Rate Enhancement of Compound I Formation of Barley Peroxidase by Ferulic Acid, Caffeic Acid, and Coniferyl Alcohol†

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ABSTRACT: Reactions of barley peroxidase 1 were studied using transient-state and steady-state kinetics at pH 3.96, 25 °C, and 0.1 M ionic strength, in both the presence and the absence of 1 mM calcium ion. The rate of compound I formation from barley peroxidase 1 and hydrogen peroxide in the absence of reducing substrate is very slow, with or without calcium. When each of the three reducing substrates ferulic acid, caffeic acid, and coniferyl alcohol is added individually, there is a striking enhancement of the rate of compound I formation by a factor of 10−40 depending on the substrate. These unique rate enhancements can be explained by the effect of tight substrate binding to the native enzyme, and they may be indicative of an activating effect of reducing substrate on barley peroxidase 1 under physiological conditions. All steady-state kinetic results can be explained by an initial tight binding of reducing substrate AH to the barley peroxidase, Peroxidase + AH ⇌ Peroxidase−AH, and substitution of the peroxidase−AH complex for native enzyme in the standard modified ping-pong mechanism for peroxidase reactions [Dunford, H. B. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. II, pp 1−24, CRC Press, Boca Raton, FL]. The dissociation constant of barley peroxidase 1 and ferulic acid was 1.4 ± 0.6 μM as determined by the change in the absorbance at the Soret maximum at the conditions mentioned above. The dissociation constant of barley peroxidase 1 and cyanide, 30 ± 5 μM, changed to 55 ± 5 μM in the presence of ferulic acid. The rate of oxidation of caffeic acid was enhanced 3-fold in 1 mM calcium.

In a comparative study of plant and fungal peroxidases, barley grain peroxidase, BP 1, showed a remarkably low specific activity at pH 5 toward a number of common substrates (Andersen et al., 1991a). Further studies disclosed that BP 1 reacted uniquely with hydrogen peroxide in two respects (Rasmussen et al., 1993a). First, it showed saturation kinetics, and second, the activity was very low at pH 5 and increased toward pH 3, in contrast with the properties of other known plant peroxidases. Ferulic acid, caffeic acid, and coniferyl alcohol are substances that are widely distributed in plants. Coniferyl alcohol is generally accepted as a precursor of lignin formation, while the roles of ferulic acid and caffeic acid are more obscure. An investigation of the oxidation of ferulic acid, caffeic acid, and coniferyl alcohol by hydrogen peroxide and BP 1 has been carried out at pH 3.96, where BP 1 is both stable and active.

BP 1 is a slightly basic peroxidase with a p*I* value of 8.5 (Hejgaard et al., 1991). It exsists in two forms, BP 1a and BP 1b, differing in glycosylation only. BP 1a is glycosylated at one site, whereas BP 1b is non-glycosylated. The M_r's of the two forms are 37 000 and 36 000, respectively. The sequence of BP 1 is less than 45% identical to those of other plant peroxidases, which indicates a different physiological function (Johansson et al., 1992). BP 1 is both tissue and developmentally specific. The mature protein and the mRNA are found only in the starchy endosperm of the grains, and the mRNA is only expressed for a few days during the grain filling period two weeks after flowering (Rasmussen et al., 1991). BP 1 is thought to be targeted to the vacuoles of the plant cell, due to the presence of a C-terminal prosequence similar to the target sequence that guides barley lectin to the vacuoles (Johansson et al., 1992). Interestingly, plant vacuoles have an acidic interior with pH 6.5−2.3 (Wink, 1993), and contain phenolic substances. Hence, the low pH optimum of BP 1 (Rasmussen et al., 1993a), and the amazing rate enhancement induced by phenolic substrates, which we describe in the present work, are likely to be of physiological significance for the function of BP 1 in barley grain.

EXPERIMENTAL PROCEDURES

Materials. Barley peroxidase 1 (BP 1) was purified from barley grains (Rasmussen et al., in preparation). The same batch of peroxidase, stored at 4 °C as an AMS precipitate, was used previously for transient-state kinetic analysis of the reaction with H_2O_2 (Rasmussen et al., 1993a). The batch contained both forms of BP 1, approximately 90% of BP 1a and 10% of BP 1b. The R_z (A_{Soret}/A_{280}) of the preparation was 2.9. Ferulic acid (FA), caffeic acid (CA), coniferyl alcohol (CAlc), and horseradish peroxidase, HRP C, were

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[1] Abbreviations: BP 1, barley grain peroxidase isoenzyme 1; HRP C, horseradish peroxidase isoenzyme C; HRP A2, horseradish peroxidase isoenzyme A2; FA, ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid); CA, caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid); CAlc, coniferyl alcohol (4-(3-hydroxy-1-propenyl)-2-methoxyphenol); AMS, ammonium sulfate; P, peroxidase; Cpd I, compound I; Cpd II, compound II; AH, reducing substrate; A•, radical product.

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from Sigma. All other chemicals were purchased from Merck. All chemicals were of analytical grade. Water was drawn from a Milli Q system from Millipore.

Buffer. A 0.05 M Na citrate buffer, pH 3.96, adjusted to 0.1 M ionic strength with K_2SO_4, was used throughout, with or without 1 mM $CaCl_2$ as indicated.

pK_A Values of Phenolic Substrates. The pK_A values of the reducing substrates were determined by titrating 75 μM substrate in 16 mM HCl with dilute NaOH from pH 1.8 to 11. Scans were taken on an 8452A diode-array spectrophotometer (Hewlett-Packard), and the pH was measured using a PHM62 Standard pH meter (Radiometer, Copenhagen) after each addition of NaOH. The pK_A values were determined from plots of the absorbance at a fixed wavelength versus the pH.

Absorptivity of FA, CA, and CAlc and Their Products. Absorptivities of FA, CA, and CAlc were determined in citrate buffer and 1 mM $CaCl_2$ at 25 °C. Three stock solutions of each substrate were made in 95% EtOH, concentrations determined by weight. From each of these stock solutions absorbance scans of a series of dilutions in buffer were recorded, repeating each experiment three times. The absorptivities were determined from the slope of linear plots of absorbance versus the concentration of substrate. The absorptivities were 15.2 ± 0.2 mM^{-1} cm^{-1} for FA at 318 nm, 15.3 ± 0.7 mM^{-1} cm^{-1} for CA at 320 nm, and 14.8 ± 0.4 mM^{-1} cm^{-1} for CAlc at 264 nm.

The extinction coefficients of the products were determined by adding H_2O_2 and BP 1 several times to different dilutions of the reducing substrate until no further change in the spectra occurred. All experiments were repeated three times. The absorptivities were calculated from linear plots of absorbance of the product versus concentration. The absorptivities were 6.0 ± 0.2 mM^{-1} cm^{-1} of the FA product at 318 nm, 4.9 ± 0.1 mM^{-1} cm^{-1} of the CA product at 320 nm, and 6.6 ± 0.2 mM^{-1} cm^{-1} of the CAlc product at 264 nm. Thus $\Delta\epsilon$ values ($\epsilon_{substrate} - \epsilon_{product}$) were 9.2 ± 0.4 mM^{-1} cm^{-1} for FA, 10.4 ± 0.8 mM^{-1} cm^{-1} for CA, and 8.2 ± 0.6 mM^{-1} cm^{-1} for CAlc. The $\Delta\epsilon$ values were used for calculating the initial rates of disappearance of the reducing substrates.

