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Unique reaction of a barley peroxidase with hydrogen peroxide

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The reaction of barley peroxidase BP 1 with H_2O_2 is markedly different from that of other peroxidases. Saturation kinetics and a strong pH dependence over the accessible pH range from 3.09 to 5.08 are observed. At pH 3.8, native BP 1 has maxima at 401, 498 and 635 nm, cpd I at 403 nm, and cpd II at 407 and 521 nm with a shoulder at 553 nm. Both cpds I and II appear to be incompletely formed. Isosbestic points between native BP 1 and cpd I occur at 365 and 416 nm, while an isosbestic point in the Soret region between cpd I and cpd II has been observed at 410 nm. Between cpd II and a not yet identified intermediate isosbestic points have been observed at 408, 455 and 526 nm.

Compound I formation; pH dependence; Saturation kinetics; *Hordeum vulgare*

1. INTRODUCTION

Peroxidases (P) catalyze the oxidation of a large number of organic substrates (HA) at the expense of hydroperoxides (HOOR), via the classical reaction cycle [1].

$$P+HOOR \rightarrow cpd\ I+ROH$$

$$cpd\ I+HA \rightarrow cpd\ II+A^{\bullet}$$

$$cpd\ II+HA \rightarrow P+A^{\bullet}+H_2O$$

Most organic substrates end as radicals A•e, the fate of which depends on the peroxidase environment. In plants, the peroxidases are encoded by an estimated 8–15 gene families [2] which fulfill different, and in most cases unknown, functions. Barley peroxidase BP 1 has been chosen for extensive biological and chemical studies due to its unusual properties. It is less than 50% identical in its amino acid sequence to other known plant peroxidases [2,3] and has a C-terminal propeptide which may target it to the vacuoles [3]. The mRNA for BP 1 is expressed only in the barley seed endosperm, and only day 15–20 after flowering [4]. It has less than 1% of the specific activity but a similar substrate profile to horseradish peroxidase HRP C at pH 5 [5]. Its binding of organic substrates has recently been characterized by 2D NMR experiments [6], and its crystal structure may soon be available [7]. The present transient state kinetics investigation of the reaction has shown (i) a very unusual pH dependence and (ii) that saturation kinetics are obtained. This has never before been observed for a peroxidase at room temperature.

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Abbreviations: BP 1, barley peroxidase 1; cpd I, compound I; cpd II, compound II; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate).

2. EXPERIMENTAL

Barley peroxidase BP 1 was purified from barley grain (*Hordeum vulgare*, cv 'Bomi' mutant 'Risø 1508') as previously described [4,8]. The preparation used for kinetic studies consisted of approximately 90% of the glycosylated form (BP 1a) and 10% of the non-glycosylated form (BP 1b) as estimated by SDS-PAGE and concanavalin A chromatography (not shown). N-Terminal sequencing of the preparation showed only one protein component. The protein was stored as an ammonium sulfate precipitate at 4°C and was dialyzed extensively against deionized water prior to use. The RZ value (A(Soret peak)/A(280 nm) of the preparation was 2.9 after dialysis, consistent with the published result [4]. Enzyme concentrations were determined using an extinction coefficient of 105 $mM^{-1}\cdot cm^{-1}$ at 400 nm. The concentration of H_2O_2 was determined and checked regularly by measuring the absorbance at 240 nm, using an extinction coefficient of 43.6 $M^{-1}\cdot cm^{-1}$ [9]. All chemicals used were of analytical grade, and water was drawn from a Milli-Q system. The pH was measured using a Fisher digital pH meter, Model 25, and standard buffers from the same supplier.

Absorption spectra scanned on a conventional time scale (s–min), both in the Soret and in the visible regions, were performed on a Beckman DU 650 spectrophotometer, using enzyme concentrations from 1 to 2.6

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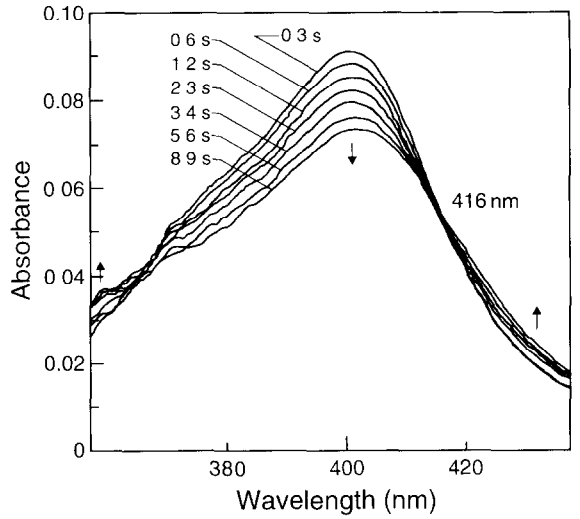

Fig. 1. Partial cpd I formation monitored by rapid-scan spectrophotometry. Spectra were obtained using a final BP 1 concentration of 1 μM, a BP 1:H_2O_2 ratio of 1.0:2.0 in sodium citrate buffer, pH 3.81, and a total ionic strength of 0.1 M. Spectra were recorded at 0.3, 0.6, 1.2, 2.3, 3.4, 5.6 and 8.9 s after mixing. The spectrum of native BP 1 is observed at 0.3 s.

