Vol. 86, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1979 Pages 190-198

THE ROLE OF PROTEIN AND PORPHYRIN IN THE REACTIVITY OF HORSERADISH PEROXIDASE TOWARD HYDROGEN DONORS

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Received November 6, 1978

SUMMARY: *The electronic ground state of the peroxidase compound I π-cation radical has been changed from $^2A_{2u}$ to $^2A_{1u}$ by substituting deuterohemin for the protohemin of the native enzyme. Although the $^2A_{1u}$ ground state is the same one as that taken by the catalase compound I π-cation radical deuterohemin horseradish peroxidase possesses no catalase activity. It thus appears that the protein and not only the ground state of the compound I π-cation radical determines the reactivities of compounds I of horseradish peroxidase toward hydrogen donors.*

INTRODUCTION: In recent years evidence has begun to accumulate that compounds I of catalase and horseradish peroxidase both contain Fe(IV) porphyrin π-cation radicals (1,2). Of the two possible porphyrin π-cation radical electronic ground states it appears that catalase compound I takes the $^2A_{1u}$ ground state and horseradish peroxidase compound I, the $^2A_{2u}$ ground state each with the characteristic visible spectrum shown in figure 1 (1). The reactivities of these enzymes toward hydrogen donors, however, differ considerably in that catalase compound I normally reacts with the two electron donor hydrogen peroxide, and horseradish peroxidase compound I with a variety of one electron donors. It was thus of interest to know whether this difference in enzymic reactivity was a function of the compound I π-cation radical ground state or unknown characteristics of the individual proteins. Accordingly horseradish peroxidase was substituted with various hemins in the hope that its π-cation radical ground state could be changed to that possessed by catalase. The complementary experiment proved impossible to perform as catalase has never been successfully reconstituted from apoprotein and protohemin (5).

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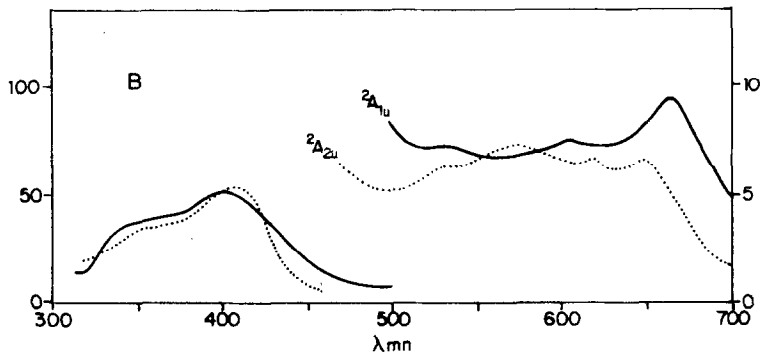


Fig. 1. Room temperature compound I spectra of catalase (———)[4] and horseradish peroxidase (------).[17]

MATERIALS AND METHODS: Horseradish peroxidase type VI protohemin and a crystalline suspension of bovine liver catalase were obtained from Sigma Chemical Co., St. Louis, Mo. 2-formyl-4-vinyl deuterohemin (*Spirographis* hemin) and 2-vinyl-4-formyl deuterohemin (Isospirographis hemin) were synthesized by the method of Clezy (6) as modified by Hamilton (7) and DiNello (8). Pure crystalline deuterohemin and diacetyldeuterohemin were synthesized by traditional methods (9,10) and purified by chromatography on polyamide as described by Chang and DiNello (11).

All other chemicals were reagent or the best commercially available grade. Protohemin was removed from peroxidase by the method of Teale (12) as modified by Yonetani (13) and the apoprotein reconstituted as described by Tamura et al (14). The time necessary for the hydrogen peroxide concentration to decrease from 10.3 mM to 9.2 mM (optical density at 240 to full from 0.45 to 0.40) was used to calculate catalase specific activity since the reaction is bimolecular and the rate depends on hydrogen peroxide concentration. Otherwise catalase and peroxidase activity assays were carried out as described in the Worthington Enzyme Manual (15).

The presence of low temperature catalase activity was assayed by incubating catalase or a substituted peroxidase (100 μgm) with hydrogen peroxide (38 μmoles) at -42° in 1 ml of a solution of 70% N,N-dimethylacetamide in

10 mM potassium phosphate buffer pH 6.0. The reaction was commenced by addition of the hydrogen peroxide in dimethylacetamide solution. Catalase activity was always accompanied by formation of gas bubbles such that the solution resembled a foam after five minutes of reaction. After one hour, 0.1 ml of the reaction solution was added to 1.9 ml of a solution of mesidine (15 mM) in 0.1 M sodium acetate pH 4.9 at room temperature. The peroxidase catalyzed oxidation of mesidine by any hydrogen peroxide remaining in solution was complete within one minute. When assaying catalase samples for low temperature activity it was necessary to add peroxidase to the mesidine solution as catalase does not oxidize mesidine.

