The Insect Hemolymph Protein HP19 Mediates the Nongenomic Effect of Ecdysteroids on Acid Phosphatase Activity*

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The activity of acid phosphatase (ACP) in insect fat bodies is stimulated by the steroid hormone 20-hydroxyecdysone (20E) in vitro. However, in fat bodies kept in culture, a factor from the hemolymph is required to enhance the ACP activity. We identified the factor as a protein with a molecular mass of 19 kDa (HP19) from the hemolymph of a lepidopteran insect, the rice moth, Corcyra cephalonica. Western analysis of hemolymph proteins with denaturing and non-denaturing PAGE using antibodies raised against HP19 suggest that this protein exists as a monomer. It is synthesized by the hind gut-associated lobular fat body of the larvae and is released into the hemolymph. The stimulatory effect of HP19 on the ACP activity is developmentally regulated and exhibits its maximal effect shortly before the onset of metamorphosis. We cloned the HP19 cDNA by immunoscreening a hind gut-associated lobular fat body cDNA expression library. Analysis of the amino acid sequence shows that HP19 belongs to the family of glutathione S-transferase (GST) like proteins. However, affinity-purified GST from Corcyra failed to show any mediation effect on 20E-stimulated ACP activity, and HP19 lacks GST enzymatic activity. Notably, HP19 mediates the hormone-stimulated ACP activity in intact fat body tissue and homogenates even in the presence of inhibitors of transcription and translation, suggesting a nongenomic mode of action. In addition, we show that HP19 inhibits the 20E-induced phosphorylation of the hexamerin receptor protein.

Insect metamorphosis, the transition from the larval to the adult stage of insects, is controlled by ecdysteroid hormones (1–3). The ecdysteroids, like the steroids in vertebrates, regulate gene transcription by binding to the nuclear receptors, which are ligand-activated transcription factors that convert the hormonal stimulus into a transcription response (4–7).

Metamorphosis involves the breakdown of larval structures and the formation of new tissues (1). As a part of cell remodeling during metamorphosis, acidic autophagic vacuoles accumulate in the cells of the fat body, and the activity of several lysosomal enzymes such as acid phosphatases increase and cause the lysis of larval tissues (8–11). The fat body fills a large fraction of the insect, and its function has been considered equivalent to the role of the vertebrate liver in the intermediary metabolism (12). It has been demonstrated that the stimulation of the lysosomal activity is governed by ecdysteroids (11, 13–16). There is an indication that in this case the hormone possibly acts on a nongenomic level (17). Although the molecular mechanism of the genomic mode of steroid action is well known, the mechanism of nongenomic steroid action remains unclear to date (18).

Earlier studies show that 20E stimulates ACP activity in fat bodies in vivo but not in vitro (19, 20). This result suggests that 20E, the active form of ecdysone, requires an additional factor (or factors) to enhance ACP activity. Hence, we have focused on the process of acid phosphatase activation by 20E in the fat body cells of our model insect, the rice moth Corcyra cephalonica. We report the appearance of a stage and tissue-specific regulated protein, HP19, in the hemolymph of Corcyra responsible for the activation of the 20E-dependent stimulation of ACP activity. This hormone-triggered activation is independent of gene transcription.

EXPERIMENTAL PROCEDURES

Insects—Larvae of the rice moth, C. cephalonica (Stainton), were reared on coarsely crushed sorghum seeds at 26 ± 1 °C, 60 ± 5% relative humidity, and a 14:10-h light:dark photo period. In the present study, the last (−Vth) instar larvae, classified into early (ELI), mid (MLI), late-larval instar (LLI) larvae and prepupae on the basis of body weight and head capsule size were used (21). The larvae were thorax-ligated behind the first pair of prolegs by slipping a loop of silk thread around the head of the larvae as described earlier (20). The fat body and other insect tissue homogenates from ligated or unligated larvae were prepared as published earlier (22) and used after protein estimation in an aliquot of the homogenate (23).

