inhibition of phosphomannose isomerase by mercury ions was studied using 50 mM Tris-HCl buffer, pH 8.0, at 37 °C. The substrate concentration was varied between 0.05 and 5 mM, and the metal concentration varied between 0 and 40 μM. The data for [I] > 1.5 μM are shown as a Lineweaver–Burk plot in Figure 3a, where the plots intersect slightly to the left of the $1/v$ axis. The replots of $(1/V_m)_{obs}$ against I and $(K_m/V_m)_{obs}$ are shown as Figure 3, parts b and c, respectively. Figure 3b shows that there is a hyperbolic component to the uncompetitive inhibition pattern.

In the initial curve fitting, a reduced data set was used where the data from the nonlinear portion of Figure 3b ([I] < 2 mM) were not included. The best fit to the data (Table I) was obtained with the mixed inhibition model giving K_m = (0.40 ± 0.07) mM, K_{is} = (6.0 ± 1.7) μM, and K_{ii} = (122 ± 61) μM. Again, the existence of an enzyme–substrate–inhibitor complex is shown by the decrease in χ^2 from 15.6 to 15.0 when the K_{ii} term is allowed in the curve fitting. An F-test shows that the probability of K_{ii} being insignificant in the curve fitting is P = 0.004 (174 degrees of freedom). The complete data set was also fitted to the model for mixed inhibition (Table I), where it gave a lower value for K_{ii} of (25.9 ± 7.1) μM. This is represented in Figure 3b as the solid line, from which it can be seen that such a fit is simply the result of linearizing the hyperbola. The data were also fitted to a partial noncompetitive (hyperbolic) inhibition model described by eq 4 (Segel, 1975). No significant improvement in the fit was achieved by the additional β term.

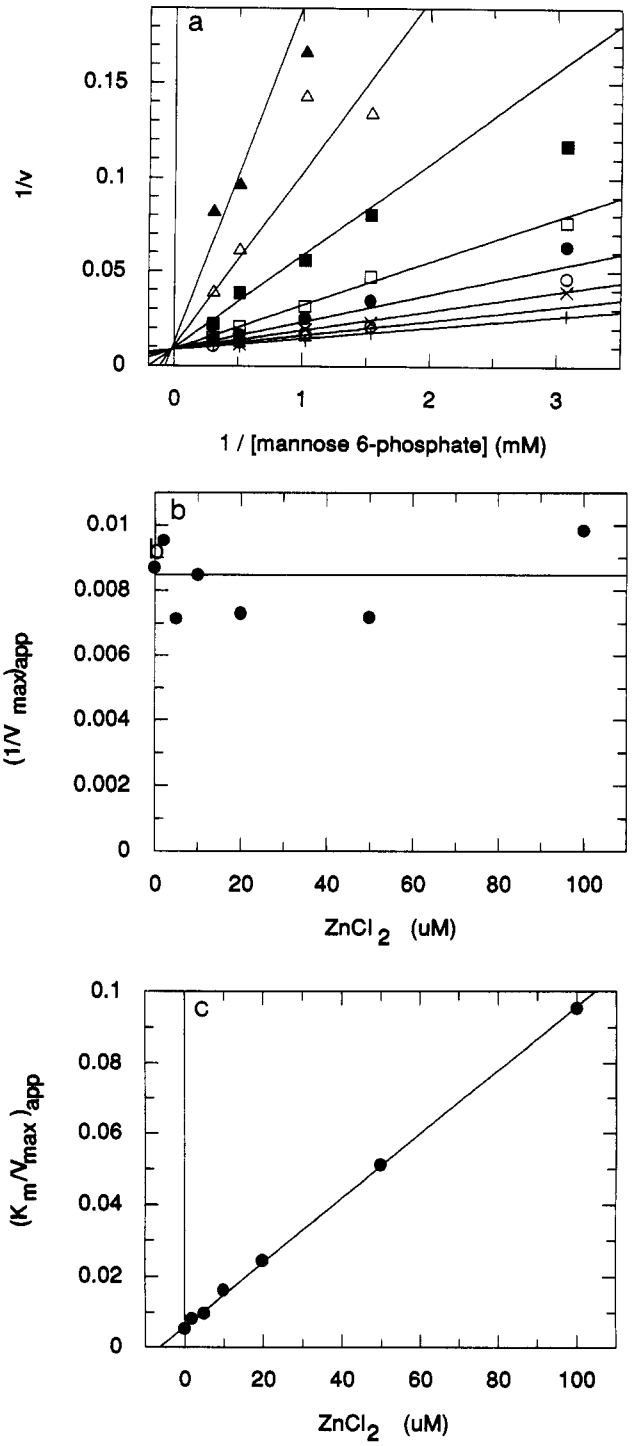


FIGURE 1: Inhibition of phosphomannose isomerase by zinc at 37 °C in 50 mM Tris-HCl, pH 8.0, buffer. (a) The initial data shown in Lineweaver–Burk transformation. Zinc concentrations were (+) 0 μM, (×) 2 μM, (○) 5 μM, (●) 10 μM, (□) 20 μM, (■) 50 μM, (△) 100 μM, and (▲) 200 μM. All points shown are the man of triplicate determinations. (b) Secondary plot showing the variation of $(1/V_m)_{app}$ with zinc concentration. (c) Secondary plot showing the variation of $(K_m/V_{max})_{app}$ with zinc concentration, showing clear competitive inhibition.

