Manganese Peroxidase from the Lignin-degrading Basidiomycete

Phanerochaete chrysosporium

TRANSIENT STATE KINETICS AND REACTION MECHANISM*

(Received for publication, September 7, 1988)

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Stopped-flow techniques were used to investigate the kinetics of the formation of manganese peroxidase compound I (MnPI) and of the reactions of MnPI and manganese peroxidase compound II (MnPII) with p-cresol and Mn++. All of the rate data were obtained from single turnover experiments under pseudo-first order conditions. In the presence of H2O2 the formation of MnPI is independent of pH over the range 3.12-8.29 with a second-order rate constant of (2.0 ± 0.1) × 10^8 M⁻¹ s⁻¹. The activation energy for MnPI formation is 20 kJ mol⁻¹. MnPII formation also occurs with organic peroxides such as peracetic acid, m-chloroperoxybenzoic acid, and p-nitroperoxybenzoic acid with second-order rate constants of 9.7 × 10^6, 9.5 × 10^6, and 5.9 × 10⁴ M⁻¹ s⁻¹, respectively. The reactions of MnPI and MnPII with p-cresol strictly obeyed second-order kinetics. The second-order rate constant for the reaction of MnPII with p-cresol is extremely low, (9.5 ± 0.5) M⁻¹ s⁻¹. Kinetic analysis of the reaction of MnI with MnPI and MnPII showed a binding interaction with the oxidized enzymes which led to saturation kinetics. The first-order dissociation rate constants for the reaction of MnI with MnPI and MnPII are (0.7 ± 0.1) and (0.14 ± 0.01) s⁻¹, respectively, when the reaction is conducted in lactate buffer. Rate constants are considerably lower when the reactions are conducted in succinate buffer. Single turnover experiments confirmed that MnI serves as an obligatory substrate for MnPII and that both oxidized forms of the enzyme form productive complexes with MnI. Finally, these results suggest the α-hydroxy acids such as lactate facilitate the dissociation of MnIII from the enzyme.

Lignin is a heterogeneous and random phenylpropanoid polymer that comprises 20-30% of woody plants (1). White rot basidiomycetes are primarily responsible for the initiation of the decomposition of lignin in wood (2, 3). When cultured under ligninolytic conditions, the white rot fungus Phanerochaete chrysosporium secretes two extracellular heme peroxidases which, along with an H2O2-generating system (2), appear to be major components of its lignin degradation system. These peroxidases, manganese peroxidase (MnP)¹ and lignin peroxidase, have been purified to homogeneity and characterized (4-9). MnP is a glycoprotein of Mr ~ 46,000 with one iron protoporphrin IX prosthetic group (8, 10). The enzyme catalyzes the H2O2- and MnII-dependent oxidation of a variety of phenols, amines, and dyes (8, 10, 11). Electronic absorption (8, 11), EPR, and resonance Raman spectral evidence (12) indicate that the heme iron in the native protein is in the high spin, pentacoordinate, ferric state with histidine coordinated as the fifth ligand.

It has also been demonstrated that MnP oxidizes MnII to MnIII and that the MnIII produced, in turn, oxidizes the organic substrates (8, 10, 11, 13). Thus the manganese ion participates in the reaction as a redox couple rather than acting as an enzyme-binding activator. Our initial optical spectral characterization of the oxidized intermediates MnPI, MnPII, and MnPIII (13) indicated that the oxidation states and catalytic cycle of MnP are similar to horseradish peroxidase and lignin peroxidase as shown below.

\[
\text{MnP} + \text{H}_2\text{O}_2 \xrightarrow{k_1} \text{MnPI} + \text{H}_2\text{O} \\
\text{MnPI} + \text{Mn}^{II} \xrightarrow{k_2} \text{MnPII} + \text{Mn}^{III} \\
\text{MnPII} + \text{Mn}^{II} \xrightarrow{k_3} \text{MnP} + \text{Mn}^{II} \\
\text{Mn}^{III} + \text{AH} \xrightarrow{k_4} \text{Mn}^{II} + \text{A}. 
\]

These experiments also demonstrated that although MnIII is a variety of phenols are capable of reducing MnPI to MnPII (Equation 2), only MnIII is capable of reducing MnPII to the native enzyme (Equation 3). Herein, we utilize transient state kinetics to determine the rate of formation of MnPI and the rate of reduction of MnPI and MnPII using MnII or p-cresol as reducing substrates. Values of \(k_1\), \(k_2\), and \(k_3\) were measured directly and reactions were conducted under pseudo-first order conditions with excess substrate, which facilitated data analysis.

