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# HEME-MODIFICATION STUDIES ON HORSERADISH PEROXIDASE

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## SUMMARY

Synthetic horseradish peroxidases (donor:$H_2O_2$ oxidoreductase, EC 1.11.1.7) were prepared from apoenzyme and modified heme. The synthetic enzymes containing proto-, meso- or deuteroheme or protoheme monomethyl ester reacted with stoichiometric amounts of hydroperoxides, to form Compound I with these synthetic enzymes. Other hydroperoxide-induced compounds such as Compounds II and III could also be formed with these synthetic enzymes. The optical spectra of these compounds were similar to those of the native enzyme, except for shifts in absorption maxima. The enzyme which contained protoheme dimethyl ester did not form these compounds under the same experimental conditions.

Except for the protoheme dimethyl ester-containing enzyme, these synthetic enzymes reacted with heme ligands such as fluoride, cyanide, and carbon monoxide to form well-defined complexes and were converted into alkaline forms on raising the pH. The affinities of the enzyme for these ligands were similar to those of native enzyme. The pH at which the alkaline transition occurred was similar to that for the native enzyme, except for the protoheme monomethyl ester-containing enzyme which showed a considerably lower p$K$ value.

The enzymatic activities of the synthetic enzymes which contained meso-, deuterohemes in the oxidation of guaiacol were similar to that of the native enzyme. The enzyme which contained protoheme monomethyl ester showed only 20% of the activity of native enzyme, and the enzyme containing protoheme dimethyl ester showed no peroxidase activity. It was concluded that the side chains at positions 2 and 4 of the porphyrin ring were not essential for the catalytic activity of horseradish peroxidase, but that the free carboxyl groups at positions 6 and 7 were essential.

## INTRODUCTION

Horseradish peroxidase (donor:$H_2O_2$ oxidoreductase, EC 1.11.1.7) is unique amongst the hemoproteins in possessing five redox states which are catalytically

Abbreviations: monoester and diester enzymes are the synthetic enzymes containing protoheme monomethyl ester and protoheme dimethyl ester, respectively.

active. Though the reaction mechanisms of this enzyme have been extensively studied, the chemical nature of these intermediates has not been well established. We have prepared synthetic horseradish peroxidases in which the prosthetic groups were chemically modified in order to observe the effect of these modifications on the properties of the various redox states.

Since Theorell[1] demonstrated the reversible cleavage of horseradish peroxidase into protoheme and the apoenzyme, the recombination of apoenzyme with modified hemins has been studied by Gjessing and Sumner[2], Theorell *et al.*[3], Paul[4] and Paul *et al.*[5]. They showed that the side chains at positions 2 and 4 of the porphyrin ring were not essential to the peroxidase activity by the recombination of apoenzyme with 2,4-substituted hemes. The effects of side-chain substitution on other properties of the proteins, for example; the optical absorption spectra and reactivity with hydroperoxide and with ligands were not reported.

Recent studies on cytochrome *c* peroxidase also indicated that substitutions at the 2 and 4 positions of the porphyrin ring do not affect the enzymic activity[6] and that modification of the carboxyl groups at positions 6 and 7 caused a drastic decrease in the enzymic activity without changing the optical and electron paramagnetic resonance properties significantly[7]. In this report, we describe the enzymic, optical and ligand-binding properties of synthetic horseradish peroxidases containing proto-, meso-, deuterohemes and protoheme mono- and dimethyl esters.

## MATERIALS

Horseradish peroxidase was purified from horseradish roots by chromatography on DEAE- and CM-cellulose columns according to the method of Shannon *et al.*[8]. The enzyme used was the main fraction adsorbed on the CM-cellulose column, and crystallized with $(NH_4)_2SO_4$. The purity index ($A_{403\ nm}:A_{278\ nm}$) of this enzyme was 3.2.

Meso- and deuterohemins were prepared from protohemin according to the methods of Fisher and Putzer[9] and Chu and Chu[10]. Protohemin monomethyl ester was obtained by partial reaction of protohemin with acidic methanol[11]. The apoenzyme was prepared from horseradish peroxidase by the acid–butanone method of Teale[12]. The synthetic enzymes containing proto-, meso- and deuterohemes were prepared by the method used for the reconstitution of cytochrome *c* peroxidase[6]. Apoenzyme was dissolved in 0.1 M Tris–HCl buffer, pH 8, to concentrations of 50–200 $\mu$M. Hemes were dissolved in a small volume of 0.1 M NaOH and the solutions diluted with distilled water to about 1 mM. The concentrations of heme solutions were determined spectrophotometrically by the pyridine hemochrome method. The solution of apoenzyme was mixed with a heme solution at a molar ratio of 1.2–1.5 and allowed to stand for 10 min at 0 °C. The mixture was dialyzed against distilled water and then against 5 mM acetate buffer at pH 4.4. The dialysate was passed through a CM-cellulose column, which was equilibrated with 5 mM acetate buffer, at 4 °C, and pH 4.4. The reconstituted enzyme was adsorbed tightly at the top of the column, and was eluted with the same buffer containing 50 mM NaCl. The main fraction was collected and rechromatographed on a CM-cellulose column.

