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NAD(P)H OXIDATION BY HYDROGEN PEROXIDE IN *ESCHERICHIA COLI*

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A protein fraction from *Escherichia Coli* soluble extracts contain a NAD(P)H:hydrogen peroxide oxidoreductase activity. This activity is compared to and found to be distinct from well-known *E. Coli* enzymes involved in the protection from peroxides: hydroperoxidase I (HPI) and its o-dianisidine peroxidase component and the alkyl hydroperoxide reductase.

Incomplete reduction of oxygen leads to reactive species such as hydrogen peroxide, superoxide and hydroxyl radicals. These oxygen metabolites can initiate peroxidation of membrane lipids, oxidize proteins and damage DNA (1).

Enteric bacteria such as *Escherichia Coli* have several enzyme activities that protect the cells from oxidative damage. (i) Superoxide dismutase, which dismutates superoxide radicals into hydrogen peroxide and oxygen, is one of the first-line defenses and has been extensively studied. (ii) Protection from hydrogen peroxide, on the other hand, is much less documented. Two slightly different catalases, named HPI and HPII, have been isolated and characterized (2,3). They efficiently decompose hydrogen peroxide to water and oxygen. Glutathione peroxidase and ascorbate peroxidase, present in mammals and plants respectively, have not been found in *E. Coli*. The only peroxidase activity observed so far is an o-dianisidine peroxidase which was found to cochromatograph with HPI during purification (2). However, the physiological relevance of this peroxidase is doubtful since no biological electron donor could be found to be able to substitute for o-dianisidine. (iii) Recently, an alkyl hydroperoxide reductase activity has been isolated from *Salmonella Typhimurium* and *E. Coli* (4). It might be involved in the detoxification of lipid or other hydroperoxides that are produced during an oxidative stress. (iv) In addition, enzymes are involved in repairing DNA lesions resulting from oxidative damage. Some of these defenses are regulated in coordinate fashion and are induced during adaptation of *E. Coli* and *S. Typhimurium* cells to hydrogen peroxide (5).

In this paper we show that *E. Coli* contains a soluble enzyme system which efficiently catalyzes the oxidation of NADPH or NADH by hydrogen peroxide. Some properties of this NAD(P)H peroxidase are characterized which demonstrate that this activity is distinct from HPI and from the alkyl hydroperoxide reductase. This is the first report of a NAD(P)H:H_2O_2 oxidoreductase in enteric bacteria and in *E. Coli*, in particular.

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MATERIALS AND METHODS

Reagents

E. Coli C 600 was the starting material for enzyme preparations. Fraction b was prepared as previously described (6). Briefly, soluble extracts were run on DEAE Trisacryl M. The 0.2 M NaCl fraction was ammonium sulfate precipitated and then chromatographed on AcA 54. The peroxidase activity came in the run-through fraction. Protein concentration was determined by the method of Bradford with BSA as a standard (7). All chemicals were of the purest grade commercially available.

Assay of NAD(P)H peroxidase

NAD(P)H peroxidase activities were determined from the disappearance of the absorbance of NADPH at 340 nm (E_{340}= 6.2 $mM^{-1}cm^{-1}$) using a UVIKON 930 spectrophotometer. Under standard conditions, the spectroscopic cuvette contained, in a final volume of 500 µl of 50 mM Hepes, pH 7.5, 0.2 mM NADPH, 20 mM H_2O_2. The reaction was initiated by adding about 100 µg of fraction b. Any additional reagent was introduced into the reaction mixture just before addition of enzyme. Incubation was made in air and at room temperature. Oxidation of NADPH was monitored for about 1 min. A short incubation time is required to avoid formation of bubbles in the cuvette due to liberation of oxygen. One unit of activity is defined as 1 nmol of NADPH oxidized per minute.

Assay of o-dianisidine peroxidase

Identical conditions were used except that NADPH was replaced by 0.2 mM o-dianisidine. The oxidation of o-dianisidine was started by adding about 50 µg of fraction b and followed by monitoring the decay of the absorbance at 460 nm for 1 min (E_{460}= 11.3 mM^{-1} cm^{-1}).