EXPERIMENTAL PROCEDURES

Manganese peroxidase was purified from the extracellular medium of acetate-buffered agitated cultures of Ph. chrysosporium as described (4, 5), except that the medium contained 0.1% Tween 80 and 6-fold concentrated trace elements (14, 15), and benzyl alcohol (6 mM) was

¹ The abbreviations used are: MnP, manganese peroxidase; MnPI, MnPII, and MnPIII, manganese peroxidase compounds I, II, and III; AH, organic reducing substrate.
between MnPI and MnPII at 397 nm. In our previous work, the origin within experimental error, indicating that the formation was irreversible. The second order rate constant ($k_{obs}$) was determined by measuring absorbance change at 420 nm.

The purified protein was electrophoretically homogeneous and had an RZ ($A_{280}/A_{230}$) value of 6.1. Enzyme concentrations were determined at 406 nm using an extinction coefficient of 129 mM$^{-1}$ cm$^{-1}$ (6). The homogeneous enzyme was dialyzed exhaustively against glass-distilled, filtered water before use.

$H_2O_2$ (30% solution) and peracetic acid (40%) were obtained from British Drug House Chemicals and FMC Corp., respectively. Other organic peroxides and $p$-cresol were purchased from Aldrich. All other chemicals were of reagent grade. Solutions were prepared using deionized water obtained from the Milli Q System (Millipore). The concentration of $H_2O_2$ was determined by the horseradish peroxidase assay (16).

Kinetic measurements were conducted using the Photal (formerly Union Giken) RA 601 Rapid Reaction Analyzer equipped with a 1-cm observation cell and interfaced with a digital computer system (Sord M200 Mark III). One reservoir contained enzyme in water, at a concentration of 1.0 mM after mixing, while the other reservoir contained the substrate ($H_2O_2$, Mn$^{II}$, or $p$-cresol) and buffer. All experiments were performed at (25 ± 0.5)°C in 20 mM sodium lactate buffer or otherwise as indicated in the text. The pH was varied from 3.1 to 6.29. MnPI was prepared by adding 0.9 eq of $H_2O_2$ to native MnP. MnPII was obtained by the successive addition of 1.0 eq of ferrocyanide and 0.9 eq of $H_2O_2$. All enzyme samples were freshly prepared for each experiment. The substrate concentrations were at least 10 times in excess to maintain pseudo-first order kinetics. The pseudo-first order rate constants were determined by a nonlinear least-squares computer analysis of the exponential traces. Experiments were conducted with stable solutions of native MnP, MnPI, or MnPII (13) placed in the stopped-flow apparatus, for the direct determination of $k_1$, $k_2$, or $k_3$. Electronic absorption spectra were recorded on a Shimadzu UV-260 or Cary 219 spectrophotometer.

RESULTS

Spectral Characteristics—Spectra of native MnP, MnPI, and MnPII in the Soret region (13) are compared in Fig. 1. Isosbestic points between native MnP and MnPI occur at 426 and 358 nm, between native MnP and MnPII at 417 nm, and between MnPI and MnPII at 397 nm. In our previous work (13) MnPII was prepared by adding 2 eq of $H_2O_2$ to the native enzyme. In the present study, with the successive addition of stoichiometric amounts of ferrocyanide and $H_2O_2$, the absorbance of the MnPII Soret peak was 10% higher than the value we obtained previously, but had the identical $\lambda_{max}$ at 420 nm.

