Serpin-derived Peptide Substrates for Investigating the Substrate Specificity of Human Tissue Kallikreins hK1 and hK2*

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Luc Bourgeois†§, Michèlle Brillard-Bourdét‡, David Depertes§, Maria A. Juliano*, Luiz Juliano*, Roland R. Tremblay¶, Jean Y. Dubé§, and Francis Gauthier‡**

From the †Laboratory of Enzymology and Protein Chemistry, CNRS EP 117, University François Rabelais, 37032 Tours, France, the §Laboratory of Hormonal Bioregulation, CHUL Research Center, Sainte Foy, Quebec, Canada, and the ¶Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil.

The third human tissue kallikrein to be identified, hK2, could be an alternate or complementary marker to kallikrein hK3 (prostate-specific antigen) for prostate diseases. Most of the hK2 in seminal plasma forms an inactive complex with protein C inhibitor (PCI), a serpin secreted by seminal vesicles. As serpin inhibitors behave as suicide substrates that are cleaved early in the interaction with their target enzyme, and kallikreins have different sensitivities to serpin inhibitors, we prepared a series of substrates with intramolecularly quenched fluorescence based on the sequences of the serpin reactive loops. They were used to compare the substrate specificities of hK1 and hK2, which both have trypsin-like specificity, and thus differ from chymotrypsin-like hK3. The serpin-derived peptides behaved as kallikrein substrates whose sensitivities reflected the specificity of the parent inhibitory proteins. Substrates derived from PCI were the most sensitive for both hK1 and hK2 with specificity constants of about 10^7 M⁻¹ s⁻¹. Those derived from antithrombin III and α₂-antiplasmin were more specific for hK2 while a kallistatin-derived substrate was specifically cleaved by hK1. hK1 and hK2 substrates of greater specificity were obtained using chimeric peptides based on the sequence of serpin reactive loops.

The main difference between specificities of hK1 and hK2 arise because hK2 can accommodate positively charged as well as small residues at P₁ and requires an arginyl residue at P₁. Thus, unlike hK1, hK2 does not cleave kininogen-derived substrates overlapping the region of N-terminal insertion of bradykinin in human kininogens.

The human kallikrein gene family contains three well-characterized members, hKLK1, hKLK2 and hKLK3 (1). These genes have been mapped to chromosome 19 (2) and are transcribed to give rise to proteases hK1, hK2, and hK3 respectively.

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** To whom correspondence should be addressed: Enzymology and Protein Chemistry Laboratory, CNRS EP 117, University François Rabelais, 2bis Bd Tonnellé, 37032 Tours Cedex, France. Tel.: 33 02 47 36 60 45; Fax: 33 02 47 36 60 46; E-mail: gauthier@univ-tours.fr.

1 The abbreviations used are: IGBP-3, insulin-like growth factor binding protein 3; ACT, α₁-antichymotrypsin, α₁-P, α₁-proteinase inhibitor; α₂-AP, α₂-antiplasmin; ATIII, antithrombin III; PCI, protein C inhibitor; SBTI, soya bean trypsin inhibitor; TFA, trifluoroacetic acid; AMC, 7-aminomethylcoumarin; Abz, o-amino benzoxyl; EDDnp, N-(3,4-dimethoxyphenyl)l-arginine; pNA, paranitroanilide; PTH, phenylthiohydantoin; Bz, benzoyl; Fmoc, N-(9-fluorenyl)methoxycarbonyl; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight.
the prostate, in seminal fluid, and at metastatic sites. As kallikreins are inhibited differently by natural inhibitors of the serpin family (17), that probably all behave as suicide substrates (20, 21), we used the peptide sequences of several serpin reactive loops to prepare specific, sensitive substrates capable of discriminating between the activities of these proteinases.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from commercial sources: DEAE-Sephadex A50 (Pharmacia Biotech Inc.); SBTI (Boehringer Mannheim); 4-methylumbelliferyl, 7-amino-4-methylcoumarin, (D)PFR-AMC, IGEPAL CA-630, 4-methylumbelliferyl-4-guanidinobenzoate (Fluka Chemie AG); EDTA, N,N-dimethylformamide, acetonitrile (Merck); and TFA (Perkin-Elmer). All other reagents were of analytical grade.