Recombination of apoenzyme with protoheme monomethyl ester was performed in an aqueous lutidine solution near 0 °C. Protoheme monomethyl ester in lutidine

(approx. 100 $\mu$M) was mixed with apoenzyme dissolved in 10 mM phosphate buffer (pH 7) at 0 °C and the mixture was gently stirred for 10 min at 0 °C. The final lutidine concentration was approx. 10% (v/v). The mixture was then dialyzed against 5 mM phosphate buffer at pH 6 for several hours to remove lutidine, and then against distilled water at 0 °C. After dialysis, the synthetic enzyme was purified on a CM-cellulose column as described above.

Protoheme dimethyl ester was combined with apoenzyme in 5 mM phosphate buffer (pH 6) containing 10% (v/v) acetone at 0 °C. Because of the low affinity of the dimethyl ester for apoenzyme, a 10-fold excess of the dimethyl ester was mixed with apoenzyme. The fractions eluted with 70 mM NaCl in 5 mM acetate buffer (pH 4.4) were used in the experiments.

## METHODS

Spectrophotometric measurements were carried out with a Cary recording spectrophotometer Model 15 and a Hitachi–Perkin–Elmer spectrophotomer Model 124 equipped with cell compartments maintained at 23 °C. The measurement of enzymic activity was carried out according to the standard method of Maehly[14]. The concentrations of horseradish peroxidase and apoenzyme were determined spectrophotometrically using extinction coefficients of 107.7 $mM^{-1} \cdot cm^{-1}$ at 403 nm[15] and 20 $mM^{-1} \cdot cm^{-1}$ at 278 nm, respectively. The pyridine hemochrome method was used for the determination of the concentration of modified hemes and of the synthetic enzymes containing the corresponding hemes[16].

## RESULTS

### *Recombination of horseradish apoperoxidase with modified hemins*

A spectrophotometric titration of apoenzyme with protoheme at pH 8 is shown in Fig. 1, where the clear inflection point can be seen. Well-defined inflection points were also obtained with meso- and deuterohemes, and with protoheme monomethyl ester. The stoichiometry of the reaction between apoenzyme and these hemes was 1:1. The preparation of protoheme monomethyl ester throughout is a mixture of 6-

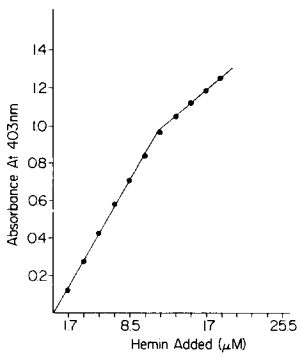


Fig. 1. Spectrophotometric titration of apohorseradish peroxidase with protoheme. Approximately 12 $\mu$M apoenzyme in 0.1 M Tris–HCl buffer, pH 8, was titrated by successive additions of protoheme.

