Purification and Characterization of Wild-type and Mutant “Classical” Nitroreductases of Salmonella typhimurium

**L33R MUTATION GREATLY DIMINISHES BINDING OF FMN TO THE NITROREDUCTASE OF S. TYPHIMURIUM**

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The **“Classical” nitroreductase of Salmonella typhimurium** is a flavoprotein that catalyzes the reduction of nitroaromatics to metabolites that are toxic, mutagenic, or carcinogenic. This enzyme represents a new class of flavin-dependent enzymes, which includes nitroreductases of Enterobacter cloacae and Escherichia coli, flavin oxidoreductase of Vibrio fischeri, and NADH oxidase of Thermus thermophilus. To investigate the structure-function relation of this class of enzymes, the gene encoding a mutant nitroreductase was cloned from *S. typhimurium* strain TA1538NR, and the enzymatic properties were compared with those of the wild-type. DNA sequence analysis revealed a T to G mutation in the mutant nitroreductase gene, predicting a replacement of leucine 33 with arginine. In contrast to the wild-type enzyme, the purified protein with a mutation of leucine 33 to arginine has no detectable nitroreductase activities in the standard assay conditions and easily lost FMN by dialysis or ultrafiltration. In the presence of an excess amount of FMN, however, the mutant protein exhibited a weak but measurable enzyme activity, and the substrate specificity was similar to that of the wild-type enzyme. Possible mechanisms by which the mutation greatly diminishes binding of FMN to the nitroreductase are discussed.

Nitroreduction is an initial step in the metabolism of a variety of structurally diverse nitroaromatic compounds, including nitrofurans, nitropyrines, and nitrobenzenes (1–5). Enzymes that catalyze this process are termed nitroreductases and are classified into two groups: oxygen-sensitive and oxygen-insensitive (6). The former enzymes, such as NADPH-cytochrome P-450 oxidoreductase (EC 1.6.2.4) and NADPH-b5 oxidoreductase (EC 1.6.2.2), catalyze the one-electron reduction of nitro moieties in which case the anion free radicals are formed (7–9). These enzymes are termed oxygen-sensitive, because the resulting radicals are easily reoxidized to the parent compounds by O₂ in a futile redox cycle, which generates superoxide. Thus, these enzymes can mediate the reduction of nitroaromatics only under anaerobic conditions (3). The latter enzymes, such as NAD(P)H-quinone oxidoreductase (formerly called DT-diaphorase, EC 1.6.99.2) and nitroreductases of enteric bacteria, catalyze the two-electron reduction of the nitro moiety through nitroso and hydroxylamine intermediates to the fully reduced amino compounds (10, 11). Although this process does not produce superoxide, some of the hydroxylamine intermediates are mutagenic and carcinogenic (12, 13).

In the strains of *Salmonella typhimurium* used in the Ames mutagenicity test, a “classical” nitroreductase plays an important role in the reductive metabolic activation process (2). In fact, *S. typhimurium* TA98NR, a nitroreductase-deficient strain, is resistant to both the killing and mutagenic effects of nitroarenes, whereas *S. typhimurium* YG1021, a nitroreductase-overproducing strain, is extremely sensitive to the effects (14, 15). The latter strain was constructed in this laboratory by introducing a multicopy number plasmid carrying the gene encoding the nitroreductase of *S. typhimurium* into an Ames tester strain TA98 (16). The enzyme is termed classical because other nitroreductases were identified in *S. typhimurium* later (17). The NfsB protein of *Escherichia coli*, which is about 90% homologous to the nitroreductase of *S. typhimurium*, has been used in antibody-directed enzyme prodrug cancer therapy, because it can activate a nitroaromatic monofunctional prodrug CB1954 (18–20).

