# Thermodynamics of Binding of the Distal Calcium to Manganese Peroxidase[†]

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ABSTRACT: We previously demonstrated that manganese peroxidase from *Phanerochaete chrysosporium* was susceptible to thermal inactivation due to release of the distal calcium, which maintained the distal heme environment of the enzyme [Sutherland, G. R. J., Zapanta, L. S., Tien, M., & Aust, S. D. (1997) *Biochemistry 36*, 3654−3662]. In this investigation the binding of calcium to the distal calcium binding site of manganese peroxidase was studied by optical absorption spectroscopy and isothermal titration calorimetry. The dissociation constant for the distal calcium binding site was 11 ± 1 $\mu$M and the Hill coefficient was 1.1 ± 0.1. The binding of calcium was accompanied by decreases in enthalpy and entropy that were large compared to those of other calcium binding proteins. The decreases were consistent with the large conformational changes proposed to occur in manganese peroxidase as a result of the binding and release of the distal calcium. Studies involving binding of the hydrophobic fluorescent probe, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt (bis-ANS), to manganese peroxidase indicated that the active, calcium-containing form of the enzyme had less exposed hydrophobic surface area, which would contribute to an increase in enthalpy and entropy upon calcium binding. Therefore, the negative changes in enthalpy and entropy associated with calcium binding were attributed to a large increase in the structural rigidity and compactness of the enzyme. The dissociation constant for calcium decreased and the rate of thermal inactivation decreased with decreasing pH. However, both the ability of calcium to prevent thermal inactivation of manganese peroxidase and the rate of calcium binding decreased as the pH decreased. Therefore it was proposed that, at lower pH, calcium binding to manganese peroxidase was more thermodynamically favorable, but the rate of calcium binding decreased because the flexibility of the calcium binding site, and in turn exposure of the ligands to the incoming ion, decreased.

Manganese peroxidase (MnP)[1] was first identified and purified from the white-rot fungus *Phanerochaete chrysosporium* (Glenn & Gold, 1985; Glenn et al., 1986; Wariishi et al., 1988). It is a component of the nonspecific, extracellular, lignin-degrading system of white-rot fungi and is believed to be essential for the degradation of both lignin and environmental pollutants by these fungi (Barr & Aust, 1994; Wariishi et al., 1988). In the presence of hydrogen peroxide, MnP catalyzes the one-electron oxidation of manganese(II) to produce complexes of manganese(III), which are proposed to promote the degradation of a wide variety of compounds by acting as diffusible one-electron oxidants (Glenn et al., 1986; Wariishi et al., 1988).

As with many peroxidases, MnP contains calcium (Haschke & Friedhoff, 1978; Poulos et al., 1993; Schuller et al., 1996; Sundaramoorthy et al., 1994; Sutherland & Aust, 1996). Calcium ions were identified in the distal and proximal heme environment of MnP by X-ray crystallographic analysis (Sundaramoorthy et al., 1994). Our laboratory demonstrated that MnP was a unique peroxidase because it was very susceptible to thermal inactivation, particularly at neutral pH, due to the release of calcium from the enzyme (Sutherland & Aust, 1996). This inactivation process was prevented and reversed by the addition of calcium, and inactive MnP contained only one calcium, which was predicted to be in the proximal calcium binding site. The structural changes that occurred as a result of thermal inactivation were predominantly in the distal heme environment and were explained by the loss of the calcium in the distal side of the heme (Sutherland et al., 1997). Crystallographic analysis of MnP showed that an amino acid (Asp47) of the distal calcium binding site was in an α-helix, helix B, which was above the heme active site (Sundaramoorthy et al., 1994). The residue adjacent to Asp47, His46, was directly above the heme iron in helix B and was proposed to be required for catalytic activity. Upon release of calcium from MnP during thermal inactivation, His 46 became a ligand to the heme iron, converting the enzyme to hexacoordinated, low-spin inactive enzyme (Sutherland et al., 1997). This proposal was further supported by analysis of a recombinant MnP in which Asp47 was altered to alanine to disrupt the distal calcium binding site. This form of MnP was inactive, had the same spectroscopic properties as thermally inactivated MnP, and contained only one calcium, predicted to be the proximal calcium (Sutherland et al., 1997).

It was not possible to use dialysis or gel-permeation chromatography to separate inactive MnP from the calcium released from the enzyme during inactivation. This was because we found that, in order for successful reactivation, the conditions (i.e., temperature, pH, enzyme concentration) needed to be kept constant throughout the inactivation and reactivation process. Therefore a novel procedure was developed that enabled the binding of calcium to inactive MnP to be studied. The thermodynamics of calcium binding to inactive MnP were analyzed to understand the character

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[1] Abbreviations: MnP, manganese peroxidase; ITC, isothermal titration calorimetry; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt; $I_{max}$, maximum fluorescence intensity.

