THE FORMATION OF THE PRIMARY COMPOUND FROM HYDROGEN PEROXIDE AND *PSEUDOMONAS* CYTOCHROME *c* PEROXIDASE

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1. Introduction

Cytochrome *c* peroxidase (cytochrome *c*-551:H_2O_2 oxidoreductase, EC 1.11.1.5) of *Pseudomonas aeruginosa* catalyzes the peroxidation of *c*-type cytochromes and azurin, a copper protein, of the same organism [1,2]. The enzyme contains two covalently bound heme *c* moieties in a single polypeptide chain [3,4]. The absorption spectrum of the enzyme is of low-spin character although one of the hemes is found to be in low-spin and the other in a high-spin state [5]. The high-spin heme has been concluded to be the primary target of H_2O_2 in the peroxidatic reaction cycle [5].

We report here on rapid scan spectra of *Pseudomonas* cytochrome *c* peroxidase compound I, obtained by observation of the reaction between partially reduced enzyme and hydrogen peroxide. Compound I had not been observed earlier due to its rapid decomposition.

2. Materials and methods

Pseudomonas cytochrome *c* peroxidase was prepared from acetone-dried cells of *P. aeruginosa* as in [3]. The ratio A_{407}/A_{280} of the preparation was 4.6. The concentration of a stock solution of the enzyme was determined spectrophotometrically [4].

Pseudomonas cytochrome *c*-551 was prepared as in [6]. The purified cytochrome preparation had a purity ratio A_{551} (red.)/A_{280} = 1.20. The concentration of the cytochrome was determined by using the value $\Delta\epsilon_{551}$ = 19.0 $mM^{-1} \cdot cm^{-1}$ [1]. Azurin was prepared from acetone-dried cells of *P. aeruginosa* as in [7]. The purity ratio of the preparation, A_{625} (ox.)/A_{280}, was 0.4. The concentration of azurin was determined spectrophotometrically by using ϵ_{625} (ox.) = 5.1 $mM^{-1} \cdot cm^{-1}$ [1]. Hydrogen peroxide solutions were prepared from 30% hydrogen peroxide (Fisher Scientific Company, NJ) and the concentration was determined spectrophotometrically at 230 nm using ϵ = 72.4 $mM^{-1} \cdot cm^{-1}$ [8].

The absorption spectra of the protein solutions were measured with a Cary 219 recording spectrophotometer. Stopped flow and rapid scan measurements were made on a Union Giken Model RA601 stopped flow spectrophotometer equipped with a 1 cm observation cell thermostated at 25 ± 0.2°C. The method of obtaining the rapid scan absorption spectra has been described [9].

3. Results

When pure *Pseudomonas* cytochrome *c* peroxidase is isolated both heme irons are in the ferric state and we shall refer to this as the totally ferric form. In the reaction between hydrogen peroxide and the totally ferric form a slow time-dependent spectral change at the Soret band was observed as examined by the rapid scan technique. The peroxidatic activity of the reaction mixture at different times was tested using reduced cytochrome *c*-551 as substrate. No peroxidatic activity could be shown towards cytochrome *c*-551.

In contrast, when the peroxidase was partially reduced with an excess of reduced azurin, the addition of H_2O_2 caused a rapid spectral change, indicating the formation of an intermediate (fig.1). Under the conditions outlined in the caption to fig.1 the primary compound is formed within 1 ms after mixing. About 100 ms after mixing the original spectrum of half-reduced enzyme reappears. These results show that the intermediate has high peroxidatic activity towards