Purification and Titration of Human Kallikreins—Kallikrein hK2 was purified from human seminal plasma after dissociation of the PCI-hK2 complex (17). Human kallikrein hK1 was purified to homogeneity from urine essentially as described (22). Briefly, 6.5 liters of human urine were concentrated to 200 ml by passage through an Amicon PM10 membrane, dialyzed against 20 mM phosphate buffer, pH 6.0, 150 mM NaCl, and applied to a DEAE A50 column (4 × 2 cm) equilibrated in the same buffer. The column was washed with 200 ml of equilibration buffer and proteins were eluted with a 50–500 mM NaCl gradient. Fractions were assayed for their enzymatic activity toward (D)PFR-AMC in the presence of SBTI. Fractions resistant to SBTI inhibition were pooled and titrated with aprotinin (23). The active site of hK2 was titrated using 4-methylumbelliferonyl-4-guanidinobenzoate (24).

Synthesis of Fluorogenic Substrates—The intramolecularly quenched fluorogenic peptides were synthesized by classical solution methods; glutamine was the C-terminal residue in all peptides due to a requirement of hK2 was titrated using 4-methylumbelliferyl-4-guanidinobenzoate (previously Nonidet P-40). Specificity constants (\(k_{cat}/K_m\)) for interaction and cleavage by the proteinase. The free inhibitor, it is unlikely that any structural constraint is present in seminal plasma mainly as a tight complex with protein C inhibitor (11), whose concentration in seminal plasma is more than 40 times that in the blood plasma (28). The rate of inhibition of hK2 in seminal plasma roughly follows that of semenogelin proteolysis after ejaculation (12). PCI also inhibits human kallikreins hK3 and hK1, forming SDS-stable complexes (29, 30). This inhibitor belongs to the serpin family and interacts with target proteinases to form enzymatically inactive, tight-binding complexes. This involves the cleavage of the reactive loop and insertion of the cleaved loop into a \(\beta\)-sheet structure of the protein, leading to the trapping of the proteinase as a stable complex (21). But the reactive loop mobility is not required for enzyme recognition, so that its blocking by mutation or with synthetic loop peptides converts the inhibitor into a substrate (31).

Kinetic Measurements—All assays were carried out at 37 °C in 50 mM Tris-HCl buffer, pH 8.3, containing 1 mM EDTA, 0.02% IGEPAL, and diluted with activation buffer. Substrate purity was checked by MALDI-TOF mass spectrometry (Tokyo-Elec, Micromass) and by reverse-phase chromatography on a C18 column eluted with a 10-min linear gradient of 0–60% acetonitrile in 0.075% TFA at 2.0 ml/min. Bz-VKKR-AMC and (D)PFF-AMC were synthesized as described previously (26).

Kinetic Measurements—All assays were carried out at 37 °C in 50 mM Tris-HCl buffer, pH 8.3, containing 1 mM EDTA and 0.02% IGEPEA (previously Nonidet P-40). Specificity constants (\(k_{cat}/K_m\)) were determined for peptidyl-AMC and Abz-peptidyl-EDDnp substrates under pseudo-first order conditions, using a substrate concentration far below \(K_m\) (27). Calculations were done using Enzfitter software (Biosoft). For AMC substrates, the spectrofluorometer excitation was 350 nm and emission was 460 nm, and experiments were carried out using 0.5 AMU AMC for calibration. Excitation and emission wavelengths were 320 and 420 nm for experiments with intramolecularly quenched fluorogenic substrates. The system was standardized using Abz-FR-OH prepared by total tryptic hydrolysis of Abz-FR-pNA, and its concentration was determined from the absorbance at 410 nm, assuming \(e_{410} = 8,800 \text{ M}^{-1} \text{ cm}^{-1}\) for p-nitroanilide. Substrate concentrations of Abz-peptidyl-EDDnp were determined by measuring the absorbance at 385 nm, assuming \(e_{385} = 17,300 \text{ M}^{-1} \text{ cm}^{-1}\) for EDDnp. Kinetic constants were measured using eight substrate concentrations (0.1–15 \(\mu\)M), the final concentrations of hK1 and hK2 were 10 nM to 10 mM, depending on the substrate used.