Assay of catalase

Decomposition of H_2O_2 was followed spectrophotometrically at 240 nm using E= 43.6 M^{-1} cm^{-1}. The incubation mixture contained, in a final volume of 500 µl of 50 mM Hepes pH 7.5, 20 mM H_2O_2, in air and at room temperature. The reaction was started by adding 20 µg fraction b. Alternatively, catalase was measured in terms of oxygen production using a Clark-type oxygen electrode.

RESULTS

Characterization of a NAD(P)H:hydrogen peroxide oxidoreductase activity in E. Coli soluble extracts.

During purification of *E. Coli* enzyme systems that protect ribonucleotide reductase from oxygen radicals (8), we discovered that a protein fraction, named fraction b, catalyzed the oxidation of NADPH by hydrogen peroxide. This fraction was obtained from soluble extracts after two chromatography steps, first DEAE followed by AcA 54. The assay was routinely made by incubating 100 µg fraction b in the presence of 20 mM H_2O_2 and 0.2 mM NADPH in Hepes buffer, pH 7.6. NADPH oxidation was monitored spectrophotometrically, as indicated in the experimental part. In the absence of enzyme, no oxidation could be detected. Addition of fraction b initiated a fast and linear decrease of the absorbance at 340 nm and resulted in the complete consumption of NADPH after a few minutes. NADH was oxidized at approximately the same rate, under identical conditions. Activities of around 40-45 units were usually obtained. Activity was a function of fraction b concentration. When H_2O_2 was omitted in the reaction mixture, a weak NADPH oxidase activity was detected (<2% of the peroxidase activity). In the absence of NADPH, fraction b was able to decompose H_2O_2 into oxygen. This catalase activity was clear both from the decay of the absorbance at 240 nm, characteristic of H_2O_2, and from oxygen evolution under a Clark-type electrode. This reaction explained the quick formation of bubbles inside the cuvettes.

As shown in Figure 1, the pH optimum of the NAD(P)H peroxidase activity was 7.5. Experiments were conducted with sulfonic acid buffers since other usual buffers such as Tris or

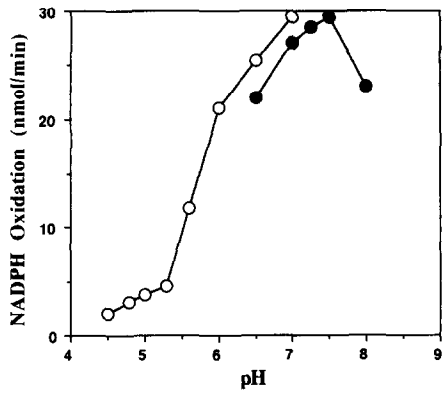


Figure 1. NADPH peroxidase activity as a function of pH. The oxidation of 0.2 mM NADPH by 20 mM H_2O_2 in the presence of 96 µg fraction b was measured in 50 mM buffers at the indicated pH. Buffering was achieved with Mes-NaOH in the pH range 4.5 to 7.0 (-○-) and with Hepes-NaOH in the range 6.5 to 8.0 (-●-).

phosphate inhibited the reaction (Table 1). Inhibition by Tris was concentration-dependent (data not shown).

In Figure 2 the dependence of the peroxidatic activity on NADPH or H_2O_2 concentrations was analyzed. From standard kinetic analysis of these data, apparent Km with respect to NADPH was found to be 30-40 µM. On the other hand, Km for H_2O_2 cannot be

Table 1. Effects of buffers, salts and metal chelators on the NAD(P)H and o-dianisidine peroxidase activities

Conditions	NAD(P)H peroxidase (%)	o-dianisidine peroxidase (%)
Complete system (Hepes)	100	100
Complete system (Tris)	46	95
Complete system (phosphate)	28	112
Complete system (Hepes)		
+ 40 mM NaCl	33	100
+ 40 mM KCl	22	93
+ 1 mM NaN_3	43	78
+ 1 mM KCN	6	24
+ 1 mM EDTA	65	100
+ 1 mM desferrioxamine	100	-
+ 1 mM ferrozine	1	-
+ 1 mM BPS	28	-

