Structural features for the mechanism of antitumor action of a dimeric human pancreatic ribonuclease variant

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Abstract: A specialized class of RNases shows a high cytotoxicity toward tumor cell lines, which is critically dependent on their ability to reach the cytosol and to evade the action of the ribonuclease inhibitor (RI). The cytotoxicity and antitumor activity of bovine seminal ribonuclease (BSRNase), which exists in the native state as an equilibrium mixture of a swapped and an unswapped dimer, are peculiar properties of the swapped form. A dimeric variant (HHP2-RNase) of human pancreatic RNase, in which the enzyme has been engineered to reproduce the sequence of BSRNase helix-II (Gln28→Leu, Arg31→Cys, Arg32→Cys, and Asn34→Lys) and to eliminate a negative charge on the surface (Glu111→Gly), is also extremely cytotoxic. Surprisingly, this activity is associated also to the unswapped form of the protein. The crystal structure reveals that on this molecule the hinge regions, which are highly disordered in the unswapped form of BSRNase, adopt a very well-defined conformation in both subunits. The results suggest that the two hinge peptides and the two Leu28 side chains may provide an anchorage to a transient noncovalent dimer, which maintains Cys31 and Cys32 of the two subunits in proximity, thus stabilizing a quaternary structure, similar to that found for the noncovalent swapped dimer of BSRNase, that allows the molecule to escape RI and/or to enhance the formation of the interchain disulfides.

Keywords: crystal structure; antitumor agents; ribonuclease; 3D-domain swapping; dimers

Abbreviations: BSRNase, bovine seminal ribonuclease; des(1–7)HP-RNase, human pancreatic ribonuclease in which the first seven residues have been deleted; HHP-RNase, covalent dimeric variant of human pancreatic ribonuclease in which four residues at the helix-II region are mutated according to BSRNase sequence (Gln28→Leu, Arg31→Cys, Arg32→Cys, and Asn34→Lys); HHP2-RNase, HHP-RNase with an additional mutation (Glu111→Gly); HP-RI, structure of human pancreatic ribonuclease complexed with ribonuclease inhibitor; HP-RNase, human pancreatic ribonuclease; MxM-BSRNase, dimeric form of BSRNase in which the chains swap their N-terminal tails; M=M-BSRNase, unswapped dimer of BSRNase; M=M-HHP2, unswapped dimer of HHP2-RNase; NCD-BSRNase, noncovalent swapped form of BSRNase; ONC, Onconase; PDB, Protein Data Bank; PM7, human pancreatic ribonuclease in which residues 1–21 are replaced by the corresponding residues of BSRNase; PM8, N-terminal swapped dimer of a variant of human pancreatic ribonuclease; RI, ribonuclease inhibitor; RMSD, root-mean-square deviation.

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Introduction

Ribonucleases (RNases) are structurally and functionally diverse enzymes with general and specific activities. Prokaryotic and eukaryotic cells are endowed with a large number of exo- and endo-RNases that catalyze RNA cleavage reactions important for RNA maturation and degradation, antiviral defense, and gene silencing. In addition to their general function, a specialized class of RNases shows cytotoxicity toward tumor cell lines. Paradigmatic examples are bovine seminal RNase (BSRNase)^{1,2} and RNase from oocytes of *Rana pipiens*^{3–5} (commercial trademark of Alfacell, USA, onconase (ONC)), both belonging to the pancreatic-like RNase family, whose prototype is the bovine pancreatic enzyme (RNase A).^{6,7} This unusual biological activity is critically dependent on the ribonucleolytic activity⁸ and on the ability of these molecules to reach the cellular cytosol and to degrade RNA by evading the action of the ribonuclease inhibitor (RI).^{9,10} It is currently accepted that RI, which binds most RNase A family members with femtomolar affinities, plays an important role in the protection of host cells from endogenous RNases.^{9–11} Only frog RNases such as OnconaseTM (ONC)¹² and BSRNase^{13–15} are resistant to human RI. The former, which is in Phase III clinical trials as an antitumor agent against malignant mesothelioma,³ escapes RI because it lacks many of the residues involved in the RNase A-RI recognition.¹⁰