Theorell–Yonetani Double-Inhibition Analysis. This method is a test of whether two inhibitors are mutually exclusive, or if they bind at different subsites within the substrate binding

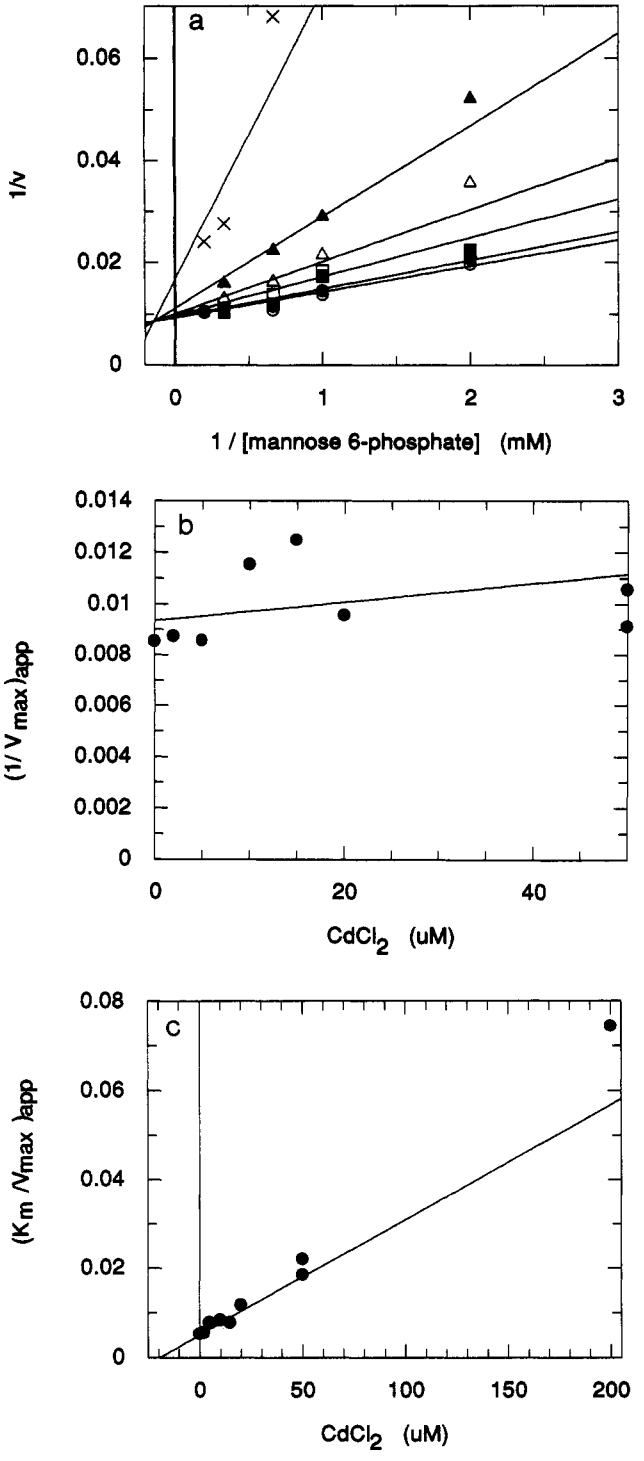


FIGURE 2: Inhibition of phosphomannose isomerase by cadmium at 37 °C in 50 mM Tris-HCl, pH 8.0, buffer. (a) The initial data shown in Lineweaver–Burk transformation. Cadmium concentrations were (○) 0 μM, (●) 2 μM, (□) 5 μM, (■) 10 μM, (△) 20 μM, (▲) 50 μM, and (×) 200 μM. All points shown are the mean of triplicate determinations. (b) Secondary plot showing the variation of $(1/V_m)_{app}$ with cadmium concentration. (c) Secondary plot showing the variation of $(K_m/V_{max})_{app}$ with cadmium concentration.