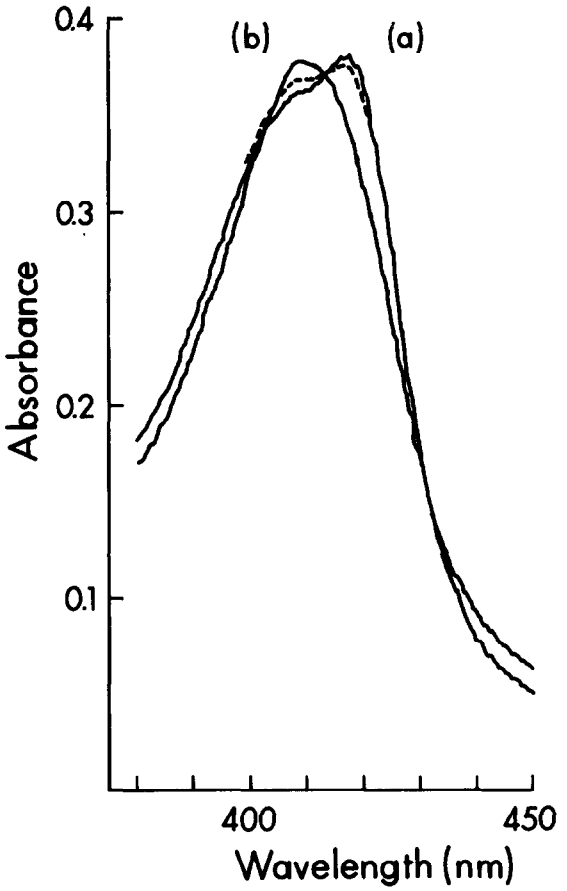


Fig.1. Rapid scan spectra on the formation of primary peroxide compound of partially reduced *Pseudomonas* cytochrome *c* peroxidase: (a) Spectrum of enzyme partially reduced with reduced azurin; (b) spectrum of peroxide-compound obtained 1 ms after mixing; (– – –) spectrum obtained 130 ms after mixing. Final concentrations of reactants: 2 μM enzyme, 20 μM azurin, 4 μM hydrogen peroxide in 0.025 M sodium phosphate buffer (pH 6.0).

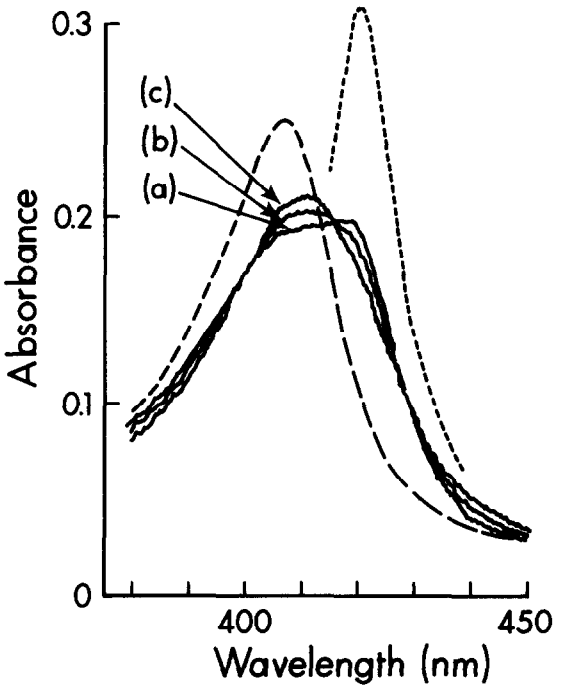


Fig.2. Rapid scan spectra on the formation of primary peroxide-compound of partially reduced *Pseudomonas* cytochrome *c* peroxidase: (– – –) Spectrum of totally ferric enzyme; (. . . .) spectrum of enzyme fully reduced with dithionite; (——) (a), enzyme partially reduced with potassium ferrocyanide; (——) (b), spectrum of peroxide-compound obtained 1 ms after mixing; (——) (c), spectrum of peroxide-compound obtained 12 ms after mixing. This spectrum was the same as that obtained 26 ms after mixing. Final concentrations of reactants: 1 μM enzyme, 40 μM ferrocyanide, 2 μM hydrogen peroxide in 0.025 M sodium phosphate buffer (pH 6.0).

reduced azurin and the hydrogen peroxide is consumed within ~100 ms. As azurin is a good electron donor in the peroxidatic reaction the intermediate is rapidly decomposed and no isosbestic point is observed in the spectra of the compound and ferric or ferrous enzyme.

When the enzyme is partially reduced with ferrocyanide, a poor substrate, its reaction with hydrogen peroxide results in a rather stable intermediate (fig.2) with an absorption maximum at 411 nm. In this case the spectra show isosbestic points with the partially reduced enzyme at 401, 415 and 429 nm indicating the presence of one intermediate only. Further, the isosbestic points are clearly different from that between totally ferric and totally ferrous forms of the enzyme at 413 nm.

When the reduction of the enzyme was increased by increasing the concentration of ferrocyanide, a broad maximum level of compound I formation was reached when the ratio of ferrocyanide to total enzyme was in the range 80:1 to 200:1 (fig.3).

