Amphibacillus xylanus NADH Oxidase and Salmonella typhimurium Alkyl-hydroperoxide Reductase Flavoprotein Components Show Extremely High Scavenging Activity for Both Alkyl Hydroperoxide and Hydrogen Peroxide in the Presence of S. typhimurium Alkyl-hydroperoxide Reductase 22-kDa Protein Component*

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The flavoprotein NADH oxidase from Amphibacillus xylanus consumes oxygen to produce hydrogen peroxide. The amino acid sequence of this flavoprotein shows 51.2% identity to the F-52a component, denoted AhpF, of the alkyl-hydroperoxide reductase from Salmonella typhimurium. AhpF also catalyzes NADH-dependent hydrogen peroxide formation under aerobic conditions, albeit at a somewhat slower rate than the Amphibacillus protein. In the presence of the 22-kDa colorless component (AhpC) of the Salmonella alkyl-hydroperoxide reductase, both proteins catalyze the 4-electron reduction of oxygen to water. Both flavoproteins are active as AhpC reductases and mediate electron transfer, resulting in the NADH-dependent reduction of hydrogen peroxide and cumene hydroperoxide. Both enzymes' kinetic values for hydrogen peroxide, cumene hydroperoxide, and NADH are so low that they could not be determined accurately. V_{max} values for hydrogen peroxide or cumene hydroperoxide reduction are >10,000 min^{-1} at 25°C. These values are almost the same as the reduction rate of the flavoprotein component by NADH. The involvement in catalysis of a redox-active disulfide of the A. xylanus flavoprotein was shown by construction of three mutant enzymes, C337S, C340S, and C337S/C340S. Very little activity for hydrogen peroxide or cumene hydroperoxide was found with the single mutants (C337S and C340S), and none with the double mutant (C337S/C340S).

Analysis of the DNA sequence upstream of the Amphibacillus flavoprotein structural gene indicated the presence of a partial open reading frame homologous to the Salmonella ahpC structural gene (64.3% identical at the amino acid sequence level), suggesting that the NADH oxidase protein of A. xylanus is also part of a functional alkyl-hydroperoxide reductase system within these catalase-lacking bacteria.

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1 The abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

aerobic bacteria from alkaline compost (1). The bacteria have unique phenotypic and chemotaxonomic characteristics (2) as well as bioenergetic properties (3) and were named Amphibacillus xylanus (2). A. xylanus, lacking a respiratory system and hemeproteins, catalase, and peroxidase, grows well and has the same growth rate and cell yield under strictly anaerobic and aerobic conditions (2). This growth characteristic of A. xylanus is due to the presence of anaerobic and aerobic pathways producing similar amounts of ATP (4). Under aerobic conditions, NADH is thought to be responsible for maintenance of the intracellular redox balance (4).

A flavoprotein functional as NADH oxidase was purified from aerobically grown A. xylanus (5). The flavoprotein is a homotetramer composed of subunits (Mr = 56,000) containing 1 mol of FAD and also catalyzes a thiol-disulfide interchange reaction, NADH:DTNB oxidoreductase (6). The complete reduction of enzyme by dithionite requires 6 electrons/subunit (6). Such behavior indicates the presence of redox centers in addition to the FAD, and these were postulated to be disulfides (6). To assess the catalytic role of disulfide in the enzyme, two of the cysteines (Cys-337 and Cys-340), which show a high degree of homology to thioredoxin reductase, had been changed to serines by site-directed mutagenesis of the cloned flavoprotein gene (individually and in a double mutant) (7). Titration of the three mutant enzymes, lacking Cys-337, Cys-340, or both cysteines, required only 2 electron eq to reach the reduced flavin state (7). The NADH:DTNB oxidoreductase activities of all mutant enzymes were <3% of the activity of the wild-type enzyme (7). These results indicate that Cys-337 and Cys-340 participate in the NADH:DTNB oxidoreductase activity in the wild-type enzyme and demonstrate the involvement of Cys-337 and Cys-340 as the redox-active disulfide (7).