Enzyme Preparation. BP 1 was prepared for kinetic studies in different ways. For steady-state experiments performed in calcium-containing buffer solutions, the enzyme was prepared by spinning down BP 1 from the AMS suspension and dissolving the pellet in 1 mM $CaCl_2$. These preparations showed an apparent R_z of 2.4, due to the content of AMS. Residual AMS in the steady-state kinetic experiments was negligible, because of the low concentrations of BP 1. For transient-state experiments performed in calcium-containing buffer, the enzyme was prepared by spinning down BP 1 from the AMS suspension and dissolving the pellet in 10 mM $CaCl_2$, followed by dialysis against 1 mM $CaCl_2$. The pH of the enzyme preparation was adjusted to pH 4 by addition of a small amount of solid citric acid. The R_z value of this preparation was 3.0. The enzyme was checked for activity against CA and H_2O_2 in a steady-state experiment and had activity similar to the preparations used for the steady-state measurements including calcium. For cyanide titrations, the enzyme was prepared as described for the transient-state experiments with calcium present, apart from the adjustment of the pH of the enzyme solution. For the steady-state and transient-state experiments without calcium, AMS-precipitated BP 1 was dialyzed against Milli Q water prior to use as described previously (Rasmussen et al., 1993a). For FA titrations, the AMS pellet of BP 1 was dissolved in 10 mM $CaCl_2$, dialyzed against 1 mM $CaCl_2$, and finally dialyzed against 0.05 M Na citrate buffer and 1 mM $CaCl_2$, pH 3.96. Apart from the lowered R_z values in the presence of AMS, the spectra showed no obvious differences in terms of shifts of peak maxima. The concentration of BP 1 was determined using an absorptivity of 105 mM^{-1} cm^{-1} of the Soret band, which is 402 nm at pH 4 (Rasmussen et al., 1993b). HRP C was prepared by centrifuging an AMS precipitate followed by dissolving the pellet in Milli Q water. The apparent R_z was 2.9 due to the content of AMS. The concentration of HRP C was determined using an absorptivity of the Soret band at 403 nm of 105 mM^{-1} cm^{-1} (Ohlsson & Paul, 1976).

Substrate Preparations. The concentrations of stock solutions of H_2O_2 in water were determined and checked regularly using an absorptivity of 43.6 M^{-1} cm^{-1} at 240 nm (Kulmacz, 1986). The concentrations of the reducing substrates were determined using the absorptivities previously noted. Stock solutions of FA were made in 95% EtOH and checked regularly. Stock solutions of CA and CAlc in 95% EtOH were made fresh every day. In the case of CAlc, oxygen was removed by flushing EtOH and buffer with argon to avoid autoxidation of the substrate. All three substrates display low solubility in Na citrate buffer at pH 3.96. The low solubility was overcome by preparing the stock solutions in 95% EtOH, which upon dilution in the aqueous solutions used for all experiments provided stable clear solutions.

Steady-State Kinetic Measurements. Kinetic experiments were performed using a Hewlett-Packard 8452A diode-array spectrophotometer equipped with a stopped-flow SFA-12 rapid kinetics accessory and an OPT-12P pneumatic drive, both from Hi-Tech Scientific. The temperature was held constant at 25 °C, using a Hewlett-Packard 89090A Peltier temperature controller. The stopped-flow instrument had a dead time of 0.1 s and a total reaction volume of approximately 400 μL. Enzyme solution was placed in one syringe, and a mixture of H_2O_2 and reducing substrate was placed in the other. Citrate buffer with 1 mM $CaCl_2$ was used. The buffer temperature was adjusted to 25 °C before use. For each concentration of reducing substrate, enzyme blank experiments were done to correct for autoxidation. The final enzyme concentration was optimized to 25 nM. Final concentrations of the reducing substrates were from 5 to 126 μM. Initial rates could usually be followed as a linear decrease in absorbance within the first 2.5 s of the reaction. The initial rate was determined as the average of at least five experiments with a deviation from the mean of less than 10%. Experiments were done in series using a fixed concentration of reducing substrate and hydrogen peroxide concentrations from 2 μM to 2 mM. Five or six series of measurements were made with different concentrations of the reducing substrate.

Transient-State Kinetic Measurements. The stopped-flow transient-state experiments were performed using a Union Giken RA-601 rapid reaction analyzer. Final enzyme concentration was 1 μM. Pseudo-first-order kinetics was obtained by using a 10−100-fold excess of H_2O_2. The experiments were carried out in citrate buffer with and without 1 mM $CaCl_2$ at 25 ± 1 °C. The reaction was followed at 411 nm, the isosbestic point between the first

and second products of the reaction of BP 1 with H_2O_2 (Rasmussen et al., 1993a). Pseudo-first-order rate constants, k_{obs}, were obtained by exponential fitting to the kinetic traces.

FA and Cyanide Titrations. Binding assays were performed by difference absorption spectral titrations in 0.05 M Na citrate buffer and 1 mM $CaCl_2$, pH 3.96, at 25 °C. The FA binding study was performed using a Cary 5 spectrophotometer. Aliquots of 1 or 2 μL of 1 mM FA (made in Milli Q water from 30 mM FA in EtOH) were added to 1100 μL of 9.6 μM BP 1 in the sample cuvette and to 1100 μL of buffer in the reference cuvette. Spectra were recorded before and after each addition of FA. The dissociation constant K_d was determined from the intercept of a plot of

$$1/[\text{FA}] = \Delta\epsilon^*[\text{BP 1}]/K_d^*\Delta A - 1/K_d \quad (1)$$

$\Delta\epsilon$ and ΔA are the differences in extinction coefficient and absorbance at 405 nm, respectively, between the complex and BP 1 (Paul & Ohlsson, 1978).

BP 1 was titrated with NaCN with and without FA present using a Hewlett-Packard 8452A diode-array spectrophotometer. The enzyme concentration was 3 μM. Experiments were performed with BP 1:FA ratios of 1:0, 1:1.1, 1:10, and 1:39, repeating each experiment twice. Absorbance scans were taken after each addition of either FA or NaCN. Plots of ΔA ($A_{402,\text{BP 1}} - A_{402,\text{CN-bound BP 1}}$) versus ΔA/[CN] were used to determine K_d according to Fersht (1985). For the experiments including FA, the absorbance at 402 nm of BP 1 with FA was used for $A_{402,\text{BP 1}}$.

Curve Fitting and Calculation of Steady-State Data. Initial reaction rates were calculated as the initial slopes of the kinetic traces, using the HP spectrophotometer calculation program. Curve fittings were performed using the Enzfitter software program (Elsevier-Biosoft, Cambridge, U.K.), a nonlinear regression data analysis program. The initial rates (v) and [H_2O_2] were fitted to an equation of the form

$$v/2[\text{E}]_o = A[\text{H}_2\text{O}_2]/(B + [\text{H}_2\text{O}_2]) \quad (2)$$

to determine the parameters A and B for each concentration of reducing substrate.