A different mechanism enables BSRNase to evade RI.¹⁶ This covalent homodimeric ribonuclease exists as an equilibrium mixture of two isomers, designated as MxM-BSRNase and M=M-BSRNase.¹⁷ They differ for the swapping of the N-termini (residues 1–15) within a substantially identical quaternary framework.^{18–20} In both isoforms, the dimeric interface is formed by the hinge peptides (residues 16–22) and the helices (residues 23–34) that comprise the four cysteines forming the two interchain disulfide bridges (Cys31-Cys32' and Cys32-Cys31') and the side chains of Leu28 and 28' that form stabilizing hydrophobic interactions across the molecular twofold axis.^{18,20} In the most abundant swapped form, MxM-BSRNase, the dimeric interface also includes the closed interface arising from the swapped elements.^{18,20} As a consequence of this latter contribution, MxM-BSRNase gives rise, upon selective reduction of the intersubunit disulfides, to a metastable noncovalent swapped dimer (NCD-BSRNase), whereas M=M-BSRNase yields a monomeric derivative.¹⁷

Although the two dimers present an almost undistinguishable external shape,²¹ the cytotoxic action is a peculiar property of MxM-BSRNase.^{13–15} Following the experimental results obtained *in vitro*, it is believed that in the reducing cytosolic compartment M=M-BSRNase dissociates into monomers, which are strongly inhibited by RI, whereas MxM-BSRNase survives as NCD-BSRNase. This dimer presents an overall

structure highly reminiscent of the covalent forms, with the cysteine residues involved in the intersubunit linkages not far away from each other.^{16,21} Because of its shape, it can easily evade interactions with RI.¹⁶ In addition, the proximity of the cysteine residues may enhance the formation of the disulfide bridges and explain the higher stability of the covalent linkages in the swapped protein with respect to the unswapped one.^{16,22}

To obtain a potential chemotherapeutic agent more efficiently tolerated by the human immune system, various attempts have been made to transfer the antitumor structural determinants of ONC and BSRNase to human pancreatic ribonuclease (HP-RNase), a monomeric enzyme very sensible to RI inactivation.^{11,23} HP-RNase, whose physiological role remains unclear, possesses 70% identity with both bovine pancreatic and seminal enzymes. Compared with RNase A, it contains a higher percentage of basic residues, it shows a C-terminal extension of four residues (EDST) beyond the terminal valine (V124) and exhibits higher activity against double stranded RNA.²⁴ Up to now, all the attempts to grow crystals of the free native enzyme suitable for X-ray analysis failed; moreover, solution NMR studies have been hampered by aggregation problems. Only very recently, the solution structure of native HP-RNase has been reported (PDB code 2K11).²⁵ The crystal structures of few variants of this enzyme have been determined. They include PM7, in which residues 1–22 are replaced by the corresponding residues of BSRNase and Pro50 by a serine (PDB code 1DZA),²⁶ and des(1–7)HP-RNase, in which the first seven residues have been deleted (PDB code 1E21).²⁷ Very recently, four mutants with a leucine zipper-like hydrophobic interface engineered to form leucine zippers in the crystal phase (PDB codes 2EoJ, 2EoL, 2EoM, and 2EoO, hereafter denoted as 2L, 3L, 4L, N4L, respectively) have been also successfully crystallized,²⁸ and the 3D-structure of HP-RNase complexed with the human ribonuclease inhibitor (HP-RI) has been reported (PDB code 1Z7X).²⁹ In all these structures, the tertiary folding of HP-RNase closely resembles that of RNase A, displaying the characteristic V-shaped central β -core motif flanked by three helices. The crystallographic structure of another mutant (PM8) (PDB code 1H8X),³⁰ in which residues 1–22 and 101 were replaced by the corresponding residues of BSRNase, revealed an unexpected domain-swapped dimer based on the interchange of N-terminal tails. Despite the strict similarity of the involved domains with respect to NCD-BSRNase, PM8 adopts a different quaternary shape that does not hamper the binding of RI.¹⁶ Moreover, this dimer is highly unstable in aqueous solution, where it is present mostly as a monomer, and its occurrence in the solid state has been probably favored by the low dielectric constant of the precipitant solution.³⁰