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of the protein conformational changes associated with the binding of calcium and the reactivation of MnP. The binding of bis-ANS, a fluorophore that binds to hydrophobic regions in proteins, was studied to compare the hydrophobic surface area of active and inactive MnP. The binding of other metals to the distal calcium binding site of MnP and the resulting effects on the structure and activity of the enzyme were also investigated.

## MATERIALS AND METHODS

*Chemicals.* Hydrogen peroxide, succinic acid, Trizma Base and chelating resin (iminodiacetic acid; Chelex 100) were purchased from Sigma Chemical Co. (St. Louis, MO). Manganese(II) sulfate, terbium(III) chloride, holmium(III) chloride, and lanthanum(III) chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium oxalate, calcium chloride, sodium succinate, Tris hydrochloride, and dibasic and monobasic sodium phosphate were purchased from Mallinckrodt Chemical, Inc. (Paris, KY). Bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt) was purchased from Molecular Probes (Eugene, OR). All solutions were prepared using purified water (Barnstead NANOpure II system; specific resistance 18.0 MΩ·cm$^{-1}$). All buffers were passed through a column of Chelex 100 (Bio-Rad, Richmond, CA).

*Enzyme.* Manganese peroxidase isozyme H4 (p*I* 4.5) was purified from liquid cultures of *P. chrysosporium* as previously described (Tuisel et al., 1990). The enzyme concentration was determined using an extinction coefficient of 127 mM$^{-1}$ cm$^{-1}$ at 406 nm (Millis et al., 1989). The activity of MnP was assayed by monitoring the production of manganese(III)−oxalate at 270 nm using conditions reported previously (Sutherland & Aust, 1996). Absorption spectra were recorded using a Shimadzu UV-2101 PC scanning spectrophotometer.

*Preparation of Thermally Inactivated MnP.* Chelating resin was used to bind the calcium that was released from MnP during thermal inactivation. Enough chelating resin was used (200 mg/mL, ∼60 mM iminodiacetic acid) to bind at least 99.7% of the calcium released from MnP. This estimate was based on the reported binding constant of 4.6 × 10$^3$ M$^{-1}$ for the binding of calcium to Chelex 100 at pH 7.0 (Blinks et al., 1978). The actual binding constant should be higher at the higher pH (7.0−8.0) used in this study. Prior to each experiment, the chelating resin was rinsed with 20 mM Tris buffer, to remove any iminodiacetate that may have leached from the resin into solution. The pH of the final solution containing 20 mM Tris buffer and chelating resin was checked immediately prior to the addition of MnP and adjusted if necessary with HCl or Chelex-treated NaOH. The enzyme mixtures, contained in plastic vials, were then incubated in a shaking water bath until the activity decreased to less than 5% of the original activity. Samples of the inactive enzyme were carefully removed from the chelating resin using a 40−200 μL Oxford BenchMate continuously adjustable pipette.

*Determination of Dissociation Constant by Optical Absorption Spectroscopy.* Inactive MnP, prepared as described above, was added to both the sample and reference cuvettes of the spectrophotometer equipped with a circulating water bath set at 30 °C. Calcium was added to the sample cuvette and the difference absorption spectrum for the enzyme was acquired. Calcium was added as 5 μL aliquots to 1.0 mL enzyme mixtures. Once equilibrium was reached (i.e., no more change in the difference spectrum), the absorbance of the difference spectra at 405 and 421 nm was recorded. The absorbance values were plotted versus concentration of calcium and the dissociation constant ($K_d$) for calcium was calculated by fitting the data to the following equation using nonlinear regression analysis (Sigma Plot, Jandel Scientific Software, San Rafael, CA). The absorbance at equilibrium

$$A = \frac{\Delta A}{[E]} \times \frac{([E]+[L]+K_d) - \{([E]+[L]+K_d)^2 - 4[E][L]\}^{1/2}}{2}$$

is $A$ for each concentration of calcium ([L]), $\Delta A$ is the maximum change in absorbance, and [E] is the concentration of MnP. The average and standard deviation for $K_d$ were determined from three data sets.

*Determination of Hill Coefficient.* The data from the difference spectra acquired during the titration of inactive MnP with calcium, as described immediately above, were plotted as log [$Y$/(1 − $Y$)] vs log [$Ca^{2+}$], where $Y$ represents the fraction of MnP with calcium bound. Values for $Y$ were calculated by dividing the absorbance at 405 nm from the difference spectrum for a given concentration of calcium by the absorbance at 405 nm of fully reactivated, calcium-bound MnP. According to the Hill equation written below, the slope of the Hill plot is equal to the Hill coefficient, $n$.

$$\log \frac{Y}{1-Y} = n \log [Ca^{2+}] - \log K_d$$

The slope of the plot was determined using Sigma Plot (Jandel Scientific Software, San Rafael, CA) and was reported as the average ± standard deviation.

