Isolation and Reconstitution of Iron- and Manganese-Containing Superoxide Dismutases from *Bacteroides thetaiotaomicron*

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Superoxide dismutase (SOD) from extracts of anaerobically maintained *Bacteroides thetaiotaomicron* was a dimer of equally sized 23,000-molecular-weight monomers joined noncovalently. A preparation with a specific activity of 1,200 U/mg contained 1.1 g-atom of Fe, 0.6 g-atom of Zn, and <0.05 g-atom of Mn per mol of dimer. The apoprotein, prepared by dialysis of iron-SOD in 5 M guanidinium chloride–20 mM 8-hydroxyquinoline, had no superoxide-scavenging activity when renatured without exogenous metal. Enzymatic activity was restored to the denatured apoprotein by dialysis against either 1 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ or 1 mM MnCl_2 in 20 mM Tris (pH 7.0). The Fe-reconstituted enzyme and the native enzyme were inhibited ~50% by 0.2 mM NaN_3 , whereas the Mn-reconstituted enzyme was inhibited 60% by 10 mM NaN_3 . Aeration of the anaerobic cells resulted in a fourfold induction of an azide-resistant SOD. The enzyme (43,000 molecular weight) isolated from aerated cells was a dimer of equally sized subunits. The metal content was 1.0 g-atom of Mn, 0.55 g-atom of Fe, and 0.3 g-atom of Zn per mol of dimer. Enzymatic activity of the denatured apoprotein from this enzyme was also restored on addition of either iron or manganese. The constitutive Fe-SOD and the O_2 -induced Mn-SOD, tested alone and in combination, migrated identically on acrylamide gels, had similar amino acid compositions, and had alanine as the sole N-terminal amino acid. These data are consistent with the synthesis of a single apoprotein in either anaerobically maintained or oxygenated cells. The apoprotein could accept either iron or manganese to form the holoproteins. We have observed a similar phenomenon with SOD from *Bacteroides fragilis* (E. M. Gregory, Arch. Biochem. Biophys. 238:83–89, 1985).

Superoxide dismutases (SODs) are a family of metalloproteins which catalyze the disproportionation of superoxide radicals to O_2 and H_2O_2 . For these enzymes, iron, manganese, or copper is the active-site prosthetic group (8, 15, 26). With the exception of the copper-containing bacteriocupreins (21, 24), SODs in procaryotes have either iron or manganese at the active site. These SODs, purified or in crude extracts, are differentiated by their characteristic inhibition with sodium azide and rate of inactivation by H_2O_2 . Iron-containing SODs are inhibited 70 to 90% by 1 mM NaN_3 and have half-lives of several minutes when incubated with 5 mM H_2O_2 . Manganese-containing SODs are inhibited 50% by 20 mM NaN_3 and are stable for at least 2 h in 5 mM H_2O_2 (17).

SOD activity in cell extracts from the anaerobe *Bacteroides fragilis* (VPI 2393) was inhibited by azide and inactivated by H_2O_2 . The enzyme isolated from anaerobically maintained cells contained iron as the prosthetic metal. The iron was removed upon denaturing the enzyme in the presence of a chelator, 8-hydroxyquinoline. Fe-SOD activity was restored to the denatured apoprotein by renaturation in an iron-containing buffer. Moreover, enzymatic activity is restored upon renaturation with Mn salt (6). Meier et al. (16) have demonstrated in situ substitution of Mn for Fe in SOD from the anaerobe *Propionibacterium shermanii*. This flexibility of accepting either Fe or Mn is uncommon among SODs.

SOD activity was induced by aeration of mid-growth-phase *B. fragilis*, but the resultant activity was characteristic of Mn-SOD, not Fe-SOD. The apoprotein from this enzyme

is reactivated with Mn, but is also reactivated with Fe. The physical properties and amino acid composition of the SODs from both anaerobically maintained and aerated *B. fragilis* cells are virtually identical except for metal content. To extend our study of the SOD synthesized by *Bacteroides* spp. and test the model we hypothesized, studies with the constitutive and O_2 -induced *Bacteroides thetaiotaomicron* were undertaken.