Chromatographic Procedures—Fluorogenic peptides (15–30 \(\mu\)M final) were incubated with purified kallikreins (0.75–1.50 nmol final) at 37 °C in 50 mM Tris-HCl buffer, pH 8.3, containing 1 mM EDTA, 0.02% IGEPEA, Aliquots (200 \(\mu\)l) were removed at different times (5 min to 10 h depending on the peptide and enzyme used). The reaction was blocked by adding 4 \(\mu\)l of TFA, and the sample was fractionated on a C18 cartridge column (5 \(\mu\)m, 30 × 2.1 mm) eluted with a linear gradient of acetonitrile (0–60%, \(\nu/\nu\)) in 0.075% TFA for 10 min at a flow rate of 2.0 ml/min with simultaneous recordings at 3 wavelengths (220, 230, and 280 nm), which allowed the direct identification of the formation of EDDnp-containing peptides prior to N-terminal sequencing. All cleavage sites of significant importance identified by HPLC were determined by N-terminal sequencing for all substrates used in this study.

The hydrolysis products of the bradykinin-containing Abz-peptide were separated on an Aquapore RP 300 cartridge column (7 \(\mu\m), 100 × 2.1\) mm), using a linear gradient of acetonitrile (5–25 min, 0–60% acetonitrile, \(\nu/\nu\)) in 0.075% TFA for 35 min at a flow rate of 1.0 ml/min. Cleavage sites were identified by N-terminal sequencing.

Amino Acid Sequence Analysis of Peptide Products—The amino acid sequences were determined using an Applied Biosystems 477A pulsed liquid sequencer with the chemicals and program recommended by the manufacturer. Phenylthiohydantoin derivatives were identified using an on-line model 120A PTH analyzer.

RESULTS AND DISCUSSION

Preliminary attempts to identify the biological targets of hK2 have shown that coagulum-forming proteins, including semenogelin and fibronectin, are cleaved at Arg sites by hK2 and at Tyr sites by hK3 in vitro (12). It is not yet clear, however, whether hK2 participates in sperm liquefaction and, if so, the extent to which it cleaves coagulum-forming proteins. hK2 is present in seminal plasma mainly as a tight complex with protein C inhibitor (11), whose concentration in seminal plasma is more than 40 times that in the blood plasma (28). The rate of inhibition of hK2 in seminal plasma roughly follows that of semenogelin proteolysis after ejaculation (12). PCI also inhibits human kallikreins hK3 and hK1, forming SDS-stable complexes (29, 30). This inhibitor belongs to the serpin family and interacts with target proteinases to form enzymatically inactive, tight-binding complexes. This involves the cleavage of the reactive loop and insertion of the cleaved loop into a \(\beta\)-sheet structure of the protein, leading to the trapping of the proteinase as a stable complex (21). But the reactive loop mobility is not required for enzyme recognition, so that its blocking by mutation or with synthetic loop peptides converts the inhibitor into a substrate (31).

Therefore, synthetic peptides derived from the variable loop structure of individual serpin inhibitors are potential substrates for serine proteinases that are inhibited by serpins. A prerequisite, however, is that the conformation of the loop within the native protein is not essential for cleavage. Because of the flexibility of the reactive loop in the free inhibitor, it is unlikely that any structural constraint is required for interaction and cleavage by the proteinase. The differences in the sequences of the reactive loops in serpin family members (Table I) indicate that it should be possible to develop specific substrates for individual serine proteinases.

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
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<tbody>
<tr>
<td>Alignment of the reactive-centre loop sequences of human serpins</td>
</tr>
<tr>
<td>The scissile bond is at P$_1$P$_2$ site.</td>
</tr>
<tr>
<td>Serpin</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>PCI</td>
</tr>
<tr>
<td>ATIII</td>
</tr>
<tr>
<td>Kallistatin</td>
</tr>
</tbody>
</table>

\(\nu/\nu\) in 0.075% TFA for 35 min at a flow rate of 1.0 ml/min. Cleavage sites were identified by N-terminal sequencing.