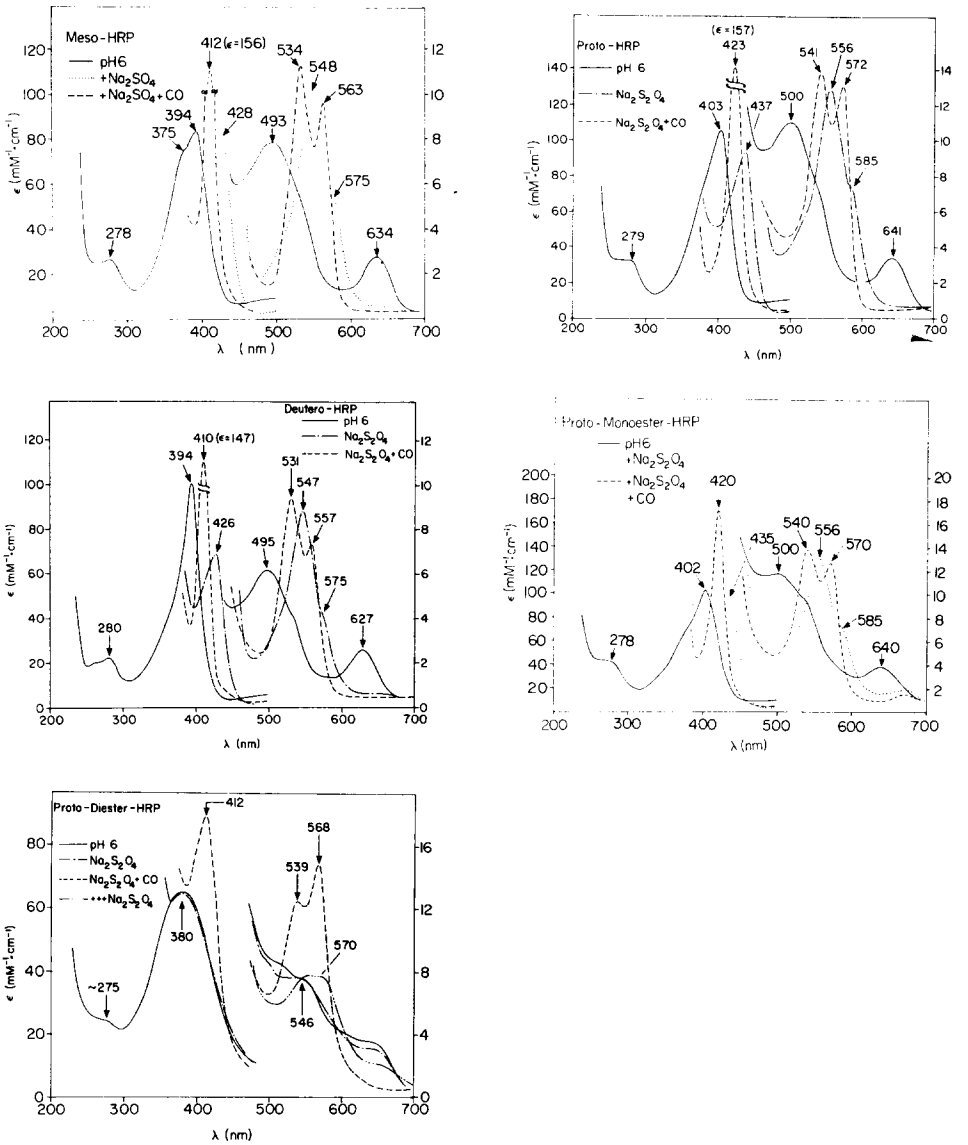


Fig. 2. Light absorption spectra of synthetic horseradish peroxidases (HRP) and their dithionite-reduced compounds in the presence and absence of CO at 23 °C.

and 7-monoesters of protoheme. The affinity of these isomers toward apoenzyme appears to be similar. However, protoheme dimethyl ester had a very low affinity toward the apoenzyme and a 10-fold excess of protoheme dimethyl ester was required for a complete reconstitution of the enzyme. The affinity of protoheme dimethyl ester toward the apoenzyme is pH independent unlike that of hemes having free carboxyl groups. Protoheme dimethyl ester was combined with apoenzyme and the product was purified at pH 6, because at pH 8 the synthetic enzyme was adsorbed irreversibly on the CM-cellulose column.