MATERIALS AND METHODS

Cell culture. *B. thetaiotaomicron* (VPI 3388) was maintained anaerobically in chopped-meat medium (7). Cells were cultured in prerduced, anaerobically sterilized peptone-yeast basal medium (7) supplemented with 1% glucose. Growth was monitored turbidimetrically (11). For oxygenation studies, 1-liter portions of mid-growth-phase cells were transferred to 2.8-liter Fernbach flasks, covered loosely with foil, and shaken at 125 rpm on a reciprocating platform shaker at 37°C. Cells were harvested either by centrifugation (16,000 $\times g$ for 10 min) or by multiple-pass filtration (Pellicon cassette system), washed once in 50 mM potassium phosphate (pH 7.0), and lyophilized for storage. SOD activity was measured and units were defined as described by McCord and Fridovich (15), except that 0.05 mM NaCN was added to the assay mixture. Inhibitors, when present, were added to the mixture to the appropriate concentration. Protein in crude extracts was measured by the A_{280} , corrected for interference by nucleic acids (25) assuming an extinction of 1 ml $\text{mg}^{-1} \text{cm}^{-1}$. The extinction at 280 nm of isolated enzymes was determined by measuring the A_{280} of samples whose protein content had been determined by the method of Lowry et al. (13). Electrophoresis of native protein was performed on gels containing 7.5 or 10% acrylamide (3). The gels were stained

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TABLE 1. Isolation of SOD from anaerobically maintained *B. thetaiotaomicron*

Step	Vol (ml)	U/ml	Total U	Protein (mg/ml)	Total protein (mg)	Sp act (U/mg)	% Recovery	Purification (fold)
100,000 × g supernatant	114	124	14,140	14.5	1,650	8.6	100	1
DE-23	63	153	9,640	5.8	365	26.4	68	3.1
DE-53 salt gradient	188	37.5	6,110	0.07	13.2	463	43	54
DE-53 pH gradient	81	48	3,890	0.04	3.2	1,220	28	141

for protein with Coomassie blue and for SOD activity by the method of Beauchamp and Fridovich (2).

Amino acid composition was determined on protein samples hydrolyzed in 6 N HCl under N₂ at 110°C for 24, 48 and 72 h, using a Beckman series 340 high-pressure liquid chromatography system with a Pickering ion-exchange column and a ninhydrin postcolumn reactor. Methionine and cysteine were oxidized to methionine sulfone and cysteic acid, respectively, by performic acid (19). Tryptophan was determined by the Edelhoch method (4). Native molecular weight was measured by gel exclusion chromatography on Sephadex G-75 calibrated with appropriate molecular weight standards. Subunit molecular weight was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Laemmli (12). N-terminal amino acid analysis was performed on hydrolysates of the dinitrophenylated proteins (18). The N-terminal residue was initially identified by comparison of its *R_f* on silica gel with those of standards and confirmed by cochromatography of the 2,4-dinitrophenol derivative with the appropriate standard in two dimensions. The solvents were toluene-pyridine-2-chloroethanol-0.8 N ammonium hydroxide (100:30:60:60 [vol/vol]) (upper phase) and chloroform-benzyl alcohol-glacial acetic acid (70:30:3 [vol/vol]) (20).

Metal removal and replacement studies were performed as previously described (5, 6). The protein was denatured by dialysis in 5 M guanidinium chloride containing 20 mM 8-hydroxyquinoline and then dialyzed for 8 h in 5 M guanidinium chloride to remove the chelator. Samples thus denatured were dialyzed in Tris buffer (20 mM, pH 7.0) containing metal salt. Excess metal salt was removed by further dialysis in the Tris buffer plus 1 mM EDTA. In each case, insoluble protein was present and was removed by centrifugation before SOD activity was assayed. Tris buffer used in the reconstitution studies was passed through Chelex resin to reduce the intrinsic metal content. In reconstitution studies with the ferrous salt, there was always a rapid oxidation of iron and subsequent precipitation of iron oxide. Metal content was determined with a Perkin-Elmer model 560 atomic-absorption spectrophotometer.