ACP Assay—The enzyme assay was carried out according to the method of Hendrickson and Clever (24). The reaction mixture contained 150 mM sodium acetate buffer (pH 5.0) and 20 μg of tissue homogenate proteins. It was incubated at 37 °C for 10 min to exclude glucose-6-phosphate activity (25). The reaction was initiated by the addition of 5 μmol of substrate, p-nitrophenyl bisiodum phosphate (Sigma) to the assay mixture followed by incubation for 1 h at 37 °C. The reaction was terminated by the addition of 0.5 ml of 0.1 M NaOH, and the color was measured at 420 nm against a substrate blank. The p-nitrophenol was used for the

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1 The abbreviations used are: 20E, 20-hydroxyecdysone; ACP, acid phosphatase; HP19, 19-kDa hemolymph protein; CcHP19, C. cephalonica HP19; CigGST, C. fumiferana GST; GST, glutathione S-transferase; HGLFB, hind gut-associated lobular fat body; ELI, early last instar larvae; MLI, mid-last instar larvae; LLI, late-last instar larvae.
preparation of a standard curve. The activity of the enzyme was expressed as nmol of 4-nitrophenol released/h/g of fat body protein.

Hemolymph Sample—Hemolymph from LLI larvae was collected into tubes pretreated with 0.025% phenylthiourea, diluted (1:20) with 10 mM Tris-HCl (pH 7.4), and spun for 3 min at 1000 × g to remove hemocytes. These hemolymph samples were used immediately after preparation.

Studies on Fat Body Cultures—Ribbon-shaped visceral fat bodies from LLI larvae were dissected 24 h after ligation under sterile conditions in cold insect Ringer’s solution and transferred to 100 μl of TC-100 insect culture medium (JRH Biosciences Inc.) with traces of streptomycin-sulfate. After rinsing, the tissue was transferred to 200 μl of fresh culture medium, and 80 nm 20E was added while an equal volume of carrier solvent (ethanol) was added to the control cultures. The hormone solutions were dissolved in ethanol (50% vol/vol), which never exceeded 0.05% in any of the experiments. To study the hemolymph effect, the diluted (1:20) or fractionated hemolymph was added to the fat body culture in the presence or absence of 80 nm 20E. Studies with glutathione S-transferase (GST) were carried out by adding purified cytosolic GST from Cecropia to the fat body cultures in the presence of hormone. These cultures were then incubated for 4 h at 25 °C with gentle shaking. At the end of incubation the tissue was removed, rinsed in ice-cold insect Ringer, homogenized, and used for ACP assay.

Fractionation and Purification of Hemolymph Proteins—Total hemolymph protein was loaded on a pre-equilibrated (10 mM Tris-HCl (pH 7.4)) Sephadex G-50 column and eluted with the equilibration buffer. The single fractions were checked for their ability to enhance the ACP activity in the fat body as well as in different tissues. These cultures were then incubated for 4 h at 25 °C with gentle shaking. At the end of incubation the tissue was removed, rinsed in ice-cold insect Ringer, homogenized, and used for ACP assay.
hp19 hormone manipulation studies. Hormone injections of 80 nM 20E, i.e. the physiological concentration (22, 36), to 24 h post-ligated LLI larvae caused a significant increase in the ACP activity in fat bodies after 24 h compared with the solvent-treated larvae (Fig. 1b).

To study the effect of hormone on the ACP activity of fat bodies kept in culture, the tissue was dissected from 24 h post-ligated larvae and cultured for 4 h in the presence of 80 nM 20E. The results show that 20E did not elicit any stimulatory effect, and the activity was more or less the same as in the controls (Fig. 1c). However, the addition of hemolymph from LLI larvae together with 20E caused a significant increase in the ACP activity (Fig. 1c). This observation suggests that the hemolymph contains a factor (or factors) required by 20E to stimulate the ACP activity in fat body cultures. When the hemolymph was treated with alcohol, heat, acid, alkali, or protease, no stimulation of the ACP activity by 20E could be observed, suggesting the proteinaceous nature of the factor (Fig. 1d).

