Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 191-197

THE NON-PHOTOSENSITIZED POTENTIATION BY THE PHOTOSENSITIZER HEMATOPORPHYRIN OF THE HORSERADISH PEROXIDASE-CATALYZED H_2O_2-MEDIATED OXIDATION OF NADPH TO $NADP^+$

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Received November 28, 1983

Horseradish peroxidase is known to oxidize NADPH in a reaction initiated by hydrogen peroxide. The present study demonstrates that the photosensitizer hematoporphyrin acting in a non-photodynamic manner, has a marked potentiating effect on the nucleotide oxidation rate. Over 90 percent of the NADPH oxidation product is enzymatically active $NADP^+$. The ratio of NADPH oxidized to NADPH added indicates the existence of a chain reaction. It is suggested that the enzymatic generation of hematoporphyrin transients which are usually generated photodynamically, may be responsible for the acceleration in rate of reduced nucleotide oxidation.

Potentiation of enzymatic activity is a topic of considerable and continued biochemical interest. Of particular interest in this regard are the peroxidases, horseradish peroxidase being the primary model protein studied. Specifically, it has been demonstrated that horseradish peroxidase (EC 1.11.1.7) can catalyze a hydrogen peroxide-initiated oxidation of NADH (1-3) and NADPH (2), and thyroxin and some thyroxin analogues have been demonstrated to markedly potentiate the oxidation of NADH by HRP-H_2O_2 (Ref. 2 and 4).

The experiments described herein utilize the well known photosensitizer hematoporphyrin (5-10) and demonstrate that a non-photodynamic interaction exists between hematoporphyrin and the system HRP-NADPH-H_2O_2. Specifically, these experiments demonstrate that hematoporphyrin can function as a potentiator of the H_2O_2-initiated

Abbreviations: HP= Hematoporphyrin dihydrochloride; IC= DL-isocitrate; ICDH= NADP-linked isocitrate dehydrogenase, HRP= horseradish peroxidase; $^1O_2$= singlet molecular oxygen.

0006-291X/84 $1.50

oxidation of NADPH to enzymatically active $NADP^+$ catalyzed by horseradish peroxidase.

MATERIALS AND METHODS

Chemicals were purchased from the following suppliers as indicated: Hematoporphyrin dehydrochloride, horseradish peroxidase type I, isocitrate dehydrogenase (pig heart) type IV, and DL-isocitrate (sodium salt), Sigma Chemical Co.; catalase, Boehringer Mannheim; H_2O_2 (30%), Fisher Chemical Co; NADPH and $NADP^+$, P-L Biochemicals Inc.

All experiments were performed in quartz cuvettes on a Cary 219 spectrophotometer at ambient temperature with buffer in the reference cell. All solutions were made with water from a Millipore Milli-RO, Milli-Q system. A typical reaction mixture contained 10 mM potassium phosphate pH 7.4, HRP 0.33 mg/ml, HP 8 μM, and NADPH 0.1 mM, unless otherwise noted. The reaction was initiated by addition of 20 μl of 6.9 mM H_2O_2 (to give a potential initial concentration of 46 μM). The final volume was 3 ml. H_2O_2 solutions were made with water instead of buffer. Control experiments demonstrated that no significant oxidation occurred in the absence of H_2O_2, that no significant oxidation occurred in the absence of HRP, that rate of oxidation correlated with HRP concentration, and that no significant oxidation occurred when albumin was substituted for HRP. No variation in rate of NADPH oxidation occurred on rotation of the reaction cuvette into and out of the spectrophotometer beam. H_2O_2 concentrations were determined by the method of Allen et al (11). Where utilized, the following components were added as follows: catalase, 25 μl of 2 mg/ml (cuvette concentration 16.7 μgm/ml (1080 U/ml)). $MgCl_2$, 10 μl of 1500 mM (cuvette concentration 5 mM). IC, 40 μl of 600 mM (cuvette concentration 8 mM). ICDH, 10 μl of 14 mg/ml (cuvette concentration 47 μgm/ml). In the cuvette the A_{340} of catalase was 0.01, and the A_{393} was 0.024.

RESULTS

Effect of HP on NADPH oxidation rate.

