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THE H_2O_2-MEDIATED OXIDATION OF NADPH TO $NADP^+$ CATALYZED BY THE HEME-UNDECAPEPTIDE FROM CYTOCHROME C

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NAD(P)H is known to be oxidized by singlet molecular oxygen, perhydroxyl radical, and hydroxyl radical. In marked contrast to these reactive oxygen species, NAD(P)H is stable in the presence of micromolar concentrations of H_2O_2. The experiments herein demonstrate that NADPH is rapidly oxidized by H_2O_2 in the presence of a heme-peptide. The oxidation product is enzymatically active $NADP^+$. In the absence of NADPH, the heme-peptide undergoes rapid degradation via reaction with H_2O_2. In the presence of NADPH, the reduced nucleotide is oxidized to $NADP^+$ and the heme-peptide is partially protected from oxidation. It is suggested that under certain conditions the reduced nucleotides may contribute to the protection of intracellular heme moieties from degradation engendered by endogenous or exogenous H_2O_2.

The pyridine nucleotide coenzymes NADH and NADPH are well known to fulfill a central role in the energy metabolism of all aerobic cells. The oxidation-reduction characteristics of these molecules facilitate the linkage of numerous metabolic pathways (1) and therefore, the elucidation of processes of reduced nucleotide oxidation has been in the past and continues to be at present, a subject of intensive biochemical interest (2).

Similarly, the role of reactive forms of oxygen in the biological processes has been a topic of intensive investigation. A linkage exists between some of these oxygen species and NAD(P)H, in that oxidation of NADH and/or NADPH has been demonstrated with the species $^1O_2$ (Ref. 3), $HO_2^{\cdot}$ (Ref. 4), and $OH^{\cdot}$ (Ref. 5). In marked contrast, NAD(P)H

Abbreviations: $^1O_2$ = singlet molecular oxygen; HO_2 = perhydroxyl radical; $OH^{\cdot}$ = hydroxyl radical.

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is relatively stable in the presence of millimolar concentrations of H_2O_2 (3,6).

The experiments reported herein demonstrate that NADPH undergoes an H_2O_2-mediated conversion to enzymatically active $NADP^+$ when the heme-undecapeptide from cytochrome c is present to catalyze the reaction.

MATERIALS AND METHODS

Chemicals were obtained from the following suppliers as indicated: isocitrate dehydrogenase (pig heart), DL-isocitrate, and heme-undecapeptide (microperoxidase-11, MP-11) (Sigma Chemical Co.); hydrogen peroxide (30%) (Fisher Scientific Co.); NADPH and NADH (P-L Biochemicals, Inc.). Catalase (beef liver) (Boehringer Mannheim).

Unless indicated otherwise, a typical reaction mixture contained 3.5 μM heme-undecapeptide, and 0.1 mM NADPH, in 10 mM phosphate buffer pH 7.4. The reaction was initiated with the addition of H_2O_2 as indicated, using aliquots of 20 μl or less. The final volume was 3 ml. The rates of oxidation were calculated from the recorded changes in absorbance at 340 nm, using a molar extinction of $6.2 \times 10^3\ M^{-1}\ cm^{-1}$. Concentration of H_2O_2 was determined according to the procedure of Allen et al (7).

RESULTS AND DISCUSSION

Oxidation of NADPH by H_2O_2 and Heme-11. Inhibition by catalase.

The data of Fig. 1 demonstrates that a rapid oxidation of NADPH occurs upon addition of H_2O_2 to a solution of NADPH and heme-undecapeptide. In control experiments without heme-undecapeptide, no NADPH was oxidized by H_2O_2 under the conditions utilized. Similarly, when NADPH was incubated with heme-undecapeptide in the absence of H_2O_2, the integrity of NADPH was maintained. These experiments indicate that all three system components are necessary for the occurrence of NADPH oxidation. The addition of catalase (2 μg/ml) during the course of NADPH oxidation resulted in cessation of the reaction, whereas addition of a protein control for catalase had no effect on the reaction rate. This suggests that H_2O_2 is necessary not only for the initiation but also the propagation of NADPH oxidation. When NADH was substituted for NADPH in the reaction system, virtually identical rates of nucleotide oxidation were observed (data not shown).

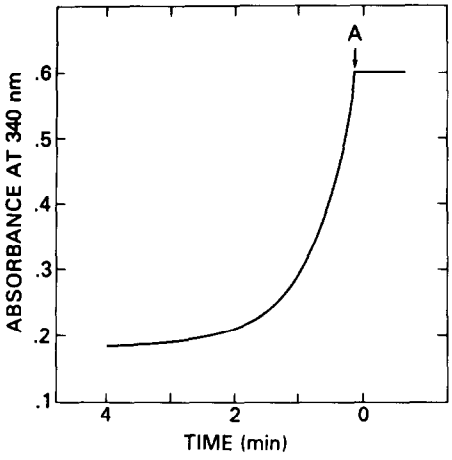

Fig 1. Oxidation of NADPH by H_2O_2 and heme-undecapeptide. Reaction mixture as under Materials and Methods. At point A, H_2O_2 was added to a final concentration in the cuvette of 46 μM.