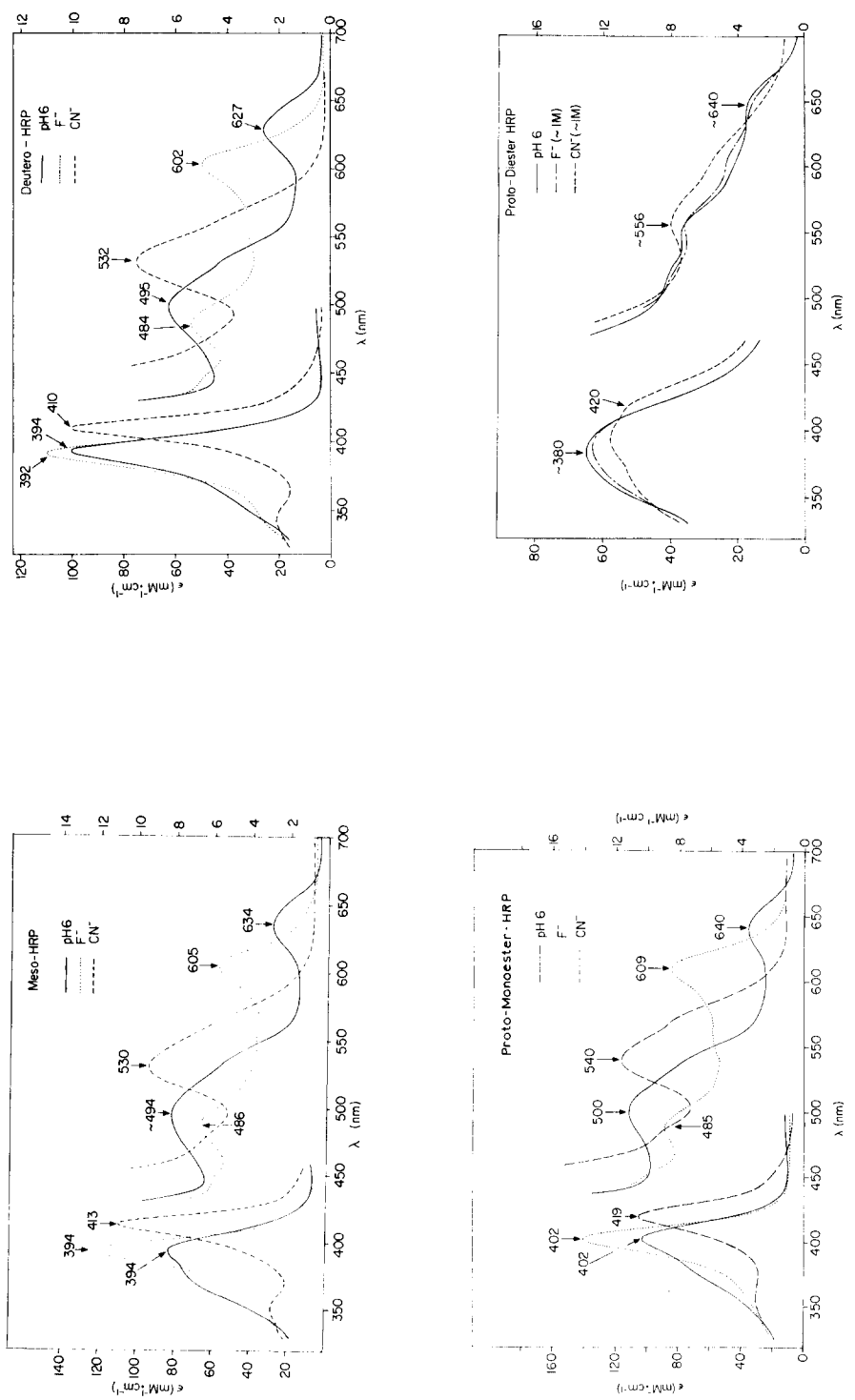


Fig. 3. Light absorption spectra of synthetic horseradish peroxidases (HRP) and their complexes with fluoride and cyanide at 23 °C.

TABLE 1

LIGHT ABSORPTION MAXIMA (nm) OF SYNTHETIC HORSERADISH PEROXIDASES AND THEIR DERIVATIVES

Except for the alkaline forms, the measurements are made in 0.1 M phosphate buffer, pH 6, at 23 °C.

| *Enzymes* | *Soret* | | *Visible* | | |
|---|---|---|---|---|---|
| Proto | 403 | 500 | | | 641 |
| Meso | 394 | 493 | | | 634 |
| Deutero | 394 | 495 | | | 627 |
| Monoester | 404 | 500 | | | 640 |
| Diester | 380 | — | | | — |
| *plus* $Na_2S_2O_4$ | | | | | |
| Proto | 437 | | 556 | | |
| Meso | 428 | | 548 | | |
| Deutero | 426 | | 547 | | |
| Monoester | 435 | | 556 | | |
| Diester | 380 | | — | | |
| *plus* $Na_2S_2O_4$ *plus CO* | | | | | |
| Proto | 423 | 541 | | 572 | |
| Meso | 412 | 534, | | 563 | |
| Deutero | 410 | 531 | | 557 | |
| Monoester | 420 | 540 | | 570 | |
| Diester | 412 | 539 | | 568 | |
| *plus* $F^-$ | | | | | |
| Proto | 404 | 490 | | | 611 |
| Meso | 394 | 486 | | | 605 |
| Deutero | 392 | 484 | | | 602 |
| Monoester | 402 | 485 | | | 609 |
| Diester | — | — | | | — |
| *plus* $CN^-$ | | | | | |
| Proto | 421 | 540 | | | |
| Meso | 413 | 530 | | | |
| Deutero | 410 | 532 | | | |
| Monoester | 419 | 540 | | | |
| Diester | — | 556 | | | |
| *plus* $OH^-$ | | | | | |
| Proto | 416 | 543 | | 575 | |
| Meso | 405 | 534 | | 565 | |
| Deutero | 404 | 533 | | 566 | |
| Monoester | 414 | 542 | | 574 | |
| Diester | — | — | | — | |

*Light absorption spectra*

Light absorption spectra of the synthetic enzymes and their derivatives are shown in Figs 2 and 3. The synthetic enzymes containing mesoheme, deuteroheme and protoheme monomethyl ester were readily reduced with dithionite. The reduced compounds formed well-defined complexes with CO. The ferric enzymes formed complexes with fluoride and cyanide. The absorption maxima of the spectra of these synthetic enzymes and their derivatives are shifted to shorter wavelengths in the order proto- > meso- > deuteroenzyme. The spectra of these synthetic enzymes and their derivatives are quite similar to those of the protoenzyme. The spectrum of the monoester enzyme, which is that of a mixture of high- and low-spin states at pH 6, is slightly different from that of the protoenzyme. However, the spectra of its reduced