4. Discussion

The mechanism of heme containing peroxidases involves the formation of a compound between hydrogen peroxide and the ferric high spin form of the enzyme. Since one of the two hemes in native *Pseudomonas* cytochrome *c* peroxidase is in high spin form this heme has been assumed to be involved in forma-

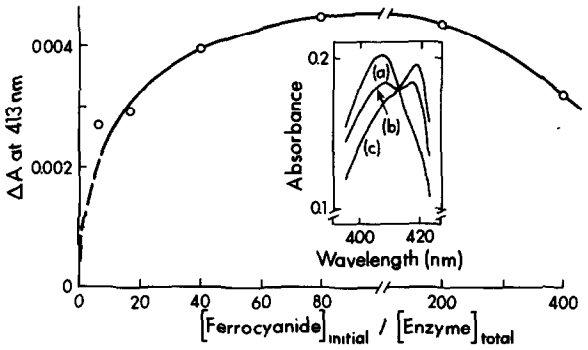


Fig.3. The inset shows the spectra of partially reduced enzyme 30 min after mixing with ferrocyanide. Total cytochrome *c* peroxidase 1 μM; ferrocyanide was (a) 3 μM, (b) 40 μM, and (*c*) 200 μM (all concentrations after mixing). The main part of the diagram shows ΔA_{413}, the isosbestic point shown in the inset when enzyme–ferrocyanide mixtures of the type above were mixed with hydrogen peroxide. Total enzyme was 0.5 μM and initial hydrogen peroxide 0.6 μM (both after mixing). The values of ΔA were obtained 50 ms after mixing and represent maximum conversion to compound I for the specified experimental conditions.

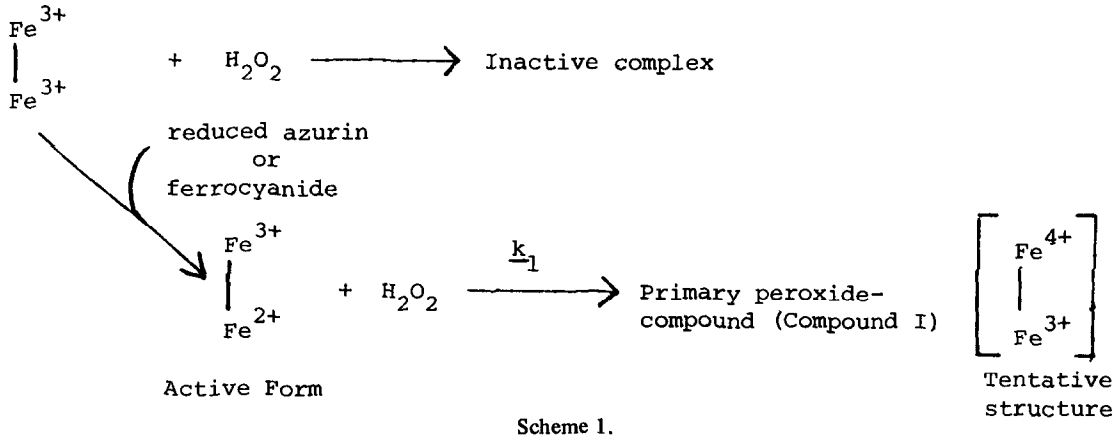
tion of such a compound [10]. The present investigation, however, shows that the intermediate formed between the totally ferric enzyme and hydrogen peroxide is not peroxidatically active towards reduced substrates like cytochrome *c*-551. This is in agreement with earlier observations made under steady-state conditions suggesting that the enzyme must be partially reduced before the peroxidation reaction begins [11].

When partially reduced enzyme reacts with hydrogen peroxide a highly reactive intermediate is formed. The rate of formation of the primary compound increases with increasing concentration of H_2O_2, and the rate of decomposition of this compound increases with increasing concentration of reduced azurin (unpublished) showing the primary compound to be active in the enzyme cycle.

It is evident from fig.3 that an optimum reduction of the enzyme is necessary for a maximal formation of the primary compound between H_2O_2 and the peroxidase. It would appear that the totally reduced enzyme is inactive. It is assumed that the active form of the enzyme has one heme in the ferrous and the other in the ferric form. On this basis, scheme 1 may be presented for the formation of the primary peroxide compound.

In principle, this mechanism is similar to that of yeast cytochrome *c* peroxidase where one of the two oxidizing equivalents of hydrogen peroxide is accepted by heme-iron, converting it from the ferric to ferryl form [12], and the other is accepted by amino acid residues [13,14]. In *P. aeruginosa* cytochrome *c* peroxidase the second oxidizing equivalent is accepted by the second heme group which appears to act more like a cytochrome than a peroxidase.

Pseudomonas cytochrome *c* peroxidase seems to be a first primitive form of peroxidases. A later evolution of the enzyme has replaced the heme-electron donor which might result in a considerable saving of biosynthetic energy. Also the ferrous heme electron donor is readily oxidized in aerobic environments and so had to be replaced in higher organisms.



Scheme 1.

Acknowledgements

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