The amount of hydrogen peroxide remaining in the assay samples was calculated using the stoichiometry of 3 molecules of hydrogen peroxide consumed for every molecule of the oxidized product of mesidine produced and an ϵ mM of 1.27×10^3 for the oxidized product of mesidine (16).

Since catalase might be inactive at -42°, but still oxidize the hydrogen peroxide present in the assay solution when added to the mesidine-peroxidase solution at room temperature, additional evidence of low temperature activity was necessary. The formation of gas bubbles in the dimethylacetamide-phosphate buffer solution was taken to be evidence of oxygen evolution. Also, addition of mesidine and peroxidase to catalase solutions at -42° produced none of the characteristic purple color of mesidine oxidation after 3 hours. Solutions of mesidine, peroxide, and horseradish peroxidase turned deep purple after 1 hour at -42°.

Low temperature spectra of compound I of hemin substituted peroxidases were obtained in 30% 3.3 mM potassium phosphate - 70% N,N-dimethylacetamide at -42°. N,N-dimethylacetamide for these experiments was distilled from anhydrous copper sulfate. A small Dewar flask with an unsilvered bottom was first charged with liquid propane. While the flask came to thermal equilibrium, the phosphate buffer (0.72 ml) containing the enzyme (approx. 0.8 mg) was mixed in the low temperature cell with the desired amount of

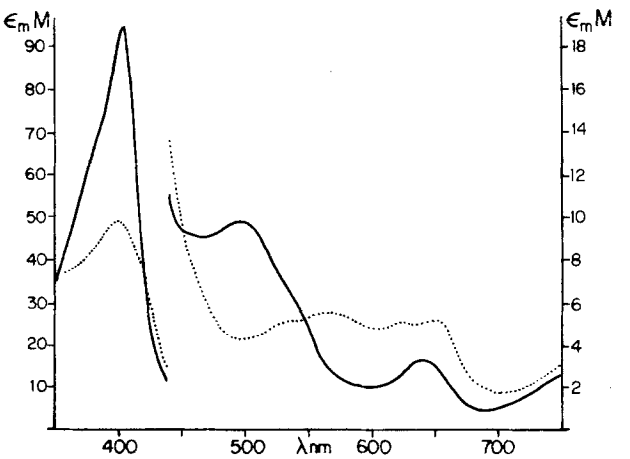
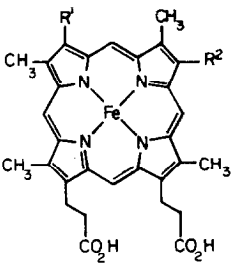


Fig. 2. Low temperature spectra of protohemin reconstituted horseradish peroxidase (———) and its compound I (------).

N,N-dimethylacetamide which had been cooled to -8 to -12° in a methanol-ice bath. The low temperature cell containing the enzyme solution was then quickly transferred to the Dewar and the Dewar placed in the modified cell compartment of a Cary 17 spectrophotometer. After recording the spectrum of the resting Fe(III) enzyme, the peroxidase was oxidized by addition of 2 µl of solution containing 1.1 eq. of H_2O_2 in N,N-dimethylacetamide. The low temperature spectrum of compound I of the enzyme was then recorded.

RESULTS: The low temperature spectrum of protohemin reconstituted horseradish peroxidase proved to be almost identical to that recorded at room temperature by Schonbaum and Lo (17) (see figures 1 and 2; see also Douzou et al 1970 (18). The protohemin reconstituted peroxidase also proved active in the peroxidation of mesidine at -42° using the visual test described in Materials and Methods. Through alternate addition of hydrogen peroxide and luminol at -42°, the enzyme could be cycled between the Fe(III) enzyme and compound I (oxidized by two electrons). After addition of luminol, the intermediacy of compound II in the reduction of compound I to the Fe(III) enzyme could be verified by low temperature spectroscopy. The catalytic



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activity and intermediate oxidized compounds of horseradish peroxidase thus remain intact in the dimethylacetamide buffer solution at -42°.

Since previous studies performed at room temperature indicated that compound I spectra of deuterohemin (**1**, $R^1=R^2=H$) and mesohemin (**1**, $R^1=R^2=CH_3CH_2-$) peroxidases were similar to those of protohemin HRP (14,19) and thus possessed the same ground state as the native enzyme, peroxidase was first substituted with hemins bearing more strongly electron-withdrawing side chains. 2-formyl-4-vinyl deuterohemin (**1**, $R^1=-CHO$, $R^2=CH_2=CH-$), 2-vinyl-4-formyl deuterohemin (**1**, $R^1=CH_2=CH-$, $R^2=-CHO$), and diacetyldeuterohemin (**1**, $R^1=R^2=CH_3\overset{O}{\overset{\|}{C}}-$), however, all possessed compound I spectra similar to that of the protohemin enzyme and thus the same ($^2A_{2u}$) electronic ground state. The compound I spectrum of deuterohemin enzyme was subsequently reexamined at -42° and found to be characteristic of a $^2A_{1u}$ ground state or catalase type π-cation radical (see figure 3). This spectrum was stable for more than an hour at -42°. The low temperature deuterohemin compound I spectrum and the catalase compound I spectrum both possess the long wavelength peak and less intense peak at shorter wavelength characteristic of $^2A_{1u}$ ground state π-cation radicals and not the broad visible absorption between 530 and 650 nm characteristic of $^2A_{2u}$ or peroxidase type π-cation radicals (1,2).

