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HORSERADISH PEROXIDASE. XXXVII.

COMPOUND I FORMATION FROM RECONSTITUTED ENZYME LACKING FREE CARBOXYL GROUPS AS HEME SIDE CHAINS

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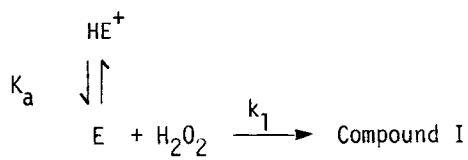
SUMMARY: Recombination of apo horseradish peroxidase with 2,4 dimethyl-deutero hemin and its mono- and dimethyl esters was performed. The number of free carboxyl side chains in these three hemins is 2, 1 and 0 respectively. Despite such a difference, all of these three reconstituted enzymes can react with H_2O_2 to produce compound I. The second order rate constants for compound I formation are 1.3×10^7 $M^{-1}s^{-1}$, 8.5×10^6 $M^{-1}s^{-1}$ and 5.9×10^6 $M^{-1}s^{-1}$. Therefore the propionate side chain of hemin has no direct role in compound I formation.

INTRODUCTION

A basic group which affects the rate of compound I formation has been reported for horseradish peroxidase (1), turnip peroxidase P_1 and P_7 (2) and cytochrome c peroxidase (3). These results have led to the following scheme for compound I formation:



The pK_a values lie between 2.5 and 5.5 for the four different peroxidases, thus the catalytic group has been regarded as a carboxylate (1-5). Artificial horseradish peroxidases (6) and cytochrome c peroxidase (7,8) reconstituted with an unnatural hemin had one or both propionate side chains esterified and also had very low enzymatic activity. Therefore the propionate side chain has been considered as a candidate for the basic

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group (2,4,9). In this paper we report on further experiments using reconstituted horseradish peroxidase designed to answer whether the propionate side chain acts as a distal group. In order to avoid subreactions with the 2,4 substituents of the hemin during esterification or hydrolysis (10) and steric hindrance between 2,4 substituents and protein (11) we used 2,4 dimethyldeutero hemin IX.

MATERIALS AND METHODS

2,4-Dimethyldeutero porphyrin IX dimethyl ester was synthesized by condensing 3,3′,4,4′,5,5′-hexamethyl-2,2′-dipyromethenium bromide and 5,5′-dibromo-3,3′-di-(2-methoxycarbonyl ethyl)-4,4′-dimethyl-2,2′-dipyrromethenium bromide in anhydrous formic acid following the procedure reported earlier (12). The crude product was esterified with methanol/H_2SO_4 and purified by chromatography on silica gel. 2,4-Dimethyldeutero porphyrin IX and its monoethyl ester were prepared by hydrolysis of the porphyrin dimethyl ester by 6N HCl (8), and were purified by chromatography on silica gel. 2,4-Dimethyldeutero hemin and its mono- and dimethyl esters were prepared by insertion of iron into the corresponding porphyrin using the $FeSO_4$/pyridine/acetic acid method (13).

Horseradish peroxidase (E.C.1.11.1.7) was purchased from Boehringer-Manheim Corp. as an ammonium sulfate precipitate (Lot Nos. 1227335 and 1366531) and was prepared for use by chromatography on Sephadex G-25. The purity numbers (PN: the ratio of absorbance at 403 nm to 280 nm) for both lots were 3.3. The major component of horseradish peroxidase obtained from Boehringer-Manheim Corp. has been determined to be isozyme C (14), according to nomenclature by Paul (15) and Shannon *et al.* (16).

Apohorseradish peroxidase was prepared according to the method of Teale (17). Recombination of apoenzyme with 2,4 dimethyldeutero hemin was performed following the method of Makino and Yamazaki (18). Since hemin esters have very low solubility in water, recombination with 2,4 dimethyldeuterohemin mono- and dimethyl esters was performed in lutidine aqueous solution, as reported by Tamura *et al.* (6) for the preparation of protohemin monomethyl ester enzyme.

