

Kinetic and spectral properties of pea cytosolic ascorbate peroxidase

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Abstract Sufficient highly purified native pea cytosolic ascorbate peroxidase was obtained to characterize some of its kinetic and spectral properties. Its rate constant for compound I formation from reaction with H₂O₂ is $4.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, somewhat faster than is typical for peroxidases. Compound I has the typical optical spectrum of an iron(IV)-porphyrin- π -cation radical, despite considerable homology with yeast cytochrome *c* peroxidase. The rate constant for compound I reduction by ascorbate is extremely fast ($8.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.8), again in marked contrast to the behavior of the yeast enzyme. The pH-rate profile for compound I formation indicates a pK_a value of 5.0 for a group affecting the active site reaction.

Key words: Compound I; Compound II; Transient state kinetics; Optical spectra; Ascorbate oxidation; pH dependence; Cytochrome *c* peroxidase, comparison

1. Introduction

By definition ascorbate peroxidase exhibits a preference for ascorbate as reducing substrate. It is believed to play a protective role in plants against oxidative damage by hydrogen peroxide. Ascorbate peroxidases were thought to exist in two different forms, one in chloroplasts, and the other in cytosol. The spinach chloroplast form was discovered first [1], followed by studies on the enzymes found in soybean root nodules [2] and in tea leaves [3]. Four different forms of ascorbate peroxidase, rather than two, have now been identified. These are the following: cytosolic ascorbate peroxidase, chloroplast stromal ascorbate peroxidase, chloroplast thylakoid-bound ascorbate peroxidase and glyoxysomal ascorbate peroxidase [4–7]. The first three have been purified to homogeneity. The last has not, but its cDNA has been cloned.

The present study was performed using the cytosolic form of pea ascorbate peroxidase, purified to homogeneity [4]. This heme enzyme is a homodimer with a molecular weight of 57 500 daltons. It is distinct from the pea enzyme found in chloroplasts. The cDNA encoding the cytosolic enzyme has been sequenced [8], and the crystal structure of the recombinant enzyme has been determined [9].

There is considerable homology between cytosolic ascorbate peroxidase and yeast cytochrome *c* peroxidase, leading to the classification of both as peroxidases of prokaryotic origin [10]. Despite the homology, yeast cytochrome *c* peroxidase has a compound I structure with the free radical located on Trp-191; and with a Trp residue in the same location, recombinant

pea cytosolic ascorbate peroxidase compound I has the free radical located on the porphyrin ring [11], as do the classical peroxidases [12]. In this paper we examine the optical spectral and kinetic properties of pure native enzyme, isolated from the cytosol of pea shoot tissue. The native pea cytosolic enzyme must be the reference point for all work on its recombinant and genetically engineered versions.

2. Material and methods

Growth of plant material, enzyme isolation and purification was carried out as previously described [4]. The native enzyme samples had R.Z. values (A_{403}/A_{280}) from 1.95 to 2.04. Enzyme concentration was determined using an extinction coefficient of $1.5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 403 nm [4].

Diluted hydrogen peroxide, obtained as a 30% stock solution from BDH Chemicals, was standardized using the horseradish peroxidase-catalyzed oxidation of iodide to triiodide [13]. Concentrations were confirmed using absorbance measurements at 240 nm where the extinction coefficient of H₂O₂ is $39.4 \text{ M}^{-1} \text{ cm}^{-1}$ [14]. L-Ascorbic acid (Sigma) and the chemicals used for the buffers (Fisher) were used without further purification. Aqueous solutions were prepared using water purified through the Milli-Q system (Millipore Corp.) and concentrations of solutes were determined by weight.