Spectral changes induced by H_2O_2 on reaction components.

The data of Fig. 2 demonstrate the changes induced by addition of H_2O_2 on the absorption spectra of the reaction mixture and its components. Curve 1 reveals the spectrum of the reaction mixture prior to addition of H_2O_2. Curve 3 is the spectrum of heme-undecapeptide

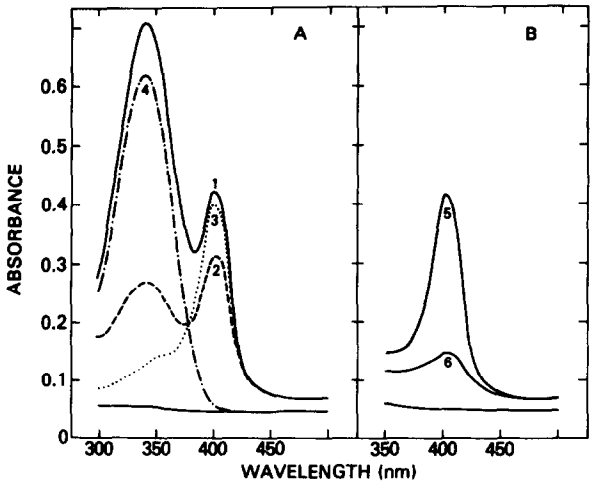

Fig. 2. Spectral changes induced by H_2O_2 on solutions of NADPH and heme-undecapeptide (A) and on heme-undecapeptide alone (B). Part A: Curve 1; reaction mixture as under Materials and Methods prior to addition of H_2O_2. Curve 2: spectrum of reaction mixture 12 minutes after addition of H_2O_2 (final concentration 46 μM). Curve 3; heme-undecapeptide, 3.5 μM. Curve 4; NADPH, 0.1 mM. Part B: Curve 5; heme-undecapeptide, 3.5 μM. Curve 6: spectrum of heme-undecapeptide (3.5 μM) four minutes after addition of H_2O_2 (46 μM). The curve at the bottom of Fig. 2A and 2B is the baseline.

alone, and curve 4 is the spectrum of NADPH alone. An examination of curve 1 relative to curves 3 and 4 demonstrate that the peak at 405 nm in curve 1 is due to heme-undecapeptide, and the peak at 340 nm in curve 1 is due primarily to NADPH. Curve 2 is the spectrum of the components of curve 1 as seen twelve minutes after the addition of H_2O_2. Curve 5 in Fig. 2B is the spectrum of heme-undecapeptide alone (identical to curve 3 in Fig. 2A). Curve 6 reveals the change induced in the heme-undecapeptide spectrum (no NADPH present) four minutes after addition of H_2O_2.

The marked H_2O_2-induced oxidation of NADPH in the complete reaction sytem is clearly seen by examination of the change in A_{340} between curves 1 and 2. In addition to the decrease at A_{340}, the absorbance at 405 nm is decreased somewhat after H_2O_2 addition to the reaction mixture and suggests a partial oxidation of heme-undecapeptide. The decrease in A_{405}, however, is much less than that occurring in the absence of NADPH. The ΔA_{405} between curves 1 and 2 is 0.1. In contrast, the ΔA_{405} between curves 5 and 6 is 0.26. These findings suggest that the presence of NADPH in the reaction mixture offers considerable protection to heme-undecapeptide.

Protection of Heme-undecapeptide Catalytic Activity by NADPH during H_2O_2 Induced NADPH Oxidation

The data of curves 3A and 3B compare the rates of NADPH oxidation after a second exposure of heme-undecapeptide to H_2O_2 under conditions where NADPH was present (3A), or absent (3B), during the initial H_2O_2 exposure. In Fig. 3A at point 1 H_2O_2 was added to give a cuvette concentration of 23 μM and the oxidation of NADPH monitored at A_{340}. An identical aliquot of H_2O_2 was added at point 2. In contrast to the rate of oxidation seen after H_2O_2 addition at point 2 in Fig. 3A, Fig. 3B demonstrates the effect of a ten minute preexposure of heme-undecapeptide to 23 μM H_2O_2 prior to addition of NADPH. At point 1 of Fig. 3B, NADPH was added to a final concentration of 0.2 mM. At point 2 of Fig. 3B, 10 μl of 6.9 mM