Hemin mono- or dimethyl ester in lutidine (0.5 ~ 1mM) was mixed dropwise with apoenzyme dissolved in 5% lutidine water, 0.1 M phosphate pH 7.0 at 0°C until a 20% excess of the hemin compound was reached and gently stirred for 20 min. The final lutidine concentration was about 10% (v/v). After lutidine was removed by dialysis, the hemin apoenzyme complex was chromatographed on CM 52 (Whatman). The reconstituted enzyme was eluted with 0.1 M acetate pH 4.4. The eluent was concentrated using Amicon Diaflo membrane filter PM 10, and was dialyzed against five times distilled water.

Spectrophotometric measurements were carried out with a Cary 219 spectrophotometer for ordinary time scale experiments, and with a Union Giken rapid reaction analyzer RA 601 for rapid reactions. All measurements were performed at 25°C and the ionic strength in reaction solution was adjusted to 0.11 by addition of KNO_3.

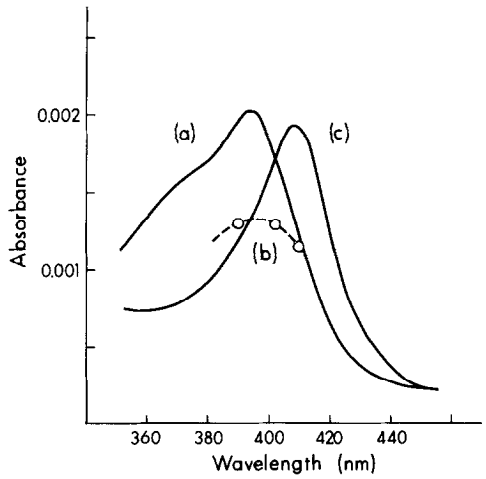

Figure 1: Spectra of 2,4 dimethyldeutero hemin dimethyl ester enzyme and its hydrogen peroxide compound. (a) Ferric enzyme, 0.4 μM, pH 7.0. (b) Compound I. Absorbance change was measured 2 sec after reaction was started by mixing 10 μM of H_2O_2. o indicates observed points. (c) Compound II, 2 min after reaction was started.

RESULTS AND DISCUSSION

Three reconstituted horseradish peroxidases containing 2,4 dimethyldeutero hemin and its mono- and dimethyl esters reacted with stoichiometric amounts of H_2O_2 to produce compound I. Fig. 1 shows that the change of spectra of 2,4 dimethyldeutero hemin dimethyl ester enzyme caused by the reaction with H_2O_2. Second order rate constants for compound I formation from the three artificial enzymes were 1.3×10^7 $M^{-1}s^{-1}$, 8.5×10^6 $M^{-1}s^{-1}$ and 5.9×10^6 $M^{-1}s^{-1}$, respectively. If the mechanism of compound I formation involves a propionate side chain acting as catalyst, the hemin diester-containing enzyme would not produce compound I. Therefore, these results imply that the propionate side chain has no fundamental significance for compound I formation.

Compound I formed from 2,4 dimethyldeutero hemin and its monomethyl ester enzymes was fairly stable in the absence of added electron donors. Less than 10% conversion into compound II was observed for these compounds I 20 min after the reaction was started with an equimolar amount of H_2O_2. However, compound I from the hemin dimethyl ester enzyme was reduced spontaneously into stable compound II with a half time of about 30 sec.

The yield of the hemin monomethyl ester enzyme was 25%, while the dimethyl ester enzyme only 1.5%. Such a low yield for the dimethyl ester enzyme however does not mean that the reconstituted enzyme included one of the other hemins as a contaminant because the properties and reactivity of this reconstituted enzyme toward ligands can be distinguished clearly from those of the others (T. Araiso and H.B. Dunford, unpublished results). These facts appear to eliminate a catalytic role for the propionate side chain and may imply that its main function is to stabilize the heme orientation.

In the amino acid sequence of horseradish peroxidase and turnip peroxidases, aspartate residue 43 is adjacent to histidine residue 42 (19,20), the imidazole group of which has been regarded as a distal base (21). Thus, the catalytic acid group is most likely the carboxylate of the side chain of aspartate residue 43. The implications of this possibility have been discussed (H.B. Dunford and T. Araiso, submitted for publication).

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