The amino acid sequence of A. xylanus NADH oxidase exhibits 51.2% identity in comparison with the alkyl-hydroperoxide reductase F-52a component (AhpF) from Salmonella typhimurium (5), a flavoprotein that has also been shown to possess NADH oxidase activity. Together with the 22-kDa protein component (AhpC) of the S. typhimurium alkyl-hydroperoxide reductase, AhpF was reported to catalyze the NADH-dependent reduction of alkyl hydroperoxides, but not H_2O_2 (5). We have now found that both S. typhimurium AhpF and A. xylanus NADH oxidase scavenge not only alkyl hydroperoxides, but also hydrogen peroxide in the presence of S. typhimurium AhpC. We describe the properties of such enzyme activities in this report.
Enzymes—The A. xylanus NADH oxidase and its mutants (C337S, C340S, and C337S/C340S) were purified as described previously (5–7). Recombinant AhpC and AhpF were purified in a manner similar to that previously published (8), using an expression system derived from the POX04 vector (9).

Reaction Stoichiometry—Experiments were performed under anaerobic conditions at 25 °C. Reaction mixtures (1.0 ml in volume), containing 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 150 mM ammonium sulfate, 2 mM NADH, 0.02% bovine serum albumin, and 97 μM (4.4 nmol) of AhpC, were loaded into anaerobic cuvettes with two side arms, in one of which was placed flavoprotein (61.6 μM flavin) and in the other varying amounts of cumene hydroperoxide or tert-butyl hydroperoxide. After establishing anaerobiosis by repeated evacuation and equilibration with oxygen-free argon and equilibration at 25 °C, the reaction was started by first mixing the two enzymes, followed by cumene hydroperoxide or tert-butyl hydroperoxide from the second side arm of the cuvette. After the reaction was complete, as judged by no further changes in the 300–400 nm region, the alkyl hydroperoxide content of the reaction mixtures, including that of control experiments without enzyme carried through the same anaerobic process, was analyzed by the methods of Tagaki et al. (10) and Jacobson et al. (11). In evaluating the stoichiometries of NADH reduction on addition of a limiting amount of hydrogen peroxide in anaerobic turnover experiments, standardization of the hydrogen peroxide was carried out by addition of 6–10 μl of a stock hydrogen peroxide solution (88 mM) to a 1-ml reaction mixture containing 0.03% o-dianisidine and 20 μg of horseradish peroxidase (12). The stoichiometry of the NADH oxidase reaction was determined aerobically under conditions of limiting NADH (21, 41, and 62 nmol), and the amount of hydrogen peroxide produced was assayed using the o-dianisidine/horseradish peroxidase system (12).

NADH-dependent Oxidase and Peroxidase Activities—Oxygen consumption by the enzymes was determined at 25 °C as described previously (5). The oxidase activity of NADH oxidase was measured at 25 °C as described previously (5). The oxidase activity of AhpF was measured in a total of 1 ml of 25 mM potassium buffer, pH 7.0, containing 0.5 mM EDTA, 300 mM ammonium sulfate, 1.12 μM flavoprotein (A. xylanus NADH oxidase or S. typhimurium AhpF), and 70.4 μM AhpC, was loaded into a tonometer. After establishing anaerobiosis by repeated evacuation and equilibration with oxygen-free argon and equilibration at 25 °C, the reaction was started by mixing the protein solution with varying mixtures of NADH and peroxide substrates, and the reaction was monitored at 340 nm. The NADH/peroxide mixture, containing 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM EDTA, 0.06–2 mM hydrogen peroxide or cumene hydroperoxide, and 150–600 μM NADH concentrations were halved (after mixing in the stopped-flow spectrophotometer), was bubbled with oxygen-free argon at 25 °C. Although most of these determinations were carried out under anaerobic conditions, to avoid possible complications from reaction with oxygen, we found that in the presence of AhpC, the peroxide reductase activities were in fact the same in air-saturated solution as those determined anaerobically. We did not bubble argon into NADH/hydrogen peroxide mixtures in the experiments in which the effect of changing salt concentrations was evaluated.