In 1990, we identified the nucleotide sequence of the gene encoding the nitroreductase of *S. typhimurium* and estimated that the enzyme is composed of 217 amino acids with a calculated M₀ of 23,955 (21). Since then, several enzymes have been reported to share similarities with the deduced amino acid sequence of the nitroreductase of *S. typhimurium*. Such enzymes include the oxygen-insensitive, flavin-dependent nitroreductases of *Enterobacter cloacae* (22) and *E. coli* (18, 20) and flavin reductases of *Vibrio fischeri* (23). In addition, significant levels of similarities to the *Salmonella* enzyme have been observed in NADH oxidase of *Thermus thermophilus* (24), DrgA of *Synechocystis* sp. (GenBank™ accession numbers L29426 and D90910) (25), a putative flavin reductase of *Heliobacter influenzae* (National Center for Biotechnology Information ID B64116) (26) and a putative nitroreductase of a Mycoplasma-like organism (GenBank™ accession number L22217). The NADH oxidase of *Thermus thermophilus* reduces a number of nitro compounds and contains flavin as a cofactor (27). DrgA controls resistance to metronidazole, a nitroaromatic compound, in Cyanobacterium, suggesting that DrgA also participates in nitroreduction. These bacterial enzymes are functionally similar to mammalian NAD(P)H-quinone oxidoreductase, in that they are oxygen-insensitive and flavin-dependent enzymes (28). However, no sequence similarities are observed between the bacterial enzymes and NAD(P)H-qui-
none oxidoreductase (29–31). In addition, the bacterial enzymes tightly associate with FMN (11, 20, 31, 32), whereas FAD is a prosthetic group in the mammalian enzyme (33, 34). Thus, it is suggested that the bacterial enzymes represented by the nitroreductase of *S. typhimurium* constitute a separate class of flavoproteins.

To investigate structure-function relation of this class of enzymes, we cloned the gene encoding a mutant nitroreductase from *S. typhimurium* strain TA1538NR and compared the enzymatic properties between the mutant and wild-type nitroreductases. The parent strain of TA1538NR, i.e. *S. typhimurium* TA98NR, was isolated as a mutant resistant to the killing effects of nitrofurantoin (35). The purified mutant nitroreductase showed a reduced affinity for flavin, suggesting that the replacement of leucine 33 with arginine, which was found in the mutant nitroreductase gene, causes destabilization of folding FMN in the enzyme. Based on the amino acid sequence similarities between the nitroreductase of *S. typhimurium* and NADH oxidase of *T. thermophilus*, we discuss the possible mechanisms by which the mutation reduces the affinity of the enzyme for FMN.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Enzymes—**NADH, NADPH, NADP⁺, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). Nitrofurazone, p-nitrophenol, p-nitrobenzoic acid, p-nitroacetophenone, and menadione were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). FMN and FAD were obtained from Boehringer Mannheim (Mannheim, Germany). Riboflavin was obtained from Wako Pure Chemical (Osaka, Japan). Restriction endonucleases and T4 DNA ligase were obtained from Nippon Gene (Toyama, Japan).

**Cloning of the Mutant Nitroreductase Gene from S. typhimurium Strain TA1538NR—**Genomic DNA of *S. typhimurium* strain TA1538NR was prepared and digested completely with restriction endonucleases EcoRI and PstI. A 5.65-kilobase pair DNA fragment was recovered from agarose gel and ligated into EcoRI- and PstI-digested plasmid pHSG399. E. coli XL1-Blue (Stratagene, La Jolla, CA) was transformed by the resulting plasmids. Plasmid DNAs were prepared from the transformants, and the restriction patterns were analyzed by digesting them with EcoRI plus PstI, followed by agarose gel electrophoresis. Plasmids carrying the identical restriction endonucleases 24-mer oligonucleotides corresponding to nucleotides 134–157 (AATGACCTATAGGACCTTCTGTCAGTCA) in the coding strand and 1027–1004 (TTCCGGCCATTGTACATTGGACAGA) in the complementary strand shown in the Fig. 1 of Ref. 21. The coding region of nitroreductase is nucleotides 299–949. Cys-dATP (Amersham Pharmacia Biotech) was used for internal labeling. Plasmid pYG220, a pBluescript-based plasmid carrying the wild-type nitroreductase gene (15, 21) were further selected by the digestion with EcoRI and the plasmid carrying the mutant nitroreductase gene was termed pYG143.