*Isothermal Titration Calorimetry.* The titration of inactive MnP, prepared as described above, with calcium was performed using a MicroCal ITC calorimeter (MicroCal Inc., Northampton, MA) at 30 °C. The instrument and its use have been previously described (Wiseman et al., 1989). The volume of the reaction cell was 1.34 mL and the titrant was added in 5 μL increments. The reference cell contained water. The titration data were corrected for the small heat changes observed for titration of calcium into buffer. The data were analyzed using the calorimetric analysis program ORIGIN (MicroCal Inc.) to obtain the binding constant ($K_b$) and the total apparent heat of binding ($\Delta H$). These values were then used to calculate the change in entropy ($\Delta S$) for the binding process using the equations $\Delta G = -RT \ln K_d = \Delta H - T\Delta S$. The largest error was in the value for $K_b$ (and in turn $K_d$) due to the low concentration of MnP used in the experiments. However, when higher concentrations of MnP were used the enzyme was very stable toward thermal inactivation, and the extent of reactivation, following addition of calcium, was limited.

*Fluorescence Measurements.* Fluorescence data were collected at room temperature using a Shimadzu RF-1501 spectrofluorophotometer equipped with a 150 W Xe arc lamp. The concentration of bis-ANS was determined spectrophotometrically using the extinction coefficient of 23 mM$^{-1}$ cm$^{-1}$ at 395 nm as specified by Molecular Probes. The data were corrected for fluorescence due to the enzyme

in buffer and bis-ANS in buffer. The apparent dissociation constant ($K_d$) and maximum fluorescence intensity ($I_{max}$) were calculated by fitting the data to the following equation using nonlinear regression analysis (Sigma Plot, Jandel Scientific Software, San Rafael, CA).

$$I = \frac{I_{max}}{[b]} \times \frac{([b] + [MnP] + K_d) - \{([b] + [MnP] + K_d)^2 - 4[MnP][b]\}^{1/2}}{2}$$

The fluorescence intensity for each concentration of MnP is $I$, and [b] is the concentration of bis-ANS. The fluorescent probe was titrated with varying amounts of MnP because bis-ANS can cause inner filter effects at concentrations above 10−15 $\mu$M (Musci et al., 1985).

## RESULTS

The presence of the chelating resin increased the rate of thermal inactivation of MnP. The absorption spectra of active ferric MnP and thermally inactivated MnP, following removal from the chelating resin, are shown in Figure 1A. The Soret absorption peak of inactive MnP had decreased intensity (68%) and was red-shifted from 407 to 411 nm. Other absorption bands also appeared at 360, 533, and 561 nm.

Calcium was added to inactive MnP and within 12 min the enzyme reactivated to 88% of its original activity. As shown in Figure 1A, the absorption spectrum of reactivated MnP was the same as that for active MnP except that the intensity was 91% of original, consistent with the enzyme activity. When calcium was not added, the enzyme did not reactivate and the absorption spectrum did not change. The reactivation process was monitored over time by acquiring difference spectra for inactive MnP to which calcium had been added minus inactive MnP (Figure 1B). The difference spectra had positive and negative absorption maxima at 405 and 421 nm, respectively.

Difference spectra were acquired for the titration of MnP, inactivated in the presence of chelating resin, with calcium and the $K_d$, with standard deviation, was calculated to be 11 ± 1 $\mu$M (Figure 2). The activity of the enzyme had the same dependence on the amount of calcium added (data not shown). The $K_d$ was also determined using the absorbance of the difference spectra at 421 nm and the same value was obtained. The Hill plot for the data was linear and the Hill coefficient, with standard deviation, was calculated to be 1.1 ± 0.1 (Figure 2, inset). In addition, the $K_d$ was determined at pH 7.25 and 8.25 and the values, with standard deviations, were 5.5 ± 0.9 $\mu$M and 23 ± 3 $\mu$M, respectively (data not shown). The average time necessary to reach equilibrium following the initial additions of calcium to inactive MnP decreased from 33 to 20 to 8 min as the pH increased from 7.25 to 7.75 to 8.25 (data not shown).

With each addition of calcium to inactive MnP during the isothermal titration calorimetry experiment the power decreased, indicating that the binding of calcium to inactive MnP was an exothermic process (Figure 3A). Shown in Figure 3B is the plot of heat absorbed per mole of calcium added. The fit to the data yielded the following parameters, with standard deviations: $K_d = 17 \pm 4$ $\mu$M, $\Delta H = -32 \pm 2$ kcal/mol, and $\Delta S = -84 \pm 7$ cal/(mol·K).