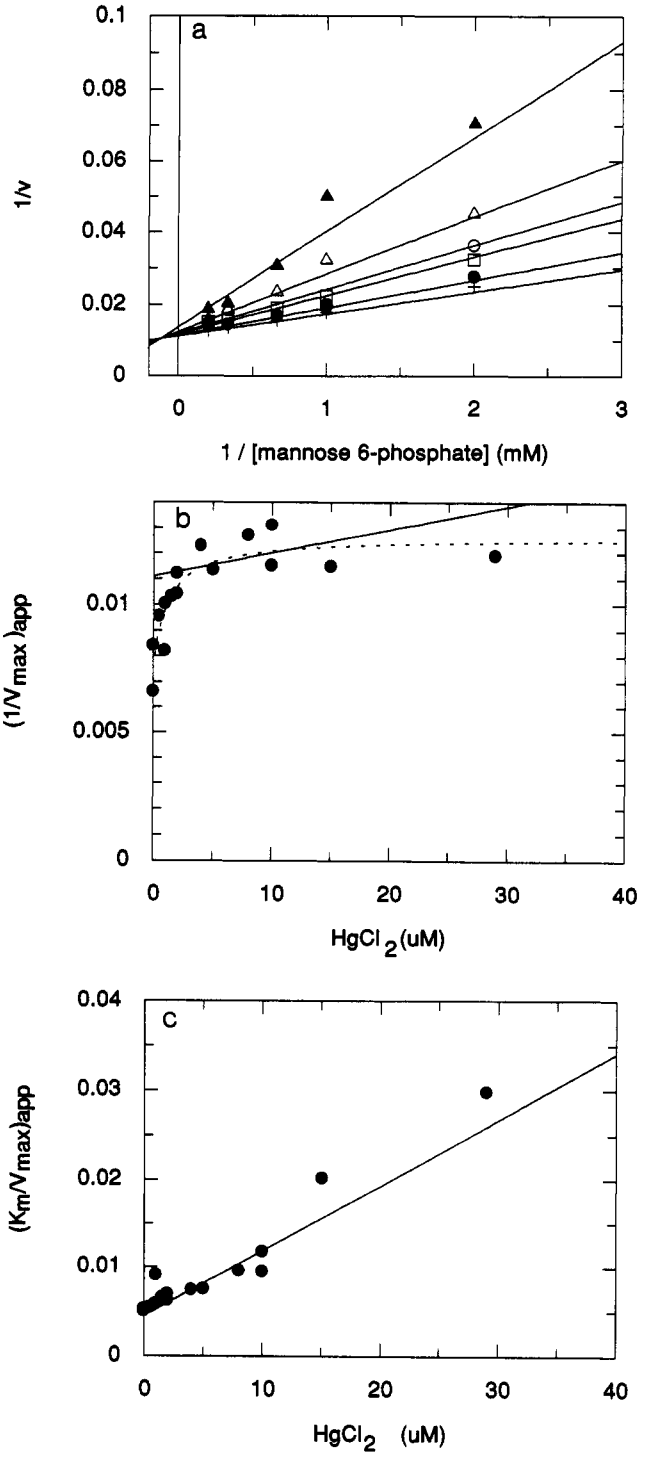


FIGURE 3: Inhibition of phosphomannose isomerase by mercury at 37 °C in 50 mM Tris-HCl, pH 8.0, buffer. (a) The initial data shown in Lineweaver–Burk transformation. Mercury concentrations shown in this figure are (+) 2 μM, (●) 4 μM, (□) 8 μM, (○) 10 μM, (△) 15 μM, and (▲) 30 mM. (b) Secondary plot showing the variation of $(1/V_m)_{app}$ with mercury concentration. The solid line shows the fit to a hyperbolic variation of $(1/V_m)_{app}$ (Segel, 1975). The dashed line shows the fit to a mixed inhibition model using the complete set. (c) Secondary plot showing the variation of $(K_m/V_{max})_{app}$ with mercury concentration.

cleft (Yonetani, 1982). The concentrations of two inhibitors I_1 and I_2 are varied in the presence of fixed subsaturating substrates. Provided that $[S]K_{is} \ll K_mK_{ii}$ for both inhibitors, the data can be fitted to eq 7.

The term α is a measure of the interaction between the inhibitors at the enzyme active site. At $\alpha = 1$, the two inhibitors bind independently and an $E{\cdot}I_1{\cdot}I_2$ complex exists. High values ($\alpha > 1$) indicate that there is an unfavorable interaction

$$v = \frac{V_{max}[S]}{K_m(1 + [I_1]/K_{is1} + [I_2]/K_{is2} + [I_1][I_2]/\alpha K_{is1}K_{is2}) + S} \quad (7)$$

between the two inhibitors. In the extreme case where α approaches infinity, the two inhibitors are mutually exclusive,