**DNA Sequencing Analysis of the Mutant Nitroreductase Gene—**DNA sequence of the cloned gene in pYG143 was determined by ALFred sequencing kit (Amersham Pharmacia Biotech) combined with diaminobenzidine-transparent technique were used to visualize the wild-type and mutant nitroreductase. The rabbit antiserum raised against the wild-type nitroreductase. Thus, this fraction was used as a source of the purified mutant nitroreductase. Type II and III nitroreductase were used for internal labeling. Plasmid pYG220, a pBluescript-based plasmid carrying the wild-type nitroreductase gene, was used as a control. Direct DNA sequencing of the mutant nitroreductase gene of strain TA1538NR was also carried out using polymerase chain reaction techniques described previously (13). In this case, strain TA1538, which carries the wild-type nitroreductase gene, was used as a control. The 24-mer oligonucleotides described above were used for amplifications.

**Purification of the Wild-type Nitroreductase of S. typhimurium—**Wild-type strain *S. typhimurium* strain YG1021 was grown in 16 liters of LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 1% NaCl) supplemented with 10 μg/ml tetracycline for 16 h at 37 °C with vigorous shaking. The cells (101 g) were washed with 50 ml Tris-HCl buffer, pH 7.5 (buffer A). All the following steps were performed on ice or at 4 °C. The washed cells were suspended in 3 volumes of buffer A containing 1 m NaCl. Dialysis was disrupted by sonication was prepared, and streptomycin sulfate was added at a final concentration of 1% to precipitate nucleic acids. After centrifugation, nitroreductase was precipitated between 40 and 60% saturation of ammonium sulfate. The precipitate was dissolved in 60 ml of buffer B plus 1 μM FMN (buffer C), followed by dialysis against buffer C. The dialyzed sample was applied to a DE52 ion-exchange DEAE-cellulose (Whatman) column (5 × 15 cm) that was preequilibrated with buffer A. Nitroreductase was eluted with a 3,000-ml linear gradient of 50–300 mM NaCl in buffer C at a flow rate of 2.5 ml/min. Fractions were subjected for enzyme assays and the analysis by SDS-polyacrylamide gel electrophoresis. The activity was eluted with about 150 mM NaCl in buffer C. The nitroreductase fractions (160 ml) were concentrated to 2 ml by Centriprep 10 concentrators. The enzyme was precipitated by the addition of ammonium sulfate to 35% saturation (buffer C + 35%). Then hydrophobic interaction chromatography was performed using a phenyl-Sepharose CL-4B (Amersham Pharmacia Biotech) column (1.5 × 30 cm). The nitroreductase activity was recovered in the pass-through fractions. The fractions (170 ml) were concentrated using Centriprep 10 concentrators (Amicon, Beverly, MA) to 4 ml. After adjustments of the concentration to 50 mM Tris-HCl, pH 7.5, and 50 mM NaCl, the sample was loaded onto a Sephacryl G-100 (Amersham Pharmacia Biotech) column (2.5 × 100 cm). The elution buffer was buffer C containing 50 mM NaCl, and the flow rate was 0.25 ml/min. The active fractions (30 ml) were pooled and concentrated to 2 ml by Centriprep 10 concentrators. The final preparation was used as purified nitroreductase.

**Amino-terminal Sequence Analysis—**Purified enzyme solution (1.5 μl) was desalted and diluted to 30 μl. The sample was loaded on to a glass fiber disc and was analyzed by Applied Biosystems 477 sequencer for the first 19 amino acids.

**Purification of the Mutant Nitroreductase—**Plasmid pYG143 was digested with NotI and SacII. A 1.0-kilobase pair DNA fragment, which contains the mutant nitroreductase gene, was isolated from agarose gel and inserted into the NotI- and SacII-digested pbScript KS+ (Stratagene). The resulting plasmid was designated as pYG149 and was introduced into the nitroreductase-deficient strain *S. typhimurium* TA1538NR. LB broth (1 liter) supplemented with 50 μg/ml ampicillin was inoculated with the transformant and incubated for 16 h at 37 °C. Purification protocols were similar to those for the purification of the wild-type enzyme, but several modifications were made because of different chromatographic behavior of the mutant enzyme. After streptomycin sulfate precipitation, the mutant nitroreductase was precipitated between 35 and 45% saturation of ammonium sulfate. The mutant nitroreductase was eluted from a DEAE-cellulose column DE52 in buffer C containing 10 mM NaCl, and the flow rate was lower than at about 0.25 ml/min for elution of the wild-type enzyme. The mutant nitroreductase tightly bound to a phenyl-Sepharose CL-4B column and could not be eluted from the column without using organic solvent. Thus, this step was omitted. Contrary to the wild-type enzyme, the mutant enzyme bound to a hydroxyapatite column and was eluted with about 70 mM sodium phosphate buffer, pH 6.8. A fraction that eluted from a hydroxyapatite column was analyzed by SDS-polyacrylamide gel electrophoresis followed by visualization with Coomassie Brilliant Blue. Thus, this fraction was used as a source of the purified mutant nitroreductase.