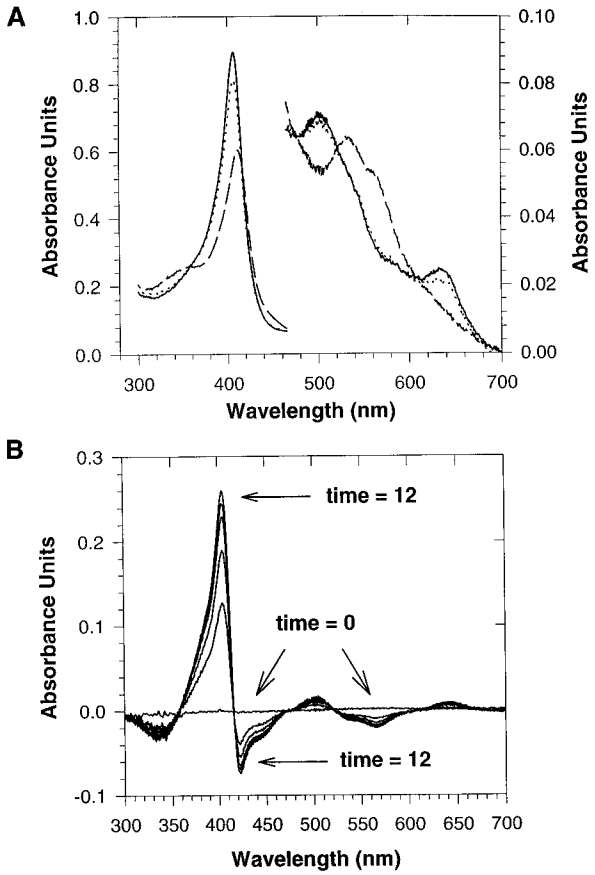


FIGURE 1: Absorption spectra of active, thermally inactivated, and reactivated MnP. (A) Absorption spectra of active MnP (—), MnP thermally inactivated in the presence of chelating resin (- - -), and calcium-reactivated MnP (•••). The MnP (7.0 $\mu$M) was thermally inactivated at 37 °C in 20 mM Tris, pH 7.5, in the presence of 200 mg/mL chelating resin for 13 min to 4% activity. The inactivated MnP was removed from the chelating resin and reactivated to 88% activity following the addition of 250 $\mu$M $CaCl_2$ and incubation at 30 °C for 12 min. (B) Difference spectra recorded during the reactivation of MnP with calcium. Inactive MnP, prepared as described for panel A, was added to both the sample and reference cuvettes. Spectra were acquired at 0, 0.5, 1.5, 3.0, 5.0, and 12 min following the addition of $CaCl_2$ to a final concentration of 250 $\mu$M to the sample cuvette mixture. Other experimental details are described under Materials and Methods.

In the presence of either active or thermally inactivated MnP, the fluorescence emission maximum of bis-ANS shifted from 500 to 485 nm and the intensity increased (Figure 4). The apparent dissociation constants for the binding of bis-ANS to active and inactive MnP were similar, 1.6 and 1.4 $\mu$M, respectively. However, the $I_{max}$ of bis-ANS was twice as large in the presence of thermally inactive MnP (900) compared to active MnP (440).

The addition of various amounts of calcium to MnP during thermal incubation at various pH values demonstrated that while calcium prevented thermal inactivation at each pH, the amount of remaining activity following incubation was higher at higher pH for each concentration of calcium (Figure 5).

As shown previously, calcium prevented the bisphasic thermal inactivation of MnP (Sutherland & Aust, 1996), but the addition of manganese(II) was also found to be equally effective at protecting enzyme activity (Figure 6). Following the addition of either calcium or manganese(II) to inactive MnP, the Soret absorption peak shifted from 411 nm back

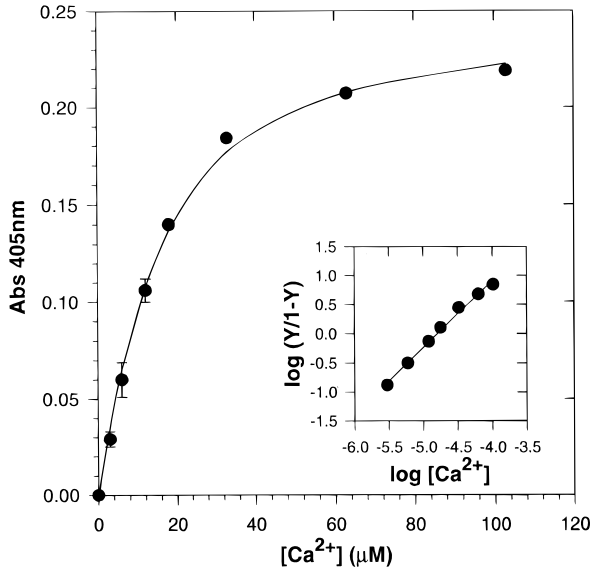


FIGURE 2: Change in absorbance of the difference spectrum of MnP during titration of inactive MnP with calcium. Manganese peroxidase (6.0 $\mu$M) was thermally inactivated with chelating resin and reactivated at 30 °C, pH 7.75, as described in the legend to Figure 1, except that the calcium was titrated into the sample cuvette containing inactive MnP. The actual data are represented by closed circles with standard deviations indicated by error bars. The solid line represents the fit to the data as explained under Materials and Methods. The inset shows the Hill plot of these data, where *Y* represents the fraction of MnP bound with calcium.

