Acta Chemica Scandinavica B 38 (1984) 79–83

The formation of the Primary Hydrogen Peroxide Compound (Compound I) of *Pseudomonas* Cytochrome *c* Peroxidase as a Function of pH

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The effect of pH on the stability and overall catalytic activity of half-reduced *Pseudomonas* cytochrome *c* peroxidase was studied over the pH range 3.5–8. The stability of the enzyme as deduced from 40 s incubation experiments is virtually unaffected by pH. However, there is a bell-shaped pH dependence for the overall catalytic reaction using H_2O_2 as oxidizing substrate and cytochrome *c*-551 as reducing substrate with maximum turnover rate at pH 6. The effects of pH on (1) rate of reduction of the totally ferric enzyme by reduced azurin over the pH range 3.5–8 and (2) the rate of compound I formation from the half-reduced enzyme and hydrogen peroxide over the pH range 4–8 were also investigated. The reduction reaction rate also appears bell-shaped with optimum rate at pH 5.6. The rate of compound I formation is virtually pH independent above pH 5 but drops dramatically as the pH is lowered from 5 to 4. The influence of an ionization with apparent pK_a value of 4.4 is implicated in compound I formation. This enzyme acid group must be deprotonated for compound I formation to occur suggesting the importance of base catalysis.

Cytochrome *c* peroxidase (cytochrome *c*-551:H_2O_2 oxidoreductase, EC 1.11.1.5) of *Pseudomonas æruginosa* catalyzes the peroxidatic oxidation of *c*-type cytochromes and azurin of the same organism.[1,2] The enzyme contains two covalently bound heme *c* moieties in a single polypeptide chain.[3,4]

Recent kinetic[5] and EPR studies[6] have shown that the active form of *Pseudomonas* cytochrome *c* peroxidase is the half-reduced enzyme in which one heme is in the ferrous and the other in the ferric state. Reaction of the half-reduced enzyme and hydrogen peroxide is fast, compound I being formed within a few milliseconds.[5] In contrast, the reaction of the totally ferric form of the enzyme with hydrogen peroxide is very slow and its effect on the peroxidation cycle is negligible.[7]

In the present investigation we report upon the rate of formation of the catalytic intermediate (compound I) from half-reduced enzyme and hydrogen peroxide as a function of pH. The effect of pH on the overall reaction rate of the enzyme was also studied. Further, the pH-stability of the enzyme was investigated, as well as the effect of pH on the rate of reduction of the peroxidase by azurin.

MATERIALS AND METHODS

Pseudomonas cytochrome *c* peroxidase was prepared from acetone-dried cells of *P. aeruginosa*.[3] The ratio A_{407} nm/A_{280} nm of the preparation was 4.5. The concentration of the enzyme was determined spectrophotometrically using A (1 %, 1 cm) equal to 12.1 at 280 nm.[4] Half-reduced enzyme was obtained by adding reduced azurin to a solution of totally ferric enzyme at a 2:1 molar ratio, the stoichiometry of the reaction being 1:1.[8] Excess of azurin was used to ensure rapid reduction of the enzyme.

Pseudomonas cytochrome *c*-551 and *Pseudomonas* azurin were prepared from acetone-dried

0302-4369/84 $2.50

cells of *P. aeruginosa* using established methodology.[9,10] The purity number of the cytochrome preparation, [A_{551} nm(red.) − A_{570} nm(red.)]/A_{280} nm, was 1.2 and that of azurin, A_{625} nm(ox.)/A_{280} nm, was 0.5. The concentration of the cytochrome was determined spectrophotometrically applying the millimolar absorptivity coefficient ε_{551}(red.-ox.)=19.0 mM^{-1} cm^{-1}, and the concentration of azurin[1] by using ε_{625}(ox.)=5.1 mM^{-1} cm^{-1}. Reduced cytochrome *c*-551 and azurin were prepared as described earlier.[2] Hydrogen peroxide solutions were prepared from 30 % hydrogen peroxide (Fisher Scientific Co.) and the concentration was determined spectrophotometrically[11] at 230 nm using ε=72.4 M^{-1} cm^{-1}.

The pH-stability of the half-reduced enzyme was investigated by incubating the enzyme at various pH-values for 40 s, after which the activity measurement was performed in 0.1 M sodium phosphate buffer at pH 6.0. The effect of pH on the overall rate of the peroxidation reaction was studied using reduced cytochrome *c*-551 as the electron donor and H_2O_2 as the acceptor in mixtures (2.0 ml) of varying pH. The reaction was started by adding half-reduced enzyme (5 μl) in 0.1 M sodium phosphate buffer pH 6.0.

The formation of compound I was followed at 413 nm with a Union Giken Model RA601 stopped-flow spectrophotometer equipped with a 1 cm observation cell thermostated at 25 °C. A solution of unbuffered half-reduced enzyme, obtained by adding reduced azurin to ferric enzyme, for which the ionic strength was adjusted to 0.1 with potassium nitrate, was mixed with buffered solutions of H_2O_2 at various pH values.