Warming of the deuterohemin HRP compound I solution to 0° for long enough to record the compound I visible spectrum (see figure 4) produced a spectrum similar to that recorded by Tamura et al (14) and Makino and

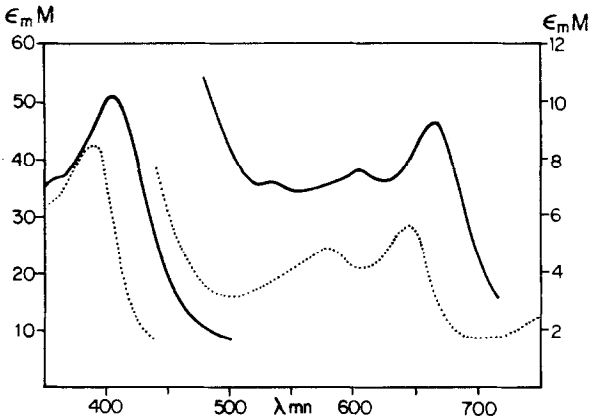

Fig. 3. Low temperature spectrum of deuterohemin reconstituted horseradish peroxidase compound I (------) and room temperature spectrum of catalase compound I (———).[4]

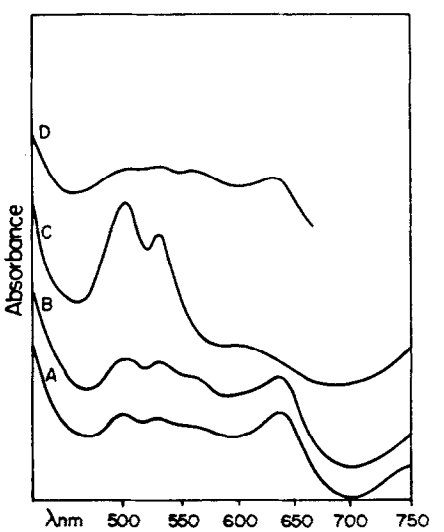

Fig. 4. A) Spectrum of deuterohemin HRP compound I prepared at -42° and warmed to 0°.

B) Spectrum of deuterohemin HRP compound I after 5 minutes at 0°.

C) Spectrum of deuterohemin HRP compound II at 0°.

D) Room temperature deuterohemin compound I spectrum recorded by Makino and Yamazaki.[19]

All spectra except D were recorded in 30% 10mM potassium phosphate pH 6.0 - 70% N,N-dimethylacetamide.

Table

Enzyme	Peroxidase activity (units/mg)	Catalase activity of reconstituted peroxidases (25°) (units/mg)	Catalase activity - μ moles H_2O_2 remaining after 1 hr incubated at -42°
Native peroxidase	3904 ± 117 n = 10	-	-
Protohemin reconstituted peroxidase	3454 ± 192 n = 10	0	38 ± 1.0
Deuterohemin reconstituted peroxidase	897 ± 52 n = 10	0	38 ± 1.0
Native catalase	-	36,200 ± 500	0.0 ± 1.0

Yamazaki (19) at room temperature. Cooling the solution back to -42°, however, did not restore the original spectrum and longer incubation of the solution at -42° produced increasing amounts of deuterohemin HRP compound II (see figure 4). It thus appears that the samples of deuterohemin HRP compound I used for the room temperature spectra recorded by other workers (14,19) (see figure 4) were contaiminated by small amounts of compound II. This hypothesis is consistent with the reported half life of deuterohemin HRP compound I (14) and the observed sensitivity of peroxidase compound I toward reducing agents which contaminate peroxidase preparations (20,21).

Although deuterohemin HRP compound I contains a $^2A_{1u}$ or catalase type π-cation radical, its activity toward hydrogen donors is unchanged from that of protohemin HRP. The table shows that both deuterohemin and protohemin HRP possess considerable activity in the peroxidation of o-dianisidine at 30°. The visual test described in Materials and Methods also showed them to be active in the peroxidation of mesidine at -42°. Neither, however, is able to decompose hydrogen peroxide at room temperature or -42°. Catalase is active in the decomposition of hydrogen peroxide at both temperatures under identical assay conditions. The activity of a compound I toward hydrogen donors is thus shown to be determined by not only the π-cation radical ground state, but the protein as well.

ACKNOWLEDGMENT: This work was supported by the United States National Institutes of Health (AM 17989).

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