Purification and Characterization of the Hemolymph Factor as a Protein Mediating 20E-stimulated ACP Activity—After loading total hemolymph protein on a Sephadex G-50 column, we eluted several fractions (Fig. 2) and checked their ability to mediate the 20E-stimulated ACP activity. We found an active protein fraction with a molecular mass of ∼22 kDa, calculated from the elution profile (Fig. 2, inset), or 19 kDa, calculated from the mobility on a SDS-PAGE (Fig. 3b). On the basis of these results we purified the active hemolymph protein first by fractionating the total hemolymph protein followed by gel filtration chromatography. The fractionation was carried out using 30- and 10-kDa cut-off filters. The filtrate from the 10-kDa cut-off filter gave a protein fraction that mediated the 20E-stimulated ACP activity of fat body cultures by an increase from 0.7 to 1.2 nmol of p-nitrophenol release/h/µg of protein (Fig. 3a). However, the protein yield in the filtrate obtained from the 10-kDa cut-off filter was insufficient to proceed for further purification. Therefore, the filtrate from the 30-kDa cut-off filters, in which the HP19 was contained, was used for gel filtration. The protein fraction eluted from the Sephadex column that mediated the 20E-dependent ACP activity first resulted in a contaminant-free pure polypeptide band of 19 kDa (Fig. 3b). Hence, the active hemolymph protein was named as HP19. Starting with 50 mg of total hemolymph protein, we obtained a 98.5-fold purification with 0.05% yield.

The Western blots presented in Fig. 3c show the specificity of the HP19 antibody both on denatured (lane 1) and non-denatured (lane 2) PAGE. A single protein band of 19 kDa suggested a monomeric structure of HP19. Southern analysis of genomic DNA (Fig. 3d) digested with EcoRI or HindIII probed with HP19-cDNA revealed HP19 as a single copy gene.

cDNA Cloning and Sequence Analysis of HP19—To identify the cDNA encoding the HP19 protein, a cDNA expression library, prepared from the RNA of HGLFB of LLI larvae, was immunoscreened. We picked 10 positive cDNA clones for detailed examination. The restriction analysis revealed 6 of the 10 clones to be of identical size. Initial sequencing study dem-
quences in the GenBank™ showed 67% identity with the four best matching invertebrate GSTs is shown in Fig. 38%. The comparison of the amino acid sequences of CcHP19 cDNA with other invertebrate GST were found to be less than 20E. The fat bodies from two 24-h post-ligated LLI larvae were fractionated on the ACP activity in fat body tissue culture in the presence of 20E. The fat bodies from two 24-h post-ligated LLI larvae were incubated with 80 nm 20E and 10 μl of fractionated hemolymph for 4 h. At the end of the incubation the fat bodies were assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. * significantly different from all other values (p < 0.05). PNP, p-nitrophenol. b, SDS-PAGE to show purification profile of HP19. The hemolymph protein was fractionated using a 30-kDa fractionator (Amicon), and the filtrate was applied on Sephadex G-50 for column purification. c, Western blot showing the specificity for HP19 (filled arrows) both in denatured (1) and non-denatured (2) PAGE. 20 μg of total hemolymph protein was loaded in each lane. d, Southern blot analysis showing single gene copy (filled arrows) of HP19. The genomic DNA (30 μg) from total larval body was digested with EcoRI (1) or HinfI (2) and probed with CcHP19 cDNA.

Fig. 3. Purification of a hemolymph protein enhancing the 20E-stimulated ACP activity in Corcyra fat body cultures. a, effect of different hemolymph fractions obtained using a 20- and 10-kDa fractionator on the ACP activity in fat body tissue culture in the presence of 20E. The fat bodies from two 24-h post-ligated LLI larvae were incubated with 80 nm 20E and 10 μl of fractionated hemolymph for 4 h. At the end of the incubation the fat bodies were assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. * significantly different from all other values (p < 0.05). PNP, p-nitrophenol. b, SDS-PAGE to show purification profile of HP19. The hemolymph protein was fractionated using a 30-kDa fractionator (Amicon), and the filtrate was applied on Sephadex G-50 for column purification. Crude hemolymph (1), proteins from filtrate of 30-kDa filter (2), protein markers in kDa (3), and active fractions eluted from G-50 column (4–8). Lanes 1–3: 10 μg; lanes 4–6, 5 μg; lanes 7 and 8, total lyophilized protein was loaded. c, Western blot showing the specificity for HP19 (filled arrows) both in denatured (1) and non-denatured (2) PAGE. 20 μg of total hemolymph protein was loaded in each lane. d, Southern blot analysis showing single gene copy (filled arrows) of HP19. The genomic DNA (30 μg) from total larval body was digested with EcoRI (1) or HinfI (2) and probed with CcHP19 cDNA.