Spectrophotometric measurements were carried out with a Cary 219 spectrophotometer at 25 °C. In all experiments, sodium acetate (0.1 M) buffers were used between 3.5 and 5.6, and sodium phosphate buffers were used between 6.0 and 8.0. pH was measured with a Fisher Acumet Model 420 Digital pH/Ion meter.

RESULTS

The stability of *Pseudomonas* cytochrome *c* peroxidase was examined in the pH region 3.0 to 8.0. The enzyme is stable, in terms of peroxidatic activity, during the incubation period of 40 s in the pH region 4.6 to 7.0 and even outside these limits the activity is about 90 % of the maximum value (Fig. 1). In contrast, as also shown in Fig. 1, the overall activity of the enzyme is maximal only at a rather narrow region around pH 6, decreasing at both the acid and alkaline side of the optimum pH.

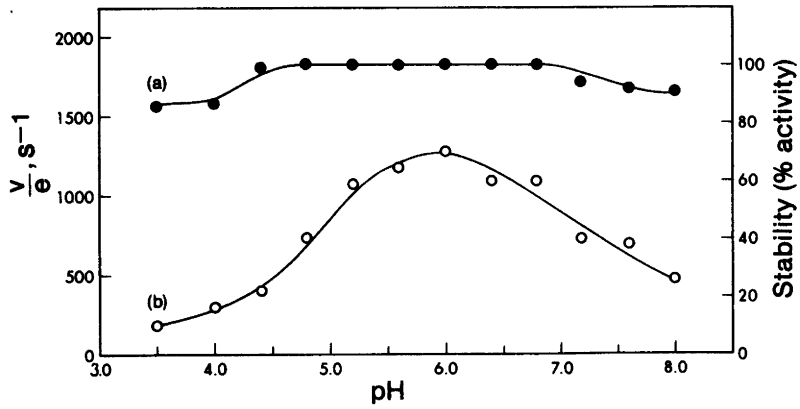

Fig. 1. The pH dependence of *Pseudomonas* cytochrome *c* peroxidase. (a) Stability of the enzyme, in terms of peroxidatic activity, after 40 s incubation in buffers of varying pH (right ordinate). Conditions in the assay mixture: enzyme 1.4 nM, H_2O_2 80.0 μM, reduced cytochrome *c*-551 10.7 μM in sodium phosphate buffer, pH 6.0, I=0.01. (b) The overall rate of the peroxidation reaction in mixtures of varying pH. *v*/*e* expressed in mol cytochrome *c*-551 oxidized per mol of enzyme per second (left ordinate). Concentrations of reactants as in (a). The points are mean values of at least 3 measurements, the error being ±10 %.

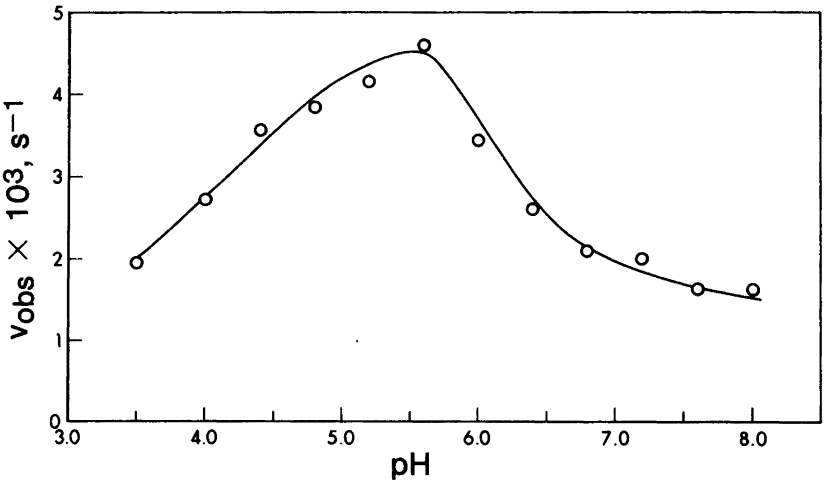

Fig. 2. Reduction of totally ferric *Pseudomonas* cytochrome *c* peroxidase by reduced azurin. Observed reaction rate (Δ absorbance at 407 nm) with 1.0 μM enzyme and 10.7 μM azurin in buffers of varying pH. The points represent the mean values of 3 measurements, the error being ±10 %.

The totally ferric form of *Pseudomonas* peroxidase is reduced to a half-reduced form by electron donors, *e.g.* reduced cytochrome *c*-551 or azurin. The effect of pH on the rate of reduction of the enzyme by azurin, followed at 407 nm, the absorption maximum of the oxidized enzyme is shown in Fig. 2. Reduction of the enzyme by azurin comprises of two reactions, one rapid and a slower one,[8] the pH dependence of the fast reaction only being considered here. The highest rate of reduction is observed at pH 5.6, the rate decreases rapidly at both acid and alkaline side of the optimum pH.