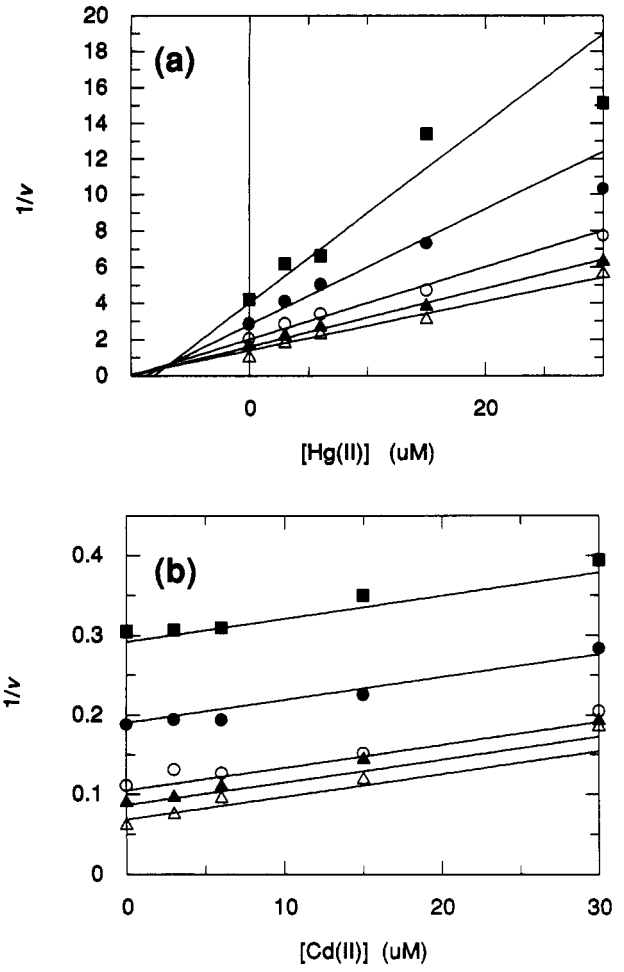


FIGURE 4: Theorell–Yonetani double-inhibition analysis. (a) Effect of (△) 0 μM, (▲) 6 μM, (○) 12 μM, (●) 30 μM, and (■) 60 μM zinc on the inhibition by mercury at 0.5 mM mannose 6-phosphate ([S] < K_m). The interesting pattern shows that the inhibitors are not mutually exclusive. (b) Effect of (△) 0 μM, (▲) 3 μM, (○) 12 μM, (●) 30 μM, and (■) 60 μM zinc on inhibition by cadmium under identical conditions. The parallel plot shows that the inhibitory effects of the two metals are mutually exclusive (α tends to infinity).

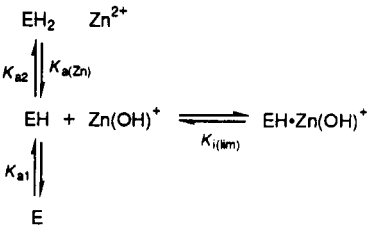
and no $E{\cdot}I_1{\cdot}I_2$ complex is formed.

To test whether zinc and mercury are mutually exclusive inhibitors of PMI, the enzyme velocity was measured in the presence of 0–120 μM zinc, 0–60 μM mercury, and 0.5 mM mannose 6-phosphate ($S < K_m$) in 50 mM Hepes buffer, pH 8.0, at 37 °C. The data where the concentration of mercury was less than 2 μM were omitted from the analysis. This ensured that the enzyme was in the linear part of the hyperbola shown in Figure 3b. Figure 4a shows an interesting series of lines, showing that the two metal ions can occupy the mannose 6-phosphate binding pocket at the same time. Direct curve fitting to eq 7 gives $\alpha = (11 \pm 3)$.

A similar experiment was carried out comparing zinc and cadmium binding. The conditions were 0–120 μM zinc, 0–120 μM cadmium, and 0.5 mM mannose 6-phosphate in 50 mM Tris-HCl buffer, pH 8.0, at 37 °C. The results are shown in Figure 4b. The pattern is clearly parallel and indicates that the zinc and cadmium ions are mutually exclusive inhibitors of phosphomannose isomerase. This observation is confirmed by direct curve fitting of the data to eq 7, where an infinite value for α is calculated.

pH Dependence of Inhibition. The pH dependence of metal ion inhibition was studied by measuring K_{is} over a range of pH values. The buffers used in this part of the study were all alkyl sulfonic acids, Mes (pH 5.5–7.0), Taps (pH 7.0–8.5), and Ampso (pH 7.8–9.8). It is expected that the formation of metal ion complexes with these buffers should be minimal at the concentrations used. (In all cases where amine buffers were used, such as Tris-HCl, slightly higher values of K_i were routinely observed). Stock solutions of 0.2 M buffer were made up, and NaCl was added to bring the final ionic strengths to 0.2 M. These buffers were diluted 4-fold in the standard assay mixture to give a final ionic strength of 50 mM. At least 15 metal ion concentrations over the range $K_{is}/5$ to $10K_{is}$ were used to determine each K_{is}. In each case, the standard error of the estimate of K_i was proportional to the magnitude of K_i, indicating that proportional weighting should be used in the secondary plots.