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kallikreins differ, probably because of the extended binding site of interaction with the substrate (32, 33). But both enzymes cleaved all PCI-derived peptides at a single site at the expected R-S bond as illustrated in Fig. 1.

Other Serpin-derived Substrates for hK1 and hK2—α₁-Pi and kallistatin are two other serpin inhibitors of hK1 (34, 35). Kallistatin is the more specific and inhibits hK1 much faster than does α₁-Pi, but its action is blocked by heparin (36). The reactive loop of kallistatin is cleaved after an FF pair (35), an unexpected feature that has been recently exploited to develop new, sensitive hK1 substrates (37). Whether hK2 is inhibited by kallistatin and cleaves after the FF pair is not known. The tissue kallikrein substrates (D)PFR-AMC and (D)PFF-AMC were assayed with hK1 and hK2, but only (D)PFR-AMC was cleaved by both enzymes (Table IV). This suggests that hK2 does not cleave the kallistatin reactive loop, at least at the FF site, and therefore that this serpin does not inhibit hK2. A kallistatin-derived peptide could therefore discriminate between hK1 and hK2 activities. Such a substrate including the FF pair (Abz-AIKFFSAQ-EDDnp) was synthesized and assayed with both enzymes. It was rapidly cleaved by hK1 with a specificity constant of about 10^5 M⁻¹ s⁻¹, but was resistant to hydrolysis by hK2 (Table II). hK1 cleaved at the expected FF-S site, as shown by N-terminal sequencing of the EDDnp-containing fragment having an absorbance peak at 360 nm (peak a).

![Fig. 1. Identification of the hK2 cleavage site within a PCI-derived substrate. Reverse-phase HPLC of Abz-FTFRSARQ-EDDnp (30 μM) incubated for 8 min at 37 °C with (solid line) and without (dotted line) human kallikrein hK2 (1 nM final concentration). Absorption spectra (200–400 nm) of the collected peaks are boxed. The peptide cleavage site was identified by N-terminal sequencing of the EDDnp-containing fragment having an absorbance peak at 360 nm (peak a).](http://www.jbc.org/)

**Table II**

<table>
<thead>
<tr>
<th>Serpin</th>
<th>Substrate</th>
<th>hK1</th>
<th>hK2</th>
<th>hK1</th>
<th>hK2</th>
<th>hK1</th>
<th>hK2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₐm (μM) hₑcat (s⁻¹) hₑcat/Kₐm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCI</td>
<td>Abz-FRSARQ-EDDnp</td>
<td>0.4 ± 0.1</td>
<td>1.70 ± 0.14</td>
<td>4.250</td>
<td>9,000</td>
<td>8.5 ± 2.0</td>
<td>24.30 ± 3.65</td>
</tr>
<tr>
<td>PCI</td>
<td>Abz-TFRSRARQ-EDDnp</td>
<td>0.4 ± 0.1</td>
<td>6.40 ± 0.50</td>
<td>16,000</td>
<td>16,000</td>
<td>5.3 ± 2.1</td>
<td>41.00 ± 3.00</td>
</tr>
<tr>
<td>PCI</td>
<td>Abz-FTFRSARQ-EDDnp</td>
<td>0.3 ± 0.1</td>
<td>3.20 ± 0.38</td>
<td>10,650</td>
<td>11,500</td>
<td>1.1 ± 0.2</td>
<td>17.60 ± 1.58</td>
</tr>
<tr>
<td>Kallistatin</td>
<td>Abz-AIKFFSAQ-EDDnp</td>
<td>1.79</td>
<td>0.54</td>
<td>304ᵃ</td>
<td>440</td>
<td>NDᵃ</td>
<td>NDᵃ</td>
</tr>
<tr>
<td>ATIII</td>
<td>Abz-VIAGRSLNPNQ-EDDnp</td>
<td>NSHᵇ</td>
<td>NSHᵇ</td>
<td>NSHᵇ</td>
<td>NSHᵇ</td>
<td>NDᵇ</td>
<td>NDᵇ</td>
</tr>
<tr>
<td>α₁-AP</td>
<td>Abz-AMIRMSILSSFSVRQ-EDDnp</td>
<td>NDᶜ</td>
<td>NDᶜ</td>
<td>NDᶜ</td>
<td>15</td>
<td>NDᶜ</td>
<td>NDᶜ</td>
</tr>
<tr>
<td>Kallistatin-</td>
<td>Abz-AIKFFSRQ-EDDnp</td>
<td>0.6 ± 0.2</td>
<td>2.00 ± 0.20</td>
<td>3,350</td>
<td>2,900</td>
<td>NDᶜ</td>
<td>NDᶜ</td>
</tr>
<tr>
<td>modified</td>
<td>PCI-Abz-TFFSARQ-EDDnp</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1,700</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ᵃ Pseudo-first order.
ᵇ From reference (35).
ᶜ ND, not determined.
ᵈ NSH, no significant hydrolysis.
taining cleavage product obtained from reverse phase HPLC (data not shown).