μM. For rapid-scan and stopped-flow experiments a Union Giken RA-601 Rapid Reaction Analyzer was used, with the final enzyme concentration always at 1 μM. In stopped-flow experiments, the change of absorbance at 411 nm (cpd I and cpd II have isosbestic points at 410 nm) due to reaction with H_2O_2, was measured as a function of time, using from 5- to 80-fold excess of H_2O_2 to give pseudo-first order kinetics. Kinetic traces, repeated at least five times for each set of conditions, were fitted by computer analysis of exponential curves to give a pseudo-first order rate constant k_{obs}, with a deviation from the mean value of less than 10%. Buffers used at pH 3.09 and 3.25 were 0.1 M glycine-HCl, all others were 0.05 M sodium citrate. The ionic strength was held constant at 0.1 M by addition of K_2SO_4. Because of the instabillity of BP 1 from pH 3.09 to 3.41, experiments were performed using pH jumps. The enzyme was dissolved in water, and H_2O_2 in buffer at the desired pH. Measurements at pH's higher than 5.08 were attempted, but the reactivity was very low and not reproducible. All experiments were performed at 25°C.

3. RESULTS

Reaction of BP 1 and H_2O_2 1.0:1.0 at pH 3.8 gave incomplete formation of cpd I. Only a 10% decrease in Soret absorption was observed as compared to an expected 50% decrease for cpd I of other peroxidases [10]. Furthermore when excess H_2O_2 was added and then destroyed by catalase after partial formation of cpd I, a return to the native enzyme was observed.

An enzyme to H_2O_2 ratio of 1.0:1.1 resulted in a mixture of cpd I and cpd II. Maximum cpd II formation was obtained within 2 min using a ratio of 1.0:2.0. Using the latter conditions, partial formation of cpd I was monitored by rapid-scan spectrophotometry as shown (Fig. 1). After 8.9 s, a 27% decrease in Soret absorption was observed. Isosbestic points between native enzyme and cpd I occur at 365 nm and 416 nm (Fig. 1). At pH 3.8 cpd I was stable for approximately 100 ms. Thereafter conversion to cpd II was observed (not shown). Cpd I and cpd II have an isosbestic point at 410 nm in the Soret region.

Furthermore, partial conversion of cpd II at pH 3.8 to a yet undefined species was observed over a period of 9 min after H_2O_2 addition, with isosbestic points at 408, 455 and 526 nm (Fig. 2a,b). The spectrum of the native enzyme which is included in Fig. 2a and b shows that these isosbestic points are not between cpd II and native BP 1. At pH 3.8, the spectrum of native BP 1 has maxima at 401, 498 and 635 nm (Fig. 2a,b); cpd I has a maximum at 403 nm (Fig. 1), while cpd II has maxima at 407 and 521 nm and a shoulder at 553 nm (Fig. 2a,b).

At pH 4.5, partial formation of cpd I was seen within 2 min using a BP 1:H_2O_2 ratio of 1.0:2.0. Cpd I formation at pH 5.08 was complicated by the need for a 5-fold excess of H_2O_2 to provide any reaction, which resulted in some bleaching of the enzyme. The Soret maximum

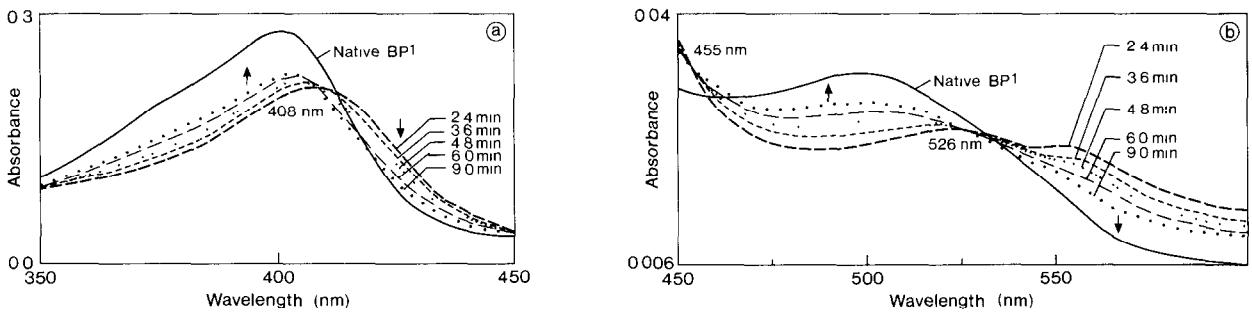

Fig. 2. Conversion of cpd II to an unknown species. Spectra in the Soret (a) and in the visible regions (b) were recorded for native BP 1 and 2.4, 3.6, 4.8, 6.0 and 9.0 min after H_2O_2 addition. Reaction conditions were as described in Fig. 1, except that a BP 1 concentration of 2.6 μM was used.