The pH dependence of $1/K_I$ will be the result of changes in both the inhibitor and the binding site on the protein. The zinc inhibition (Figure 5a) clearly shows the involvement of several ionizable groups in zinc inhibition. The hydrated zinc is hydrolyzed into $Zn(OH)^+$ with a pK_a of approximately 9 at 25 °C (Sillen & Martell, 1964). Below this value, the plot of log $(+1/K_i)$ or pK_i against pH has a slope of +1, consistent with the predominant mode of inhibition being due to $Zn(OH)^+$ binding the active form of the enzyme. Below pH 7, the slope of the pK_i versus pH plot has a slope of +2, and above pH 9 it shows a slope of −1, indicating two further ionizations involving the zinc binding site on the enzyme. The pH dependence of pK_i will be determined by the following scheme:



According to this scheme, the expression for the pH dependence of the zinc inhibition of PMI catalysis is given by

$$1/K_i = 1/K_{i(lim)}/\{(10^{pH-pK_{a1}} + 1 + 10^{pK_{a2}-pH})(1 + 10^{pK_{a(Zn)}-pH})\} \quad (8)$$

The data were fitted to eq 8 proportional weighting and gave values for $K_{i(lim)} = (0.63 \pm 0.06)$ μM, $pK_{a1} = pK_{i(lim)} = (8.95 \pm 0.05)$, and $pK_{a2} = (6.65 \pm 0.10)$. As can be seen from Figure 5a, the data fit well to the equation, and there is no trend in the residuals. No improvement in the reduced χ^2 was obtained by allowing pK_{a1} and $pK_{i(lim)}$ to vary independently.

For cadmium inhibition (Figure 5b), the plot of log $(+1/K_i)$ against pH has a slope of 1 and shows a pK_a above pH 9. The data were fitted to a model assuming a single ionization within the metal binding site, using eq 9:

$$1/K_i = 1/K_{i(lim)}/(10^{pK_{a1}-pH} + 1) \quad (9)$$

The data fit well to this simple model giving values of $K_i = (0.59 \pm 0.08)$ μM and $pK_a = (9.1 \pm 0.08)$. Analysis of the residuals show no significant trend, confirming the validity of the model (Figure 5b).

Models for the pH dependence involving two ionizations within the metal binding site were also tested. First, since the pK_a for the formation of $Cd(OH)^+$ is at pH 10.0 at 25 °C (Baes & Mesmer, 1976), it is possible that the hydrolysis of the cadmium ion plays a significant part in the inhibition. This process is modeled by two pK_as, one on the inhibitor and