Different approaches have been proposed to confer cytotoxicity to HP-RNase; they include (a) site-directed mutagenesis to weaken its interaction with RI,²³ (b) conjugation of HP-RNase to protein, peptide, and antibodies, which could enhance the uptake by target cells,^{31,32} (c) transformation of the protein into a stable dimer capable to evade RI.³³ Following the latter strategy, Piccoli et al.³⁴ have mutated four residues at the helix-II region according to BSRNase sequence (Gln28→Leu, Arg31→Cys, Arg32→Cys, and Asn34→Lys) and have produced a covalent dimeric variant (HHP-RNase), enzymatically active and selectively cytotoxic for several malignant mouse and human cell lines. The elimination of a negative charge on the HHP-RNase surface (Glu111→Gly) has led to a second dimeric mutant, HHP2-RNase, which is more powerful as a cytotoxic agent than BSRNase and is selective for malignant cells.³⁵ This result was attributed to the increased positive net charge, which might favour the binding to the extracellular matrix and/or the transfer to cytosol through membranes.³⁵ Both HHP-RNase and HHP2-RNase fold in the unswapped and swapped forms, the latter being considerably less abundant.³⁵ On the basis of BSRNase data,^{16,22} the high antitumor action exhibited by the unswapped form of these mutants is surprising, especially because the complex between RI and the monomeric enzyme HP-RNase was found to be extremely stable ($K_d = 2.9 \times 10^{-16} M$ and $t_{1/2} = 81$ days).²⁹ The cytotoxic activity of the unswapped dimers has been ascribed to the unusual stability of the interchain disulfide bridges that makes them more similar to MxM-BSRNase and allows their survival as dimers in the cytosolic compartment.³⁵

To elucidate on structural ground the different behavior of BSRNase and human dimeric RNases, we have solved the structure of the unswapped form of HHP2-RNase (M=M-HHP2). Within a quaternary framework very similar to that of BSRNase dimers, the solved structure revealed that the hinge region, which is highly disordered in M=M-BSRNase¹⁹ as well as in its monomeric derivative,³⁶ adopts well-defined conformations in both subunits. This result suggests that the hinge peptides may provide an efficient anchorage to a transient noncovalent dimer, which helps to maintain Cys31 and Cys32 of the two subunits in proximity. This can explain the stability of the interchain disulfides and can be at the basis of the enzyme cytotoxicity.

Results

Overall structure

The combined use of PEG, salts, and sugar (sodium chloride, ammonium sulfate, and threoside) successfully produced M=M-HHP2 crystals suitable for X-ray diffraction measurements. Although the crystals were grown using the equilibrium mixture of the swapped and unswapped form of HHP2-RNase, the well-

defined electron density corresponding to the hinge peptide regions clearly indicates that only the unswapped form is present in the crystal state.

The structure was solved by molecular replacement, using des(1–7)HP-RNase as starting model,²⁷ and refined to 2.60 Å resolution. The quality of the electron density maps allowed a description of the whole molecule, with the exception of only few side chains. Most of the mutated residues were clearly defined in the omit density maps. The final model [Fig. 1(A)], containing 1950 protein atoms, 162 water molecules, and 5 sulfate ions, has been refined to *R*-factor/*R*-free values of 21.4/30.6. The statistics of refinement are listed in Table I.

The two subunits, linked by the two interchain disulfide bridges, share a strong structural similarity, as judged from their low *C*^α root mean square deviation (rmsd = 0.85 Å). They are related by a local twofold axis with a rotation angle of 178° and a small screw component of 0.64 Å. The major deviations are observed for the external loops 66–71 and 87–96, involved in different packing interactions. The subunits show the typical topology of pancreatic-like RNases. They are very similar to the structure of HP-RNase in the HP-RI complex,²⁹ with a rmsd of the *C*^α atoms of 1.1 Å, and to the other known structures of HP-RNase variants^{26–28} (rmsd in the range 0.8–1.5 Å).