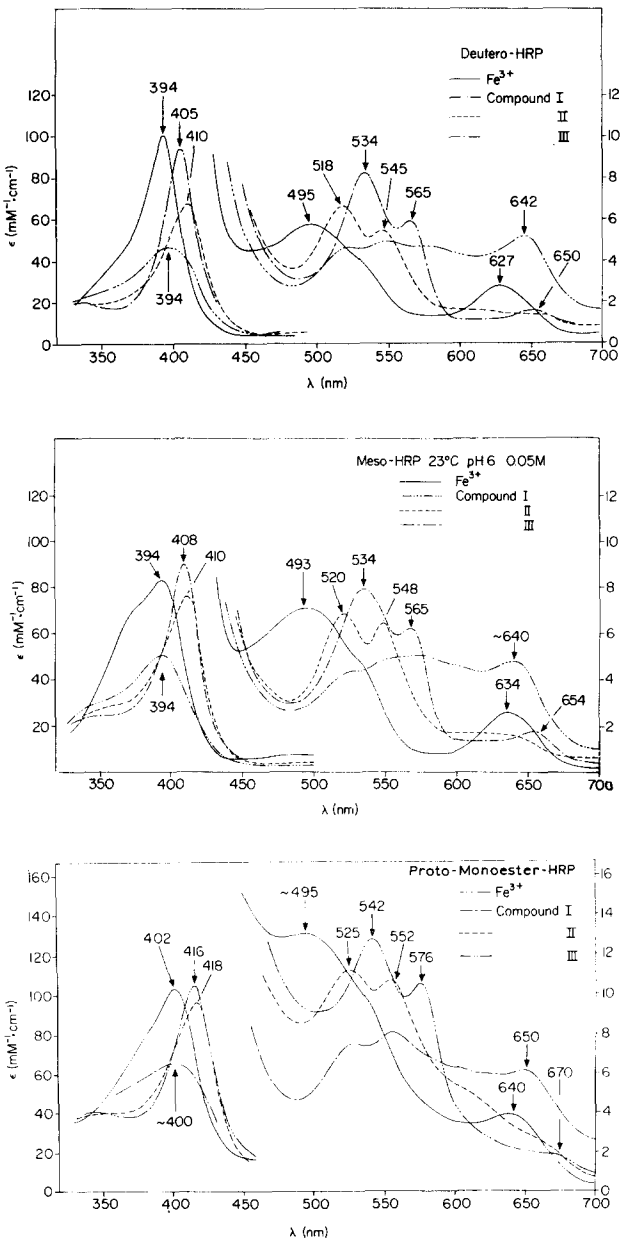


Fig. 4. Light absorption spectra of $H_2O_2$-induced compounds of synthetic horseradish peroxidases (HRP). The spectra of Compound II were recorded at 3 °C and those of Compounds I and III at 23 °C.

form and derivatives were almost the same as those of native enzyme. The optical properties of the diester enzyme are completely different from those of the native enzyme. This synthetic enzyme is less reactive with cyanide and fluoride, and is re-

duced more slowly by $Na_2S_2O_4$ than the native enzyme. The absorption maxima of native and synthetic enzymes are compared in Table I.

*Reactions with hydroperoxide*

The synthetic enzymes, except for the diester enzyme, react rapidly with hydroperoxide to form the green primary compound, Compound I, the light absorption spectra of which are shown in Fig. 4. Compound I is gradually transformed into the red secondary compound, Compound II, in the absence of hydrogen donors (Fig. 5). Compound I formed from either the proto- or the mesoenzymes is very stable with half time of decomposition ($t_{\frac{1}{2}}$) of more than 1 h; however, that of the monoester enzyme is relatively unstable ($t_{\frac{1}{2}} = 2$ min), and that of deuteroenzyme has an intermediate stability ($t_{\frac{1}{2}} = 15$ min). The stability of the peroxide complex except for those formed from the monester enzyme was enhanced by pretreatment with $H_2O_2$. The stoichiometry of the reaction between hydroperoxide and the synthetic enzyme is 1:1.

The spectra of Compound II (Fig. 4) were measured during the steady state of the peroxidatic oxidation of ascorbate. A prolonged steady state lasting for more than 5 min can be attained near 0 °C using low concentrations of the enzyme (approx. 3 $\mu$M). The formation of Compound III during the steady state was prevented by using ethyl peroxide instead of $H_2O_2$ as the electron acceptor. The absorption spectra