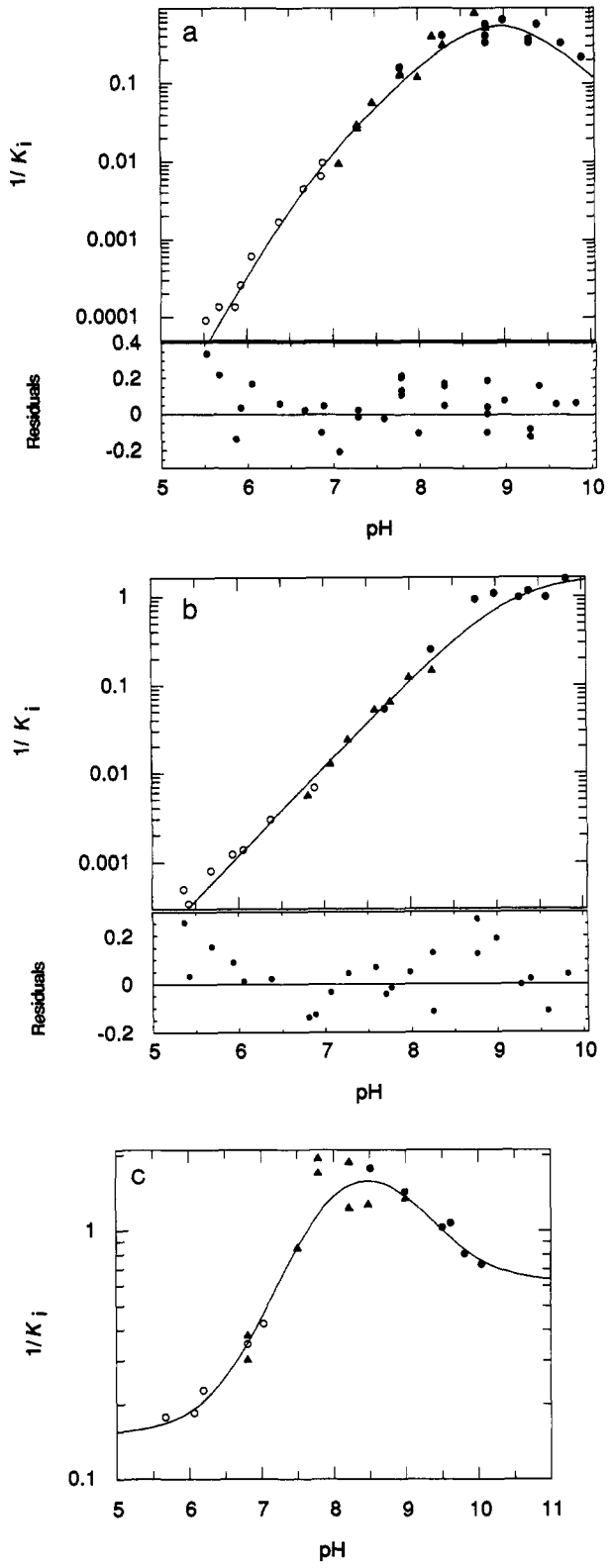


FIGURE 5: (a) pH dependence of K_{is}(zinc) at 37 °C in 50 mM (I = 0.05 M) buffer. The buffers used were Ampso (●), Taps (▲), and Mes (○). Data were fitted to a triple ionization model (eq 8). Each point on the graph represents a K_{is} determination involving at least 24 data points. The residual plot shows the difference between experimental and calculated values for K_i at each of the pH values used. (b) pH dependence of K_{is}(cadmium) at 37 °C in 50 mM buffer (I = 0.05 M). The buffers and experimental conditions are identical to panel a, and the data are fitted to the single ionization model (eq 9). (c) pH dependence of K_{is}(mercury) at 37 °C in 50 mM (I = 0.05) buffers. The buffers and experimental conditions are identical to panel a, and the data are fitted to the model described in the text (eq 10).

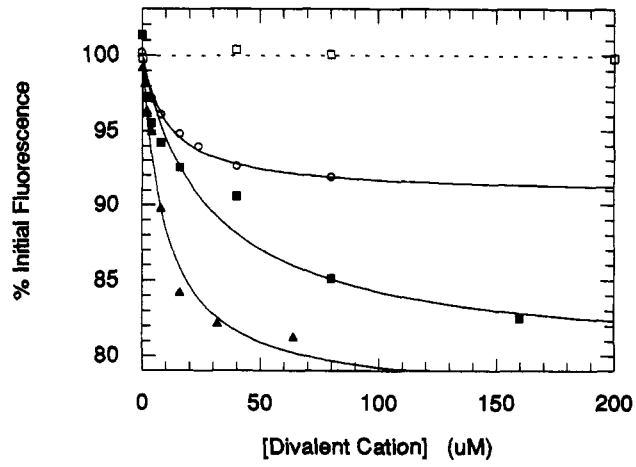
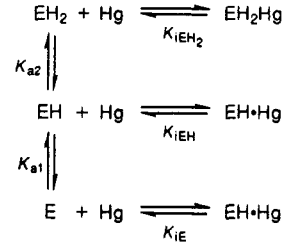


FIGURE 6: Fluorescence titration of phosphomannose isomerase with inhibitory metals. Binding of zinc (▲), cadmium (○), and mercury (■) were studied at 37 °C in 50 mM Hepes, pH 8.0. The effect of calcium is shown as a control (□).

the other on the enzyme: $1/K_i = 1/K_{i(lim)}/\{(10^{pH-pK_{a(PMI)}} + 1)(1 + 10^{pK_{a(Cd)}-pH}\}$. Altenatively, two ionizations could take place on the protein itself: $1/K_i = 1/K_{i(lim)}/(10^{pH-pK_{a1}} + 1 + 10^{pK_{a2}-pH})$. Neither of these models gave a significant decrease in the reduced χ^2 as measured by an F-test.

Initial experiments studying the pH dependence of the inhibition of PMI by mercury ions showed no significant dependence of K_{is} on the reaction pH over the range studied. However, more precise measurements (Figure 5c) show that there is small variation in K_i over the range studied. In contrast to the results for zinc and cadmium, the K_i value does not vary by more than a factor of 10 over the pH range studied. Initial studies fitting the data used the two pK_a model; however, it was clear that mercury will still inhibit PMI at low pH values. To accomodate this inhibition, it is assumed that there are two protein ionizations in the mercury binding site and that the mercury can bind to and inhibit all three enzyme species. This is described by the equations



According to this scheme, the expression for the pH dependence of the mercury inhibition of PMI catalysis is given by eq 10:

$$1K_i = (1/K_{iEH2}10^{pK_{a2}-pH} + 1/K_{iEH} + 1/K_{iE}10^{pH-pK_{a1}})/(10^{pK_{a2}-pH} + 1 + 10^{pH-pK_{a1}}) \quad (10)$$

The data were fitted using proportional weighting and gave values of pK_{a1} = (9.1 ± 0.3), pK_{a2} = (7.74 ± 0.16), K_{iEH2} = (6.5 ± 0.9) μM, K_{iEH} = (0.48 ± 0.08) μM, and K_{iE} = (1.6 ± 0.4) μM.