Formation of MnP Compound I—The rate of compound I formation was determined by measuring absorbance change at 397 nm, the isosbestic point between MnPI and MnPII. Thus, the possible subsequent conversion of MnPI to MnPII did not interfere with the rate measurement. All kinetic traces were of a single exponential character (Fig. 2, inset). The observed rate constants ($k_{obs}$) were linearly proportional to the $H_2O_2$ concentration from 20 to 100 times in excess. The plot of $k_{obs}$ versus $H_2O_2$ concentration (Fig. 2) passed through the origin within experimental error, indicating that the formation of MnPI was irreversible. The second order rate constant ($k_1$) was found to be $(2.0 ± 0.1) \times 10^8$ M$^{-1}$ s$^{-1}$ (Fig. 3). A plot of log $k_1$ versus pH is shown in Fig. 3. The formation of MnPII exhibited no pH dependence over the pH range 3.12-8.29. This value did not change significantly when the reaction was conducted in the presence of high ionic strength buffers. MnP activity is stimulated significantly by $\alpha$-hydroxy acids such as lactate or citrate (4, 8, 11, 13) presumably because these acids chelate the Mn$^{II}$ generated during the reaction. Therefore, as predicted the same rate of formation of MnPI was observed in citrate, succinate, or phosphate buffer (Fig. 3). The second order rate constants for MnP compound I formation with $H_2O_2$ and various organic peroxides are listed in Table 1.

Activation Energy for MnPI Formation—Using $H_2O_2$ as the substrate the rate of MnP compound I formation was measured over the temperature range of 3.5-34.0°C. The temperature dependence of the second order rate constants is presented as an Arrhenius plot (Fig. 4). The activation energy calculated from the slope of the plot was $(4.9 ± 0.1)$ kcal mol$^{-1}$ or $(20.7 ± 0.5)$ kJ mol$^{-1}$.
Mechanism of Manganese Peroxidase

**FIG. 3.** The pH dependence of MnPI formation. Experiments were conducted as described in Fig. 2. $k_{(app)}$ was obtained from the slope of the plot, $k_{obs}$ versus [H$_2$O$_2$]. Each $k_{obs}$ was calculated from a trace of the change in absorbance at 397 nm (Fig. 2, inset). Buffers used were sodium lactate ($\mu = 0.02$) (○); sodium succinate ($\mu = 0.02$) (■); potassium phosphate ($\mu = 0.02$) (△); and sodium lactate ($\mu = 0.1$) (●). For the latter buffer, K$_2$SO$_4$ was added to adjust the ionic strength to 0.1.

**TABLE I**
Rate of manganese peroxidase compound I formation with various peroxides

<table>
<thead>
<tr>
<th>Peroxide</th>
<th>Rate constant $M^{-1} s^{-1}$</th>
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<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>$(2.0 \pm 0.1) \times 10^6$</td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>$(9.7 \pm 0.2) \times 10^5$</td>
</tr>
<tr>
<td>$m$-Chloroperoxybenzoic acid</td>
<td>$(9.5 \pm 0.2) \times 10^4$</td>
</tr>
<tr>
<td>$p$-Nitroperoxybenzoic acid</td>
<td>$(5.9 \pm 0.1) \times 10^4$</td>
</tr>
</tbody>
</table>

**FIG. 5.** Reaction of MnPI with $p$-cresol. One drive syringe contained MnPI in H$_2$O and the other syringe contained $p$-cresol in sodium lactate buffer, pH 4.51 ($\mu = 0.02$ after mixing). $k_{obs}$ was determined from the exponential change in absorbance at 417 nm (the isosbestic point between native MnP and MnPII) as a function of time. The linear plot of $k_{obs}$ versus $p$-cresol concentration was a nonlinear least squares fit of the data. Equation 6 in the text was used for the curve fit.

**FIG. 6.** Reaction of MnPI with Mn$^{II}$. Experimental conditions were as in Fig. 5, except that Mn$^{II}$ was the reducing substrate. The buffers used were 20 mM sodium lactate, pH 4.51 (○——○), and 20 mM sodium succinate, pH 4.49 (●——●). The plot of $k_{obs}$ versus Mn$^{II}$ concentration was a nonlinear least squares fit of the data. Equation 6 in the text was used for the curve fit.