Unlike hK1, hK2 is inhibited by several other serpin inhibitors, including ATIII and α2-AP (17). We used the sequences of the reactive loops of these serpin inhibitors to develop hK2 substrates that are resistant to hK1 hydrolysis. They were assayed under similar conditions with identical amounts of active-site titrated enzymes. Results were in accordance with the inhibitory properties of the serpin inhibitors. Peptide substrates derived from ATIII and α2-AP were cleaved by hK2 but not, or far slower, by hK1, possibly because of the presence of small or charged residues at P2 (Table II). The ATIII-derived peptide was cleaved at the GR-S bond by hK2 which corresponds to the sensitive bond in the protein. But the α2-AP-derived peptide was cleaved at the SR-M bond, which flanks the RM-S sensitive bond in the serpin. The value of the specificity constant for this substrate was altered however, due to additional cleavage by hK2 at the NR-Q bond, which further demonstrates the P1/Arg specificity of this kallikrein. This cleavage appeared because of the prolonged incubation that was necessary to measure kinetic data. However, the R-Q bond is not present in α2-AP or any other serpin reactive loop but was introduced here by the accelerated procedure of multisyntesis which uses Q-EDDnp as starting material (25).

The absence of an arginyl residue from α1-P, and ACT-derived sequences may explain why hK2 does not cleave these substrates (not shown). Unlike α1-P, however, ACT binds to hK2 in vitro (17). It could be that hK2 cleaves the ACT reactive loop at a site remote from L-S chymotrypsin cleavage site, which is not present in the peptide. Cleavage at several sites within the α1-P reactive loop has already been demonstrated (38).

Chimeric Peptides to Improve Substrate Sensitivity and Specificity—Though specific for hK1, the kallistatin-derived substrate is less well hydrolyzed by this protease than those derived from the PCI reactive loop. We attempted to improve its sensitivity by changing the P2 Ala to Arg (Abz-AIKFSSRQ-EDDnp), as the presence of Arg at P2 or P3 greatly improves hydrolysis by hK1 (39). Cleavage occurred at the same site as before, but the specificity constant was significantly greater. However, this chimeric substrate was also cleaved to some extent by hK2 although cleavage was at the R-Q bond, as for the α2-AP-derived peptide (Table II).

As the Phe at P1 in the kallistatin-derived peptide is mainly responsible for the limited specificity of hK1, we replaced the P1 Arg with Phe in the sensitive PCI-derived substrate Abz-TFRSSARQ-EDDnp. The resulting substrate, Abz-TFFSARQ-EDDnp, was completely resistant to hK2 hydrolysis but remained an excellent hK1 substrate, though of slightly lower sensitivity. It thus allowed complete discrimination between the two kallikreins (Table II).