Routine absorbance measurements were made on a Beckman DU-650 spectrophotometer equipped with thermally-jacketed 1-cm cuvette holders. Rapid spectral scans and kinetic measurements were performed using the SX.17 MV microvolume stopped-flow spectrofluorimeter (Applied Photophysics). Time-dependent spectra were analyzed using the GLint application software. A 1-cm pathlength was used for absorbance measurements. Citrate buffers were used for the pH range 3.2 to 4.9; phosphate buffers for the pH range 5.2 to 7.8; and for pH 8.9 bicarbonate was used. Ionic strength was maintained at 0.1 M based on buffer concentrations. All pH measurements were made using a Fisher Accumet Model 25 digital pH meter. Experiments were conducted at 20°C.

3. Results

The native enzyme showed no spectral change over a period of 10 min at both pHs 7.8 and 5.1. Preliminary spectral investigations conducted on the Beckman spectrophotometer showed that an equimolar mixture of native enzyme and hydrogen peroxide formed some transient compound I which, within 60 s, was converted to compound II. Addition of 5 mM ascorbate rapidly reduced compound II back to the native enzyme.

Rapid spectral scan experiments conducted on the Applied Photophysics instrument showed that for a 1:1 ratio of native enzyme and hydrogen peroxide, compound I formation was incomplete after 100 ms. For a 1:10 ratio compound I formation was complete after 20 ms. The native enzyme Soret peak is at 403 nm and the peak of reduced intensity for compound I is at 404 nm. The compound II spectrum was verified by the traditional twin peaks at 528 and 558 nm; its Soret region peak occurred at 413 nm. Spectra of native enzyme and com-

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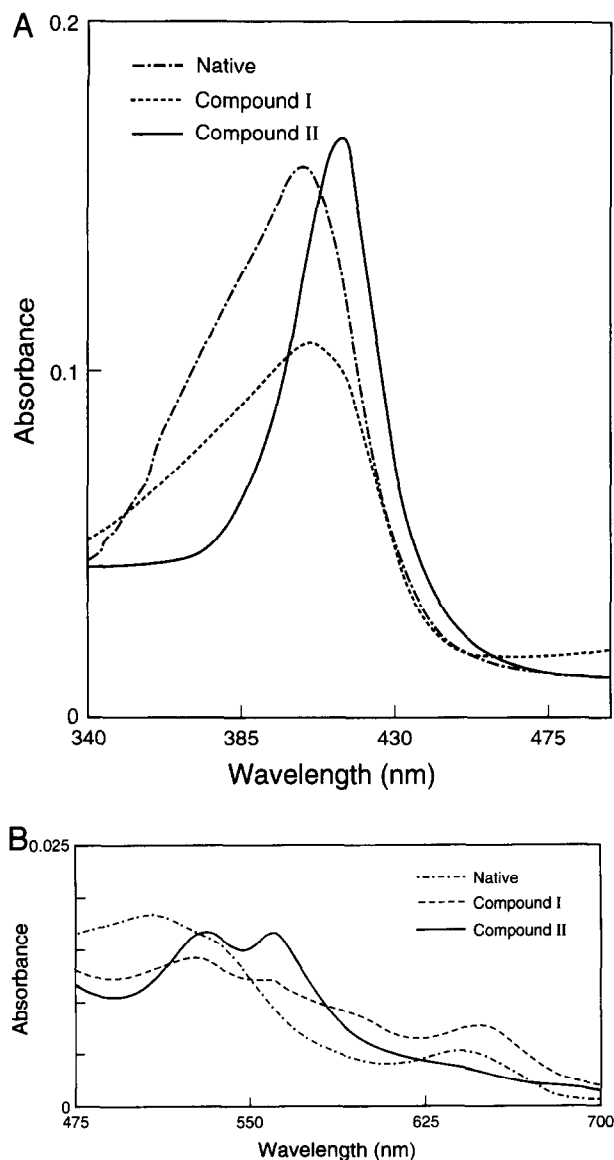


Fig. 1. (A) Soret region spectra of native pea cytosolic ascorbate peroxidase and its oxidized intermediates, compounds I and II. All scans were taken using 1 μM native enzyme in phosphate buffer at pH 7.8. Compound I was formed by adding 1 μM H_2O_2 to native enzyme. Compound II was formed by adding 10 μM H_2O_2 to native enzyme. (B) Spectra in the visible region.

pounds I and II are shown in Fig. 1. The following isosbestic points were observed in the Soret region: between native enzyme and compound I, 424 nm; between native enzyme and compound II, 407 nm; and between compounds I and II, 398 nm.