RESULTS

NADH Oxidase Activity—Under aerobic conditions, the purified A. xylanus NADH oxidase consumes oxygen to produce hydrogen peroxide (5), and the Vmax value is 119 s–1 at pH 7.0 and 25 °C (6). Our analyses of the purified recombinant AhpF protein of the alkyl-hydroperoxide reductase system of S. typhimurium indicate that, like A. xylanus NADH oxidase, this flavoprotein also catalyzes the NADH-dependent reduction of oxygen to hydrogen peroxide, at a rate of 1.23 units/mg at pH 7.0 and 25 °C, under standard assay conditions (200 μM NADH and 260 μM O2). This corresponds to an apparent turnover number of 1.07 s–1, which is much lower than the kcat value (obtained by extrapolation to infinite concentrations of both substrates) of 119 s–1 for the Amphibacillus flavoprotein. While a complete kinetics study has not yet been done with the Salmonella flavoprotein, it would appear that it is indeed less reactive with O2 than the Amphibacillus enzyme since under the same assay conditions, the latter enzyme has an observed turnover of 15 s–1. As with the NADH oxidase, a stoichiometry of 1.06 ± 0.10 (n = 3) mol of hydrogen peroxide produced per mol of NADH oxidized was observed with AhpF. Free FAD also stimulates this measured oxidase activity as previously demonstrated for A. xylanus NADH oxidase (turnover increases to 6.4 and 10.1 s–1 on addition of 5 and 10 μM FAD, respectively).

NADH Peroxidase Activity for Hydrogen Peroxide—The ability of NADH oxidase and AhpF to catalyze the reduction of hydrogen peroxide in the presence or absence of S. typhimurium AhpC was tested using an anaerobic stopped-flow assay system allowing continuous monitoring of NADH oxidation in the presence of peroxide substrates such as hydrogen peroxide. Although earlier reports using an aerobic assay system stated that hydrogen peroxide was not a substrate for the S. typhimurium alkyl-hydroperoxide reductase system, our results clearly indicate that hydrogen peroxide is reduced through electron transfer from NADH that requires both AhpF and AhpC (Fig. 1). Furthermore, the A. xylanus flavoprotein is able to substitute for AhpF from S. typhimurium in catalysis of hydrogen peroxide reduction to give a turnover number of similar magnitude (Fig. 1). The Vmax values for the A. xylanus flavoprotein and S. typhimurium AhpF were 185 and 249 s–1, respectively, extrapolated to saturating AhpC concentrations.

The dependence of activity on the AhpC concentration for NADH:hydrogen-peroxide reductase activities of A. xylanus NADH oxidase and S. typhimurium AhpF were 118 and 145 s–1, respectively.

![Fig. 1. Time course for the oxidation of NADH by hydrogen peroxide in the presence of A. xylanus NADH oxidase or S. typhimurium AhpF.](image)

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were too low to allow accurate determination of values; the rates were the same with NADH in the concentration range 75–300 mM and with H2O2 in the range 0.03–1 mM. From the shapes of the progress curves, such as those of Fig. 1, upper limit $K_m$ values of 10 mM for H2O2 and 20 mM for NADH can be estimated. The stoichiometry of NADH oxidation relative to limiting hydrogen peroxide added from a standardized stock solution was 113.5 nmol of NADH to 105.4 nmol of hydrogen peroxide (1.08:1) in assays containing both AhpF and AhpC.

Studies employing an oxygen electrode to directly monitor oxygen consumption also confirmed hydrogen peroxide-producing oxidase activities for both the NADH oxidase and AhpF flavoproteins in the absence of the AhpC component, although considerably more AhpF was required in these studies to achieve rates similar to the oxidase (2.3 nmol of AhpF gave rates comparable to those with 0.22 nmol of NADH oxidase). On addition of catalase following full reaction with limiting oxygen, excess NADH (600 mM), and NADH oxidase (Fig. 2) or AhpF (data not shown), oxygen was produced, indicating the formation of hydrogen peroxide during turnover with oxygen. When the same experiments were repeated in the presence of AhpC, however, no oxygen was detected following addition of catalase (Fig. 2), indicating that no hydrogen peroxide accumulated under these conditions. That oxygen consumption was the result of 2-electron reduction in the presence of flavoprotein only or 4-electron reduction in the presence of AhpC and either flavoprotein was confirmed by determination of the stoichiometry of NADH reduction relative to oxygen consumption in the presence of limiting NADH amounts. Thus, in experiments conducted with AhpF in the absence or presence of AhpC, the ratios of NADH oxidation relative to oxygen consumed were 1.11 and 2.35, respectively. The overall reaction catalyzed by both flavoproteins (AhpF and A. xylanus NADH oxidase) in the presence of AhpC is envisaged to occur as outlined in Scheme 1 (see "Discussion" for further details).