to that of active MnP (407 nm; Figure 6, inset), and enzyme activity increased to 76% and 77%, respectively. However, the reactivation process, and thus rebinding, was complete in 10 min following the addition of calcium but took 50 min with manganese(II). The presence of terbium(III) accelerated the rate of thermal inactivation, while the addition of terbium plus excess calcium prevented thermal inactivation (Figure 6). The amount of terbium(III) used in this experiment did not have any effect on the activity or absorption spectrum of MnP prior to thermal incubation. Other lanthanide metals such as holmium(III) and lanthanum(III) also accelerated the rate of thermal inactivation of MnP (data not shown). The addition of terbium(III) to inactive MnP did not reactivate the enzyme and had a much different effect on the absorption spectrum of the enzyme. The Soret absorbance of the heme decreased in intensity and shifted to a broad peak with a maximum at 405 nm (Figure 6, inset).

## DISCUSSION

As has been shown with other chelators of calcium (Sutherland & Aust, 1996), the rate of thermal inactivation of MnP increased in the presence of chelating resin. The absorption spectrum of MnP inactivated in the presence of the resin was identical to that of MnP inactivated in the presence of other chelators or no chelator, and it was readily reactivated following the addition of calcium at a rate comparable to that of MnP inactivated in the presence of EGTA (Sutherland et al., 1997). Following the removal of inactive MnP from the chelating resin, no enzyme reactivation occurred unless calcium was added. Therefore, the chelating resin must have bound essentially all the calcium removed from MnP during thermal inactivation and thus prevented calcium from rebinding to the distal site.

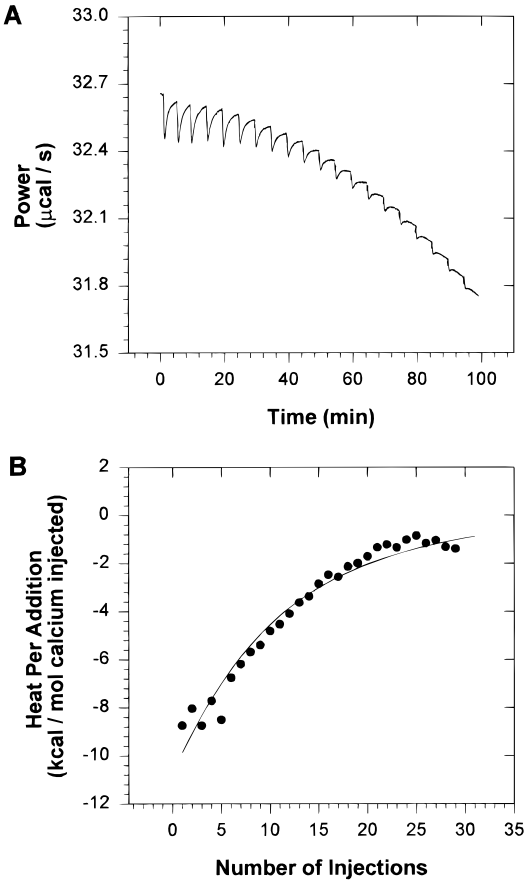


FIGURE 3: Isothermal titration calorimetry data for the titration of calcium into inactive MnP. (A) Raw data for the titration of 7.0 $\mu$M inactive MnP with 0.535 mM $CaCl_2$ at 30 °C. Inactive MnP was prepared as described in the legend to Figure 1. (B) Integration of the data in panel A yielded the total heat as a function of total added calcium (●). The solid line represents the fit of the data.

By titrating MnP, inactivated in the presence of chelating resin, with calcium and recording the absorption difference spectrum for inactive MnP in the presence and absence of calcium, the $K_d$ was determined to be $11 + 1$ $\mu$M. This indicated that the calcium binding site had a moderately high affinity for calcium and was comparable to other extracellular calcium binding proteins that bind calcium to increase structural stability (Strynadka & James, 1989). The linear Hill plot, with $n = 1.1 \pm 0.1$, indicated that the changes in the absorption spectrum of MnP during reactivation were due to the binding of calcium to one site. This was consistent with our hypothesis that thermal inactivation and the calcium-dependent reactivation of MnP were due to the dissociation and association of one calcium at the distal calcium binding site (Sutherland et al., 1997). The $K_d$ determined by ITC corresponded well with the value determined by difference spectroscopy. As has been observed for many calcium binding proteins, the binding of calcium was associated with a decrease in enthalpy and entropy and was thus enthalpically driven (Kuroki et al., 1992).