From the single-mixing stopped flow experiments on rates of compound I formation using excess hydrogen peroxide, the following results were obtained. At both pHs 7.8 and 5.1, single exponential curves were observed, indicating pseudo-first-order kinetics. Plots of the first-order rate constants, k_{obs} , versus hydrogen peroxide concentration were linear with small but finite intercepts. The slopes are equal to the second-order rate constants. At pH 7.8 the rate constant for compound I formation, k_1 , is $8.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The intercept has a value of 40 s^{-1} (Fig. 2). At pH 5.1, k_1 is $3.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and the intercept is 83 s^{-1} (data not shown).

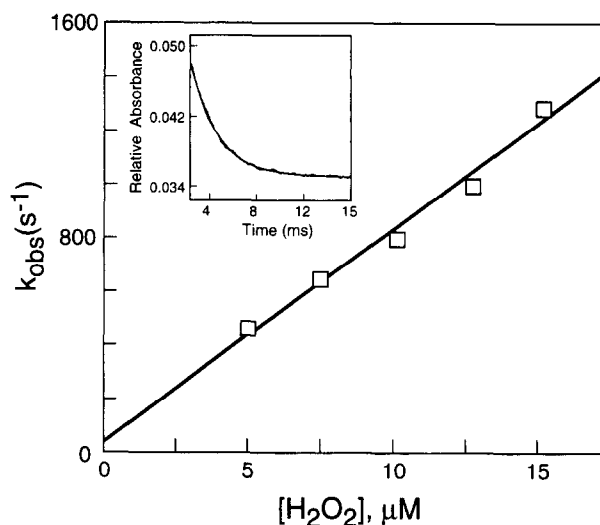


Fig. 2. Pseudo-first-order rate constants for compound I formation from native pea cytosolic ascorbate peroxidase at pH 7.8. One syringe of the stopped flow apparatus contained 1 μM ascorbate peroxidase while the other contained at least a 10-fold excess of H_2O_2 in the same buffer. The second-order rate constant for compound I formation was obtained from the slope of the plot. The inset shows a typical trace for compound I formation followed as a decrease in absorbance at 403 nm. The experimental curve is fit by a single exponential equation (solid line).

The pH dependence of the pseudo-first-order rate constant for compound I formation, obtained using a 10-fold excess of hydrogen peroxide, is shown in Fig. 3. With a 10-fold excess of hydrogen peroxide compound II is more completely formed at pH 7.8 than at 5.1 (Fig. 4A). The rate constant for conversion of compound I to II, k_2 , at pH 7.8 in the presence of hydrogen peroxide as the only substrate, is $1.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 4B).

From sequential mixing experiments, rate constants for reactions with ascorbate at pH 7.8 are as follows. For compound I, k_2 is $8.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 5); and for compound II, k_3 is $3.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (data not shown).

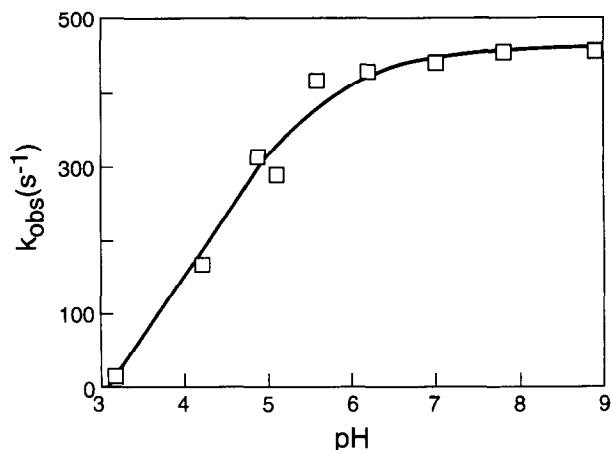


Fig. 3. pH dependence of ascorbate peroxidase compound I formation. Final concentrations in the reaction mixture are 0.5 μM native enzyme and 5 μM H_2O_2 in 0.1 M buffer. Pseudo-first-order rate constants are obtained as described in Fig. 2. The inflection point occurs at pH 5.0.