The quaternary association of M=M-HHP2 is very similar to that of the covalent dimeric forms of BSRNase^{18–20}: after the superimposition of the core of one subunit, a further rotation of only 8.2° and 7.3° is required to fit the core of the second subunit of M=M-BSRNase and MxM-BSRNase, respectively. Thus, the interchain disulfide bonds and the two mutations Gln28→Leu and Asn34→Lys reproduce in M=M-HHP2 the same helix-II/helix-II interface characteristic of the seminal ribonuclease.^{18–20} This similarity extends to the two Leu28 side chains, which make stabilizing hydrophobic interactions across the molecular twofold axis. The side chains of Lys34 are completely disordered, as in M=M-BSRNase and MxM-BSRNase.^{18–20}

The two active sites are very well defined and show minor structural differences [Fig. 1(B)], probably related to the crystallization pH (6.8), which is close to the *pK*_a of the catalytic histidines. In one subunit, the side chain of His119 adopts the B conformation ($\chi^1 \approx -80^\circ$),³⁷ stabilized by the interaction with the sulfate ion, which in turn is linked to His12, Phe120, and Gln11. In the other subunit, the sulfate is displaced toward the entrance of the active site. Here, it is anchored only by a hydrogen bond with His119, which adopts the A conformation ($\chi^1 \approx 160^\circ$),³⁷ stabilized by the strong interaction with the neighboring Asp121.

The hinge peptides and the modeling of a putative transient noncovalent dimer

The electron density associated with the two hinge regions (residues 16–22) is well defined [Fig. 1(C)].

Figure 1. (A) A ribbon diagram showing the secondary structural elements in M=M-HHP2. The intersubunit disulphide bridges are drawn in ball-and-stick representation. (B) Superimposition of active site region of the two subunits of M=M-HHP2: the groups adopting a different conformation in B subunit are drawn in cyan. (C) OMIT Fo-Fc map of the two hinge peptides (first subunit on the top) contoured at 3.5 σ (I). (D) Space-filled model of the putative noncovalent unwrapped dimer obtained by reduction of intersubunit disulphide bridges of M=M-HHP2. In this representation, the two subunits are colored in cyan, the hinge peptides in blue, the two Leu28 in red, and the Cys31 and 32 of the two subunits in yellow. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The two loops adopt a conformation very similar to that found in the structure of des(1–7)HP-RNase (rmsd 0.33 Å on the average)²⁷ and in the mutant N4L (rmsd 0.29 Å),²⁸ and different from that adopted by the mutant 2, 3, and 4L (rmsd in the range 2.43–2.54 Å)²⁸ and observed for the HP-RI complex (rmsd 2.54 Å).²⁹ In the latter structure, the hinge peptide is characterized by the formation of a type IV β -turn (residues 18–21) stabilized by three hydrogen bonds.²⁹

In M=M-HHP2, a type IV β -turn motif encompasses residues 20–23 and is stabilized by hydrogen bonds formed by the carbonyl oxygen of Ser20 with the amide nitrogen of Ser23 (3.3 Å for both subunits) and with the OG of Ser23 (2.6 and 2.3 Å, for the two subunits, respectively). The peptide is further tethered to the body of the molecule by the hydrogen bond bridging the amide nitrogen of Ser22 with the carbonyl oxygen of Thr99 (3.1 and 2.8 Å, respectively). The structural features of the peptide are significantly

different from those observed for the M=M isomer of the seminal enzyme, where the peptide is characterized by a pronounced flexibility that does not allow to

Table I. Refinement Statistics

Resolution range (Å)	40.0–2.60
Total Number of reflections used	8539
Number of reflection in the test set	462
R-factor/R-free (%)	21.4/30.6
Number of protein atoms	1950
Water sites	162
Sulfate anions	5
R.m.s deviation from stereochemical target values	
Bond length (Å)	0.009
Bond angles (°)	1.17
Average B-factor (Å ²)	
Main chain	19.5
Side chain	20.3
Ion atoms	31.6
Solvent atoms	23.9

model the region 19–21. Moreover, the remaining part of the hinge loop is characterized by a conformation of Gly16 not accessible to a non-Gly residue (Asp in HHP2-RNase) and stabilized by a hydrogen bond with the Arg80 side chain.¹⁹