Xanthine, cytochrome *c* (type III), EDTA, riboflavin, Tris, bovine serum albumin, ovalbumin, myoglobin, hemoglobin, tetrazolium salts, and 2-(N-morpholino)ethanesulfonic acid were purchased from Sigma Chemical Co. Ultrapure guanidinium chloride was obtained from Heico, Inc. DEAE-cellulose (DE-53) was obtained from Whatman, Inc., Chelex 100 was from Bio-Rad Laboratories, and phenyl Sepharose was from Sigma. All other reagents were used without further purification. 2,4-Dinitrophenyl amino acids used as standards were purchased from Sigma.

RESULTS

Induction of SOD in *B. thetaiotaomicron*. SOD activity (0.9 U/mg) in crude extracts from anaerobically maintained cells

was inhibited 74% when 1 mM NaN₃ was included in the assay, a characteristic of Fe-SODs (17). Aeration of the mid-growth-phase cells for 2 h resulted in a fourfold increase in SOD activity (3.8 U/mg). Incubation of cells with either rifampin (1 µg/ml) or chloramphenicol (500 µg/ml) 30 min before aeration prevented the increase in SOD activity. SOD in extracts of aerated cells was inhibited 19 and 51% by inclusion of 1 and 10 mM NaN₃, respectively, in the assay, characteristic of Mn-SODs (17). Enzymatic activity in extracts from cells removed at intermediate times was progressively more resistant to azide inhibition with increased time of aeration. Crude homogenates from control or aerated cells, individually or in combination, were separated on gels containing 7.5 or 10% acrylamide. In each case, only a single, identically migrating SOD activity band was observed. Further studies were performed with the enzymes purified from the anaerobic or O₂-induced cells.

Isolation of SODs from anaerobically maintained *B. thetaiotaomicron*. Six grams of lyophilized cells was rehydrated in 125 ml of 50 mM potassium phosphate-1 mM EDTA (pH 7.8) and disrupted with two 2.5-min bursts of sonic oscillation through the microtip of a Branson W185D sonifier set at 70 W. The slurry was clarified by centrifugation at 100,000 × g for 1 h. These and subsequent operations were at 4°C. Supernatant was applied to a DE-23 column (2.5 by 40 cm) equilibrated in 20 mM Tris-1 mM EDTA (pH 8), and the SOD activity was isocratically eluted with this buffer. The eluate (approximately 125 ml) was applied to a DE-53 column (2.4 by 40 cm) equilibrated in the Tris buffer. The column was washed with 125 ml of the Tris buffer and eluted with a linear KCl gradient in Tris buffer (0 to 25 mM KCl, 800 ml of each component). Fractions (8.2 ml) with SOD activity were pooled, dialyzed in the 20 mM Tris buffer, and applied to a DE-53 column (1 by 10 cm). This column was eluted with a gradient of decreasing pH established with 400 ml of the Tris buffer and 400 ml of 40 mM morpholine-ethanesulfonic acid (pH 5.5). Fractions (4.8 ml) with SOD activity were pooled, concentrated under N₂ over a YM-10 Ultrafilter, and stored frozen. SOD was purified 140-fold from the initial crude extract to a specific activity of 1,220 U/mg (Table 1). The initial specific activity of the extract from lyophilized cells (4 U/mg) was 10-fold greater than that from freshly harvested or frozen cells. Decreased solubilization of some protein, but not SOD activity, in the lyophilized cells was the apparent cause. Appropriately stained gels, containing either 7.5 or 10% acrylamide, revealed a single zone of SOD activity (2 U applied) migrating coincidentally with a single band of protein (40 to 70 µg applied) after electrophoresis of the isolated enzyme.

The native molecular weight was 46,000, determined by gel exclusion chromatography, and the denatured protein migrated as a 23,000-dalton polypeptide in sodium dodecyl sulfate-acrylamide gels independently of 2-mercaptoethanol treatment. The observation of a single protein band is consistent with a homogeneous enzyme preparation. An

TABLE 2. Amino acid composition of *B. thetaiotaomicron* SOD

Amino acid	Residues per mol of dimer (72-h hydrolysis, unless noted)	
	Fe-SOD	Mn-SOD
Lys	32.6	31.6
His	4.2	5.3
Arg	9.7	10.3
Asp	44.9	45.0
Thr ^a	16.1	15.2
Ser ^a	19.2	17.8
Glu	36.7	36.5
Pro	15.6	13.9
Gly	31.2	33.4
Ala	36.0	32.2
Cys ^b	0.6	0.6
Val	26.9	24.4
Met ^b	7.0	7.8
Ile	18.5	16.0
Leu	31.7	31.7
Tyr	12.6	15.0
Phe	15.2	15.3
Trp ^c	8.6	8.9

^a Extrapolated to zero hydrolysis time from 24-, 48-, and 72-h hydrolysis data.