The complete system contained 100 µg fraction b, 0.2 mM NADPH, 20 mM H2O2 in 0.5 ml of 50 mM Hepes or Tris-HCl or phosphate buffer, pH 7.5. Additional reagents were salts and metal chelators, where indicated. NADPH and o-dianisidine oxidations were measured spectrophotometrically, as described in Materials and Methods. 100 % activity corresponds to 35-40 units. BPS= Bathophenanthroline-sulfonate.

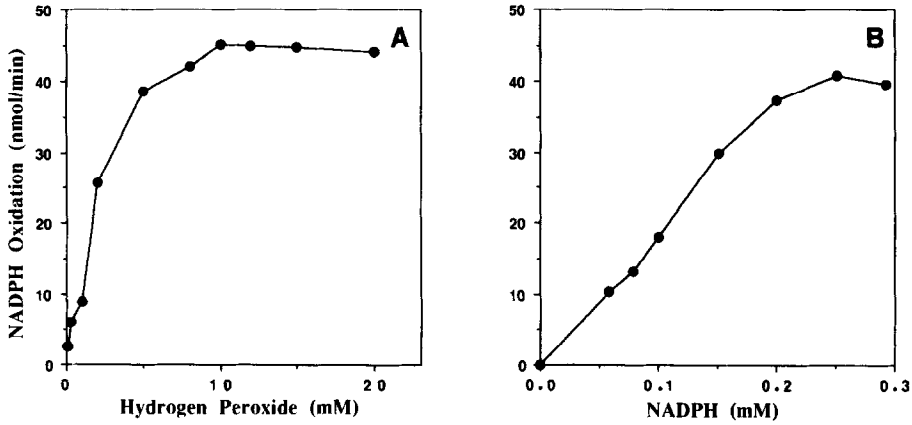


Figure 2. Dependence of the NADPH peroxidase activity on H_2O_2 (A) or NADPH (B) concentrations. Oxidation of NADPH was assayed in the presence of 96 µg fraction b as described in Materials and Methods. In panel A, NADPH concentration was fixed at 0.2 mM. In panel B, H_2O_2 concentration was fixed at 20 mM.

accurately determined because of the fast unspecific decomposition of hydrogen peroxide. Complete kinetic characterization of the NAD(P)H peroxidase requires further purification.

Oxidation of NADPH by hydrogen peroxide is due to an enzyme that is distinct from HPI

Fraction b was also found to contain a o-dianisidine peroxidase activity, similar to that previously found in *E. Coli* and shown to be identical to HPI. Experiments were thus conducted in order to test whether the NAD(P)H peroxidase is distinct or not from HPI.

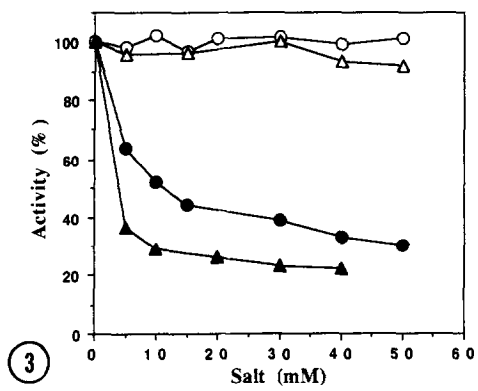
pH optimum of o-dianisidine peroxidase in fraction b was found at pH 6-6.5, as expected, and thus different from that of the NAD(P)H peroxidase. Table 1 shows that o-dianisidine peroxidase was not inhibited by Tris or phosphate buffers. Moreover, the new peroxidase activity was very sensitive to chloride salts (table 1 and Figure 3) while o-dianisidine peroxidase was not affected during incubation in the presence of the same salts. This inactivation of the NAD(P)H peroxidase was reversible since the activity was fully recovered after extensive dialysis of salted fraction b. Cyanide and, to a lesser extent, azide were efficient inhibitors of NAD(P)H peroxidase while o-dianisidine peroxidase was much more resistant. A characteristic feature of the NAD(P)H peroxidase is the strong inhibition by ferrous chelators such as ferrozine and bathophenanthroline-sulfonate (Figure 4). On the other hand, ferric chelators such as desferrioxamine had no effect on the enzyme (Table 1).