Another characteristic of hK1, which is shared by its mammalian homologues having kininogenase properties, is that it prefers a large hydrophobic residue at P2 for substrate recognition and cleavage (40). This holds true for serpin inhibitors of hK1 that all have a phenylalanyl residue at P2. Unlike hK1, hK2 cleaves ATIII and α2-AP-derived peptides at sites having a small residue at P2 (Table II). Previous experiments carried out on several members of the rat tissue kallikrein family showed that some of them, which also accommodated small, uncharged residues at P2, cleaved substrates with positively charged residues at that position (41, 42). We measured the hydrolysis of a similar substrate, Bz-VKKK-AMC, by hK1 and hK2. The substrate was totally resistant to hK1, but was readily cleaved by hK2 (Table III). The peptide sequence was therefore used as the P fragment of an intramolecularly quenched fluorogenic substrate having the P′ sequence of the PCI-derived substrates to enhance the sensitivity and specificity to hK2. The resulting chimeric sequence, Abz-VKKSARQ-EDDnp, was hydrolyzed by hK2 at the R-S site with a kcat/Km about 50 times greater than that for the AMC derivative (Table III). However, hK1 also cleaved this substrate, mainly at the K-K site, in keeping with its P2 specificity and secondarily at the R-Q site. We removed the Val at P3 in this substrate to reduce cleavage by hK1. Abz-KKRSARQ-EDDnp was hydrolyzed more slowly by hK1 but dramatically faster by hK2, so that it is the most sensitive substrate for hK2 (Table III).

P1 Specificity and Kininogenase Properties—All cleavages of serpin-derived substrates by hK2 were at a P1/Arg site (Table II). In contrast, hK1 hydrolyzes kallistatin and kallistatin-derived substrates at a P1/Phe site and also cleaves kinogenins at a P1/Met site to release Lys-bradykinin. The apparent P1/Arg restricted specificity of hK2 suggests that this protease does not release a vasoactive kinin from its precursors, unless cleavage occurs at a different site. This could happen because the P2 specificity of hK2 is broader than that of hK1. We investigated this possibility by measuring the activities of hK1 and hK2 under similar experimental conditions using fluorogenic substrates that reproduced the sequences at both ends of the bradykinin moiety in human kinogenins (Table IV) and on a peptide spanning the complete bradykinin sequence with N- and C-terminal extensions (Abz-LGMISLMKRPPGFSPFRSS-RIL-NH2) (33). Since the latter peptide was too long for efficient quenching of the intramolecular fluorescence, it was labeled only N-terminally with Abz, and cleavage products were analyzed by reverse-phase HPLC and protein sequencing (Fig. 2). Both propeptases cleaved at the C-terminal cleavage site of bradykinin (R-S site), generating the Abz-LGMISLMKRPPGFSPFRSS peptide. This peptide was further processed by hK1 (but not by hK2) to generate Lys-bradykinin after cleavage at the M-K bond (Fig. 2). The intramolecularly quenched fluorogenic substrate (Abz-LGMISLMKRPPQ-EDDnp) was specifically cleaved by hK1 at the same M-K bond (Table IV). These results suggest that hK2 has no kininogenase properties of biological relevance.

The kinetic approach used here shows that the specificity of hK2 differs significantly from that of kallikrein hK1, and therefore that it has different biological targets. But these targets remain to be identified. The hK2 concentration in seminal
plasma is estimated at 0.1 μM,2 much like that of prostasin (43), but at least 100 times lower than that of hK3/prostate-specific antigen (44). But hK2 is rapidly bound by PCI in seminal plasma, so that almost no free, active enzyme is present in this fluid. The rate at which hK2 is inhibited by PCI is roughly the same as that of the degradation of the coagulum-forming proteins, whereas considerable hK3 remains free after protein degradation (12). Though hK2 hydrolyzes semenogelins and fibronectin in vitro (12), it is not known whether the process also occurs in vivo, where the concentration of hK3 is far higher. If hK2 participates in sperm liquefaction or any other process generating biologically active peptide from coagulum-forming proteins, it would be a unique example of the regulation of proteolytic activity by the simultaneous delivery of appropriate proteins, it would be a unique example of the regulation of generating biologically active peptide from coagulum-forming proteins.

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References


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