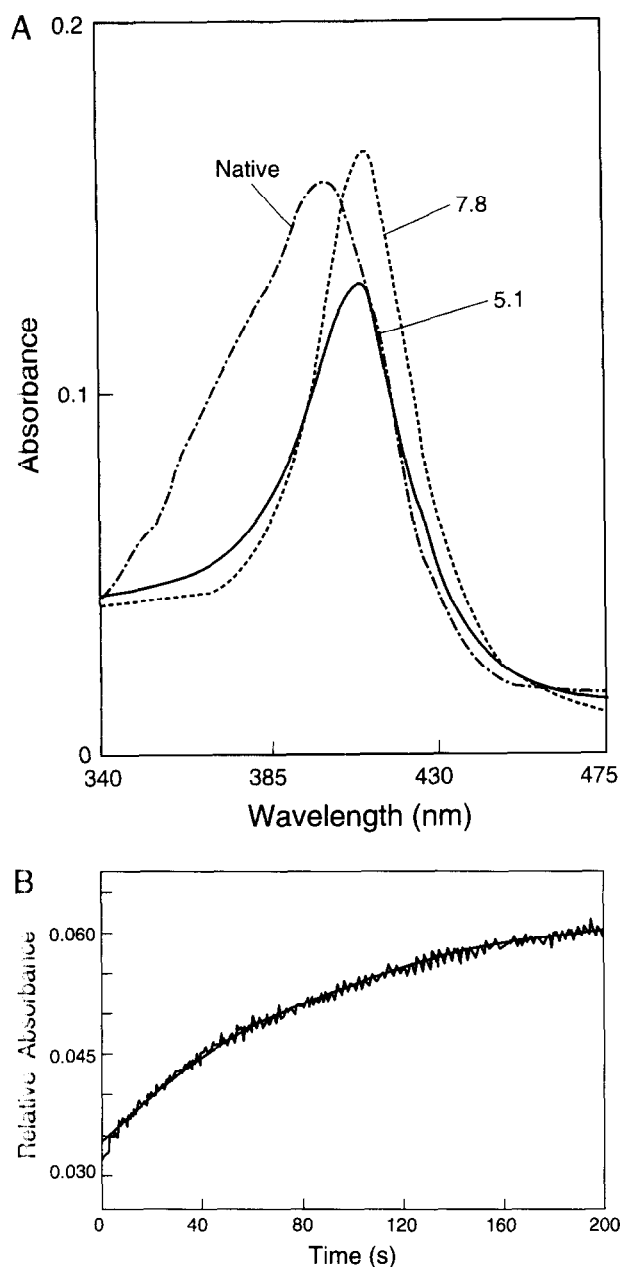


Fig. 4. (A) Soret region spectra of ascorbate peroxidase compound II in 0.1 M phosphate buffer at pHs 5.1 and 7.8 obtained by mixing 0.5 μM native enzyme with 10 μM H_2O_2 . (B) is a kinetic trace of compound II formation at pH 7.8 obtained at 420 nm, close to the iso-bestic point between native enzyme and compound I. The solid line is a single-exponential curve fit.

4. Discussion

The spectral and kinetic results of the present study prove that ascorbate acts as a one-electron reductant for both compounds I and II. The product must be the monodehydroascorbate radical. The rate constant for reduction of compound I by ascorbate at pH 7.8 is extremely fast: $k_2 = 8.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Its accurate measurement was made possible by use of the sequential mixing mode of the Applied Photophysics instrument. Compound II reduction by ascorbate at pH 7.8 is comparatively slow: $k_3 = 3.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. If speed of enzyme turnover is crucial, a different reductant from ascorbate for compound II might speed recycling. The inability to obtain a

high yield of compound II at pH 5.1 (Fig. 4A) is likely an indication that compound II reacts faster at lower pH, and hence its partial reduction begins as fast as it is formed.