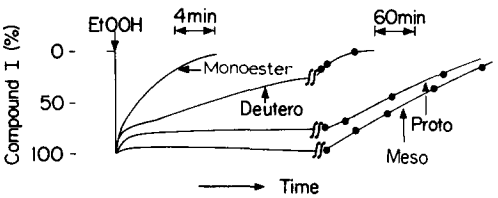


Fig. 5. Time course of the transformation from Compound I to Compound II in the absence of hydrogen donor. The reactions were initiated by the addition of an equimolar ethyl peroxide in 50 mM phosphate buffer, pH 6, at 23 °C and followed by measuring the absorption changes at the isosbestic point between ferric state and Compound II. These isosbestic points were 411 nm (protoenzyme), 403 nm (mesoenzyme), 404 nm (deuteroenzyme), and 410 nm (monoester enzyme). EtOOH = ethyl peroxide.

of Compound II formed from the synthetic enzymes are similar to those of the protoenzymes. In the absence of hydrogen donors, Compound II of monoester enzymes decomposed into the ferric state with a half time of about 30 min, whereas that formed from the other synthetic enzymes was more stable ($t_{\frac{1}{2}}$ = approx. 2 h).

The absorption spectra of Compound III were recorded in the presence of a 100-fold excess of $H_2O_2$ and 50 $\mu$M ascorbic acid. In the absence of ascorbic acid, a mixture of Compound I and III was formed[17]. The absorption maxima of these peroxide compounds of the synthetic enzymes containing meso- and deuterohemes and protoheme monomethyl ester were shifted to shorter wavelengths. The small absorption observed in a range from 650 to 700 nm in the presence of excess $H_2O_2$ might be due to the formation of Compound IV, which has not been well characterized[18,19]. The diester enzyme formed no compounds with hydroperoxide. The absorp-

TABLE II

LIGHT ABSORPTION MAXIMA (nm) OF THE HYDROPEROXIDE-INDUCED COMPOUNDS OF SYNTHETIC HORSERADISH PEROXIDASES

| *Compound* | *Soret* | | *Visible* | |
|---|---|---|---|---|
| Proto | | | | |
| I | 400 | | | ≈650 |
| II | 418 | 524 | 555 | |
| III | 417 | 542 | 576 | |
| IV | | | | 670 |
| Meso | | | | |
| I | 394 | | | ≈640 |
| II | 410 | 520 | 548 | |
| III | 408 | 534 | 565 | |
| IV | | | | 654 |
| Deutero | | | | |
| I | 394 | | | ≈642 |
| II | 410 | 518 | 545 | |
| III | 405 | 534 | 565 | |
| IV | | | | 650 |
| Proto-monoester | | | | |
| I | 400 | | | ≈650 |
| II | 418 | 525 | 552 | |
| III | 416 | 542 | 576 | |
| IV | | | | 670 |

tion maxima of these hydroperoxide-induced compounds of the synthetic enzymes are summarized in Table II.

*Reactivity towards ligands*

Fig. 6 shows the formation of the cyanide complex of the synthetic enzymes. The affinity of cyanide is decreased in the order of proto monomethyl ester, protoheme, deuteroheme and mesoheme. The diester enzyme has an extremely low affinity for cyanide (*cf.* Fig. 3). The absorption spectra of the synthetic enzymes at various

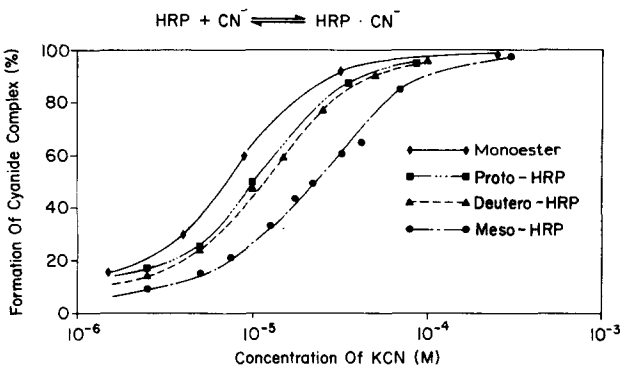


Fig. 6. Titrations of the formation of the cyanide complexes of the synthetic horseradish peroxidases (HRP) in 0.1 M phosphate buffer, pH 6, at 25 °C. The end-point was obtained by the addition of 10 mM cyanide. Enzyme concentrations used were approximately 10 $\mu$M.