onstrated significant sequence similarity among these clones. Furthermore, they showed homology with invertebrate GSTs. One of our clones was sub-cloned and totally sequenced (GenBank™ accession number AY369240). This HP19 cDNA was 634 nucleotides long, with an open reading frame of 585 bp, which encodes a protein of 195 amino acids. The calculated molecular mass of the translated unmodified protein was 22.95 kDa, which is close to the mass of HP19 detected in HGLFB, the tissue that synthesizes the protein (see Fig. 6e). The polypeptide comprises 12.3% basic (9 Arg, 1 His, and 14 Lys) and 13.3% acidic residues (10 Asp and 16 Glu) but no Cys residue. The estimated isoelectric point (pI) is 5.36. Comparison of the C. cephalonica HP19 (CcHP19) cDNA with the sequences in the GenBank™ showed 67% identity with Choristoneura fumiferana GST (CGGST) (38). Similarities of HP19 cDNA with other invertebrate GST were found to be less than 38%. The comparison of the amino acid sequences of CcHP19 with the four best matching invertebrate GSTs is shown in Fig. 4. Although the CcHP19 cDNA sequence revealed 67% identity with CGGST, affinity-purified GST from Corcyra had no enhancing effect on the 20E-dependent ACP activity when compared with purified HP19 or recombinant HP19 (Fig 5a). Furthermore, the hemolymph as well as the purified HP19 had negligible GST activity (Fig. 5b).

Tissue-specific Appearance of HP19—Co-culturing of different larval tissues with fat body demonstrates that the HGLFB is the only HP19-synthesizing tissue. A stimulation of the ACP activity by 20E was only observed when it was co-cultured with HGLFB (Fig. 6a). The hemolymph used in all experiments was cell-free, and, therefore, cannot be the site of HP19 synthesis. The tissue specificity of HP19 biosynthesis was further confirmed by immunohistochemical staining of different tissue sections using HP19 antibody. Again, HP19 was found to be localized only in HGLFB (Figs. 6, 5 and c). Western analysis of proteins from different tissues also revealed the presence of HP19 only in HGLFB (Fig. 6e, lane 2) and total larval body protein (Fig. 6e, lane 5). However, the apparent mass of HP19 in these was ~5 kDa higher than the HP19 present in hemolymph (Fig. 6e, lane 1). Northern hybridization with total RNA from different tissues and from total larval body displayed the tissue-specific expression of HP19 gene in HGLFB (Fig. 6f, lane 2). The faint band of HP19 in the Western blot (Fig. 6e, lane 5) and the apparent absence of HP19-mRNA in the total larval body, which comprises the HGLFB, is probably due to the limitation of the methods used.

Developmental Regulation of HP19—The developmental profile studies of HP19 in Corcyra during the last (=Vth) instar larval development suggest that only the hemolymph of LLI larvae could mediate the 20E-stimulated ACP activity (Fig. 7a). Western analysis of proteins from hemolymph (Fig. 7b) and HGLFB (Fig. 7c) of different developmental stages of Vth instar larvae show that HP19 is present at a maximal concentration in LLI (lane 3). HP19 is present in the hemolymph at all the developmental stages tested (Fig. 7b, lanes 1–4) but was not detectable in the HGLFBs of prepupae (Fig. 7c, lane 4). The intensity of the HP19 band in ELI and MLI was low compared with LLI (lanes 1–3). These results suggest that HP19 is synthesized throughout the complete last larval stage in HGLFB and is released into the hemolymph. The synthesis rate is low in ELI larvae, and the secretion into the hemolymph is rapid with exception of the LLI stage, when the HP19 synthesis is accelerated and is paralleled by the activation.