The binding of bis-ANS to both active and inactive MnP caused a blue shift in the fluorescence emission maximum and an increase in the fluorescence intensity of the fluorophore, as has been observed for other proteins (Musci et al., 1985). The apparent $K_d$ for the binding of bis-ANS to active and inactive MnP and the fluorescence emission maximum for the two complexes were similar, but $I_{max}$ was twice as high for the inactive MnP−bis-ANS complex. This indicated

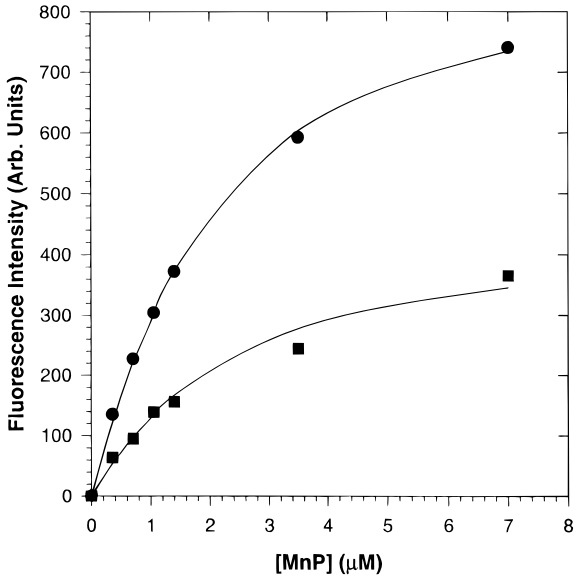


FIGURE 4: Effect of active and thermally inactivated MnP on the fluorescence intensity of bis-ANS. The fluorescence intensity was recorded at 500 nm ($\lambda_{ex}$ = 374 nm) in mixtures containing 20 mM Tris buffer, pH 7.9, 1.0 $\mu$M bis-ANS, and various amounts of active (■) or thermally inactivated (●) MnP. Thermally inactivated MnP was prepared as described in the legend to Figure 1. The solid lines represent the fit to the equation as described under Materials and Methods.

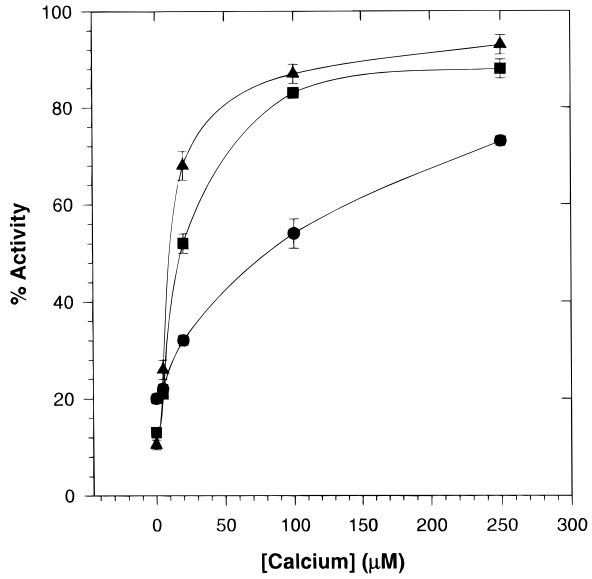


FIGURE 5: Effect of calcium on the amount of MnP activity remaining following thermal incubation at various pH. Manganese peroxidase (0.18 $\mu$M) was incubated at 43.5 °C in 20 mM sodium phosphate buffer, pH 5.5 (●), 6.5 (■), and 7.5 (▲) in the presence of 0, 5, 20, 100, and 250 $\mu$M $CaCl_2$. No chelating resin was utilized in this experiment. The remaining activity was determined following incubation at 43.5 °C for the amount of time required to inactivate MnP in the absence of calcium to less than 20% activity (5.5 h at pH 5.5, 6 min at pH 6.5, or 1 min at pH 7.5). The error bars represent standard deviations.

that there was greater exposure of hydrophobic surface area in inactive MnP. This could in part be due to the decreased α-helical content of inactive MnP (Sutherland et al., 1997).