Finally, based on preliminary attempts to purify the NAD(P)H peroxidase, it was found that this activity could be separated from the o-dianisidine peroxidase on ion-exchange chromatography (Mono Q), gel filtration (Superose 6), and ammonium sulfate fractionnation (data not shown).

Substrate specificities

We have shown that H_2O_2 can support the oxidation of NADPH, NADH and o-dianisidine in fraction b. Ascorbate and glutathione could not be oxidized (data not shown).

On the other hand, NADPH in the presence of fraction b could reduce 2,6-dichloroindophenol, methylene blue and ferricyanide. However, cumyl and t-butyl



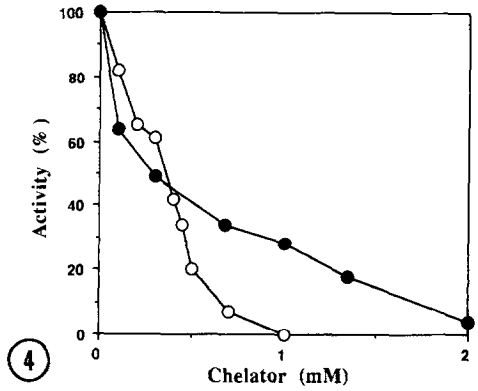


Figure 3. Inhibition of the NADPH peroxidase (closed symbols) and the o-dianisidine peroxidase (open symbols) activities by potassium (△ , ▲) or sodium (○ , ●) chloride.Reactions were carried under standard conditions described in Materials and Methods.

Figure 4. Inhibition of the NADPH peroxidase activity by ferrozine (-○-) and bathophenanthroline-sulfonate (-●-). Reactions were carried under standard conditions described in Materials and Methods.

hydroperoxide were not substrates indicating that the NAD(P)H peroxidase is distinct from the alkyl hydroperoxidase. It should be noted that H_2O_2 was not a substrate of the latter enzyme (4).

DISCUSSION

Enzyme defenses against hydrogen peroxide toxicity in *E. Coli* involve two catalases purified and characterized by I. Fridovich and named HPI and HPII. HPI has been shown to possess a dianisidine peroxidatic activity. We have detected a new activity in partially purified soluble fractions of *E. Coli* C 600 cells. It can be defined as a NAD(P)H:H_2O_2 oxidoreductase or a NAD(P)H peroxidase, since it catalyzes the oxidation of NADPH or NADH by hydrogen peroxide. We have clearly demonstrated that this activity is distinct from HPI in terms of pH optimum, sensitivity towards various salts, Tris and phosphate buffers. Preliminary experiments indicate that it is separable from the o-dianisidine peroxidase. It is also different from the alkyl hydroperoxide reductase (4) since alkyl hydroperoxides could not serve as substrates.The great inhibition of the NAD(P)H peroxidase by any chloride salt and by classical buffers certainly explain why this activity is so difficult to detect in bacterial extracts and why it has not been described earlier.

Several bacteria lacking de novo heme biosynthesis such as *Streptococcus faecalis* contain a NADH peroxidase which catalyzes the decomposition of hydrogen peroxide normally achieved by catalase (9). The enzyme from *S. faecalis* has been extensively studied and found to be a FAD-containing flavoprotein (9). It is too early for claiming that the new NAD(P)H peroxidase activity in *E. Coli* is related to such enzymes or not. Fraction b is still very crude and purification of this activity has to be revisited.Nevertheless it is tempting to suggest that *E. Coli* does not contain only one system to get rid of toxic concentrations of hydrogen peroxide and that NAD(P)H peroxidases could participate in such an important function.

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