Fluorescence Titrations. For all three transition metals studied, the binding of the metal ions caused a decrease in the tryptophan fluorescence signal. This binding is saturable and can be described by a simple two-state model. Since the metal binding does not cause a shift in the emission maximum, it

can be fitted to the equation

$$\phi_{obs} = \phi_e - (\phi_e - \phi_{ei})[I]/([I] + K_s) \quad (11)$$

where ϕ_{obs} is the observed fluorescence, and ϕ_e and ϕ_{ei} are the fluorescence of the free enzyme and the enzyme–metal complex, respectively. K_s is the dissociation constant for the metal from the enzyme–metal complex. The data are shown in Figure 6. The largest fluorescence change is produced by zinc, where the ratio $\phi_{ei}/\phi_e = (0.77 \pm 0.02)$ and $K_s = (7.8 \pm 1.4)$ μM. For cadmium, $\phi_{ei}/\phi_e = (0.91 \pm 0.01)$ and $K_s = (8.1 \pm 0.7)$ μM. Mercury causes a decrease in fluorescence, $\phi_{ei}/\phi_e = (0.80 \pm 0.01)$, with $K_s = (27 \pm 4$ μM). In the mercury binding experiments, the fluorescence change is slow after the metal ion addition, and the samples was left for at least 2 min before reading the fluorescence. Rapid reaction studies show that on adding 10 μM $HgCl_2$ under identical conditions, the rate of change of fluorescence is 0.025 s^{-1}, whereas the fluorescence change accompanying zinc binding is complete within 1 s (Timothy N. C. Wells, unpublished observations). The effect of ionic strength was controlled for by adding $CaCl_2$, to a maximum concentration of 8 mM. No effect was seen below 4 mM, and a small (1%) decrease was seen at 8 mM.

DISCUSSION

The data presented here show that there are at least three different binding sites for transition metal ions within phosphomannose isomerase, all of which significantly alter the enzyme activity.

The first site contains the essential zinc, which when removed by chelating agents leads to a loss of activity (Gracy & Noltmann, 1968b). This site has an affinity for zinc in the low picomolar range (Timothy N. C. Wells, unpublished observations). However, the location of the essential zinc within the protein and its role in the phosphomannose isomerase reaction have not yet been defined. The pH optimum and [^{3}H]-exchange data (Gonzalez de Galdeano & Simon, 1970) support an enzyme mechanism that proceeds by way of an ene-diol intermediate, with two bases of pK_a 6.0 and 8.8 (Gracy & Noltmann, 1968a). It is possible that water coordinated to zinc may be acting as one of these bases. The alternative is that it may simply be required to maintain the structure of the protein. Active-site modification experiments (Timothy N. C. Wells, unpublished results) show that the enzyme has an essential arginine and histidine within the active site, favoring a structural role.

In addition to this essential zinc, this study demonstrates the existence of inhibitory metal ion binding sites that are specific for zinc, cadmium, and mercury. These are within the mannose 6-phosphate binding pocket and have micromolar affinity constants. The existence of inhibitory zinc binding sites within the active sites of zinc-dependent enzymes has been shown for several proteases [for example, thermolysin (Holmquist & Vallee, 1974); collagenase (Mayalla & Van Wart, 1989); and carboxypeptidase A (Larsen & Auld, 1989)]. The inhibitory effects of excess zinc on the reactions of carboxypeptidase A and PMI are similar. Inhibition is competitive with respect to substrate, and pH studies show that the major inhibitory species is $ZnOH^+$.

The ability of metal ions to inhibit the phosphomannose isomerase reaction is limited to the triad of zinc, cadmium, and mercury. Divalent cations from group II in the periodic table either show inhibition in the millimolar range or else show no detectable inhibition at 10 mM (Table I). Other transition metals such as Co(II) and Mn(II) also require millimolar concentrations to produce inhibition. This confirms

Table II: Inhibition of PMI Activity by Metal Ions, Concentration of Metal Monohydroxide Complexes, and Ionic Radii

metal cation	K_i (μM)	[$MeOH^+$][a]	ionic radius (Å)[b]
Be	2650	−5.4	0.31
Mg	18000	−11.44	0.65
Ca	15000	−12.85	0.99
Zn(II)	6.7	−8.96	0.74
Cd(II)	19.5	−10.08	0.97
Hg(II)	6.0	−3.40	1.10
Co(II)	3500	−9.65	0.74
Mn(II)	1550	−10.59	0.80

[a] Logarithm of the dissociation constant for the first hydrolysis of the hydrated cation (Baes & Mesmer, 1976). [b] Values are Pauling radii (Cotton & Wilkinson, 1980).

that the inhibitory effect is neither a simple function of ionic radius nor the dissociation constant for hydrolysis of the hydrated cation (Table II). Pb(II) also shows no significant inhibition of phosphomannose isomerase in the millimolar range. This result contrasts with carboxypeptidase A where Pb(II) has $K_i = 48$ μM and is mutually exclusive with Zn(II) ($K_i = 24$ μM) as an inhibitor of peptide hydrolysis (Larsen & Auld, 1991).