Curve Fitting and Calculation of Transient-State Data. The pseudo-first-order rate constants, k_{obs}, were obtained by exponential fitting to the kinetic traces using the Union Giken calculation program. Curve fittings of plots of k_{obs} versus [H_2O_2] were performed by using the Enzfitter software program.

RESULTS

pK_A Values of Reducing Substrates. The pK_A values were determined to be 4.40 ± 0.04 and 9.0 ± 0.1 for FA, 4.60 ± 0.01 and 8.7 ± 0.1 for CA, and 9.40 ± 0.05 for CAlc. Hence these substrates are largely uncharged during the kinetic experiments at pH 3.96 and therefore showed low solubility.

FA Oxidation by BP 1. FA absorbs in the UV region with a maximum at 318 nm and a shoulder at 290 nm in citrate buffer, pH 3.96 (Figure 1A). FA was oxidized only in the presence of both H_2O_2 and enzyme. The isosbestic point between FA and oxidized FA is 360 nm. The spectrum of the product has a maximum at 286 nm and a broad shoulder around 320 nm. A similar experiment was performed using HRP C for comparison. The spectra of the products of

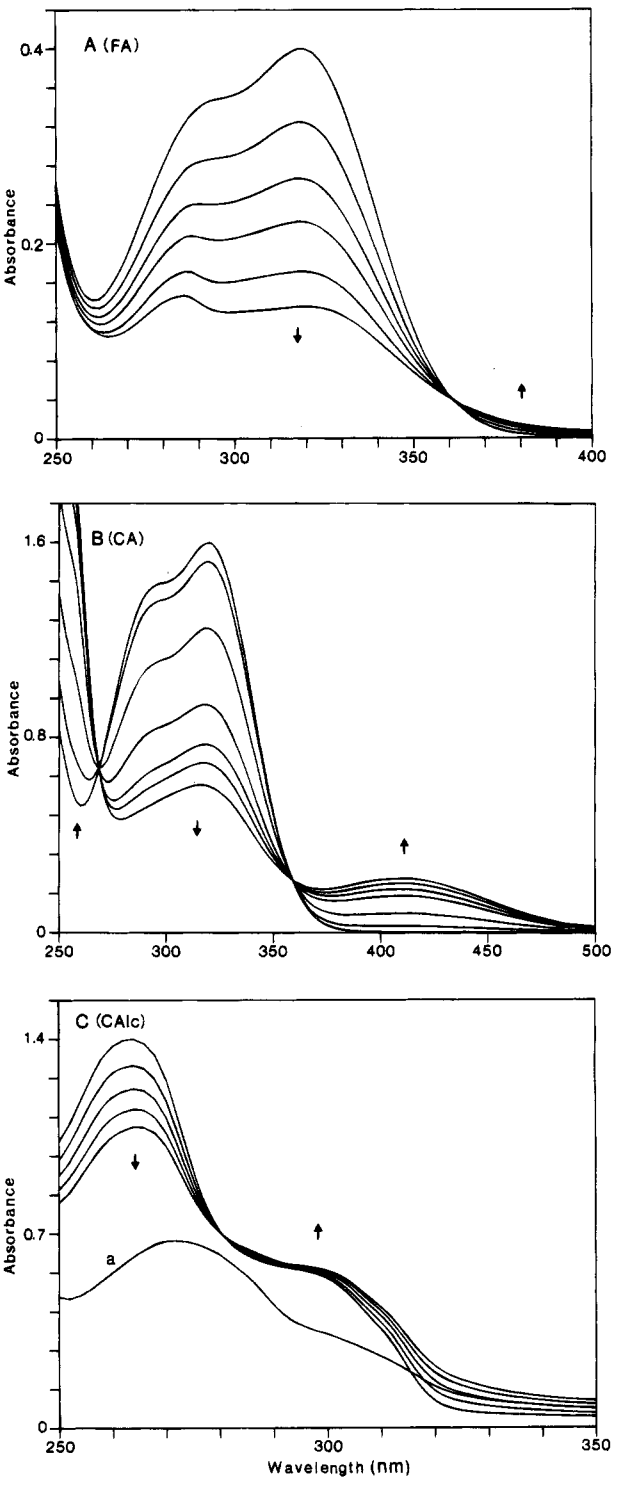

FIGURE 1: Oxidation of FA, CA, and CAlc by BP 1 and H_2O_2. The arrows indicate the development of the spectra. All spectra were recorded at 25 °C in 0.05 M Na citrate buffer and 1 mM $CaCl_2$ at pH 3.96 and 0.1 M ionic strength adjusted by addition of K_2SO_4. (A) Oxidation of FA. Scans were taken at 0, 8, 20, 32, 60, and 120 s. An isosbestic point is seen at 360 nm. Experimental conditions: 25 μM FA, 670 μM H_2O_2, 15 nM BP 1. (B) Oxidation of CA. Scans were taken at 0, 10, 30, 50, 70, 90, and 120 s. Isosbestic points are seen at 268 and 360 nm. The reaction shown is 85% complete. Experimental conditions: 100 μM CA, 50 μM H_2O_2, 25 nM BP 1. (C) Oxidation of CAlc. Scans were taken at 0, 1.4, 2.8, 4.2, and 5.6 s. An isosbestic point at 280 nm is seen between scans from 0 to 5.6 s. Experimental conditions: 100 μM CAlc, 50 μM H_2O_2, 25 nM BP 1. The final spectrum of the CAlc oxidation product corresponding to infinite time (a) was obtained by adding BP 1 and H_2O_2 several times to the solution.