^b As cysteic acid and methionine sulfone after performic acid oxidation.

^c Spectrophotometric analysis (4).

enzyme sample with a specific activity of 1,220 U/mg contained 1.1 g-atom of Fe, 0.6 g-atom of Zn, and <0.05 g-atom of Mn mol of dimer⁻¹. The extinction coefficient at 280 nm was 2.0 ml mg⁻¹ cm⁻¹, based on UV absorbance of a sample whose protein content was determined by the method of Lowry et al. (13). Bovine serum albumin was the standard. Amino acid composition of the Fe-SOD is shown in Table 2. Alanine was identified as the sole N-terminal residue in the dimeric protein. Fe-SOD was inhibited 78% by the inclusion of 1 mM NaN₃ in the assay but was not inhibited by 1 mM NaCN (Table 3). Incubation of the enzyme in 5 mM H₂O₂ (23°C) resulted in a time-dependent inactivation with a half-life of 3.5 min.

Characterization of O₂-induced SOD. SOD from aerated cells was isolated by a previously established procedure (6a), which is virtually identical to that shown for Fe-SOD (Table 1). The enzyme was purified 114-fold from a specific activity of 11.6 U/mg in the crude cell extract to a specific activity of

TABLE 3. Azide inhibition of native and metal-reconstituted SODs from *B. thetaiotaomicron*

Sample	% Inhibition of SOD activity by NaN ₃ concn (mM):						
	0.1	0.2	0.5	1.0	5.0	10.0	20.0
Constitutive Fe-SOD							
Native enzyme	31	51	56	78			
Fe reconstituted	24	48	63	82			
Mn reconstituted				17	30	60	59
Induced Mn-SOD							
Native enzyme				7	17	51	51
Mn reconstituted				5	4	35	38
Fe reconstituted	56	80	81	86			

TABLE 4. Resolution-reconstitution studies with constitutive Fe-SOD and O₂-induced Mn-SOD

Reconstituting metal	Initial ^a U	Recovered ^b U	% Recovery	% Inhibition ^c of recovery by Zn
Constitutive Fe-SOD				
Fe(NH ₄) ₂ (SO ₄) ₂ (1 mM)	315	30	9.5	
Fe(NH ₄) ₂ (SO ₄) ₂ + ZnSO ₄ (10 μM)	315	6.8	2.1	78
MnCl ₂ (1 mM)	315	75	28.8	
Mn + ZnSO ₄ (10 μM)	315	27	8.5	65
No metal	315	<0.1		
O ₂ -induced Mn-SOD				
MnCl ₂ (1 mM)	510	27	5.3	
MnCl ₂ + ZnSO ₄ (10 μM)	510	15	3.0	43
Fe(NH ₄) ₂ (SO ₄) ₂ (1 mM)	510	31	6	
Fe(NH ₄) ₂ (SO ₄) ₂ + ZnSO ₄ (10 μM)	510	10	2	67

^a Total SOD units dialyzed in denaturant.

^b Total SOD units recovered after renaturation and removal of excess metal.

^c Percentage of difference in recoverable units with and without ZnSO₄ in the buffer.

1,330 U/mg. A single protein band and a corresponding SOD activity band were observed on appropriately stained gels containing either 7.5 or 10% acrylamide. The molecular weights of the native enzyme (43,000) and of the denatured subunits (20,000) were similar to that of the Fe-SOD. However, metal analysis of a preparation with a specific activity of 1,330 U/mg revealed 1.0 g-atom of Mn, 0.55 g-atom of Fe, and 0.3 g-atom of Zn mol of dimer⁻¹. Extinction at 280 nm was 2.1 ml mg⁻¹ cm⁻¹, as determined from UV absorbance. The amino acid composition of the Mn-SOD is shown in Table 2. Alanine was the sole N-terminal amino acid. Mn-SOD was inhibited 7% on addition of 1 mM NaN₃ and 51% on addition of 20 mM NaN₃ to the assay. Enzymatic activity was stable for at least 2 h in 5 mM H₂O₂ (23°C).