Although zinc, cadmium, and mercury ions are all micromolar inhibitors of phosphomannose isomerase, four major distinctions can be made between the zinc and the cadmium inhibition on the one hand and mercury inhibition on the other.

First, there are differences in the type of inhibition. The inhibition is predominantly caused by formation of enzyme–inhibitor rather than enzyme–substrate–inhibitor complexes for all three metal ions, with $K_{ii} > 10K_{is}$. However, there is a hyperbolic effect on the uncompetitive inhibition curve for mercury. The simplest explanation would be that the mercury species can bind to the enzyme–mannose 6-phosphate complex, producing a ternary complex with reduced activity. However, the model used in eq 3 also predicts a hyperbolic dependence for the competitive plot, which is not observed. The explanation for this may lie in the simplicity of the model used, which assumed equilibrium binding. The fluorescence change accompanying mercury binding is slow ($k_{on} = 0.025$ s^{-1}) compared to that for zinc or cadmium.

Second, the Theorell–Yonetani experiments show that zinc and cadmium ions are mutually exclusive inhibitors. However, it is possible to get synergistic inhibition with zinc and mercury ions. This means that the mannose 6-phosphate binding pocket can contain both metal ions at the same time. From the Theorell–Yonetani plot, the calculated repulsion term, or α-value, is 11. This indicates that the binding of one metal complex weakens the binding of the second metal by a factor of 11. The free energy of this repulsive interaction between the two metals is given by $\Delta G_{rep} = RT \ln(\alpha)$, or 1.41 kcal/mol.

Third, over the range of maximal enzyme activity (pH 6–8.5) the pH variation of pK_i shows a unit slope for zinc and cadmium and relative pH independence for mercury. The pK_a of the first hydrolysis of zinc is at 9.1 and 10.0 for cadmium (Baes & Mesmer, 1976). A ligand pK_a is detected at pH 8.95 in the zinc inhibition, showing that phosphomannose isomerase is principally inhibited by the monohydroxide $Zn(OH)^+$. Similar results have been observed for zinc inhibition of carboxypeptidase A (Larsen & Auld, 1989), a study that very clearly shows that inhibition is only caused by the monohydrate species $Zn(OH)^+$. The pH range studied is not sufficiently wide to be able to detect the pK_a of $Cd(OH)^+$ formation; the

coupling assay is not sufficiently stable to allow accurate kinetic measurements above pH 9.8.

In both cases, the pH dependence is consistent with an enzyme ionization at pH 9.0. The catalytic reaction has an upper pK_a of 8.8 (Gracy & Noltman, 1968b), and it could be that the same group is responsible for both pH effects. However, chemical modification studies have shown this pK_a to be due to arginine (Timothy N. C. Wells, unpublished results), a ligand that has not been reported in zinc binding sites (Vallee & Auld, 1990).

The inhibition by mercury shows no pH dependence since above pH 4 the predominant mercury species is the neutral $Hg(OH)_2$ (Baes & Mesmer, 1976). The small pH dependence seen is caused by two ionizations within the active site that are not sufficiently close to the inhibitory metal binding site to prevent the mercury binding, but merely perturb the K_i 13.5-fold and 3.3-fold. The ionization at pH 9.1 is probably caused by the same group responsible for the pK_a in the zinc and cadmium inhibition studies (pH 8.95 and 9.1, respectively). The lower pK_a at pH 7.7 is significantly different from any of the other ionizations seen and is, therefore, due to a previously uninvolved group on the enzyme.

Finally, the fluorescent studies show that the binding of any of the three metal ions can change the conformation of the enzyme, a property not shown by other divalent cations such as calcium. Mercury ions bind slowly, with $K_s = (27 \pm 4)$ μM, a value 5-fold higher than the inhibition constant K_{is}. Zinc and cadmium ions bind rapidly and show binding constants equal to and lower than their inhibition constants. This change in fluorescence provides a tool by which the kinetics of the reaction and its inhibition can be studied on a millisecond time scale.

In order to understand the detailed structure of the inhibitory binding sites, it will be necessary to solve the three-dimensional structure of the enzyme by X-ray crystallography. In the absence of such data, the most likely explanation is that the hydrated metal ions are binding to the hydrogen bond donors and acceptors that normally bind the sugar hydroxyls of the mannose 6-phosphate. If this is the case, it is easy to see that the mercury and zinc complexes could bind to nonexclusive sites within the mannose 6-phosphate binding pocket. It is possible that the two sites are for the monohydroxide and dihydroxide, rather than being simply metal specific. This also underlines the importance of understanding the hydrolysis state of the inhibitory metal ions.

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SUPPLEMENTARY MATERIAL AVAILABLE

Equations for a simple case, a bell-shaped pH curve, an inhibition including metal ion hydrolysis, and a mercury ion inhibition (3 pages). Ordering information is given on any current masthead page.

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