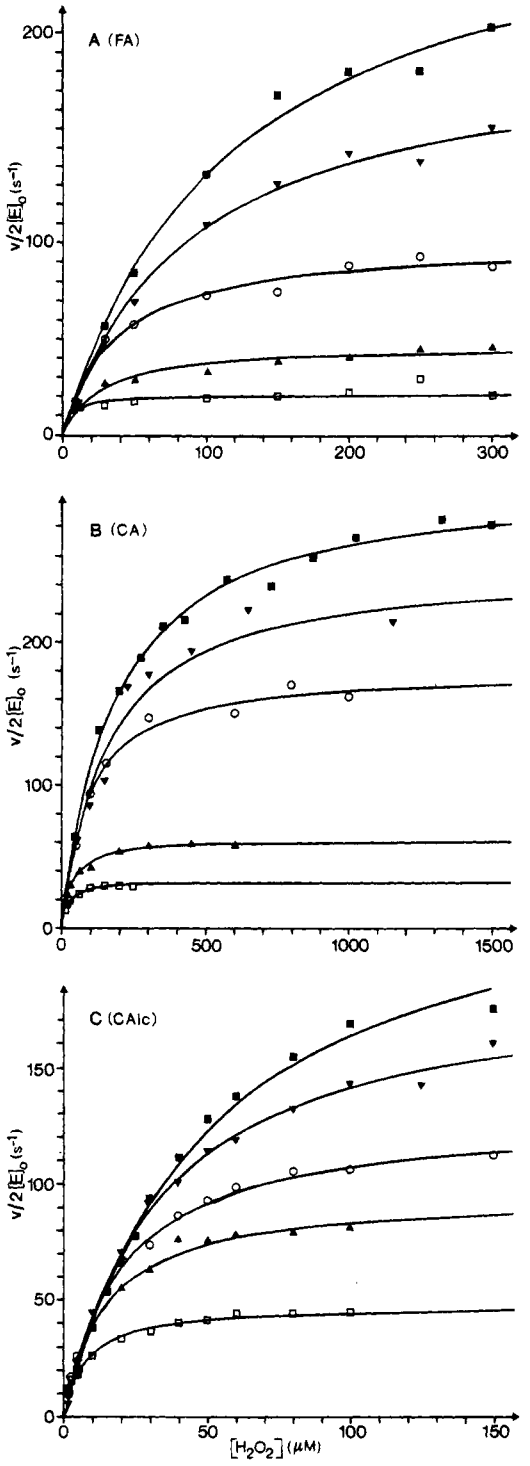

FIGURE 2: Steady-state initial rates of the oxidation of FA, CA, and CAlc by BP 1 and H_2O_2 at different concentrations of the reducing substrates. Initial rates divided by $2[E]_0$ are plotted against $[H_2O_2]$ according to eq 2. Experiments were performed at 25 °C in 0.05 M Na citrate buffer and 1 mM $CaCl_2$ at pH 3.96 and 0.1 M ionic strength with 25 nM BP 1. (A) Oxidation of FA: (□) 5.7, (▲) 11.9, (○) 30.8, (▼) 61.1, and (■) 125.7 μM FA. The results from experiments using 94.4 μM FA were very close to those obtained at 125.7 μM and are omitted for clarity. (B) Oxidation of CA: (□) 11.5, (▲) 19.4, (○) 58.3, (▼) 77.0, and (■) 100.0 μM CA. (C) Oxidation of CAlc: (□) 20.0, (▲) 40.0, (○) 60.0, (▼) 80.0, and (■) 100.0 μM CAlc. Each data point is the average value of at least five measurements, all deviating less than 10% from the mean. Error bars are omitted for clarity. The theoretical curve and parameters *A* and *B* were obtained by fitting eq 2 to the data points. Parameters *A* and *B* and rate constants k_1 and k_3 calculated for each concentration of reducing substrate are listed in Table 1.

oxidation of FA by HRP C and H_2O_2 using the same experimental conditions were very similar (not shown), except that the isosbestic point of the reaction using HRP C was less well defined and drifted from 360 to 354 nm within 40 s of reaction.

CA Oxidation by BP 1. CA absorbs in the UV region with a spectrum very similar to that of FA. CA has a maximum at 320 nm and a shoulder at 290 nm in citrate buffer, pH 3.96. No oxidation of CA occurred when only H_2O_2 or BP 1 was present. The spectrum of the product has maxima at 408 and 318 nm. The isosbestic points between substrate and product are 268 and 360 nm (Figure 1B). The reaction shown is 85% complete.

CAlc Oxidation by BP 1. CAlc absorbs in the near-UV region with a maximum at 264 nm and a broad shoulder at 300 nm in citrate buffer, pH 3.96. In oxygen-free buffer, CAlc is only oxidized when both H_2O_2 and BP 1 are present. The oxidation of CAlc is not so straightforward as that of FA and CA. During the initial stages of the reaction spectra have an isosbestic point at 280 nm (Figure 1C). The spectrum of the final product is easily reproduced, but several sets of isosbestic points are evident during the reaction. In Figure 1C, the first 5.6 s of the reaction and the final spectrum of the product are shown. The later steps before the final product are omitted for clarity. The spectrum of the final product has a maximum at 290 nm and a broad shoulder around 310 nm.

Steady-State Rate Measurements of the Oxidation of FA, CA, and CAlc by BP 1 and H_2O_2. The initial rate of disappearance of reducing substrate was calculated by applying Beer's law to the change in absorbance as a function of time. The extinction coefficients used for rate calculations were the $\Delta\epsilon$ values ($\epsilon_{substrate} - \epsilon_{product}$) listed in Experimental Procedures. The rates of disappearance of reducing substrate versus $[H_2O_2]$ were fitted for each concentration of reducing substrate (Figure 2) according to the generally accepted mechanism for peroxidase reaction (Dunford, 1991). k_1 is the rate constant for compound I formation, and k_2 and k_3 are the rate constants for disappearance of compounds I and II, respectively (Appendix). Assuming $k_2 > k_3$,

$$\frac{-d[AH]/dt}{2[E]_0} = \frac{k_3[AH][H_2O_2]}{(k_3/k_1)[AH] + [H_2O_2]} \quad (3)$$

Thus the parameters from eq 2 become

$$A = k_3[AH] \quad (4)$$

$$B = (k_3/k_1)[AH] \quad (5)$$

Plotting *A* versus *B* gives straight lines with slopes of k_1 if the classical mechanism is followed. In the case of mechanisms involving reversible steps, the *A*:*B* ratio becomes a hyperbolic function of concentration of reducing substrate (Bakovic & Dunford, 1993). For BP 1, plots of *A* versus *B* gave straight lines for FA (Figure 3A) and CA (Figure 3B). The slopes, and hence the k_1 values, were $(2.5 \pm 0.2) \times 10^6$ M^{-1} s^{-1} for FA and $(1.7 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for CA. k_3 can be calculated as the slope of parameter *A* plotted versus the concentration of the reducing substrate according to eq 4. The k_3 value is $(2.9 \pm 0.2) \times 10^6$ M^{-1} s^{-1} for oxidation of FA and $(3.2 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for oxidation of CA. The parameters *A* and *B* and the calculated values for k_1 and k_3 for all concentrations of FA, CA, and CAlc are listed in