It was previously reported that the binding of calcium to proteins was thermodynamically driven by the change in entropy associated with the release of water molecules from hydrated calcium and the enzyme calcium binding site (Kuroki et al., 1992). Kuroki and co-workers estimated $\Delta H$

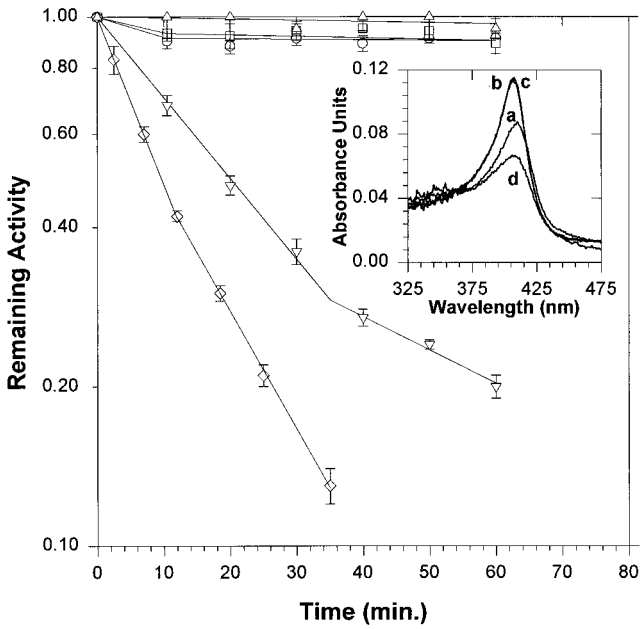


FIGURE 6: Effect of calcium, manganese(II), and terbium(III) on the thermal inactivation of MnP. Manganese peroxidase (0.14 $\mu$M) was incubated at 37 °C in 20 mM sodium phosphate buffer, pH 6.5, in the presence of no added cation (▽), 100 $\mu$M $CaCl_2$ (○), 100 $\mu$M $MnSO_4$ (□), 100 $\mu$M $TbCl_3$ (◇), and 100 $\mu$M $TbCl_3$ plus 500 $\mu$M $CaCl_2$ (△). No chelating resin was utilized in this experiment. Aliquots of the incubation mixture were removed periodically and assayed for activity at room temperature. The data are represented as the average and standard deviation of three values. The inset shows the effect of calcium, manganese(II), and terbium(III) on the absorption spectrum of thermally inactivated MnP. Manganese peroxidase (1.0 $\mu$M) was inactivated as described in the legend to Figure 1, except that only 125 mg/mL chelating resin was used. The inactive MnP was separated from the chelating resin and divided to four 1.0 mL aliquots. The samples were incubated at 30 °C and received (a) no addition, (b) $CaCl_2$ to 1 mM, (c) $MnSO_4$ to 1 mM, and (d) $TbCl_3$ to 1 mM. Spectrum a was recorded at the time of addition of the cations but did not change over the course of the experiment. Spectra b and c were recorded 60 min after the addition of the metals. Spectrum d was recorded 1 min after the addition of terbium(III) and corrected for absorption due to $TbCl_3$.

and $\Delta S$ for the release of water molecules upon calcium binding to calculate $\Delta H$ and $\Delta S$ for protein conformational changes that occurred as a result of calcium binding (Kuroki et al., 1992). The values of $T\Delta S$ and $\Delta H$ for the binding of calcium to several calcium binding proteins were plotted ($T\Delta S$ vs $\Delta H$) by Kuroki et al. (1992) and a linear relationship was observed for both the raw data (i.e., the entire binding process) and for the corrected values (i.e., only the protein conformational changes) (Figure 7). The line that was fit to the plot of the corrected values had a slope of approximately 1 and passed through the origin. Therefore $\Delta G \cong 0$ for the protein conformational changes, indicating that the entire binding process must have been driven by the release of water (Kuroki et al., 1992). Linear relationships between enthalpy and entropy, often referred to as the enthalpy−entropy compensation, have been observed for many proteins in aqueous environments undergoing similar processes via similar mechanisms (Leffler & Grunwald, 1963; Lumry & Rajender, 1970; Mateo et al., 1986).

The values of $T\Delta S$ and $\Delta H$ for the binding of calcium to MnP fit on the appropriate lines constructed from the data for the other calcium binding proteins (Figure 7) but the values were more negative for MnP. The large negative

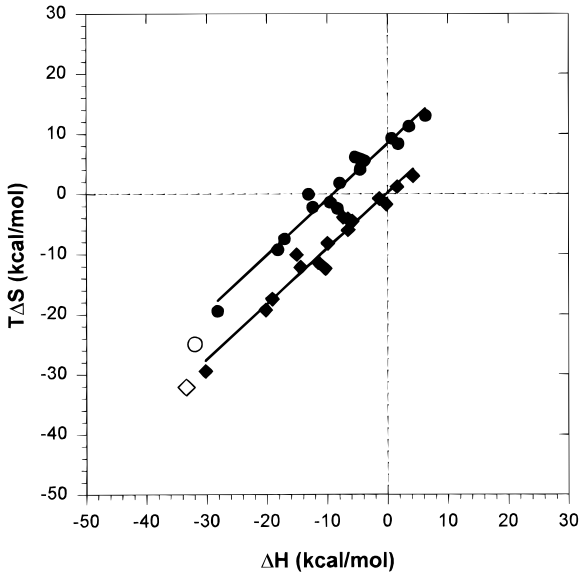


FIGURE 7: Plot of $T\Delta S$ versus $\Delta H$ for the binding of calcium to various calcium binding proteins. Both the actual data (●) and the data corrected for $T\Delta S$ (10 kcal/mol) and $\Delta H$ (2 kcal/mol) due to release of water from calcium in solution (◆) are plotted. The corresponding values for MnP are also shown (○ and ◇). The correction for MnP was only $^5/_7$ that for other proteins because two fewer water molecules were released upon binding since the distal calcium of MnP was bound by five ligands from amino acids and two water ligands. The data for the other proteins and the appropriate correction were reported previously (Kuroki et al., 1992).