Reduction of MnP Compound I—The reduction of MnPI to MnPII was followed at 417 nm, the isosbestic point between native MnP and MnPII (Fig. 1). Pseudo-first order conditions were employed using an excess of reducing substrate, $p$-cresol, or Mn$^{II}$ (as MnSO$_4$) at pH 4.51, the pH optimum for MnP activity (8, 10, 11, 13). A linear dependence of $k_{obs}$ on $p$-cresol concentration was observed between 0–1.0 mM (Fig. 5). The second order rate constant, $k_{(app)}$, for the reaction of MnPI with $p$-cresol, calculated from the slope of the plot in Fig. 5, was found to be $(1.8 \pm 0.2) \times 10^3 M^{-1} s^{-1}$.

In contrast, with Mn$^{II}$ as the reducing substrate, the plot of $k_{obs}$ versus [Mn$^{II}$] leveled off at high Mn$^{II}$ concentrations (Fig. 6). This behavior can be described by a simple binding interaction between reactants according to Equations 5 and 6,

\[
\text{MnPI} + \text{Mn}^{II} \xrightarrow{K_1} \text{MnPI} - \text{Mn}^{II} \xrightarrow{k_b} \text{MnPII} + \text{Mn}^{IIn}
\]

\[
k_{\text{obs}} = \frac{k_b}{1 + [\text{Mn}^{II}]/K_1}
\]
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FIG. 7. Reaction of MnP with MnIll. MnP was prepared by the successive addition of 1.0 eq of ferrocyanide and 0.9 eq of H₂O₂ to native MnP in water. MnIll in sodium lactate, pH 4.51 (µ = 0.02) after mixing, was added to the enzyme, and the exponential change in absorbance was measured at 420 nm as a function of time, kobs, was determined from a mean of four to six experimental traces. The plot of kobs versus MnIll concentration is a nonlinear least squares fit of the data. Equation 9 in the text was used for the curve fit.

where kₐ is a first order rate constant (s⁻¹) and Kᵢ is an apparent dissociation constant (M) given by Equation 7.

$$Kᵢ = \frac{[\text{MnP]} [\text{MnIll}]}{[\text{MnP} - \text{MnIll}]}$$

(7)

The constants, kₐ and Kᵢ, were calculated from Equation 6 using a nonlinear least squares fit to the data. The calculated curve is shown in Fig. 6; kₐ and Kᵢ were determined to be (0.7 ± 0.1) s⁻¹ and (3.3 ± 0.8) × 10⁻⁴ M, respectively. The reduction of MnP by MnIll was also performed in sodium succinate (µ = 0.02 M) at pH 4.49 where saturation kinetics were again observed (Fig. 6). With this buffer which cannot chelate MnIll readily (8, 11), values of kₐ and Kᵢ were (0.13 ± 0.01) s⁻¹ and (2.3 ± 0.2) × 10⁻⁴ M, respectively. The curve fit computed from Equation 6 is also shown in Fig. 6. Finally, when the reaction was conducted in citrate buffer (µ = 0.02 M) at pH 4.42, the values for kₐ and Kᵢ were similar to those found with lactate buffer.

Reactions of Compound II—Reduction of MnP with MnIll to native MnP was followed at 420 nm at pH 4.51 under pseudo-first order conditions with reducing substrate, p-cresol, or MnIll in excess. With p-cresol as the reducing substrate a linear relationship between kₐ and substrate concentration (0–10 mM) was observed (data not shown). The second order rate constant (kₐ app) for the reaction of MnP with p-cresol was calculated to be 9.5 ± 0.5 M⁻¹ s⁻¹. This is an extremely small value for a peroxidase compound II reduction. The initial rate of substrate oxidation in a reaction mixture consisting of 1.0 µg/ml enzyme, 0.1 mM phenolic substrate, and excess H₂O₂ would be approximately 1 × 10⁻⁴ nmol min⁻¹ µg⁻¹. With such a slow rate of reduction of MnP, the catalytic cycle of the enzyme would be stopped. This explains why MnP requires MnIll to complete its catalytic cycle (13).