**Western Blot Analysis—**Samples were separated by SDS-polyacrylamide gel electrophoresis and blotted on nitrocellulose sheets (BA83, Schleicher & Schuell, Dassel, Germany) by semidry electrophoretic transfer. The rabbit antiserum raised against the wild-type nitroreductase was prepared by Takara-shuzo Co. (Osua, Japan). The nitroreductase antiserum and horseradish peroxidase-linked anti-rabbit antibody (Amersham Pharmacia Biotech) combined with diaminobenzidine-staining technique were used to visualize the wild-type and mutant nitroreductases (36).

**Enzyme Assays—**Nitrofurazone reductase activity was determined by the method of McCalla et al. with modifications (37). Reaction was performed in 0.6 ml buffer B and NaCl. In buffer C, the initial reaction velocity was determined by monitoring the decrease in absorption at 375 nm of nitrofurazone. The extinction coefficient of 1.5 × 10⁴ M⁻¹ cm⁻¹ was used to calculate the amount of nitrofurazone reduced per min per mg of protein. Because the absorbance of NADPH overlaps that of nitrofurazone at 375 nm, the NADPH-generating system was employed to maintain constant concentrations of NADPH. When a fixed concentration of NADH or NADPH was used, the decrease in absorption at 400 or
The purification of the wild-type and mutant nitroreductases of *S. typhimurium* was achieved using a combination of ammonium sulfate precipitation, DEAE-Sepharose column chromatography, hydroxyapatite chromatography, and gel filtration. The purification was monitored by SDS-polyacrylamide gel electrophoresis and Western blotting with antiserum against the wild-type nitroreductase. The specific activity of the purified enzymes was determined using nitrofurazone as a substrate. The purification was effective, with most of the enzyme activity being recovered in the final fractions. The purified enzymes were used for further experiments, including the determination of cofactor requirements and the effect of various compounds on the enzyme activity.
L33R Mutant Nitroreductase of S. typhimurium

The purified enzymes were run on 12% SDS-polyacrylamide gel and stained with Coomassie Blue R-250 or subjected for Western blotting analysis. Lane 1, crude extract (18.6 μg); lane 2, cytosol (16.1 μg); lane 3, precipitate from ammonium sulfate (8.6 μg); lane 4, pooled fractions from the DEAE-cellulose column (1.27 μg); lane 5, pooled fractions from the phenyl-Sepharose column (0.37 μg); lane 6, unbound fractions from the hydroxyapatite column (0.3 μg); lane 7, pooled fractions from the Sephadex G-100 column (0.29 μg). The amount of the sample in each lane contains the nitroreductase at the activity of 10 nmol/min nitrofurazone reduction. B, purification of the L33R nitroreductase. Lane 1, crude extract (18.5 μg); lane 2, cytosol (16.5 μg); lane 3, precipitate from ammonium sulfate (2.9 μg); lane 4, pooled fractions from the DEAE-cellulose column (0.62 μg); lane 5, pooled fractions from the hydroxyapatite column (0.39 μg). C, Western blotting analysis of the wild-type and the L33R mutant nitroreductases. Lanes 1 and 2, 0.1 and 1 μg of the L33R mutant nitroreductase, respectively; lanes 3, 4, and 5, 0.01, 0.1, and 1 μg of the wild-type nitroreductase, respectively.

The absorption maxima in the visible light region are at 370 and 455 nm, whereas those for free FMN are 375 and 448 nm.

Comparison of Enzymatic Properties between the Wild-type and Mutant Enzymes—If the major deficit of the mutant nitroreductase is its reduced affinity for FMN, which is an essential cofactor for nitroreductase activities.