NADH Peroxidase Activity for Cumene Hydroperoxide—Peroxidase assays using cumene hydroperoxide as substrate under anaerobic conditions were also carried out to confirm the ability of A. xylanus NADH oxidase to substitute for AhpF in alkyl-hydroperoxide reductase assays with AhpC (Fig. 3). Again, neither flavoprotein nor AhpC alone supported facile reduction of the hydroperoxide by NADH (data not shown), while both flavoproteins were able to catalyze such reduction in the presence of AhpC (Fig. 3). The extrapolated $V_{max}$ values for the A. xylanus flavoprotein and AhpF at saturating concentrations of AhpC were 170 and 238 s$^{-1}$, respectively. These $V_{max}$ values were also almost the same as the reduction rate of the flavoprotein by NADH. The $K_m$ values for AhpC in its reaction with the A. xylanus flavoprotein and AhpF were 15 and 23 mM, respectively. However, the $K_m$ values for cumene hydroperoxide and NADH were again too low to allow their determination, with upper limit values of 10 and 20 mM, respectively.

Stoichiometric electron transfer by A. xylanus NADH oxi-
Activities of wild-type and mutant C340S, C337S, and C340S/C337S were measured under anaerobic conditions at 25 °C. 0.56 μM A. xylanus NADH oxidase (dashed line), mutant C340S (solid line), mutant C337S (dotted line), or mutant C340S/C337S (dashed-dotted line) and 35.2 μM AhpC were mixed with 0.5 mM hydrogen peroxide and 150 μM NADH and monitored at 340 nm. Conditions are described under “Experimental Procedures.” The hydrogen-peroxide reductase activities of wild-type and mutant C340S, C337S, and C340S/C337S A. xylanus NADH oxidases were 117, 0.4, 0.2, and 0.0 s⁻¹, respectively.

As a direct indication of the stoichiometric transfer of electrons from NADH to AhpC via the A. xylanus NADH oxidase, anaerobic NADH titration of AhpC in the presence of a catalytic amount of the flavoprotein was carried out, followed by DTNB assay to detect protein thiols. Anaerobic titration of AhpC in the presence of a 200-fold lower amount of the A. xylanus flavoprotein resulted in the oxidation of 1.06 eq of NADH/subunit of AhpC (data not shown). Thus, alkyl-hydroperoxide reductase activity is also supported by the A. xylanus NADH oxidase flavoprotein in the presence of AhpC. As a direct indication of the stoichiometric transfer of electrons from NADH to AhpC via the A. xylanus NADH oxidase, anaerobic NADH titration of AhpC in the presence of a catalytic amount of the flavoprotein was carried out, followed by DTNB assay to detect protein thiols. Anaerobic titration of AhpC in the presence of a 200-fold lower amount of the A. xylanus flavoprotein resulted in the oxidation of 1.06 eq of NADH/subunit of AhpC (data not shown). The DTNB assay for free thiols indicated the generation of 1.83 thiol/subunit of AhpC, fully consistent with results obtained earlier using the AhpF flora-protein from S. typhimurium instead of the A. xylanus flavoprotein (8).

NADH Peroxidase Activity of Mutant NADH Oxidases—We had postulated that Cys-337 and Cys-340 of the A. xylanus flavoprotein are involved in the flow of electrons from NADH to a second redox disulfide center (6). To test our hypothesis, we had constructed three mutant enzymes, C337S, C340S, and C337S/C340S, in which Cys-337 and Cys-340 had been replaced by alanine. These results parallel the loss of DTNB reductase activity for these mutants and indicate that Cys-337 and Cys-340 are involved in the NADH-peroxide reductase activity in the wild-type enzyme in the presence of AhpC.