changes may arise because the binding of calcium to MnP causes conformational changes that result in a more compact, rigid enzyme structure with more hydrogen bonds and van der Waals interactions. They could also be due to exposure of hydrophobic residues upon calcium binding, which would cause water to form ordered clathrate structures. However, binding studies involving bis-ANS indicated that there was more hydrophobic surface area in inactive MnP. Therefore, the binding of $Ca^{2+}$ to MnP, and its reactivation, should decrease the solvent-accessible hydrophobic area of MnP and contribute an increase in enthalpy and entropy. Since calcium binding caused a net decrease in enthalpy and entropy, the predominant contribution to the thermodynamic parameters must have been the formation of a more compact and less flexible MnP structure. This is typical for calcium binding proteins that bind calcium for structural stability and supports our proposal that the binding of calcium to the distal calcium binding site of MnP causes relatively large conformational changes that confer structural stability to the heme environment of the enzyme (Sutherland et al., 1997).

At lower pH the rate of thermal inactivation of MnP, and thus the rate of calcium release, decreased (Sutherland & Aust, 1996) and the affinity of calcium for MnP increased. However, the rate of binding of calcium to inactive MnP decreased at lower pH such that even though the process was thermodynamically more favorable, there was a kinetic barrier that decreased the rate. Consistent with this proposal, the addition of calcium was less efficient at preventing thermal inactivation of MnP at lower pH. We propose that these phenomena can be explained by the effects of pH on the flexibility of the distal calcium binding site of MnP. Rate constants for the binding of calcium to proteins are predominantly dependent on the flexibility of the binding site, which enables exposure of coordinating oxygen ligands to the incoming cation (Falke et al., 1994). It is predicted that at lower pH MnP is more stable because there is less conformational mobility around the distal calcium binding site, preventing calcium release and inactivation. But once the calcium was released, the decreased flexibility of the binding site at lower pH decreased the rate of calcium binding and reactivation, even though the binding of calcium to MnP was thermodynamically more favorable.

Manganese(II) prevented thermal inactivation of MnP as effectively as calcium and reactivated MnP to the same extent as calcium, although at a slower rate. Therefore, it appears that manganese(II) can bind to the distal calcium binding site of MnP and maintain the correct conformational structure of MnP required for activity. Manganese(II) is thus important to the overall activity of MnP in fungal cultures not only as an inducer and substrate/redox mediator of MnP but also for its ability to stabilize the enzyme from thermal inactivation (Brown et al., 1990; Glenn et al., 1986; Sutherland & Aust, 1996; Wariishi et al., 1988).

The addition of terbium(III), a fluorescent probe that has been shown to replace calcium in many proteins (Evans, 1990), actually accelerated the rate of thermal inactivation of MnP. This appeared to be due to the binding of terbium(III) to the distal calcium binding site, either displacing calcium or preventing it from rebinding, rendering MnP inactive. The absorption spectrum of inactive MnP changed immediately following the addition of terbium(III), indicating that a binding event must have altered the heme environment and rendered the enzyme inactive. Terbium(III) did not affect MnP prior to thermal incubation, and excess calcium prevented the effect of terbium(III) on thermal inactivation. Therefore, it appears terbium(III) only affected the heme environment of MnP following the release of calcium and was therefore either binding at the distal calcium site or a site that was only accessible in inactive MnP. While the replacement of calcium with lanthanide ions in horseradish peroxidase did yield active enzyme (Morishima et al., 1986), terbium(III) also appeared to disrupt the heme environment of cationic peanut peroxidase (Maranon et al., 1993).

In conclusion, a method was developed to study the binding of calcium to a binding site that controlled the heme environment and activity of MnP. The reactivation of thermally inactivated MnP involved the binding of one calcium with moderately high affinity. Calcium binding caused large decreases in entropy and enthalpy, which were attributed to the ability of calcium binding to increase the structural rigidity and compactness of the enzyme. This was consistent with the proposed role of the distal calcium to stabilize the structural integrity of the enzyme, particularly the distal heme environment (Sutherland et al., 1997). Manganese(II) may also act to stabilize MnP *in vivo* since manganese(II) was also able to bind to the distal calcium binding site of MnP and the enzyme was structurally and functionally equivalent to active MnP.

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