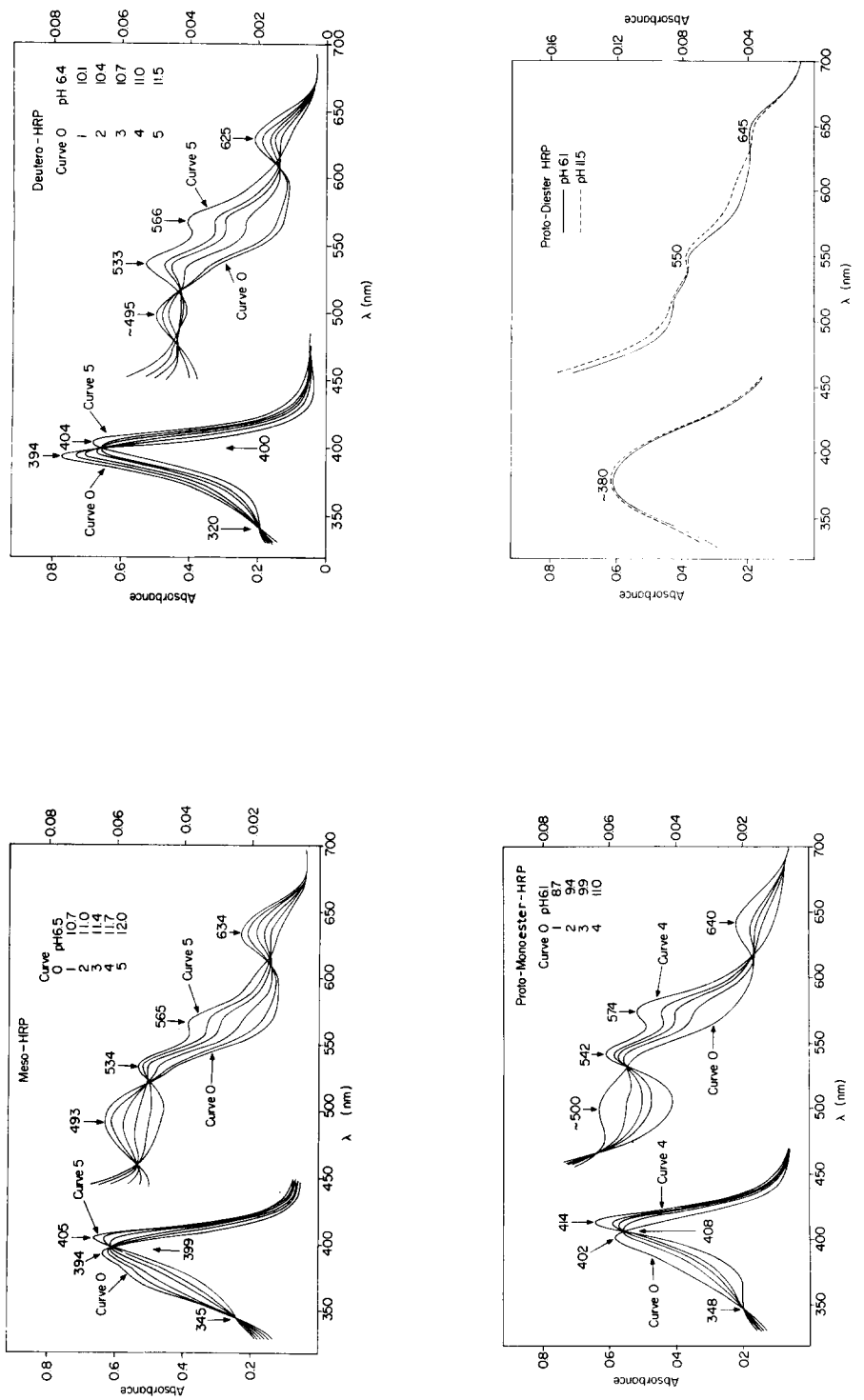


Fig. 7. Effect of pH on absorption spectra of the synthetic horseradish peroxidases (HRP) at 23 °C.

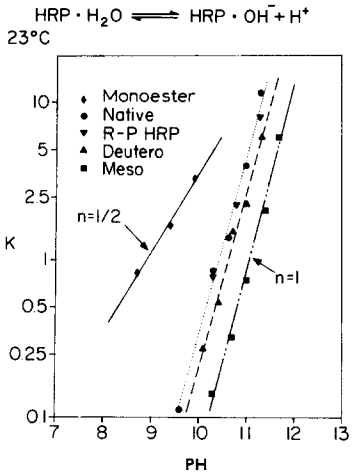


Fig. 8. The "Hill plots" of the formation of the alkaline form of the synthetic horseradish peroxidases (HRP), which were obtained from the results of Fig. 7. R-P HRP indicates the reconstituted protoenzyme. $K = Y/1-Y$ where Y is a fractional formation of alkaline form.

pH values are shown in Fig. 7. Except for the diester enzyme, the absorption spectrum of which is essentially unchanged between pH 6 and 11.5, the alkaline forms of synthetic enzymes have similar absorption spectra showing well defined $\alpha$ and $\beta$ bands. Fig. 8 shows the "Hill plot" of the formation of the alkaline form obtained from Fig. 7. From the slope of the curve, the "Hill constant" $n = 1$, was obtained for the synthetic enzymes containing proto-, meso- and deuterohemes, while that of the monoester enzyme is 0.5, suggesting that this enzyme might be a mixture of the two different components having different p$K$ values. The order of the p$K$ values is the same as that of the formation of the cyanide complex (*cf.* Fig. 6). The dissociation constants of cyanide and alkaline complexes of these synthetic enzymes were summarized in Table III.