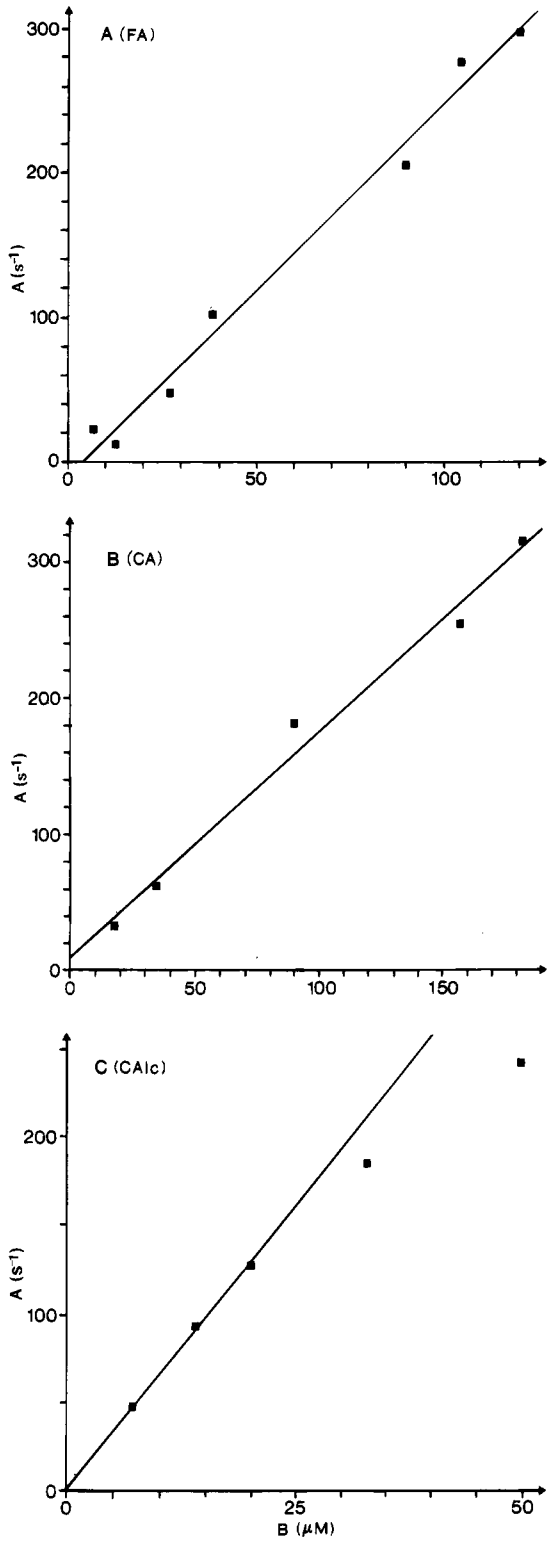

FIGURE 3: Plots of parameter A versus B. The slopes yield k_1 for the formation of compound I from H_2O_2 reaction with the complex of BP 1 and reducing substrate. (A) BP 1−FA complex, (B) BP 1−CA complex, and (C) BP 1−CAlc complex. Values of k_1 are $(2.5 \pm 0.2) \times 10^6$ M^{-1} s^{-1} for FA, $(1.7 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for CA, and $(6.7 \pm 0.6) \times 10^6$ M^{-1} s^{-1} for the data points at the three lowest concentrations of CAlc.

Table 1. In contrast to FA and CA, the plot of A versus B for CAlc did not give a straight line (Figure 3C). As is seen from Table 1, the major difference between CAlc and the other two reducing substrates is seen for the k_1 values. First, the k_1 values obtained in the presence of CAlc are higher

Table 1: Steady-State Parameters[a] (see Appendix) for the Reaction of BP 1 with H_2O_2 and FA, CA, or CAlc at pH 3.96, 25 °C

reducing substrate, concn (μM)	parameters[b]		rate constants[c]	
	A (s^{-1})	B (μM)	k_1 (μM^{-1} s^{-1})	k_3 (μM^{-1} s^{-1})
FA, 5.7	22 ± 1[d]	7 ± 2	3.1 ± 1.5	3.9 ± 0.1
FA, 11.9	48 ± 3	27 ± 7	1.8 ± 0.8	4.0 ± 0.3
FA, 30.8	102 ± 5	38 ± 7	2.7 ± 0.8	3.3 ± 0.2
FA, 61.1	206 ± 11	90 ± 13	2.3 ± 0.5	3.4 ± 0.2
FA, 94.4	278 ± 12	105 ± 11	2.7 ± 0.4	3.0 ± 0.1
FA, 125.7	299 ± 18	120 ± 18	2.5 ± 0.6	2.4 ± 0.1
CA, 11.5	32 ± 0.3	18 ± 0.8	1.8 ± 0.1	2.8 ± 0.1
CA, 19.4	62 ± 1	35 ± 3	1.8 ± 0.2	3.2 ± 0.1
CA, 58.3	181 ± 4	90 ± 9	2.0 ± 0.3	3.1 ± 0.1
CA, 77.0	254 ± 13	157 ± 26	1.6 ± 0.4	3.3 ± 0.2
CA, 100.0	316 ± 6	182 ± 13	1.7 ± 0.2	3.20± 0.02
CAlc, 20.0	48 ± 0.9	7 ± 0.6	6.8 ± 0.7	2.4 ± 0.1
CAlc, 40.0	95 ± 2	14 ± 1	6.8 ± 0.7	2.38± 0.03
CAlc, 60.0	129 ± 2	20 ± 1	6.5 ± 0.4	2.15± 0.03
CAlc, 80.0	188 ± 4	33 ± 2	5.7 ± 0.5	2.35± 0.05
CAlc, 100.0	245 ± 9	50 ± 4	4.9 ± 0.6	2.5 ± 0.1

[a] Parameters were obtained from plots of initial rates of the disappearance of reducing substrate versus $[H_2O_2]$, at constant concentrations of reducing substrate. All experiments were performed at 25 °C in 0.05 M Na-citrate buffer, 1 mM $CaCl_2$, 0.1 M ionic strength, pH 3.96. [b] Parameters A and B were obtained by fitting eq 2 to the experimental data. [c] Calculated from $k_1 = A/B$, and $k_3 = A/[AH]$. [d] Standard deviation.

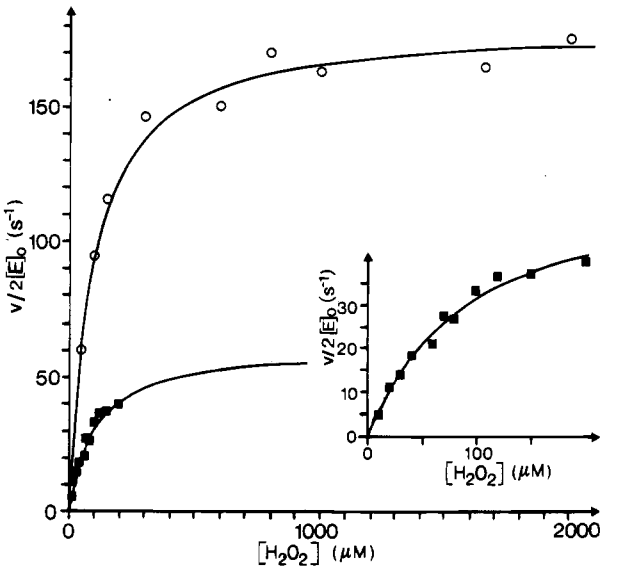

FIGURE 4: Calcium ion dependence of steady-state rates of oxidation of CA by BP 1 and H_2O_2. (○) Oxidation of 58.3 μM CA as described in Figure 2B, i.e., in 1 mM $CaCl_2$. (■) Oxidation of 58.3 μM CA using both enzyme and buffer solutions prepared with no added $CaCl_2$; all other conditions are as described in Figure 2B. The inset shows an expanded plot of this experiment.

than the k_1 values obtained in the presence of FA and CA, and second, the k_1 values decrease as [CAlc] increase above 60 μM. The values of k_3 for the reaction of CAlc are constant over the concentration range used with an average of $(2.4 \pm 0.1) \times 10^6$ M^{-1} s^{-1}.