The contrast with the lower reactivity of horseradish peroxidase is striking. Compound I of horseradish peroxidase reacts with ascorbate with a maximum rate constant of $2.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at pH 4.5. Compound II of horseradish peroxidase and ascorbate react with a rate constant of approximately $3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at the same pH. Both rates decrease rapidly with increasing pH [15].

From the only measurement of yeast cytochrome *c* reactivity with ascorbate, a k_{cat} value of 3 s^{-1} and a K_m value of 11 mM was reported for pH 6, very low reactivity indeed [16]. Thus, ascorbate peroxidase and cytochrome *c* peroxidase have comparable rates of reaction with hydrogen peroxide [17], but markedly different reactivities with the small reducing substrate, ascorbate.

The inflection point in the pH-rate profile for compound I formation from ascorbate peroxidase indicates a $\text{p}K_a$ value of 5.0 for an acid group affecting the active site chemistry, with the basic form of the enzyme reacting with hydrogen peroxide. The profile is of similar shape to that for other peroxidases. However, the $\text{p}K_a$ value is considerably higher than for horseradish peroxidase [18], and may be higher than that for the more closely related yeast cytochrome *c* peroxidase when specific salt effects for the latter enzyme are taken into account [17].

Identification of compound I for recombinant ascorbate peroxidase was obtained by EPR [11]. The EPR spectrum is similar to that obtained for catalase [19] and horseradish peroxidase compounds I [20,21] and is indicative of an iron(IV)- π -cation radical. In the present paper we show that compound I of native pea cytosolic ascorbate peroxidase exhibits the

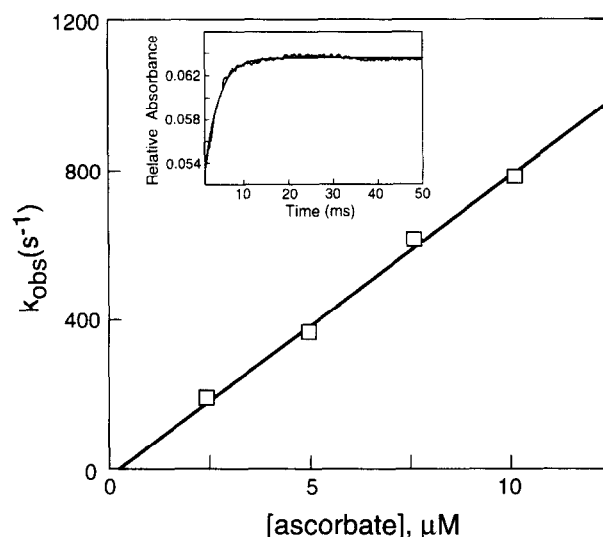


Fig. 5. Plot of pseudo-first-order rate constants versus ascorbate concentration for the reaction of ascorbate peroxidase compound I with ascorbate at pH 7.8, obtained using the sequential mixing mode of the stopped flow apparatus. 1 μM of native enzyme was reacted with 1 μM H_2O_2 in the pre-mixing cell. After a 50 ms delay the newly formed compound I was reacted with varying concentrations of ascorbate. The second-order rate constant for the reaction ($8.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) is equal to the slope of the plot. The reaction was followed at 407 nm, the isosbestic point between native enzyme and compound II. The inset is a typical trace of the reaction which is fit to a single exponential equation (solid line).

classic reduced Soret region spectrum typical of the compounds I of most peroxidases and catalase. Therefore both the native and recombinant enzymes have the classical compound I structure, despite the homology with yeast cytochrome *c* peroxidase. The recombinant enzyme has a potassium or calcium ion binding site near Trp-179, which would be the site of the protein radical if compound I of ascorbate peroxidase were the same as for the yeast enzyme [9].

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