In the seminal enzyme the different properties of the M=M and MxM forms have been rationalized in terms of their behavior under reducing conditions^{16,22}: the former yields a monomeric enzyme, whereas the latter gives rise to a metastable noncovalent dimer (NCD-BSRNase), characterized by a structure similar to that of the parent species. The possibility that a similar noncovalent transient dimeric species could be formed also for HHP2-RNase has been investigated using a docking procedure inspired by the 3D structures of NCD-BSRNase. The relative orientation of the M=M-HHP2 subunits in the dimer was changed by rotating about an axis orthogonal to the molecular dyad and passing through the midpoint of the Leu-Leu interchain contact. This procedure brings the two hinge peptides in close contact to each other, while the cysteine residues move about 4 Å away. The overall shape of the final model [Fig. 1(D)] closely resembles that of NCD-BSRNase. The area buried at the interface is significantly larger than that of the HHP2-RNase, although its values (~ 720 Å²) lies in the lower limit of the values usually needed to stabilize a dimeric form.³⁸ It should be noted, however, that at the serine rich hinge region interface several hydrogen bonds can be formed between the side chains of Ser17 of one subunit and Thr24 of the other, and vice versa, and between the OG atoms of the two Ser20 residues.

Discussion

RI binds multiple members of the RNase A superfamily with equilibrium dissociation constants in the femtomolar range, forming one of the tightest noncovalent interactions among biomolecules.^{12,29,39} This broad specificity may reflect the role of RI as a sentry to protect cells from all of the pancreatic RNase superfamily enzymes that can pass through the cellular membrane. Therefore, the capability to evade RI may confer cytotoxic properties to the molecule and have potential medical applications.²³ Dimerization seems to be a possibility and BSRNase has been suggested to have exploited it by forming a dimer whose quaternary structure interferes with the binding of RI.^{16,22} In the reducing environment of the cytosolic compartment, the swapping phenomenon plays an essential role, as it favours the formation of a metastable dimer, which confers a greater stability to the interchain disulfides bridges, by facilitating the reformation of the Cys-Cys bonds,^{16,22} or because it has by itself the right shape to escape the protein inhibitor.¹⁶

Monomeric HP-RNase is not cytotoxic and forms with RI a particularly tight complex with a K_d value of $2.9 \times 10^{-16} M$.²⁹ The dimeric mutant HHP2-RNase, designed to adopt a quaternary structure similar to

that of BSRNase, is scarcely prone to swap³⁵; however, its unswapped form is hyperactive as a cytotoxic agent.^{34,35} This property has been ascribed to the stability of the interchain disulfides that is significantly larger than that of M=M-BSRNase.^{34,35} Surprisingly, the structural data clearly show that Cys31 and Cys32 have in the two enzymes similar accessible surface area, similar contacts and environments in a range of about 10 Å. The only notable structural difference lies in the two hinge peptides, which are disordered in M=M-BSRNase^{19,36} while adopting a well-defined conformation in M=M-HHP2. The substitution Gly16-Asp, Asn17→Ser, and Arg80→His occurring in M=M-HHP2 with respect to BSRNase can be responsible for the observed structural behavior. In this regard, it should be noted that the monomeric form of BSRNase, where the segments connected by the hinge peptide bears a relative topology strictly similar to those in M=M-BSRNase, the disorder is even more pronounced.³⁶ Vice versa, in MxM-BSRNase, the hinge peptides adopt definite conformations. Similarly, in PM7, which has the BSRNase sequence in the hinge region, this peptide is disordered,²⁶ whereas it is fully ordered in PM8, the closely related swapped dimer.

Thus, despite the similarity in the quaternary assembly, the dimeric form of HHP2-RNase has a small tendency to swap with respect to the seminal enzyme, and the M=M isomer properties are more similar to those of MxM-BSRNase than to the ones exhibited by M=M-BSRNase. To explain these properties, we hypothesize that the ordered hinge peptides, together with the hydrophobic interactions between the Leu28 side chains, provide a good anchoring surface that stabilizes in reducing conditions a transient dimeric structure, even in the absence of the swapping phenomenon. A molecular modeling of the noncovalent dimer has provided a good support to this hypothesis. Although the interacting surface is not large, the fact that the hinge regions have already a well-fixed conformation can make the difference with M=M-BSRNase, where the dimer formation would require an additional energetic cost to immobilize the disordered region.