Effect of Ionic Strength on Peroxidase Activities—The peroxidase activity of NADH oxidase/AhpC mixtures was markedly dependent on ionic strength. All the data above were obtained in the presence of optimal concentrations of ammonium sulfate (150 mM). The V_max and K_M values for AhpC of 185 s⁻¹ and 13.5 μM quoted above were obtained under these conditions. When the NADH-hydroperoxide reductase activity was measured in the absence of ammonium sulfate, but with a high concentration of AhpC (35.2 μM), the activity was only ~20% of that in the presence of 150 mM ammonium sulfate, and the activity was found to be markedly dependent on the salt concentration (Fig. 5). Fig. 5 also shows the effect of other salts on the basal activity obtained with the standard buffer (50 mM phosphate buffer, pH 7.0) used throughout this work, expressed as a function of the total ionic strength. While there is clearly a general ionic strength effect, the stimulation effect is greatest with phosphate and least with chloride. This order is the same as that found for enhancing hydrophobic interactions (13) and suggests that the activity stimulation by salts is due to enhancing the hydrophobic interaction between the flavoprotein and the simple disulfide protein, AhpC. The interaction between the flavoprotein component of AhpF from S. typhimurium and AhpC from the same organism is less dependent on ionic strength. While we have not performed a complete study, in the absence of additional salt, the V_max value for the AhpF-AhpC system for both hydrogen peroxide and cumene hydroperoxide was 80 s⁻¹. This V_max value was almost the same as that in the presence of 150 mM ammonium sulfate. However, the K_M values for AhpC in hydrogen peroxide and cumene hydroperoxide assays were 50 and 62 μM, respectively, over twice as great as the corresponding values reported above in the presence of 150 mM ammonium sulfate. Thus, with both enzyme systems, the interaction between the flavoprotein and...
AhpC appears to be significantly dependent on hydrophobic interactions.

**DISCUSSION**

The A. xylanus NADH oxidase flavoprotein has unique functional properties that are different from other known NADH oxidases (14–20). The flavoprotein was shown to catalyze electron transfer between NADH and DTNB, and the complete reduction of the enzyme by dithionite required 6 electron eq/mol of enzyme-bound flavin (6). Such behavior indicates the presence of redox centers in addition to the FAD, and these were shown to be two disulfides (7).

The alkyl-hydroperoxide reductase of S. typhimurium was first recognized through the isolation of mutant cells that were resistant to mutagenesis by alkyl hydroperoxide (11), and its scavenging activity for hydrogen peroxide was not found in early research (11). The A. xylanus NADH oxidase is thought to function in vivo to regenerate NAD from NADH produced in the aerobic pathway, and hydrogen peroxide was shown to be the final product in the isolated NADH oxidase reaction (5). These two enzyme systems were presumed to be unable to reduce hydrogen peroxide. However, in the presence of saturating concentrations of AhpC, the V_{max} values for hydrogen peroxide and alkyl hydroperoxide are almost the same as the reduction rate by NADH of the enzyme-bound FAD in the NADH oxidase, suggesting that these values may be limited by the reduction rate of the flavoprotein component. K_{m} values for hydrogen peroxide, cumene hydroperoxide, and NADH are too low to allow accurate determination of their values in these experiments. Several enzymes that show scavenging activity for hydrogen peroxide and alkyl hydroperoxides have been purified and characterized from bacteria or mammalian sources (9, 11, 21–26). The V_{max} values for the flavin-containing NADH oxidase from Streptococcus faecalis is 121 s^{-1} at pH 5.4 (9). The K_{m} values for hydrogen peroxide of this enzyme are 130 μM at pH 5.4 and 10.1 μM at pH 7.5 (9). The K_{m} value for hydrogen peroxide of horse liver catalase is 1.1 μM (21). The K_{m} value for cumene hydroperoxide with the glutathione peroxidase from pig erythrocytes is 40 μM at pH 8.0 (22). None of these enzymes has been reported to show such high turnover numbers and low K_{m} values for both hydrogen peroxide and alkyl hydroperoxide as the enzymes described here.