Influence of Calcium Ion on Steady-State and Transient-State Reaction Rates. Initial rates of oxidation of CA were measured with and without calcium present. By plotting the rate of disappearance of CA versus $[H_2O_2]$ at 58 μM CA in experiments without calcium ion (Figure 4, lower curve), we obtained the parameters $A = 61 \pm 4$ s^{-1} and $B = 95 \pm 13$ μM by fitting to eq 2 as described in the previous section. The parameters for the experiment with calcium present in

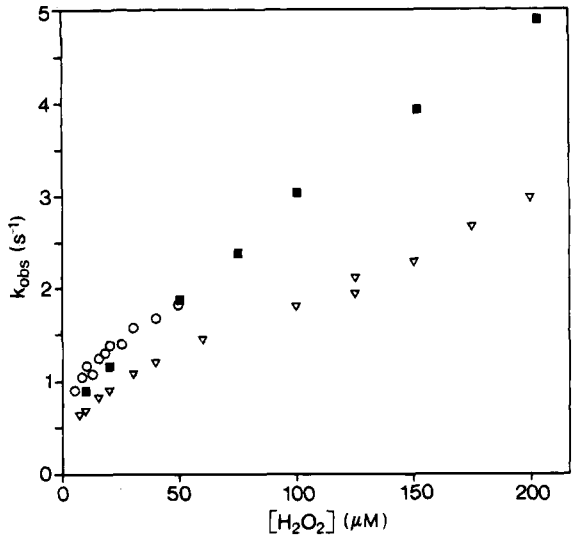

FIGURE 5: Transient-state reaction of BP 1 and H_2O_2. The pseudo-first-order rate constants, k_{obs}, obtained by exponential curve fitting of the kinetic traces, were plotted against H_2O_2 concentration. The experiments were carried out at 25 ± 1 °C in 0.05 M Na citrate buffer, 0.1 M ionic strength: (■) pH 3.96, buffer and enzyme both prepared with 1 mM $CaCl_2$; (○) pH 3.85 and (▽) pH 4.12, buffer and enzyme prepared without $CaCl_2$. Data points at pH 3.85 and 4.12 below 60 μM H_2O_2 are from Rasmussen et al. (1993a).

the buffer and during the enzyme preparation (Figure 4, upper curve) are $A = 181 \pm 4\ s^{-1}$ and $B = 90 \pm 9\ \mu M$. Therefore, both k_1 and k_3 seem to be enhanced by a factor of 3 in the presence of calcium ion.

Pseudo-first-order rate constants obtained at pH 3.96 with 1 mM $CaCl_2$ and at pH 3.85 and 4.12 without calcium present are shown from 5 to 200 μM H_2O_2 in Figure 5. The data points in each series can be interpreted in two ways, either as an extreme sensibility of BP 1 toward oxidative damage by hydrogen peroxide under transient-state conditions or as a mechanism including a reversible step.

$$\text{BP 1} + H_2O_2 \rightarrow \text{product}$$

$$\downarrow\uparrow + H_2O_2$$

$$\text{BP 1-}H_2O_2 + H_2O_2 \rightarrow \text{product}$$

Only a small effect of added calcium ion may be observed for $[H_2O_2]$ above 75 μM, suggesting a slight increase in stability, and thus a lower sensitivity toward oxidative damage by hydrogen peroxide. At lower concentrations of hydrogen peroxide there is no observed effect by the added calcium ion. The initial slopes are $2.3 \times 10^5\ M^{-1}\ s^{-1}$ at pH 3.85 and $1.1 \times 10^5\ M^{-1}\ s^{-1}$ at pH 4.12, which in both cases of interpretation represent the true rate of compound I formation from undamaged BP 1. For either interpretation the difference in rate due to calcium ion is obviously insufficient to account for the 10−40-fold increase in k_1 value observed under steady-state conditions as compared to transient-state conditions.

Binding of FA to BP 1. The complex formation of FA and resting-state BP 1 was examined by absorption difference spectroscopy at pH 3.96 (Figure 6), as the reducing substrates change k_1 10−40-fold. The peak in the difference spectra observed at 405 nm indicates that the Soret maximum observed at 401 nm for resting-state BP 1 is an apparent Soret maximum. Calculating K_d from these data according to eq 1 gives 1.4 ± 0.6 μM, which is a very tight binding for a reducing substrate and a peroxidase. K_d for HRP C and guaiacol is 10 mM (Hosoya et al., 1989), for example.

Binding of Cyanide to BP 1. The possible influence of FA on cyanide binding to BP 1 was analyzed. Titrations of BP 1 with NaCN showed a small increase of the dissociation constant K_d in the presence of FA. K_d was 30 ± 5 μM in the absence of FA; with a BP 1:FA ratio of 1:1.1, K_d was 30 ± 5 μM; with a ratio of 1:10, K_d was 60 ± 5 μM; and with a ratio of 1:39, K_d was 55 ± 5 μM.

DISCUSSION

Barley grain peroxidase, BP 1, is an unusual peroxidase in many ways. The small percentage of sequence identity to other known plant peroxidases and its specific tissue and developmental expression indicate a distinct physiological role in the barley grain. The oxidation by hydrogen peroxide and barley peroxidase of three phenolic substrates naturally occurring in plants, ferulic acid (FA), caffeic acid (CA), and coniferyl alcohol (CAlc) has been investigated to shed more light on the unique properties of BP 1.

Rate Enhancement of Compound I Formation by FA, CA, and CAlc. The most striking result obtained in this study is the enhancing effect of reducing substrate on the rate of compound I formation. In absence of reducing substrate, the rate of compound I formation was measured using transient-state kinetics. New experiments performed in the presence of calcium essentially confirmed the earlier results which were obtained in the absence of calcium (Rasmussen et al., 1993a). Calcium had no effect until $[H_2O_2]$ exceeded 75 μM (Figure 5). In the presence of each of the reducing substrates, the rate of compound I formation determined by steady-state kinetics is enhanced 10−40 times depending on the substrate (Table 1). Therefore, compound I formation in BP 1 is dependent upon the presence of reducing substrate, which to the best of our knowledge has never before been observed for a peroxidase. Reducing substrates could function to activate BP 1 and thus have a significant physiological role.

For FA and CA, the plots of parameters A versus B for the steady-state results were linear (Figure 3A,B). Superficially, this linear correlation could be taken to indicate that both FA and CA fit the classical peroxidase mechanism (Appendix eqs 9−11). However, the large enhancement of the rate of compound I formation by reducing substrate remains to be explained. The simplest explanation of rate enhancement of compound I formation by the reducing substrates is a tight binding of reducing substrate and BP 1, which is strongly supported by the very low K_d of 1.4 ± 0.6 μM for the BP 1−FA complex. Therefore, the normal peroxidase cycle is most likely preceded by

$$\text{BP 1} + \text{FA} \rightleftharpoons \text{BP 1-FA} \qquad (6)$$

Replacement of BP 1 by BP 1−FA or BP 1−CA (Appendix) gives a mathematical description compatible with the steady-state kinetic data observed for FA and CA.