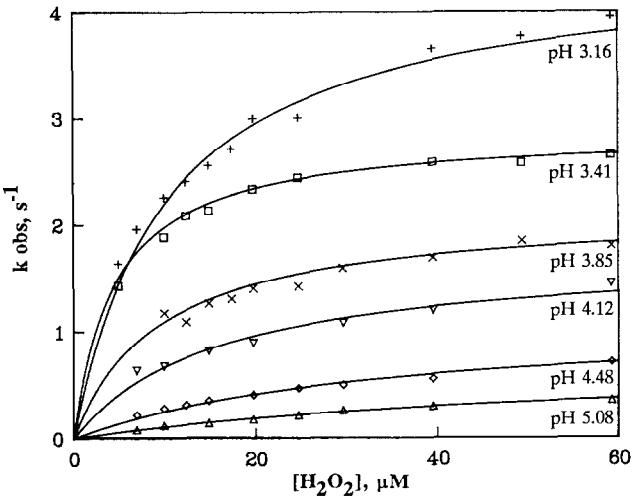

Fig. 3. Reaction of BP 1 and H_2O_2 as a function of pH. The pseudo-first order rate constant k_{obs}, obtained by exponential curve fitting of pseudo-first order kinetic traces, is plotted vs. H_2O_2 concentration. The theoretical curves were fitted by assuming Michaelis–Menten kinetics. Experiments were carried out in 0.05 M sodium citrate buffer with the ionic strength adjusted to 0.1 M by addition of K_2SO_4 at pH 3.16 (+), pH 3.41 (□), pH 3.85 (×), pH 4 12 (▽), pH 4.48 (◇), and pH 5.08 (△). Experiments carried out at pH 3.32 in sodium citrate buffer and pH 3.25 in glycine-HCl buffer gave results similar to those at pH 3.41 (not shown). Experiments performed at pH 3.09 in glycine-HCl buffer gave results similar to those at pH 3.16 (not shown).

of native BP 1 shifted to 398–399 nm at pH 5, the value reported earlier [4].

The pseudo-first order rate constant k_{obs}, for formation of cpd I on reaction of BP 1 with a 5–80-fold excess of H_2O_2, is shown as a function of H_2O_2 concentration. In the region of BP 1 reactivity from pH 3.09 to 5.08 saturation curves were observed (Fig. 3). These curves could be fitted with two parameters which we have labelled K_m^{app} and k_{cat}^{app}. For this curve fitting we used the non-linear regression data analysis program Enzfitter (Biosoft). K_m^{app} and k_{cat}^{app} changed with pH as shown in Table I.

Table I

Values of K_m^{app} and k_{cat}^{app} with standard errors obtained from the saturation kinetics of BP 1 reacting with H_2O_2 as a function of pH

pH	K_m^{app} (μM)	k_{cat}^{app} (s^{-1})	Number of k_{obs} values
3.09	9.5 ± 1.2	4.4 ± 0.2	7
3.16	10.4 ± 1.3	4.5 ± 0.2	12
3.25	3.3 ± 0.2	2.7 ± 0.02	6
3.32	6.9 ± 0.6	3.1 ± 0.1	7
3.41	4.4 ± 0 5	2.9 ± 0.1	11
3.85	9.3 ± 1.3	2.1 ± 0.1	13
4.12	15.7 ± 3.1	1.7 ± 0 1	7
4.48	32.9 ± 3.8	1.1 ± 0.1	10
5.08	53.2 ± 5.4	0.7 ± 0.04	9

4. DISCUSSION

The first published enzymatic characterization of BP 1 [5] was carried out as steady-state kinetic measurements with H_2O_2 as the oxidizing substrate, and among others, ferulic acid and ABTS as reducing substrates. All of these measurements were performed at pH 5, and a very low specific activity of BP 1 was observed. In the present work, BP 1 has been shown to possess two unique features when compared to other known peroxidases. First, the increased rate of cpd I formation as the pH is lowered from 5.08 to 3.09 is in marked contrast to other peroxidases, which display a constant high rate of cpd I formation as the pH is lowered from the neutral to the acid range; at still lower pH values the rate may decrease, as shown for horseradish [11,12], turnip [13] lacto- [14], and chloroperoxidases [15]. Second, all plots of k_{obs} vs. $[H_2O_2]$ show saturation kinetics over the accessible pH range at 25°C. The only other results in which saturation kinetics were observed for cpd I formation were obtained using cryokinetics [16,17] or very high pH were the enzyme is largely in its alkaline form [18]. The cryoenzymology results led to the proposal of cpd 0, an intermediate between native peroxidase and cpd I [17]. Thus, we suggest that an intermediate complex or compound of BP 1 is formed during cpd I formation. We also propose that only a protonated form of BP 1 is fully active, since activity increases with decreasing pH.

As shown in Table I, both K_m^{app} and k_{cat}^{app} are affected by pH, but in opposite directions. Further work is in progress which we hope will lead to a detailed mechanism for BP 1 cpd I formation, and which might be rationalized when a high resolution crystal structure for BP 1 becomes available [7].

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