Activities of crude extracts of strain TA1538NR harboring plasmid pYG143 carrying the mutant nitroreductase gene with those of crude extracts of strain TA1538NR without plasmids. Only the crude extract of strain TA1538NR harboring pYG143 exhibited an elevated nitroreductase activity in the presence of 0.1 mM FMN (data not shown), suggesting that the mutant nitroreductase itself has an FMN-dependent nitroreductase activity. Another formal possibility that the activity was due to trace amounts of the wild-type nitroreductase mistranslated from the mutant nitroreductase gene seems unlikely. Even if the wild-type enzyme were contaminated in the crude extracts, it could be efficiently removed from the L33R enzyme during the purification, in particular, in the step of hydroxyapatite chromatography. From these results, we concluded that the major deficit of the mutant nitroreductase is its reduced affinity for FMN, which is an essential cofactor for nitroreductase activities.

The Mutant Nitroreductase Has a Reduced Affinity for FMN—The wild-type nitroreductase showed the characteristic yellow color of flavin, and the color could hardly be removed by simple dialysis or ultrafiltration. However, the mutant nitroreductase was colorless. In an attempt to reconstitute the holoenzyme, the colorless preparation was incubated with excess FMN, overnight at 4 °C, followed by dialysis or ultrafiltration to remove free FMN. Nevertheless, we could only recover colorless, inactive enzyme after this procedure. To examine the possibility whether the mutant nitroreductase has a reduced affinity for FMN, the enzyme activities were assayed in the presence of FMN (0.015 and 0.1 mM). Under the conditions, the mutant enzyme exhibited a weak but measurable nitrofuranzone reductase activity: in the presence of 0.015 and 0.1 mM FMN (data not shown), suggesting that the mutant enzyme exhibited an elevated nitroreductase activity in the presence of 0.1 mM FMN. The values represent the results of three independent experiments. To exclude the possibility that the activity was due to different nitroreductases contaminated in the preparation of the purified enzyme, we compared the activities of crude extracts of strain TA1538NR harboring plasmid pYG143 carrying the mutant nitroreductase gene with those of crude extracts of strain TA1538NR without plasmids. Only the crude extract of strain TA1538NR harboring pYG143 exhibited an elevated nitroreductase activity in the presence of 0.1 mM FMN (data not shown), suggesting that the mutant nitroreductase itself has an FMN-dependent nitroreductase activity. Another formal possibility that the activity was due to trace amounts of the wild-type nitroreductase mistranslated from the mutant nitroreductase gene seems unlikely. Even if the wild-type enzyme were contaminated in the crude extracts, it could be efficiently removed from the L33R enzyme during the purification, in particular, in the step of hydroxyapatite chromatography. From these results, we concluded that the major deficit of the mutant nitroreductase is its reduced affinity for FMN, which is an essential cofactor for nitroreductase activities.

Comparison of Enzymatic Properties between the Wild-type and Mutant Enzymes—If the major deficit of the mutant nitroreductase is its reduced affinity for FMN, it may exhibit weak but similar enzymatic properties to the wild-type enzymes. The wild-type nitroreductase showed the characteristic yellow color of flavin, and the color could hardly be removed by simple dialysis or ultrafiltration. However, the mutant nitroreductase was colorless. In an attempt to reconstitute the holoenzyme, the colorless preparation was incubated with excess FMN, overnight at 4 °C, followed by dialysis or ultrafiltration to remove free FMN. Nevertheless, we could only recover colorless, inactive enzyme after this procedure. To examine the possibility whether the mutant nitroreductase has a reduced affinity for FMN, the enzyme activities were assayed in the presence of FMN (0.015 and 0.1 mM). Under the conditions, the mutant enzyme exhibited a weak but measurable nitrofuranzone reductase activity: in the presence of 0.015 and 0.1 mM FMN, the mutant enzyme exhibited 0.02 and 0.14% nitrofuranzone reductase activity, respectively, as compared with the wild-type enzyme. These results suggested that the mutant enzyme has weak but similar enzymatic properties to the wild-type nitroreductase. In other words, the mutant enzyme could be an oxygen-insensitive NAD(P)H-nitroreductase associated with quinone reductase and flavin reductase activities, with a reduced affinity for FMN.
Nitrofurazone reductase activity of the nitroreductase after reconstitution from the apoenzyme and flavins (FMN, FAD, and riboflavin). Apoenzyme (0.05 mM) was incubated with excess amounts of flavins (0.1 or 5 mM) overnight at 4 °C and analyzed for its nitrofurazone reductase activity. Apoenzyme was prepared using potassium bromide as described under “Experimental Procedures.” The experiments were repeated twice to confirm the reproducibility of the results. 