The CAlc kinetic data are more complicated as the values of k_1 decrease for the two highest concentrations of CAlc (Table 1). Tentatively we suggest a second, less tight binding site for CAlc on BP 1 which inhibits compound I formation.

$$\text{BP 1} + \text{CAlc} \rightleftharpoons \text{BP 1-CAlc} \qquad (7)$$

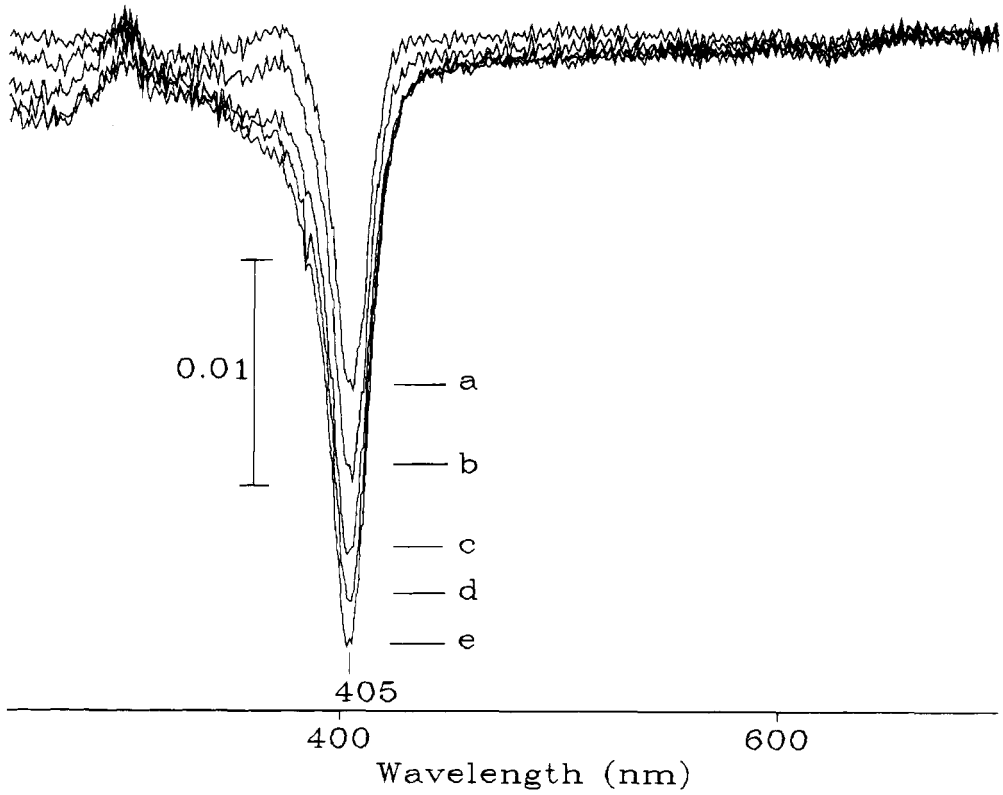

FIGURE 6: Absorption difference spectra obtained by titration of BP 1 with ferulic acid in 0.05 M Na citrate buffer at 0.1 M ionic strength, pH 3.96, and 25 °C. One or 2 μL of 1 mM FA was added to 1100 μL of 9.6 μM BP 1 sample and 1100 μL of buffer reference. Subsequently, the spectrum of BP 1 was subtracted, giving the spectra shown for final concentrations of FA of 0.94 (a), 1.87 (b), 3.73 (c), 5.59 (d), and 7.44 μM (e). Plots of 1/[FA] versus [BP 1]/ΔA_{405} according to eq 1 (not shown) gave $K_d = 1.4 \pm 0.6$ μM for two independent experiments.

$$\text{BP 1-CAlc} + \text{CAlc} \rightleftharpoons \text{CAlc-BP 1-CAlc} \quad (8)$$

The ternary complex formed in eq 8 is less active than the binary complex.

The k_1 values obtained from steady-state kinetic data were $(2.5 \pm 0.2) \times 10^6$ M^{-1} s^{-1} for FA, $(1.7 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for CA, and $(6.7 \pm 0.6) \times 10^6$ M^{-1} s^{-1} for CAlc before the onset of inhibition by high [CAlc]. These k_1 values for BP 1 can be compared to k_1 values for other peroxidases: for HRP A2, $(1.76 \pm 0.05) \times 10^6$ M^{-1} s^{-1} (Kato et al., 1984); for HRP C, $(1.55 \pm 0.05) \times 10^7$ M^{-1} s^{-1} (Dunford et al., 1978) or $(1.68 \pm 0.05) \times 10^7$ M^{-1} s^{-1} (Kato et al., 1984); for *Coprinus cinereus* peroxidase, $(7.1 \pm 0.1) \times 10^6$ M^{-1} s^{-1} (Andersen et al., 1991b); and for turnip peroxidases 1 and 7, $(1.17 \pm 0.17) \times 10^7$ and $(1.6 \pm 0.2) \times 10^6$ M^{-1} s^{-1}, respectively (Job et al., 1978). Thus BP 1, which reacts extremely slowly in the presence of H_2O_2 only, forms compound I at a rate within an order of magnitude of the other peroxidases because of the unique enhancing ability of the reducing substrates FA, CA, and CAlc.

The enhanced rate of oxidation of BP 1 can be explained by assuming an overlap between the molecular π-orbitals of the heme and the substrate; as both are electron-donating species (with heme being the most efficient), such an overlap would lower the oxidation potential of the heme. Furthermore, the essential planarity of both heme and reducing substrate makes such an overlap plausible. Similar effects are well known in chemical systems such as the organic superconductors (Jerome et al., 1980). The fact that the k_1 values obtained from oxidation of CAlc are significantly higher than those obtained with FA and CA supports this hypothesis, since CAlc, lacking the carbonyl group, is a better electron donor than FA and CA. An alternative explanation of the reduced reactivity of BP1 in the absence of reducing substrate is that BP1 is especially sensitive to oxidative damage by hydrogen peroxide. When reducing substrate is present, then only steady-state concentrations of oxidized forms of the enzyme are present, and these are rapidly removed in reaction with the reductant. We thank one of the referees for this suggestion. Oxidative inactivation is observed in the mammalian peroxidase prostaglandin H synthase in the absence of reducing substrate (Smith et al., 1992; Bakovic & Dunford, 1994).

Cyanide Binding. The binding of cyanide to peroxidase is generally accepted to mimic the initial binding of hydrogen peroxide to peroxidase. Cyanide titration of BP 1 shows that the binding of cyanide is slightly reduced in the presence of FA, indicating that their binding sites at the heme are different and that steric hindrance may occur. Therefore, it appears that the last part of the reaction with hydrogen peroxide, the oxidation of BP 1, is the step enhanced by the reducing substrates.

Rate of Compound II Reduction. The values of k_3 obtained from plots according to eq 4 are $(2.9 \pm 0.2) \times 10^6$ M^{-1} s^{-1} for FA, $(3.2 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for CA, and $(2.4 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for CAlc. These values are independent of the concentration of reducing substrate within experimental error (Table 1).