With MnIll as the reducing substrate for MnP, saturation kinetics were observed. This reaction can be described by Equations 8–10 which are analogous to Equations 5–7.

$$\text{MnP} + \text{MnIll} \overset{kₐ}{\rightarrow} \text{MnP} - \text{MnIll} \overset{kₐ}{\rightarrow} \text{MnP} + \text{MnIll}$$

(8)

$$k_{obs} = \frac{kₐ}{1 + [\text{MnIll}]}$$

(9)

$$Kᵢ = \frac{[\text{MnP}] [\text{MnIll}]}{[\text{MnP} - \text{MnIll}]}$$

(10)

Using Equation 9 and the same nonlinear least squares analytical method, the first order rate constant (kₐ) and dissociation constant (Kᵢ) were found to be (0.14 ± 0.01) s⁻¹ and (4.4 ± 0.8) × 10⁻⁴ M. The fit of the calculated curve to the data is shown in Fig. 7. Table II summarizes the kinetic parameters obtained from these transient state kinetic studies on the reductions of MnP and MnP.

DISCUSSION

Manganese peroxidase is a heme-containing enzyme isolated from the extracellular medium of ligninolytic cultures of the white rot basidiomycete P. chrysosporium (4, 8, 10, 11). In the presence of MnIll, the enzyme catalyzes the H₂O₂-dependent oxidation of a variety of amines, organic dyes, and phenols including phenolic lignin model compounds (4, 8, 10, 11, 13, 17). The catalytic activity is dramatically stimulated by α-hydroxy acids such as lactate and citrate, and it has been suggested that α-hydroxy acids complex MnIll, thus stabilizing its high redox potential (8, 10, 13). In recent studies (8, 10, 13, 17) we have shown that the enzyme oxidizes MnIll to MnP and that the MnP then acts as an obligatory redox couple, oxidizing various organic substrates. The prosthetic group of MnP is iron protoporphyrin IX (8, 12). In this respect MnP resembles other fungal and plant peroxidases. Recently we were able to prepare the oxidized intermediates of MnP, compounds I, II, and III, in the presence of stoichiometric amounts of H₂O₂ (8, 13). Each of these intermediates has spectral characteristics similar to those of horseradish peroxidase (18) and lignin peroxidase (19). In addition, titration of MnP with ferrocyanide demonstrated that MnP was reduced back to the native enzyme via two single electrons.

Table II

<table>
<thead>
<tr>
<th>Oxidized enzyme</th>
<th>Substrate</th>
<th>Second order rate constanta (M⁻¹ s⁻¹)</th>
<th>First order rate constant (s⁻¹)</th>
<th>Apparent dissociation constant (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnP</td>
<td>MnIll</td>
<td>0.7 ± 0.1</td>
<td>(3.3 ± 0.8) × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-Cresol</td>
<td>(1.8 ± 0.2) × 10⁻³</td>
<td>(2.2 ± 0.2) × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>MnP II</td>
<td>MnIll</td>
<td>0.14 ± 0.01</td>
<td>(4.4 ± 0.8) × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-Cresol</td>
<td>9.5 ± 0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Given as kₐ app and kₐ in the text.
b Given as kₐ and Kᵢ in the text.
c Given as Kᵢ and kₐ in the text.
d The data obtained in succinate buffer.
steps (13) with MnPII as an intermediate. Furthermore, we demonstrated that although both MnII and phenols are able to reduce MnPI to MnPII, only MnII is capable of efficiently reducing MnPII to the native enzyme (15). These findings indicate that the catalytic cycle of MnP is similar to that of horseradish peroxidase and lignin peroxidase as shown in Equations 1–3, and they explain the absolute dependence of the catalytic activity on MnII. To understand more fully the mechanism of MnP and the role of MnII and α-hydroxy acids in the catalytic cycle, the transient state kinetics of MnP formation and of MnPI and MnPII reductions were studied at the pH optimum (4.5) and under pseudo-first order conditions with substrate in excess.