Amphibacillus sp. are Gram-positive microorganisms and taxonomically far distant from Gram-negative microorganisms, such as Salmonella sp. However, the amino acid sequences of Amphibacillus NADH oxidase and Salmonella AhpF show a high identity of 51.2% (5). In the absence of AhpC, Salmonella AhpF also shows NADH oxidase activity that is accelerated in the presence of additional free FAD, in a manner similar to that of A. xylanus NADH oxidase. Furthermore, in the presence of S. typhimurium AhpC, both enzymes catalyze the NADH-dependent 4-electron reduction of oxygen to water (Scheme 1) or the 2-electron reduction of hydrogen peroxide or alkyl hydroperoxides to water or the respective alcohol products (Scheme 2). The two peroxidase activities of NADH oxidase and AhpF in the presence of AhpC give turnover numbers of similar magnitude. The efficiency of the two-protein components of Schemes 1 and 2 deserves comment. The overall rate of NADH:peroxide reductase activity, with H_{2}O_{2} or cumene hydroperoxide, appears to be limited by the rate of flavin reduction by NADH. The bimolecular rate constant for the binding of NADH in this reaction, derived from k_{cat}/K_{m(NADH)} is at least 1 × 10^{7} M^{-1} s^{-1} at 25 °C. What is even more remarkable, however, is the efficiency of electron transfer between the reduced flavin and the Cys-337–Cys-340 disulfide of the flavoprotein and the thiol-disulfide interchange with the disulfide of AhpC. All of these events must occur considerably faster than the limiting rate of flavin reduction, i.e. >200 s^{-1} at 25 °C.

With A. xylanus, which lacks a respiratory chain, NADH oxidase should regenerate NAD from NADH formed in the aerobic pathway (Scheme 3), and a lot of hydrogen peroxide is presumed to be produced in aerobic growing cells. Despite lacking catalase, A. xylanus can grow well under aerobic conditions (2). The location of a partial open reading frame upstream of the NADH oxidase locus (from positions −15 to −312; Ref. 5) with homology to S. typhimurium AhpC (64.3% identity of the deduced amino acid sequences; Ref. 34 and Fig. 6) leads us to suggest that the A. xylanus NADH oxidase protein also exists as one component of an alkyl-hydroperoxide reductase system in A. xylanus.

The region of the NADH oxidase surrounding Cys-337 and Cys-340 is highly conserved with respect to Escherichia coli thioredoxin reductase (6, 28). This region of thioredoxin reductase contains the redox-active disulfide, which is composed of Cys-135 and Cys-138 (29). The steady-state kinetic analysis of two active-site mutants of thioredoxin reductase, Ser-135,Cys-138 and Cys-135,Ser-138, shows 10 and 50%, respectively, of the thioredoxin reductase activity of the wild-type enzyme (30). Williams and co-workers (30) suggested that the remaining thiol can carry out interchange with the disulfide of thioredoxin, and the resulting mixed disulfide can be reduced by NADH via FAD. On the other hand, the single mutants of NADH oxidase (C337S and C340S) show very little activity for hydrogen peroxide and cumene hydroperoxide, and no activity was observed in the double mutant (C337S/C340S). These results indicate that neither the remaining thiol nor the second disulfide alone has thiol-disulfide interchange activity with AhpC. It is clear that thiol-disulfide interchange reactions also differ from that of thioredoxin reductase, in spite of the high degree of conservation around the active-site cysteines. We found that the mutants require only 2 electron eq to reach the reduced flavin state (7), while the complete reduction of the wild-type enzyme requires 6 electron eq (6). Thus, electrons from FADH_{2} must pass through Cys-337 and Cys-340 to reduce

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the second disulfide of the NADH oxidase or the disulfide of AhpC.

Rhee and co-workers (31-34) have purified a 25-kDa protein from yeast and rat brain that can participate in the reduction of hydrogen peroxide or alkyl hydroperoxides and named this protein thiol-specific antioxidant. The 25-kDa protein, whose sequence is 40% identical to that of AhpC (33, 34), was shown to be a peroxidase that reduces hydrogen peroxide and alkyl hydroperoxides with the use of reducing equivalents derived from thioredoxin, thioredoxin reductase, and NADPH (35). No inactivation by peroxides was observed in the NADH oxidase-AhpC or AhpF-AhpC systems, and the peroxidase reductase activity for both substrates appears to be limited in rate by the reduction by NADH of the flavin in the NADH oxidase component.2

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