Metal replacement studies with Fe-SOD and Mn-SOD. Anaerobically maintained *B. thetaiotaomicron* synthesized an Fe-SOD, whereas the aerated cells synthesized an Mn-SOD. Despite the differences in prosthetic metal at the active site, the two proteins are markedly similar if not identical in size, amino acid composition, and physical properties. It is thus possible that the organism synthesizes a single apoprotein capable of accepting either Fe or Mn at the active center. The specificity of metal replacement in the denatured apoprotein from either metalloprotein was tested. Enzymatic activity was restored to the denatured Fe-SOD apoprotein during dialysis in buffer containing the iron salt (Table 4). Of the total 315 U denatured, 9.5% was recovered on dialysis in the iron salt and subsequent removal of excess metal ions. Enzymatic activities of both the Fe-reconstituted and constitutive Fe-SOD were similarly inhibited (51 and 48%, respectively) by 200 μM NaN₃. Removal and replacement of the metal intrinsically bound to the enzyme is not uncommon. However, enzymatic activity was also restored upon dialysis in MnCl₂ of the denatured apoprotein from Fe-SOD. Of the initial 315 U of SOD denatured, 24.8% was

recovered. Reconstitution with Mn of the denatured apoprotein derived from Fe-SOD conferred resistance to NaN_3 . Addition of 10 mM NaN_3 to the assay inhibited enzymatic activity by 60% (Table 3).

The denatured apoprotein from Mn-SOD was also reactivated on dialysis in either MnCl_2 or $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (Table 4). During reconstitution of this enzyme, 5.3% of the total activity units was recovered on dialysis with MnCl_2 , and 6.0% was recovered on dialysis with the ferrous salt. Addition of Mn to the apoprotein resulted in an enzymatic activity whose inhibition by NaN_3 was similar to that of the native Mn-SOD. However, addition of Fe to the apoprotein conferred azide sensitivity. Enzymatic activity of Fe-reconstituted Fe-SOD was inhibited 56% upon addition of 100 μM NaN_3 .

Zinc, present at 0.3 to 0.6 g-atom mol of dimer⁻¹ in the native SODs, inhibited reconstitution with either Fe or Mn of the denatured apoproteins. Reconstitution with iron of the apoprotein from Fe-SOD was inhibited 78% by 10 μM ZnSO_4 , whereas 65% inhibition of the Mn-dependent reconstitution was observed. Reconstitution of the apoprotein derived from the Mn-SOD was likewise inhibited (Table 4). Metal content of the renatured samples was not measured.

DISCUSSION

SODs in procaryotes typically contain either iron or manganese as the active center metal. Removal of the metal results in an inactive apoprotein, and in the majority of the SODs tested, activity is reconstituted only with the metal that was intrinsically bound to the holoprotein (9). The Fe-SOD and Mn-SOD of *Escherichia coli* are differentiated in crude extracts based on strict metal requirements for reconstitution. Thus, Fe-SOD apoprotein regains activity only when reconstituted with iron, and Mn-SOD apoprotein regains activity only when reconstituted with manganese (9). Similarly stringent metal requirements are observed with SOD from *Streptococcus sanguis* and *Acholeplasma laidlawii* (9). Such stringency was lacking in the iron- and manganese-containing SODs from the anaerobe *B. fragilis*. Fe-SOD isolated from anaerobically maintained cells and Mn-SOD isolated from aerated cells lost all superoxide-scavenging activity when denatured in the presence of 8-hydroxyquinoline, a metal chelator (6), but enzymatic activity was restored to the denatured apoproteins on dialysis with the salt of either iron or manganese. The reconstituted Fe-SOD from either apoprotein was inhibited by low concentrations of azide, whereas the reconstituted Mn-SOD from either apoprotein resisted azide inhibition. Zinc was present in these SODs and has been found as a constituent metal in SODs from *Plectonema boryanum* (1), *Thermoplasma acidophilum* (23), and *Methanobacterium bryantii* (10). Based on studies with the *B. fragilis* SODs, we proposed a model consistent with the insertion of either Fe or Mn into a single apoprotein synthesized by the cell (5). In anaerobically maintained *B. fragilis*, Fe-SOD was formed virtually exclusively. Upon aeration of the cells, synthesis of the apparently identical apoprotein was increased, but Mn, rather than Fe, was inserted. The holoenzyme from aerated *B. fragilis* contains Mn, Fe, and Zn (5). The simplest model consistent with the data involves a single binding site per subunit, a site which accepts Fe, Mn, or Zn. Binding of Zn results in an inactive protein. A more complex model involves distinct but mutually exclusive binding sites for each metal.