The ability of HP to potentiate the oxidation of NADPH by HRP and H_2O_2 is demonstrated in Figure 1, utilizing the standard reaction mixture except for varying concentrations of HP. Curves A,B,C, and D contain 0, 8, 16, and 24 μM HP respectively. It can be seen that as the concentration of HP is increased the rate of oxidation of NADPH is increased. Similar results were obtained when NADH was utilized in place of NADPH (data not shown).

$NADP^+$ as the reaction product.

An NADPH regenerating system utilizing NADP-linked isocitrate dehydrogenase was utilized to determine if the oxidation product of the HP accelerated reaction (HP-HRP-H_2O_2-NADPH) was enzymatically active $NADP^+$. This is demonstrated in Figure 2. At arrow 1 H_2O_2 was added to initiate the oxidation, and at arrow 2 catalase was added to terminate the

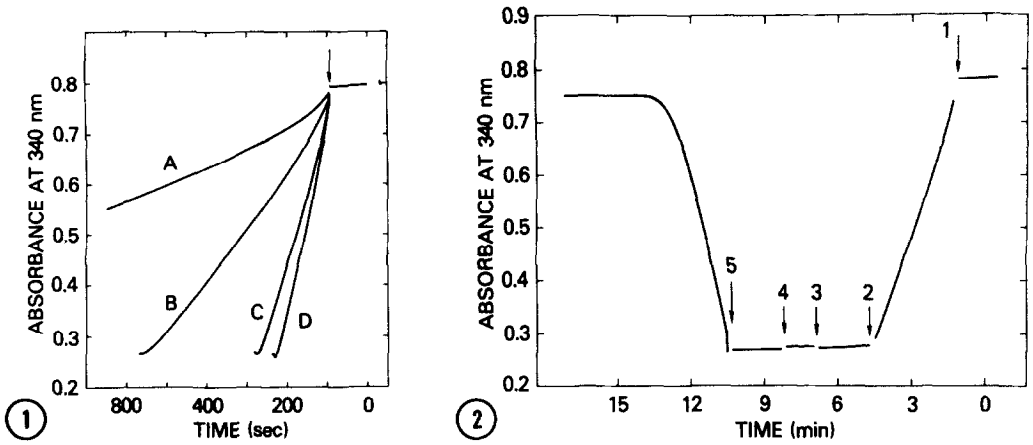

Figure 1. Effect of HP concentration on rate of NADPH oxidation by HRP-H_2O_2. In each case the cuvette contained the standard reaction mixture (as defined under Materials and Methods) except for varying concentrations of HP. The arrow signifies addition of H_2O_2 (see Materials and Methods). The HP concentrations were 0, 8, 16, and 24 μM, in A,B,C, and D, respectively. The initial A_{340} varied with HP concentration but was vertically adjusted so all curves could be presented in one figure.

Figure 2. Recovery of NADPH oxidation product as $NADP^+$. The initial reaction mixture and H_2O_2 addition was as defined under Materials and Methods. Arrow 1 signifies H_2O_2 addition to the standard reaction mixture. Other additions as follows as defined under Materials and Methods: Arrow 2, catalase. Arrow 3, $MgCl_2$. Arrow 4, IC. Arrow 5, ICDH.

oxidation. Arrows 3,4, and 5 indicate the respective additions of Mg^{++}, IC, and ICDH. As can be seen, the data in Figure 2 demonstrate that over 93% of the oxidation product was enzymatically active $NADP^+$. Recoveries of this order were a constant finding. Additionally, Figure 2 indicates that the HP induced increase in ΔA_{340}/min is due to potentiation of NADPH oxidation and not to oxidation of HP.

Spectral changes induced during NADPH oxidation.

Figure 3A demonstrates the absorption spectra of the various individual reaction mixture components (curve 1, HRP 0.33 mg/ml; curve 2, NADPH 0.1 mM; curve 3, HP 7 μM) and of all components collectively (curve 4). Figure 3B demonstrates the absorption spectra of the reaction mixture before (curve 4) and 5 minutes after (curve 5) the addition of H_2O_2. Catalase was added 5 minutes after H_2O_2 addition to prevent further oxidation. As can be seen, the major alteration in the spectrum lies at wavelengths consistent with NADPH oxidation. In contrast, the spectrum in