Effect of Added Calcium Ions. Calcium ion plays an important role in stabilizing the structure of plant peroxidases. In searching for an explanation of the very low rate of compound I formation from BP 1 and H_2O_2 observed earlier (Rasmussen et al., 1993a), we suspected that calcium ion might have been lost during enzyme preparation, or storage

in AMS. Therefore, we repeated the earlier transient-state experiments with the difference that calcium ion was added to the BP 1 solutions. Except for the results obtained for $[H_2O_2]$ in excess of 75 μM, there were essentially no changes in rates (Figure 5). Therefore loss of calcium ion was not the cause of the low rates of compound I formation. Comparison of the steady-state results, obtained in the presence of reducing substrate, to the transient-state results, obtained in the absence of reducing substrate, with all experiments performed with added calcium ion, showed that the difference between the two measurements of compound I formation rates could be accounted for almost entirely by the enhancement effect of reducing substrate.

Added calcium ion did enhance the rate of oxidation of CA (Figure 4). From these data it seems that both k_1 and k_3 are affected by a factor of 3 by the presence of calcium ion. Therefore, we suggest that the activated form of BP 1 with reducing substrate bound is more sensitive toward the presence of calcium ion than the resting state.

Summary. The reaction of BP 1 with hydrogen peroxide to form compound I is enhanced 10−40-fold in the presence of reducing substrate. This enhancement in rate, so far unique in peroxidase kinetics, can be explained by the effect of tight substrate binding to the native enzyme. The enhancement appears to occur in the oxidation step of compound I formation, not as an effect on the initial binding of hydrogen peroxide. Added calcium ion accelerates 3-fold the rate of oxidation of caffeic acid.

ADDED IN PROOF

The K_d for BP 1 and FA reported in this study was affected by the minute amounts of ethanol used for dissolving FA. From titration studies similar to the experiments performed in Figure 6, with either FA or ethanol, we find $K_d = 7$ μM for BP 1 and FA and $K_d = 2$ mM for BP 1 and ethanol. The effects of FA and ethanol were additive.

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APPENDIX

Mechanism:

$$P + H_2O_2 \xrightarrow{k_1} \text{Cpd I} + H_2O \quad (9)$$

$$\text{Cpd I} + AH \xrightarrow{k_2} \text{Cpd II} + A^{\bullet} \quad (10)$$

$$\text{Cpd II} + AH \xrightarrow{k_3} P + A^{\bullet} + H_2O \quad (11)$$

Steady-state conditions:

$$d[P]/dt = k_3[\text{Cpd II}][AH] - k_1[P][H_2O_2] = 0 \quad (12)$$

$$d[\text{Cpd I}]/dt = k_1[P][H_2O_2] - k_2[\text{Cpd I}][AH] = 0 \quad (13)$$

$$d[\text{Cpd II}]/dt = k_2[\text{Cpd I}][AH] - k_3[\text{Cpd II}][AH] = 0 \quad (14)$$

Mass balance:

$$[E]_o = [P] + [\text{Cpd I}] + [\text{Cpd II}] \quad (15)$$

$$[E]_o = \{(k_3[AH]/k_1[H_2O_2]) + (k_3/k_2) + 1\}[\text{Cpd II}] \quad (16)$$

Initial rate expression:

$$-d[AH]/dt = k_2[\text{Cpd I}][AH] + k_3[\text{Cpd II}][AH] \quad (17)$$

Combining eqs 14 and 17 leads to

$$-d[AH]/dt = 2(k_3[\text{Cpd II}][AH]) \quad (18)$$

Combining eqs 16 and 18 yields

$$-d[AH]/dt = 2[E]_o k_3[AH]/\{(k_3[AH]/k_1[H_2O_2]) + (k_3/k_2) + 1\} \quad (19)$$

Assuming $k_2 > k_3$ and rearranging eq 19,

$$(-d[AH]/dt)/2[E]_o = (k_3[AH][H_2O_2])/\{(k_3/k_1)[AH] + [H_2O_2]\}$$

$$= A[H_2O_2]/(B + [H_2O_2]) \quad (20)$$

$$A = k_3[AH]; \quad B = (k_3/k_1)[AH]; \quad A/B = k_1$$

REFERENCES

Andersen, M. B., Johansson, T., Nyman, P. O., & Welinder, K. G. (1991a) in *Biochemical, Molecular and Physiological Aspects of Plant Peroxidases* (Lobarzewski, J., Greppin, H., Penel, C., & Gaspar, Th., Eds.) pp 169−173, University of Geneva, Switzerland.

Andersen, M. B., Hsuanyu, Y., Welinder, K. G., Schneider, P., & Dunford, H. B. (1991b) *Acta Chem. Scand. 45*, 1080−1086.

Bakovic, M., & Dunford, H. B. (1993) *Biochemistry 32*, 833−840.

Bakovic, M., & Dunford, H. B. (1994) *Biochemistry 33*, 6475−6482.

Dunford, H. B. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 2, pp 1−24, CRC Press, Boca Raton, FL.

Dunford, H. B., Hewson, W. D., & Steiner, H. (1978) *Can. J. Chem. 56*, 2844−2852.

Fersht, A. (1985) *Enzyme Structure and Mechanism,* pp 176−192, W. H. Freeman and Company, New York.

Hejgaard, J., Petersen, J. F., Veitch, N. C., Pedersen, B. J., & Welinder, K. G. (1991) in *Biochemical, Molecular and Physiological Aspects of Plant Peroxidases* (Lobarzewski, J., Greppin, H., Penel, C., & Gaspar, Th., Eds.) pp 49−53, University of Geneva, Switzerland.

Hosoya, T., Sakurada, J. Kurokawa, C., Toyoda, R., & Nakamura, S. (1989) *Biochemistry 28*, 2639−2644.

Jerome, D., Mazaud, A., Ribault, M., & Bechgaard, K. (1980) *J. Phys. Lett. 41*, L95.

Job, D., Ricard, J., & Dunford, H. B. (1978) *Can. J. Biochem. 56*, 702−707.

Johansson, A., Rasmussen, S. K., Harthill, J. E., & Welinder, K. G. (1992) *Plant Mol. Biol. 18*, 1151−1161.

Kato, M., Aibara, S., Morita, Y., Nakatani, H., & Hiromi, K. (1984) *J. Biochem. (Tokyo) 95*, 861−870.

Kulmacz, R. J. (1986) *Arch. Biochem. Biophys. 249*, 273−285.

Ohlsson, P.-I., & Paul, K.-G. (1976) *Acta Chem. Scand. B30*, 373.

Paul, K.-G., & Ohlsson, P.-I. (1978) *Acta Chem. Scand. B32*, 393−404.

Rasmussen, C. B., Bakovic, M., Welinder, K. G., & Dunford, H. B. (1993a) *FEBS Lett. 321*, 102−105.

Rasmussen, C. B., Bakovic, M., Dunford, H. B., & Welinder, K. G. (1993b) in *Plant Peroxidases Biochemistry and Physiology* (Welinder, K. G., Rasmussen, S. K., Penel, C., & Greppin, H., Eds.) pp 155−158, University of Geneva, Switzerland.

Rasmussen, S. K., Welinder, K. G., & Hejgaard, J. (1991) *Plant Mol. Biol. 16*, 317−327.

Smith, W. L., Elling, E. T., Kulmacz, R. J., Marnett, L. J., & Tsai, A. L. (1992) *Biochemistry 31*, 3−7.

Wink, M. (1993) *J. Exp. Bot. 44*, 231−246.

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