*Enzyme activities*

The enzymic activities of the natural and synthetic enzymes in the peroxida-

TABLE III

SOME ENZYME CHARACTERISTICS OF HORSERADISH PEROXIDASES

The R.Z. value is the ratio of absorbance at 278 nm to that at the maximum of the Soret band. The enzyme activities are measured according to the method of Maehly[14] at 23 °C. The value of $k_4$ is the rate constant of the reaction between Compound II and hydrogen donor. $A_{Soret}$ is the millimolar extinction coefficient ($cm^{-1} \cdot mM^{-1}$) of the Soret band at pH 6.

| *Enzymes* | *R.Z.* | $A_{Soret}$ | *Activity (%)* | $K_{CN^-}$ *(M)* | $pK_{OH^-}$ |
|---|---|---|---|---|---|
| Native | 3.2 | 107 | 140 | | 10.5 |
| Reconstituted proto | 3.4 | 105 | 100 | $1 \cdot 10^{-5}$ | 10.5 |
| Meso | 3.1 | 83 | 92 | $2.2 \cdot 10^{-5}$ | 11.1 |
| Deutero | 4.4 | 100 | 90 | $1.1 \cdot 10^{-5}$ | 10.6 |
| Monoester | 2.4 | 103 | 18 | $7.4 \cdot 10^{-6}$ | 8.9 |
| Diester | 2.5 | 65 | $\approx$ 0.01 | $>1$ | — |

tion of guaiacol are compared in Table III. The enzyme activities, the values of $k_4$, (second-order rate constant of the reaction between Compound II and hydrogen donor) of the native and synthetic enzymes which contain proto-, meso- and deuterohemes are very similar; however, that of the monoester enzymes is only 20% of the activity of the protoenzyme. The diester enzyme has an extremely low peroxidase activity.

## DISCUSSION

The comparison of the peroxidase activities of the synthetic enzymes shows that the side chains at positions 2 and 4 of the prophyrin ring are not essential for the enzymic activity, whereas the carboxyl groups at positions 6 and 7 are essential. The deuteroenzyme, which was reported to be less than 50% active[4], retains full activity under the conditions employed here. The EPR spectrum of the diester enzyme had no rhombic splitting in the $g = 6$ region. This splitting is characteristic of the native enzyme in a high-spin state. The other synthetic enzymes showed rhombic splitting at $g = 6$ like the native enzyme.

The presence of one free heme carboxyl group appears to be essential to the enzymic activity. The diester enzyme lacks various properties of the native enzyme. For example its optical absorption spectrum is substantially different from that of the native enzyme, its reactivities with ligands are diminished and it does not react with hydroperoxide. However, the monoester enzyme retains the optical properties and ligand reactivities of the native enzyme except for a low enzymic activity (*cf.* Figs 2 and 3). In addition, this synthetic enzyme also forms the hydroperoxide compounds, Compounds I, II and III.

Brunori *et al.*[20] reported the effect of substitutions at positions 2 and 4 of heme on the formation of the alkaline form of sperm whale metmyoglobin. The effect was small. Horseradish peroxidase gives similar results. However, the monoester enzyme shows a remarkable shift of the p$K$ value towards a lower pH. It is interesting to note that the slope of the "Hill plot" of the formation of the alkaline form of the monoester enzyme gives $n = 0.5$, whereas those of the synthetic enzymes containing proto-, meso- and deuterohemes give $n = 1$. This result suggests that the sample of the monoester enzyme contains two components which behave differently towards the proton. It is likely, therefore, that the synthetic enzymes containing one free carboxyl group at position 6 or 7 might have different p$K$ values of the acid–alkaline transition. Synthetic cytochrome *c* peroxidase containing diester heme retains almost all the properties of the native enzyme.

Substitutions at positions 2 and 4 of the heme ring strongly affect the $O_2$-binding properties of hemoglobin, whereas the lack of free carboxyl groups at positions 6 and 7 does not[20–22]. Similar results were obtained with myoglobin (refs 23–25 and M. Tamura and T. Yonetani, in preparation). Horseradish peroxidase Compound III which is an oxygenated ferrous form like oxyhemoglobin[17,26,27], might not be affected by the substitutions at positions 2 and 4 and the blocking of one free carboxyl group. This result may also reflect the presence of some differences between the oxygenated form of horseradish peroxidase and those of hemoglobin and myoglobin[26,28]. Hemoglobin and myoglobin are active in the ferrous state, while horseradish and cytochrome *c* peroxidases are enzymatically active in the ferric state. The dif-

ferent effects of heme modification on these hemoproteins might be caused by the difference in their valency states. It will be interesting to compare the effects of the heme substitutions on both valency states of these hemoproteins. Such experiments are planned and will be reported elsewhere.

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