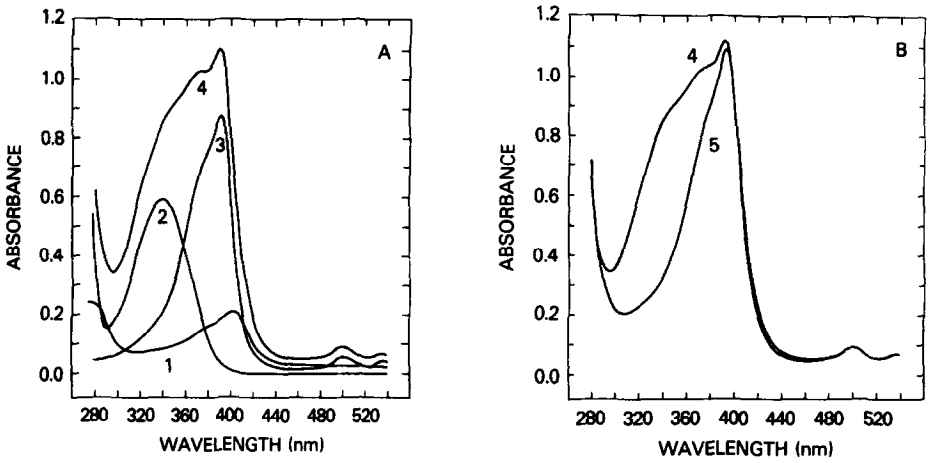

Figure 3. Absorption spectra of individual reaction mixture components, complete reaction mixture, and reaction mixture 5 minutes after oxidation initiated with H_2O_2 (oxidation halted at 5 minutes with catalase). Figure 3A: Curve 1, HRP 0.33 mg/ml. Curve 2, NADPH 0.1 mM. Curve 3, HP 8 μM. Curve 4, solution of components of curves 1,2, and 3 together. This is the standard reaction mixture except for concentration of HP. Figure 3B: Curve 4 is identical to curve 4 in Fig. 3A. Curve 5 is absorption spectrum of reaction mixture of curve 4 after addition of H_2O_2 and then catalase 5 minutes after addition of H_2O_2.

the neighborhood of the A_{393} peak is altered but slightly, suggesting relatively little modification of HP or HRP during the course of oxidation of NADPH.

Spontaneous versus catalase induced termination of oxidation.

Figure 4 demonstrates the pattern of change in A_{340} in the presence and absence of catalase. Arrow 1 in each of the four curves denotes the addition of H_2O_2 to the reaction cuvette. Curves A and B reveal the spontaneous (A) and catalase induced (B) termination of NADPH oxidation by HRP-HP-H_2O_2. Curve C reveals the effect of H_2O_2 addition to a solution of HRP and HP (without NADPH), and curve D reveals the absence of effect on A_{340} from addition of H_2O_2 to HRP alone. Experiments utilizing $NADP^+$ instead of NADPH were performed to determine if $NADP^+$ is degraded by HRP-HP-H_2O_2. $NADP^+$ 0.1 mM was substituted for NADPH in the standard reaction mixture, H_2O_2 added, and the decrease in A_{340} (identical to curve C) monitored until no further decrease occurred. Recovery of $NADP^+$ as NADPH (utilizing ICDH, Mg^+, and IC) was within 5% of that from a solution of 0.1 mM $NADP^+$ alone, indicating that $NADP^+$ is not altered by

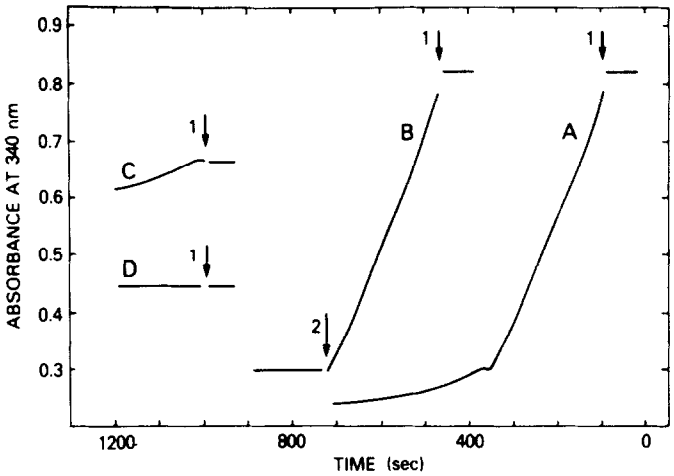

Figure 4. Spontaneous versus catalase induced termination of NADPH oxidation by HRP-HP-H_2O_2. Addition of H_2O_2 to HRP-HP alone, and to HRP alone. Arrow 1 in all cases signifies H_2O_2 addition. Component concentrations as defined under Materials and Methods. Curve A: complete reaction mixture with spontaneous termination of oxidation (no catalase added). Curve B: complete reaction mixture with reaction terminated by catalase addition at arrow 2. Curve C: change in A_{340} following addition of H_2O_2 to solution of HRP and HP. Curve D: Lack of change in A_{340} following addition of H_2O_2. The initial absorbance of all curves was vertically adjusted for purpose of clarity.