Nongenomic Regulation of 20E-stimulated ACP Activity by HP19—When the fat bodies kept in culture were incubated with 20E and HP19 for 4 h, we observed that the stimulation of the ACP activity remained unaffected in the presence of transcriptional or translational inhibitors (Fig. 8a). The results in Fig. 8b further indicate the nongenomic regulation of 20E-stimulated ACP activity by HP19 because the addition of protein directly to the fat body homogenate also mediated the steroid-stimulated action. This effect was rapid and could be observed within 30 s to 1 min (Fig. 8b). We further confirmed the nongenomic regulation of 20E-dependent ACP activity by incubating the fat bodies kept in culture first with [3H]methionine for 2 h followed by incubation with hormone, HP19, and transcriptional or translational inhibitors. The results (Fig. 9) show that the total protein synthesis is induced under the influence of 20E, and this induction is inhibited by actinomycin D and cycloheximide (Fig. 9a). However, the inhibitors did not affect the ACP activity. The HP19 enhanced the 20E-stimulated ACP activity (Fig. 9b).

Phosphorylation of the Hexamerin Receptor by 20E—We have recently reported that 20E stimulates the phosphorylation of the Corcyra hexamerin receptor, which is partly mediated by a tyrosine kinase at a nongenomic level (22). Therefore,
it was pertinent to check whether HP19 had any effect on hexamerin or tyrosine kinase phosphorylation. In vitro phosphorylation of fat body homogenate proteins from Corcyra resulted in the phosphorylation of mainly three proteins of masses 120, 60, and 48 kDa (Fig. 10). The addition of HP19 either in the absence (lane 3) or in the presence of 20E (lane 4) inhibited the basal as well as 20E-stimulated phosphorylation of the 120-kDa protein, which was identified earlier as the hexamerin receptor (22).

**DISCUSSION**

Insect development and reproduction are mainly controlled by a complex interaction between two groups of hormones, the ecdysteroids and the sesquiterpenoid juvenile hormones. The prominent functions of ecdysteroid hormones are their ability to trigger the temporal sequence of developmental processes underlying molting and metamorphosis (1, 2), one of the most dramatic events in the animal kingdom. The hemolymph, i.e., the insect "blood," is known as a source of several factors that do not only regulate ecdysteroid synthesis but also mediate ecdysteroid-dependent actions in larvae and pupae (39–45). 20E has been shown to elicit effects on autophagic processes of the fat body by stimulating the lysosomal enzyme activity of ACP (10, 11, 13–16).

Thorax ligation that renders the posterior part in larvae relatively free of endogenous hormone showed a gradual decline in larval fat body ACP activity, pointing to the hormonal dependence of ACP activity (Fig. 1). The injection of a physiological dosage of exogenous 20E into ligated larvae caused a stimulation of ACP activity. However, when 20E alone was added to fat bodies kept in culture the hormone could not stimulate the enzyme activity. Only co-treatment of the cultured fat bodies with 20E together with hemolymph enhanced the ACP activity. This result confirmed our earlier findings of the presence of some hemolymph factor that mediated the 20E-dependent ACP activity in vivo (20). In this study we identified and characterized this hemolymph factor as a 19-kDa protein (HP19) and studied its synthesis as well as its developmental regulation.

The novel function of the protein prompted us to devise a protocol for purification. Although a few nanograms of the protein were found to be sufficient for stimulation even in crude or partially purified fractions, the yield of the purified protein was very low. Other limitations were the requirement of a large quantity of hemolymph of a specific developmental stage (LLI) and removal of the major contaminating protein, hexamerin, which constitutes 75–80% of total hemolymph protein (46). Therefore, an antibody against HP19 was raised by electroeluting HP19, and its specificity against HP19 was...
confirmed. When this antibody was added together with hemolymph to cultures of LLI fat body, the hemolymph failed to mediate the 20E-dependent action. Similarly, when the hemolymph was first immunoprecipitated using HP19 antibodies and the resulting complex and the supernatant were added to the fat body cultures, HP19 action was suppressed (data not shown).

Western analysis of denatured as well as non-denatured PAGE demonstrated that HP19 is a monomeric protein in Corcyra and is obviously the product of a single copy gene (Fig. 3d). Three independent methods, co-culturing, Western analysis, and immuno-histochemistry, revealed that HP19 is synthesized by the HGLFB, from where it is released into the hemolymph. This is further confirmed by the tissue-specific gene expression only in HGLFB. Western analysis provides evidence of a difference of ~5 kDa in the mass of HP19 in HGLFB (i.e. 24 kDa) and in hemolymph (19 kDa). The predicted mass of the unmodified translated protein from the HP19 cDNA was close to the HP19 synthesized in HGLFB. Furthermore, the 0.66-kilobase HP19-mRNA (Fig. 6f) indicates a mass of about 24 kDa synthesized in HGLFB.
pared the sequence with those of related proteins, particularly we produced and characterized CcHP19-cDNA clones and com-
mon e is unclear at this point.