Lane 1, purified nitroreductase; lane 2, apoenzyme alone; lane 3, apoenzyme incubated with 0.1 mM FMN; lane 4, apoenzyme incubated with 5 mM FMN; lane 5, apoenzyme incubated with 0.1 mM FAD; lane 6, apoenzyme incubated with 5 mM FAD; lane 7, apoenzyme incubated with 0.1 mM riboflavin; lane 8, apoenzyme incubated with 5 mM riboflavin. Numbers in parentheses represent the relative activities to the intact wild-type enzyme.

**DISCUSSION**

It has been suggested that there is an oxygen-insensitive NAD(P)H nitroreductase/flavin reductase family among bacteria, because oxygen-insensitive NAD(P)H nitroreductase of *E. cloacae*, nitroreductase of *E. coli*, i.e. NfsB, NAD(P)H flavin reductase of *V. fischeri*, NADH oxidase from *T. thermophilus*, and the nitroreductase of *S. typhimurium* share sequence similarities at the amino acid level (18, 20–22, 38, 40). These enzymes require flavin as a cofactor, and the most effective flavin is FMN. The amino acid sequences of the bacterial enzymes do not share significant similarity with other proteins such as FMN-binding protein in bacteria (41) or NAD(P)H/quinone oxidoreductase in higher organisms (29, 31, 42). Thus, we suggested that the enzymes belonging to the bacterial nitroreductase family may have common structural characteristics necessary to bind FMN tightly.

In this study, we identified a transversion mutation at nucleotide 396 of the gene encoding the nitroreductase of *S. typhimurium* TA1538NR, a pKM101-removed derivative of TA98NR (Fig. 1), and characterized the purified mutant enzyme (Tables I, II, and III). TA98NR is a strain deficient in the nitroreductase and was isolated as a mutant resistant to the killing effects of nitrofurans (14, 35). The purified mutant protein, in which leucine 33 is replaced with arginine (L33R), showed no detectable level of nitrofurazone nitroreductase activity under the standard assay conditions. In addition, even the crude extract of the strain *S. typhimurium* TA1538NR harboring plasmid pYG143 highly expressing the mutant protein exhibited an extremely low activity. From these results, we concluded that the mutation of L33R is responsible for the nitroreductase deficiency of *S. typhimurium* TA98NR.

The nitroreductase protein having the mutation of L33R exhibited low but significant nitroreductase activities when FMN (0.1 mM) was present in the assay mixture. The mutant nitroreductase easily lost FMN by dialysis or ultrafiltration. These findings suggest that the replacement of leucine 33 with arginine reduces the affinity for FMN. Why is Leu33 essential for FMN binding? One possibility is that this residue forms part of the cofactor binding site itself. Alternatively, Leu33 may be distant from the binding site, but critical for determining the overall native conformation of the wild-type enzyme. It appeared that the mutation slightly affected the protein structure, because the mutant protein exhibited different chromatographic behavior from the wild-type enzyme. However, the mutant enzyme as well as the wild-type could use both NADPH and NADH as electron donors and exhibited quinone reductase and flavin reductase activities (Tables II and III). These results raised the possibility that the mutant protein has a structure similar to that of the wild-type enzyme but with a reduced affinity for FMN.

Fine structures of nitroreductase of *S. typhimurium* or closely related enzymes such as NfsB of *E. coli* are not available at present (43, 44). However, x-ray crystallographic analysis of
NADH oxidase from *Escherichia coli* (45) shows that the enzyme consists of two identical subunits associated with symmetry. This association creates two deep clefts between the subunits. Each cleft contains an active site where an FMN binding region (see text). A possible binding site. Further structural analysis of the wild-type and the L33R enzyme of *Salmonella* will shed light on the structure-function relation of this FMN-dependent nitroreductase family.

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