HRP-HP-H_2O_2. Collectively, the data of Figure 4 and the recovery experiments suggest that the behavior of curve A subsequent to the phase of rapid decrease in A_{340} is due to an interaction of HRP, HP, and H_2O_2 (or an H_2O_2 derived species) and not to oxidation of $NADP^+$. A control experiment demonstrated that no recovery of A_{340} was obtainable in the experiment of curve C with the use of ICDH, IC and Mg^{++}.

Evidence for chain reaction.

Yokota and Yamazaki have demonstrated that a chain reaction exists in the oxidation of NADH by HRP-H_2O_2 at pH 5.4 (Ref. 12). Table 1 indicates that a cnain reaction similarly exists for the HP potentiated oxidation of NADPH by HRP-HP-H_2O_2 at pH 7.4. If the reaction were a stoichiometric two-electron oxidation of NADPH by H_2O_2, then the NADPH/H_2O_2 ratio would be 1.

DISCUSSION

The evidence in Figures 1 and 2 indicate that HP potentiates the rate of oxidation of NADPH catalyzed by HRP and H_2O_2, and that the predominant

Table I. Evidence for a chain reaction in the HP potentiated oxidation of NADPH by HRP-HP-H_2O_2

Experiment	$[H_2O_2]$ mM	[NADPH] mM Initial	[NADPH] mM Oxidized	$\frac{[NADPH]\ oxidized}{[H_2O_2]\ added}$
1.	0.012	0.1	0.08	6.2
2.	0.023	0.2	0.17	7.3
3.	0.023	0.3	0.18	7.8

Experimental conditions as described under Materials and Methods except for variation of H_2O_2 and NADPH concentrations. The $[H_2O_2]$ values represent the potential initial concentration of H_2O_2 in the reaction cuvette after dilution of the addition aliquot.

oxidation product is enzymatically active $NADP^+$. There are a number of possible explanations for this potentiation. It is possible that HP causes a structural change in HRP which increased its catalytic efficacy. A second possibility is that HP may act as an oxidation-reduction cofactor for the reaction. In this event, the HP derived radicals $HP^{\ddagger}$ and/or $HP^{\mp}$ may serve as intermediates. Evidence for the generation of $HP^{\ddagger}$ from the reduction of tryptophan by the photogenerated HP triplet has been reported (8,9). With regard to $HP^{\mp}$ evidence has been presented for the generation of this species from the anaerobic reduction of photoexcited HP by reducing agents such as glutathione (13,14), pyrogallol (14,15), and ascorbate (14,15). Evidence for the generation of $HP^{\mp}$ and $HP^{\ddagger}$ by pulse radiolysis has similarly been presented (16,17). A third possibility is that HP is raised to the excited singlet state, which then decays to the excited HP triplet by intersystem crossing. The HP triplet can generate singlet oxygen by energy transfer (5-7), and $^1O_2$ is known to oxidize NADPH (7). The generation of eosin fluorescence has been demonstrated in the non-photodynamic NADH-oxidizing system $HRP/Mn^{++}/O_2$ indicating the elevation of eosin to the excited state singlet (18), and eosin accelerates NADH oxidation. In addition to the three mechanisms noted above, other potential mechanisms also exist. The elucidation of the HP potentiation mechanism is a topic for further experimental analysis. Derivatives of

hematoporphyrin are undergoing considerable research as photosensitizers in the photochemotherapy of malignant tumors (19). It will be of interest to determine if the non-photodynamic HP potentiation effect exists for these clinically useful HP derivatives, and similarly, to determine if the non-photodynamic potentiation effect exists with mammalian enzymes such as myeloperoxidase, thyroid peroxidase, and intestinal peroxidase (20).

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