Results of these studies with SODs from the anaerobe *B.*

thetaitaomicron are consistent with the model proposed for *B. fragilis*. The anaerobically maintained *B. thetaiotaomicron* cells synthesize an Fe-SOD exclusively. Aeration of the mid-growth-phase cells resulted in the induction of an Mn-SOD which, except for metal content, was quite similar or identical to the constitutive Fe-SOD. The two proteins migrated identically in acrylamide gels, had virtually identical native and subunit molecular weights, eluted from DE-53 ion-exchange resin at the same KCl concentration, and had strikingly similar amino acid compositions. Each had alanine as the single N-terminal amino acid residue. Determination of isoelectric point by isofocusing in ampholytes was not possible because of artifacts generated in the system. Each enzyme was resolved into multiple active bands over a 5 pH unit span in the isofocusing gel. The multiple bands may have resulted from binding of ampholytes to the proteins. Marklund (14) observed similar artifacts with beef heart mitochondrial Mn-SOD.

Denatured apoprotein derived from either Fe-SOD or Mn-SOD accepted Fe or Mn with restoration of superoxide-scavenging activity. Reconstitution of Fe-SOD-derived apoprotein with Fe or Mn-SOD-derived apoprotein with Mn yielded enzymes with electrophoretic and azide inhibition characteristics identical to those of the holoproteins. Reconstitution of the apoproteins with the alternate metal, that is, of Fe-SOD-derived apoprotein with Mn and Mn-SOD-derived apoprotein with Fe, did not alter electrophoretic properties but did impart azide sensitivity characteristic of the metal. Recovery of enzymatic activity after reconstitution of these enzymes was considerably less than the recovery we observed for the Fe-SOD and Mn-SOD from *B. fragilis* (5, 6), even with dialysis to remove the organic chelator. Nevertheless, sufficient activity was recovered to replicate the chemical inhibition studies. The total metal ions bound to the native Fe-SOD (1.7 g-atom mol of dimer⁻¹) and to Mn-SOD (1.9 g-atom mol of dimer⁻¹) suggests one metal-binding site per subunit. These data do not differentiate between a single binding site whose ligands accept either iron or manganese or mutually exclusive binding sites, each specific for iron, manganese, or zinc. Regulation of the type of metallo-SOD formed may be at the posttranscriptional level in these organisms. Pugh and Fridovich (22) suggested that superoxide, present in aerated cells, reduces ferric to ferrous iron, whereas Mn^{2+} is oxidized to Mn^{3+} . Mn^{3+} may compete better for the apoprotein than does Fe^{2+} . Moody and Hassan (18) postulated an Fe-specific repressor protein for control of the *E. coli* Mn-SOD. We suggested that the amount of Fe available in the anaerobe cell may diminish because of sequestration of Fe^{3+} , whereas the cellular Mn concentration may remain constant (5). Alternatively, differential transport of the metal ions into the cell during aeration may be a factor.

Approximately 30% of the metal content of Mn-SOD isolated from O_2 -induced cells was Fe. If the Fe was present as residual Fe-SOD, 1 mM NaN_3 should have inhibited that component by 80% (Table 3). Thus, the amount of inhibition of the native Mn-SOD observed with 1 mM NaN_3 should have been approximately 25%. The observed inhibition was 7%, suggesting that the Fe present either did not have SOD activity or was present in a form that resisted inhibition. The Fe may have been present as Fe-Mn hybrid dimers. These heterodimers would be physically indistinguishable from either of the homodimers but might have altered enzymatic activity of anomalous inhibition characteristics. The preparation and characterization of such dimers are in progress.

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