The formation of the primary compound between hydrogen peroxide and the half-reduced enzyme was measured at 413 nm, the isosbestic point between the totally reduced and the totally oxidized enzyme. The effect of pH on the rate of formation of compound I is shown in Fig. 3. The observed reaction rate is constant between pH 5

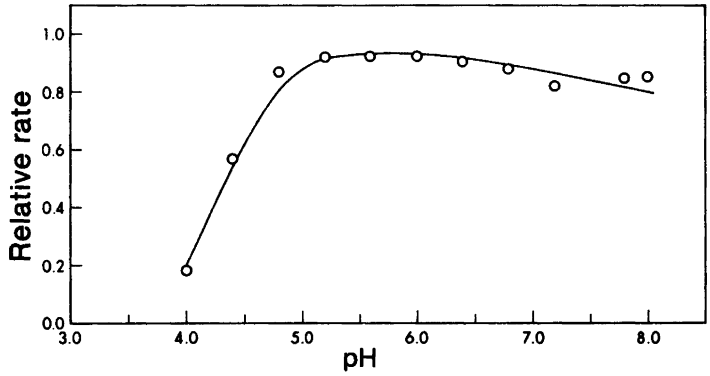

Fig. 3. Relative observed reaction rate (Δ absorbance at 413 nm) for the formation of compound I from half-reduced *Pseudomonas* cytochrome *c* peroxidase and H_2O_2 as a function of pH. Enzyme concentration, 1 μM, reduced azurin, 2 μM, and hydrogen peroxide, 1.2 μM, after mixing. The points represent the mean values of at least 5 measurements, the error being ±10 %. The ordinate scale is arbitrary.

and 7 but decreases with decreasing pH. On the alkaline side the effect of pH on the rate of compound I formation is rather small compared with the change observed in peroxidatic activity.

DISCUSSION

The similarity of the pH-dependence of the overall enzyme activity (Fig. 1) and the formation of compound I (Fig. 3) suggests that in the pH range 4–6 the enzyme activity is limited by the rate of formation of compound I. The pH *vs.* activity curve is, however, also dependent on the complex formation between the peroxidase and reduced cytochrome *c*-551. *Pseudomonas* peroxidase (pI 6.7)[3] and *Pseudomonas* cytochrome *c*-551 (pI 4.7)[12] bear opposite charges only between pH 4.7 and 6.7, the complex formation being hampered outside these limits. The slight decrease in the rate of formation of compound I above pH 7 may be caused by the decreased stability of the enzyme.

Both enzyme activity and reduction of the enzyme by azurin (Fig. 2) show a clear dependence on pH, the reduction rate falling off more rapidly than the overall reaction rate at values above pH 6. This difference may be explained by reduced azurin existing in two conformers, a redox-active and redox-inactive one.[13] The redox-active conformer is a protonated form, the pK of the equilibrium[14] being about 7.

The rate-pH profile of compound I formation of *Pseudomonas* cytochrome *c* peroxidase indicates the involvement of an acid group with an apparent value of pK_a of 4.4. The group has to be deprotonated for compound I formation to occur. It has been suggested[15] that the peroxidases in general have an acid group appropriately positioned to accept one proton from hydrogen peroxide resulting in the formation of peroxo anion, HO_2^-. This implies a large shift in the pK value of H_2O_2 upon binding, a characteristic feature of acid-base catalysis. The redox reaction proceeds further *via* nucleophilic attack of the peroxo anion on the ferric heme iron. At pH 6.0 the rate constant for compound I formation[7] from *Pseudomonas* cytochrome *c* peroxidase and H_2O_2 is 1.2×10^8 M^{-1} s^{-1}.

A probable mechanism for the reaction between a peroxide molecule and yeast cytochrome *c* peroxidase leading to compound I has recently been presented.[16] In the first step the peroxide forms a short-lived intermediate in which a singly ionized peroxide molecule is bound to the heme iron atom with the hydrogen ion transferred to a distal histidine residue.

In *Pseudomonas* cytochrome *c* peroxidase only one of the two heme *c* moieties primarily reacts with hydrogen peroxide.[6] The peroxidatically active heme is pentacoordinated. The identity of the acid distal group functioning as the proton acceptor is at present unknown. The apparent pK_a value of 4.4 determined for *Pseudomonas* cytochrome *c* peroxidase is close to the corresponding pK_a of horseradish peroxidase determined under similar conditions and which has been assumed to derive from a carboxylate.[17] It seems that also in *Pseudomonas* cytochrome *c* peroxidase base catalysis is a necessity in the formation of compound I.

Acknowledgements. A postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada (to M.R.) and a travelling grant from *Societas Scientiarum Fennica* (to N.E.) are gratefully acknowledged.

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Received February 7, 1983.