An independent support to this hypothesis come from the recently published data on the formation of the PM8 swapped dimer.⁴⁰ The effect of the temperature on the dimerization strongly suggests that PM8 dimerizes following a mechanism in which a non-swapped dimer is first formed. This rate-limiting dimerization step includes the stabilization of the open interface (hinge region) and prepares the dimer to evolve into the swapped form. In the case of HHP2-RNase, the hinge peptides are already well ordered, and the addition of the hydrophobic patch provided by Leu28 can further stabilize the dimeric form of the molecule, particularly in the overcrowded compartment of the cytosol. Indeed, more recently, it has also been shown by NMR investigations that the native

enzyme itself in solution is predominantly dimeric above 1 mM concentration,²⁵ although no indication regarding the quaternary structure of the dimer has been given.

As a final comment, we note that, although the remarkable biological properties of BSRNase and HHP2-RNase can be possibly due to several factors, at least in part they appear to be based on the existence of a similar transient noncovalent dimeric form: in the former the dimer is stabilized by the swapping phenomenon, whereas in the latter the dimer is favored by the accessibility of the unswapped hinge peptide to an ordered conformation. In both cases, in a reducing environment the hydrophobic patch created at the interface by the Leu28 side chains has an important role in determining an appropriate quaternary shape either to evade the interaction with the RI, or to favor by the proximity effect the formation of the interchain disulfides bridges.

Methods

Crystallization and data collection

Protein production and purification were performed as previously described.^{34,35} Crystals were obtained by sitting drop vapor diffusion method at 277 K. A protein solution of 10 mg/mL in 10 mM tris acetate pH 7, 300 mM NaCl was equilibrated against a solution containing 27% (w/v) polyethylene glycol and 0.2M ammonium sulfate and 100 mM cacodylate buffer pH 6.5. After several weeks, each drop contained long needle crystals. The addition of threose (100 mM) to the trials induced an increasing of the crystallization time (about 3 months) and an improvement of the crystal quality. A thin-plate like crystal was harvested and directly flash-frozen in liquid nitrogen. Data were collected at ELETTRA beamline at 100 K and were indexed, scaled, and reduced using DENZO and SCALEPACK.⁴¹ The intensities were truncated to amplitudes using TRUNCATE.⁴² Detailed data-processing statistics are given in Table II.

Structure determination and refinement

Initial phases were determined by the molecular replacement method as implemented in AMoRe,⁴³ employing a search model derived from the crystal structure of des(1–7)HP-RNase.²⁷ In this search model, the hinge peptide residues (13–23) were deleted. The solution was then subjected to several cycles of coordinate minimization and B-factor refinement with CNS.⁴⁴ Each run was alternated with manual model building using O.⁴⁵ The correctness of the model has been checked using several validation criteria. Among these, omit (Fo–Fc) maps were calculated for all residues of the structure, including the loop regions. Model validation was conducted using PROCHECK⁴⁶ and WHATCHECK.⁴⁷ Analysis of the Ramachandran plot showed that all residues lie in the

Table II. *Crystal Parameters and Data Collection Statistics*

Space group	P2 ₁ 2 ₁ 2 ₁
<i>a</i> (Å)	48.85
<i>b</i> (Å)	78.25
<i>c</i> (Å)	80.69
Resolution limits (Å)	30.0–2.6
No. of observations	185910
No. of unique reflections	9128
Completeness (%)	98.7 (98.5)
<i>I</i> / σ (<i>I</i>)	20 (9)
R-merge (%)	9.0 (16.9)
Mosaicity	0.8

Note: Values in parentheses correspond to the highest resolution shells (2.66–2.60 Å).

allowed regions. The coordinates of the structure have been deposited in the PDB (code 3F8G).

Modeling of a putative noncovalent HHP2-RNase dimer

The model of the putative dimer has been obtained using the program X-PLOR,⁴⁸ using a three-step procedure. In the first run, the molecule was highly idealized using a tight restrain to the original crystallographic coordinates to obtain a model with a final rmsd of about 0.1 Å from the initial model. The interchain disulfide bridges were then broken and complementary anchoring points were located on the 16–22 regions of the two chains. The distances between the two hinge peptide regions were restrained to contact values using a harmonic potential. This run produced essentially a coupled rigid rotation of the two chains, normal to the molecular dyad, pivoted on the mean position between the Leu28 of the two subunits. In this run, the formerly bonded cysteine residues move about 4 Å apart, while the regions 16–22 come closer together. Contacting side chains were then manually rotated to optimize interchain hydrogen bond interactions.

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