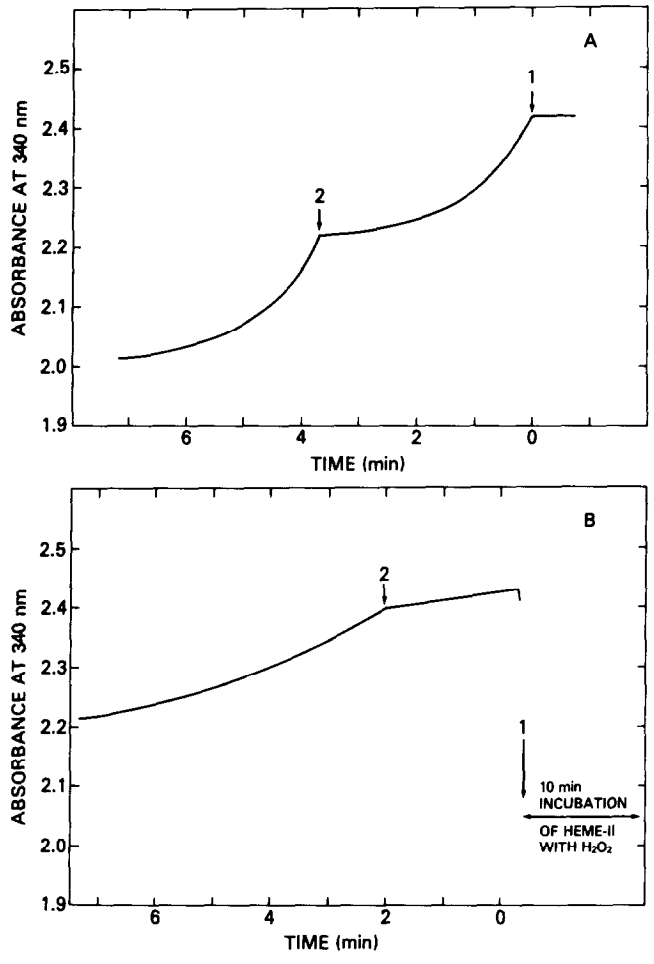

Fig. 3. Effect of NADPH on the preservation of catalytic activity in heme-undecapeptide after exposure to H_2O_2. A: Reaction mixture as under Materials and Methods except for 0.2 mM NADPH (instead of 0.1 mM). At point 1 and again at point 2, H_2O_2 was added (10 µl of 6.9 mM). B: 3.5 µM heme-undecapeptide was incubated with 0.023 mM H_2O_2 for 10 minutes. At point 1, NADPH was added (in 100 µl) to a final concentration of 0.2 mM. At point 2, 10 µl of a 6.9 mM solution of H_2O_2 was added to the cuvette.

H_2O_2 was added. A comparison of Fig. 3A with Fig. 3B with regard to the difference in ΔA_{340}/min following H_2O_2 addition at point 2 demonstrates the protective effect of NADPH oxidation against the H_2O_2 induced degradation of catalytic activity in heme-undecapeptide.

NADP$^+$ as a Product

The nucleotide product of the H_2O_2-mediated oxidation of NADPH catalyzed by the heme-undecapeptide was analyzed enzymatically.

Table 1. Estimation of enzymatically active $NADP^+$ produced in oxidation of NADPH

Expt.	[NADPH] Initial mM	$[H_2O_2]$ Initial mM	ΔA_{340} Oxidation	ΔA_{340} Recovery	% recovery in ΔA_{340}
1	0.1	0.1	-0.54	0.49	% 91
2	0.2	0.023	-0.22	0.21	95
3	0.2	0.046	-0.41	0.37	90

The procedure for oxidation of NADPH is described under Materials and Methods. For determination of $NADP^+$ produced, 10 µl of 1.5 M $MgCl_2$, 20 µl of 0.6 M DL-isocitrate, and 10 µl of NADP-dependent isocitrate dehydrogenase (14 mg/ml) were added to the reaction mixture cuvette after completion of NADPH oxidation (as determined by monitoring the decrease in absorbance at 340 nm. Regeneration of NADPH from enzymatically active $NADP^+$ was determined from the increase in A_{340} after addition of the NADPH regenerating system.

Isocitrate and NADP-dependent isocitrate dehydrogenase were added to the reaction mixture after cessation of NADPH oxidation. Table I shows that approximately 90% of the oxidized nucleotide was in the form of enzymatically active $NADP^+$.

The experiments in this study demonstrate that when heme undecapeptide is present to act as a catalyst, NADPH is rapidly oxidized by the two-election reduction product of oxygen, H_2O_2. Significantly, the NADPH oxidation product is enzymatically active $NADP^+$. The precise identification of the specific distal oxidant species responsible for NADPH oxidation is a topic for further experimental analysis. Among the alternative possibilities are a derivative of H_2O_2, a derivative of heme-peptide, and a derivative consisting of a heme-peroxide complex. Heme-undecapeptide is isolated from the parent protein by proteolytic hydrolysis (11-13), and is known to possess peroxidative activity (14-16).

The integrity of the heme moiety is essential for cell survival, and H_2O_2 is known to mediate heme degradation (8-10). Glutathione peroxidase and catalase are acknowledged to provide the primary defenses

against H_2O_2-mediated toxicity. However, the results presented herein suggest the possibility that under certain circumstances the reduced nucleotides may contribute to a final distal defense in the protection of intracellular heme moieties from the degradative effects of H_2O_2. The oxidation product $NAD(P)^+$ can be enzymatically re-reduced back to NAD(P)H, and thus further contribute in a cyclic manner to heme protection. A similar proposal has been made for the potential protective role of NADPH against toxicity to cytoplasmic components mediated by $^1O_2$ (Ref. 17).

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