The primary reaction product of peroxidases with H2O2 is the oxidized intermediate compound I. This intermediate accepts both oxidizing equivalents of H2O2 and thus contains 2 oxidizing eq over the native enzyme (18, 20, 21). The electronic absorption spectrum of MnPI, Soret maximum at 406 nm with reduced intensity (Fig. 1) (13), and additional maxima at 558 and 650 nm (13) is very similar to that of horseradish peroxidase (18) and lignin peroxidase (19, 22). The reduced Soret intensity suggests the π→cation radical nature of the MnP compound I porphyrin ring (20, 25). The activation energy for MnPII formation (4.9 kcal mol⁻¹) is also in the same range as that of other peroxidases: 3.5 kcal mol⁻¹ for horseradish peroxidase (24), 5.9 kcal mol⁻¹ for lignin peroxidase (25), and 2.9 and 5.4 kcal mol⁻¹ for turnip peroxidase isoenzymes P7 and P1, respectively (26).

The pH dependence of the formation rate of compound I has been studied with a variety of peroxidases. These studies indicate that peroxidases possess a distal ionizable group which controls the pH dependence of compound I formation. Its pK value has been reported to be in the range of 3.0 to 5.3 (8, 27–31). Based on the crystal structure of cytochrome c peroxidase, Poulos and Kraut (32) proposed that the distal histidine serves as an acid base catalyst participating in the heterolytic cleavage of H2O2. Although MnPI is similar to other peroxidases in its spectral features and activation energy of formation, the second order rate constant for MnPI formation using H2O2 as the substrate (kI) is independent of pH over the range 3.12–5.29 (Fig. 5). This may account for the decreased rate of MnPI formation (kI) was found to be 2.0 × 10⁶ M⁻¹ s⁻¹, which is 5–10 times smaller than for most other peroxidases (18, 29–31). The same pH independence was observed for lignin peroxidase compound I formation, which also has a smaller kI value (22, 25). Since either ionic strength or nitrate or acetate have been reported to shift the apparent dissociation constants for native MnP complexes were determined from different spectra between native MnP and MnP plus substrate in lactate buffer at pH 4.5: Kd ~ 4.5 μM for MnII and ~120 μM for p-cresol (data not shown). These data for the native enzyme are in accord with our findings for MnPI and MnPII, where MnII forms a complex which affects the observed kinetics, whereas p-cresol does not.

The reduction of MnPI with MnII was dependent on the buffer used; kI was >5 times larger in lactate and citrate than in succinate. In contrast, the dissociation constants Kd are approximately the same in either buffer (Table II). This suggests that α-hydroxy acids act to facilitate the dissociation of the enzyme-manganese complex by chelating MnIII. A similar effect was seen for the reduction of MnPII; however, in succinate buffer the rate was too small to obtain accurate values (data not shown). It is also likely that α-hydroxy acids act by stabilizing the MnIII at a high redox potential (11, 13). If the stimulation of enzyme activity by α-hydroxy acids occurs in part by accelerating the dissociation of the enzyme–manganese complex, then productive manganese–enzyme complexes are implied. The possibility of nonproductive complexes with horseradish peroxidase compounds has been discussed (35).

The addition of MnII and p-cresol to MnPI led to a rate of reduction which was the sum of the individual rates in the presence of a single reducing substrate. This relationship was demonstrated with various concentrations of reducing substrates, indicating that MnII does not act as an enzyme-binding activator and that the reaction with both substrates

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present probably does not occur via a ternary complex. Although the spectral characteristics of the oxidized inter-
mediates of MnP and its catalytic cycle are similar to those of
other peroxidases, this enzyme has some unusual proper-
ties. Mn$^{3+}$ is the preferred substrate especially for MnPII
and the potential of this redox couple falls conveniently be-
tween those of the enzyme’s oxi-
dized states and the terminal organic substrates (11, 13). The
Mn$^{3+}$-lactate complex generated is able to oxidize a variety of
organic compounds including lignin substructures (8, 11, 13, 17).
The effect of $\alpha$-hydroxy acids apparently occurs via inter-
action with Mn$^{3+}$. These Mn$^{3+}$-$\alpha$-hydroxy acid complexes
are stable (11, 13), yet have a high redox potential (37).
Furthermore, our present results suggest that $\alpha$-hydroxy acids help to facilitate the dissociation of the manganase-enzyme
complex.

Additional spectroscopic and kinetic studies on the inter-
action of the enzyme, manganese, $\alpha$-hydroxy acids, and the
terminal organic substrates are planned.

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