molecular mechanism of activation of ACP by ecdysteroid hor-
activity of HP19 is developmentally regulated. However, the
notable that only hemolymph from this developmental stage is
the hemolymph. The maximal HP19 concentration in the tissue
the total last larval instar. The protein is rapidly released into
cells.

cates that the protein can be secreted from the synthesizing
homogenate protein was incubated with an equal volume of 0.05%
ethanol (filled arrow

To gain more insight into the nature and function of HP19,
we produced and characterized CcHP19-cDNA clones and com-
pared the sequence with those of related proteins, particularly
with that of CGGST, which exhibits 67% identity. The CcHP19
does not contain specific amino acids that are responsible for
 glutathione binding or are involved in modulating the specific
activity of mammalian μ, γ, and σ GSTs (38, 48–50). The HP19
sequence also showed other putative post-translational modi-
fication sites like protein kinase C (189–191), casein kinase II
(81–90, 124–127, 142–145, and 161–164), and tyrosine kinase
(19–25 and 33–41) phosphorylation sites as well as N-myrist-
toylation (138–143 and 150–155) sites. However, in vitro phos-
phylation studies with hemolymph did not reveal any phos-
phylation of HP19 (data not presented).

GSTs with hormone-regulating actions are unknown, al-
though few studies on vertebrate GSTs speculate about their
steroid binding properties or their developmental and hormo-
regulation (51–54). In the present study we also checked
the possibility of GST exhibiting HP19 function. For this pur-
pose we tried to replace HP19 with affinity-purified GST and
tested whether GST can exhibit the same effect as of HP19. The
CGGST shows >60% sequence identity with HP19 (38), and the
CGGST antibody revealed immunocross-reactivity with differ-
tent tissue proteins in the mass range of HP19 (data not pre-
ented). The affinity-purified GST from Corycra did not show
any potentiation in 20E-stimulated ACP activity like that of
HP19 purified from the hemolymph or recombinant HP19. In
addition, the GST activity in the hemolymph was very low and
negligible in the purified HP19. All these studies together show
that HP19 has no GST activity. During the purification of
cytosolic GST of Corycra by affinity chromatography, the flow-
through fractions that did not bind to the affinity matrix me-
diated the 20E effect on ACP activity, further indicating that
HP19 is different from GSTs (data not presented).

A wealth of data on the molecular mechanism of ecdysteroid
action shows that the transcriptional cascade leading to mol-
ting and metamorphosis is initiated when 20E binds to its
nuclear receptor complex (2). For about four decades evidence
has accumulated that some of the hormonally induced effects
seemed to be too rapid for the classical model (18, 55). This
evidence casts doubt on the so-called genomic pathway as the
sole mode of steroid action. Today, several modes for non-
genomic steroid actions are examined. Most of them are
thought to continuously modulate the long term program al-
lowing cells or organs to adapt rapidly to environmental
changes. Numerous experiments with many different species
show that insect metamorphosis in general is under the genetic
control of ecdysteroids. A few studies indicate that some events
necessary for and accompanying metamorphosis are controlled
by 20E at a nongenomic level. However, studies on these mechanisms are restricted to a small number of experimental systems, e.g., the activation of lysosomal enzymes and the hexamerin receptor (17, 22, 37).

To learn more about the molecular mechanisms of ecdysteroid in regulating the HP19-assisted ACP activity, the fat bodies in culture were incubated for several time periods with HP19 and 20E. A minimum incubation of 4 h was essential for the stimulation of the enzyme activity by 20E, time enough for a genomic hormone action. However, the measured stimulation was unaffected by transcriptional or translational inhibitors, indicating the independence of gene activation. Furthermore, the in vitro study with fat body homogenate showed a rapid stimulation (within seconds to 1 min) of the enzyme activity, indicating the independence of gene activation. Furthermore, the in vitro study with fat body homogenate showed a rapid stimulation (within seconds to 1 min) of the enzyme activity, indicating the independence of gene activation. Furthermore, the 

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